Chapter 1

Literature Review

1.1. The importance of food protein modification

1.1.1. Fermentation

Fermentation was one of the first technique used to produce and preserve foods and had been practiced for thousands of years. It provides ways to preserve food products, enhance nutritive value, destroy undesirable factors, to make safer products, to improve appearance and taste of some foods, to salvage raw material that otherwise not usable for human consumption, and to reduce energy used for cooking (Peredes-Lopez and Harry, 1988). At present, fermentation continues to play an important role in providing variety of quality foods for human and domestic animal consumption. It is estimated that fermentation industries represent $30-50 billion in the world market.

In fermentation, raw materials are converted into products through the act of micro-organisms (bacteria, yeasts, and moulds). The process can be a natural process, in which desirable micro-organisms grow preferentially or in a controlled process in which isolated and characterised fermentative micro-organism (starter culture) is added to the raw material in large number and under controlled condition. The controlled fermentations can be further categorised into single-culture fermentations in which the inoculum (starter culture) consists of only one micro-organism and mixed-culture fermentations in which the inoculum consist of two or more organisms. The mixed-culture fermentations are, therefore, quite similar to the ancient traditional fermentation albeit controlled ones (Hasseltine, 1992).

Different human societies have developed fermentation/proteolytic processes to produce food stuffs which are often intimately associated with cultures in different countries (Adler-Nissen, 1986). In Europe, France in particular, milk is a more common substrate for fermentation with a range cheeses as its main product. In Asia, milk, soy bean, and fish are more common fermentation substrates with a range of products such as yogurt, soy sauce/paste, and fish sauce. Fermented soy bean products such as 'shoyu' and 'miso' in Japan, 'oncom' and 'tempeh' in Indonesia, 'tofu' in China are well known products in respective countries. In Africa, locust bean is used as in Nigerian fermented product called 'dawadawa' (Hesseltine, 1979; Winarno, 1979; Reddy et al., 1982).

As mentioned above the substrates used in fermentation processes differ from place to place hence the varying end products, however, the nature of the process is common. It includes one fundamental step of inoculating the raw materials with proteolytic enzymes, enzyme-producing micro-organism or, in some cases, the enzymes may already present in the raw material (Adler-Nissen, 1986).

Fermented food products may be divided into two major categories: the submerged culture-fermentations (SCFs) and the solid substrate fermentations (SSFs). In the SCFs, microbial activity occurs in a liquid phase and at a relatively low biomass concentration. In the SSFs, however, microbial activity and product formation occurs at the surface of solid substrates (Tengerdy, 1985). Examples for SCFs are soy sauce, fish sauce, keffir beer, palm wine, and rice wine. Examples of SSFs are tempeh, miso, pozol, oncom and natto (Peredes-Lopez and Harry, 1988).

1.1.2. Enzymatic modification of food proteins

Degradation or hydrolysis of protein molecules into smaller peptides and then into amino acids, their building blocks, can be achieved through the use of acid, base or enzymes (Adler-Nissen, 1986). The use of enzymes, the proteases, is by comparison the milder application in food industry. By definition, enzymic hydrolysis of food proteins is the hydrolysis of food proteins by the action of isolated enzymes with a view to applying the hydrolysed proteins as a food ingredient (Adler-Nissen, 1986), and the products are called protein hydrolysates. This, therefore, excludes all traditional fermented food products. The list of commonly used enzymes is seen in Table 1.1.

Proteins are macromolecules and highly complex polymers with functional diversity mostly related to their chemical structures. Unlike polysaccharides and nucleic acids, biopolymers that are made-up of one or more monomers, proteins and polypeptides are made-up 19 different amino acids and one imino acid (proline) combinations. Thus, numerous proteins with specific structures and functions can be synthesised by varying the amino acid composition and sequence (Damodaran, 1997).

The amino acids in protein molecules are linked together through amide bonds or peptide bonds (Damodaran, 1997). During hydrolysis, these bonds are delinked or cleaved to produce peptides (Adler-Nissen, 1986). The resulting peptides, therefore, depend on the amino acids composition and sequence of the proteins and the degree of hydrolysis (DH) which is the percentage of the peptide bonds cleaved (Adler-Nissen, 1976). This entity reflects the catalytic action of proteases (Adler-Nissen, 1986).

In general the proteolytic hydrolysis of food proteins can be shown in the following equation (Adler-Nissen, 1986; Whitaker, 2003a).

$$-HN\text{-}CHR_1\text{-}CO\text{-}NH\text{-}CHR_2\text{-}CO- + H_2O \xrightleftharpoons{\text{Protease}} NH\text{-}CHR_1\text{-}COOH + {}^{+}H_3N\text{-}CHR_2\text{-}CO-.$$

This reaction is reversible, however, in aqueous solutions and suspensions of protein the equilibrium shifts to the right and degradation or hydrolysis but not synthesis of larger molecules is favoured thermodynamically. In an irreversible or denatured substrate (e.g., cooked proteins), the number of accessible peptide bonds increases with a broader initial molecular weight distribution. When irreversible aggregation and insolubilisation occurs, the hydrolytic process can be more complicated (Adler-Nissen, 1986).

The enzymes used in modification of food proteins can be classified according to their origin (animal, plant or microbial), their catalytic action (endopeptidase or exopeptidase), and the nature of the catalytic site. Endopeptidases are always used in food protein hydrolysis, although a combination with exopeptidases is occasionally employed (Adler-Nissen, 1986). The endopeptidases can be further classified based on their catalytic site into serine proteases, cysteine proteases, metalloproteases and aspartic proteases (Whitaker, 2003a; Whitaker, 2003b), while recently a group of threonine peptidases has also been discovered (Barret, 2001). The serine proteases are well studied having a maximum activity at alkaline pH and –OH group in the catalytic centre. The cysteine proteases are similar to serine proteases but having –SH instead of –OH group in the catalytic centre. These enzymes are sensitive towards oxygen. The metalloproteases contain a metal atom, usually zinc and have optimum pH around neutral. The aspartic proteases are characterised by having a carboxyl group from aspartic acid in the active centre and maximum activity at acid pH (Adler-Nissen, 1986).

Table 1.1: List of Proteolytic Enzymes for Food Protein Hydrolysis

Source	Type of Proteases	Common Names, Trade names[a]	Typical pH range[b]	Preferential Specifity[c]
Animals				
Ox, Pig	Aspartic protease	Pepsin, pepsin A	1-4	Aromatic-COOH and –NH2, Leu-, Asp-, Glu-COOH
	Serine Protease	Trypsin	7-9	Lys-, Arg-COOH
	Serine Protease	Chymotrypsin	8-9	Phe-, Tyr-, Trp-COOH
	Mixture of trypsin, chymotrypsin, elastase and carboxypeptidase A or B	Pancreatin	7-9	Very broad specificity
Calf	Aspartic Protease	Chymosin, renin	3-6	Rennet specificity
Plants				
Papaya fruit	Cysteine Protease (Papaya fruit)	Papain pure	5-7	Lys-, Arg-, Phe-X-COOH
	Mixture of papain, chymopapain and Lysozyme	Papain crude	5-9	Broad specificity
Fig latex	Cysteine Protease	Ficin	5-8	Phe-, Tyr-COOH
Pineapple stem	Cysteine Protease	Bromelain	5-8	Lys-, Arg-, Phe-, Tyr-COOH

Adapted from: Adler-Nissen, 1986.
[a] Various other also available.
[b] Application conditions.
[c] Referring to the carbonyl terminal end after cleaving.

Table 1.1. List of Proteolytic Enzymes for Food Protein Hydrolysis (Cont.)

Source	Type of Proteases	Common Names, Trade names[a]	Typical pH range[b]	Preferential Specifity[c]
Bacteria				
Bacillus subtilis	Metalloprotease	Neutrase	6-8	Leu-, Phe-NH and others
	Serine protease	Subtilisin Novo, Subtilisin BPN	6-10	Broad specificity, mainly hydrophobic-COOH
	Mixture of the above	Bioprase, Nagase,	6-9	Broad specificity
Bacillus licheniformis	Serine protease	Rapidermase, Rhozyme P 53, MKC protease	6-10	Broad specificity, mainly hydrophobic-COOH
Bacillus sp. Alkalophilic	Serine protease	Subtilisin Carlsberg Alcalase, Maxatase, Optimase	7-12	Broad specificity, mainly hydrophobic-COOH
Billus athermoproteolyticus	Metalloprotease	Esperase, Savinase, Highly alkaline protease	7-9	Ile-, Leu-, Val-, Phe-NH_2
Fungi		Thermolysin, Termoase		
Aspergillus oryzae	Mixture of aspartic protease, metalloprotease, serine protease, and carboxypeptidase	Takadiastase, Fungal Protease, Sumyzyme LP, Veron P, Panazyme, Prozyme, Biozyme A, Sanzyme	4-8	Very broad specificity

Adapted from: Adler-Nissen, 1986.

[a] Various other also available.

[b] Application conditions.

[c] Referring to the carbonyl terminal end after cleaving.

Table 1.1. List of Proteolytic Enzymes for Food Protein Hydrolysis (Cont.)

Source	Type of Proteases	Common Names, Trade names[a]	Typical pH range[b]	Preferential Specifity[c]
A. niger	Aspartic Protease, usually mixed with carboxypeptidase	Aspergillopeptidase A (Pure asoaric protease), Sumyzyme AP, Proctase, Molsin, Pamprosin	2.5-5	Pure aspartic protease: as pepsin Mixed preparation: broad specificity
Rhizopus sp.	Aspartic protease	Sumyzyme RP, Newlase	3-5	As pepsin
Streptomyces griseus	Mixture of alkaline and Neutral peptidase plus Aminipeptidase and Carboxypeptidase Aspartic protease	Pronase	7-9	Very broad specificity
Mucor miehei	Aspartic protease	Rennilase, Fromase, Marzyme, Morcurd	3-6	Rennet specificity
M. pacillus	Aspartic protease	Emporase, Meito rennet, Noury Lab.	3-6	Rennet specificity
Endothia parasitica	Aspartic protease with	Surecurd, Suparen	3-6	Rennet specificity and some unspecific activity
Penicillium duponti	Some carboxypeptidase	*P. duponti* protease	2-5	Broad specificity
Trametes sanguinea	Aspartic protease with Some carboxypeptidase	*P. trangunea* protease	2-4	Broad specificity

Adapted from: Adler-Nissen, 1986.
[a] Various other also available.
[b] Application conditions.
[c] Referring to the carbonyl terminal end after cleaving.

The enzymic reaction of major class proteases, the serine proteases, takes place in three steps (Svendssen, 1976). The first step is the formation of Michaelis complex between protein or peptide chain as substrate and the enzyme (ES). This is followed by the cleavage of the peptide bond to liberate one of the two peptides. The third step includes nucleophilic attack on the remainder of the complex to split off the other peptide and to reconstitute the enzyme (Adler-Nissen, 1986). The enzymic reaction of cysteine (sulfhydryl) protease, such as papain and bromelain, follow that of the serine protease (Withaker, 2003a).

Papain and bromelain belong to the cysteine proteases and are of plant origin (from papaya latex and pineapple stem, respectively). Papain is an endoprotease (Kristinsson, 2006) with a single polypeptide of 212 amino acids and molecular weight of 23,350 Daltons, containing three disulfide bonds and exceptionally heat stable at neutral pH (Whitaker, 2003b). The optimum pH for papain (including crude papain) is 5-9. The preferential specificity of papain is Lys- Arg- and Phe-X-COOH at the terminal amino acid (Adler-Nissen, 1986).

Bromelain is a collective name for proteolytic enzymes or proteases found in pineapple plant (Doko, et al., 1991) most of which is in the stem. It is also an endopeptidase with an optimum pH range of 5-9 at temperature range of $50^{o}C$ – $60^{o}C$ (Kristinsson, 2006). The preferential specificity of bromelain is Lys-, Arg-, Phe- and Tyr-COOH at the terminal amino acid (Adler-Nissen, 1986).

Flavourzyme™ is a commercial name for a mixture of enzymes of fungal (*Aspergillus oryzae*) origin. According to Adler-Nissen (1986), *Aspergillus oryzae* produced a mixture of aspartic protease, metalloprotease, serine protease and carboxypeptidase with optimum pH ranges of 4-8 and had a very broad specificity. Flavourzyme™ (of

Novozymes) is a complex of endopeptidases and exopeptidases used in the food functionality industry for extensive hydrolysis of protein and debittering process (http://www.novozymes.com).

1.2. Food proteins as source of bioactive peptides

Milk, meat, fish and seafood, and some other plant origin food stuffs such as soy bean have the main sources of proteins required for human and animal growth. The first protein source human ever consumed come from the milk either of human origin (breast milk) or, in many cases, of bovine origin. Milk is an excellent source of nutrients, and since it is easily available, its composition is well studied. Milk proteins have been the subject of many biochemical investigations and their fraction and primary structure are well characterised (Swaisgood, 1993). The protein composition of bovine milk, the most consumed milk, consist of six major proteins namely α_{S1}-casein, α_{S2}-casein, β-casein, κ-casein, β-lactoglobulin, and α-lactalbumin. Apart from these six major proteins, milk proteins also include some other minor proteins that are, with respect to amino acid nutrition, too small to be considered as important (Swaisgood, 1995).

The caseins contribute about 29.5 g/l, while the whey account for about 6.3 g/l. Caseins as the main proteins in milk is well accepted as the main physiological source of amino acid required for the growth of the new born (Silva and Malcata, 2005). However it was found that the dominant physiological feature of casein micelle system is to prevent pathological calcification of mammary gland (Holt, 1997). The specific physiological property of milk proteins has not been well understood. In the last few decades, however, milk proteins have been identified to possess various peptide fragments that

are not active within the parent proteins, but will show various bioactivities once they are released through the action of enzymatic hydrolysis or fermentation.

These bioactive peptides have been identified in almost all fractions of milk proteins. The α_{S1}-casein, for instance, contained various antihypertensive peptides encrypted in its primary structure. It also contains various opioid, immunomodulatory, and antimicrobial peptides. The α_{S2}-casein also contains antihypertensive, immunomodulatory, and antimicrobial peptides. Other casein fractions also possess various bioactive peptides as mentioned above (Silva and Malcata, 2005; Dziuba and Darewicz, 2007; Dziuba et al., 2009). Casein also contained nutritionally active peptides such as caseinophosphopeptides and glycomacropeptides that are able to sequester calcium and other minerals hence function as carrier of minerals (Silva and Malcata 2005). Other milk proteins such as α-lactalbumin, β-lactoglobulin, and lactoferrin have also been reported to carry bioactive peptides in their sequences.

While the above information is mostly obtained from research with bovine milk proteins, various studies with milk proteins of other mammalian species have positively demonstrated the production of bioactive peptides through enzymatic hydrolysis or fermentation. These include antihypertensive peptides derived from murine and rat milk proteins (Kohmura et al., 1990a), and from human milk proteins (Kohmura et al., 1990a; Kohmura et al., 1990b; Kim et al., 1999), and some other milk proteins. These simply indicates that milk protein fraction of various mammalian species have some common physiological features encrypted within their fragments that are of specific roles as to provide both nutrients as well as protective agents for the benefits of their neonates.

Another major source of food proteins is muscle foods. Muscle food proteins (meat, poultry and fish) have always been classified into three groups based on their solubility

characteristics. They are sarcoplasmic, myofibrillar and stromal proteins (Suzuki, 1981; Ockerman, 1983; Bandman, 1987; King and Macfarlane, 1987). These groups make up approximately 35, 60 and 15% of estimated 1,000 or so total proteins present in muscle tissue as revealed by proteomic analysis (Lametsch and Bendixen, 2001).

According to Sikorski et al. (1990) fish meat contained 11 - 24% protein, while (Suzuki, 1981) gave a range from 15 to 24% protein. The protein contents of terrestrial animals vary. However, this variation is not as big as the variation of protein content of fish and seafood. The protein contents of beef, lamb, pork, and chicken ranged from 19 – 23% (Varnam and Sutherland, 1995). Generally the sarcoplasmic fraction comprises about 30% of the total amount of proteins in fish muscle and it is higher in pelagic fish than in demersal fish muscles. The myofibrillar protein constitutes about 40 - 60%, while the stromal protein comprises from 3% in teleosts fish and up to 10% in elasmobranch (Sikorski et al., 1990; Venugopal and Shahidi, 1996). The content of protein fractions of animal muscle proteins is approximately 33% sarcoplasmic, 56% myofibrillar, and 11% stromal proteins (Ranken, 2000).

The sarcoplasmic proteins or the proteins of the muscle fibre sarcoplasm are those that are found in solution of the intercellular fluid (King and Macfarlane, 1987). This group of proteins consist of many kinds of water soluble proteins called myogen (Suzuki, 1981) as well as many soluble enzymes that involved in anaerobic metabolism, protein of mitochondria which comprises the enzymes of tricarboxylic cycle and those of the electron transport system (Bandman, 1987). It also includes globins, microsomes and sarcoplasmic reticulum (Ockerman, 1983). These proteins are soluble in low ionic strength at neutral pH and contribute to cellular metabolism and can be extracted by simply homogenizing muscle in water (King and Macfarlane, 1987) or by simply

pressing the meat (Suzuki, 1981). This group of proteins consisted of at least 500 individual proteins (Pearson and Young, 1989; Venugopal and Shahidi, 1996; Xiong, 2004). Sarcoplasmic proteins are nutritionally inferior to myofibrillar proteins, although many studies have been done to collect this quite abundant fraction for use as feed (Suzuki, 1981).

Myofibrillar proteins are salt-soluble proteins from myofibril in the muscle fibre which contain major proteins such as myosin, actin and regulating proteins such as tropomyosin, troponin, actinin (Suzuki, 1981), C-protein, M-protein, F-protein, I-protein, myomesin, the filament of protein desmin, Z-protein, titin (Bandman, 1987; Venugopal and Shahidi, 1996) and are an integral part of the array of filaments in the muscle (King and Macfarlane, 1987). This group of proteins is the most abundant fraction of muscle protein but consists of only around one hundred proteins (Lametsch and Bendixen, 2001; Xiong, 2004). Myosin is the major myofibrillar protein chain with four light chains linked noncovalently (King and Macfarlane, 1987) and forms thick filament (Suzuki, 1981). Vertebrate skeletal myosin consists of two large polypeptides called heavy chains, each with a molecular weight of about 200 kDa and four small subunits called light chains in the molecular weight range of 16 kDa to 30 kDa (Weeds and Lowey, 1971), while the molecular weight of the native myosin is about 480 kDa (Godfrey and Harrington, 1970) and consist of approximately 4,500 amino acids (Xiong, 2004).

Myosin constitutes from 50 to 60% of the myofibrillar fraction and can be extracted easily from fish-mince in one to three minutes with 3% salt solution (Sikorski et al., 1990) and is a very important protein responsible for the functional properties of fish protein. However, myosin can easily be insolubilised as well. According to Mazo et al.

(1994) myosin was the first protein to be insolubilised when formaldehyde reacted with actomyosin, followed by actin then troponin and myosin light chain and lastly tropomyosin.

Actin makes about 15 to about 20% of the myofibrillar fraction. Actin can be extracted similar to myosin except that it needs longer extraction time. When fish mince is treated with neutral salt solution, actin is extracted together with myosin in the form of actomyosin (Suzuki, 1981; Sikorski et al., 1990). Stromal proteins are the proteins which form connective tissues, cannot be extracted by water, acid or alkaline solution and neutral salt solution of 0.01 to 0.1 M (Suzuki, 1981), play an important role in living muscle in addition to having an important part in determining meat tenderness. Stromal protein consists of collagen, elastin, or both. Collagen is the major structural component of all connective tissue consisting of Type-I, Type-II, and Type-III, occur predominantly in fibrous form in the extracellular matrix (Bandman, 1987)

The collagen level in fish muscle ranges from one to 12% of the crude protein or about 0.2 to 2.2% of the wet weight of the meat. About 1.7 to 4.6% of the collagen in fish is found in the skin (Sikorski et al., 1984; Sato et al., 1986). Collagen is responsible for the toughness of fish meat. The higher the level of collagen, the tougher the meat. This is, however, not a problem for fish processor because of its lower levels in fish as compared to that in livestock meat. Collagen is considered to have a low nutritional value. However, fish collagen differed from bovine meat collagens in having significantly higher amount of seven essential amino acids (Kimura, 1990) and a considerable lower concentration of hydroxyproline residues (Venugopal and Shahidi, 1996).

Muscle food proteins, like milk proteins, have been shown to encrypt active peptides in their sequences. These peptides do not exert any activity while remaining in their parent

proteins, but once correctly released either through fermentation or enzymatic hydrolysis, or to a lesser extent through acid or basic degradation, may show various physiological activities. These active peptides have shown various physiological activities such as antihypertensive peptide from beef (Jang and Lee, 2005), antimicrobial peptides from chicken and turkey (Evans et al., 1995), and antithrombotic peptide from pork (Shimizu et al., 2009).

Apart from milk and meat/fish proteins, other protein sources have also been reported to produce bioactive peptides. Among these protein sources, soy bean is the most promising source since it contains reasonably high protein and is a well known plant protein source. Soy bean forms part of human diet, especially in East Asia, for more than 2000 years and is consumed in the form of cooked whole soy, soy milk, soy been curd (tofu), fermented tofu, fermented soy bean (natto), and half-fermented soy bean (tempeh) (Fukushima, 2004). Soy bean contains 38% protein, 18% oil, 15% insoluble carbohydrate (dietary fibre), 15% soluble carbohydrate (including oligosaccharides), and 14% moisture, ash, vitamins, and minerals (Hoogenkamp, 2005), indicating a power house of nutrients.

The soy bean proteins exist mostly (approximately 90%) as storage proteins and consist of mostly β-conglycinin and glycinin (Koshiyama and Fukushima, 1976). β-Conglycinin is a glycoprotein and a trimer that consists of three subunits with a molecular mass of 150-200 kDa. The three major subunits are α^1-, α-, and β- having 72, 68, and 52 kDa molecular weights, respectively. In addition to the three major subunits, there is also a minor subunit called γ- in β-conglycinin (Thanh and Shibasaki, 1977). The amino acid sequences of these subunits are similar with both α^1-and α- subunits have one cysteine residue near the N-terminal end, while the β- subunit does not have this cysteine residue.

All subunits have no cystine residue in their sequence (Utsumi et al., 1997). Glycinin is a hexamer of 300-380 kDa molecular weights. All subunits are composed of acidic polypeptide (~35 kDa) and basic polypeptide (~20 kDa) that are linked together by a disulfide bond (Yokoyama et al., 1992; Hsieh, 2006; Yoshie-Stark et al., 2006; Yoshikawa and Abba 2006). Identified subunits in glycinin are grouped into two groups: group I that consists of subunits $A_{1a}B_{1b}$ (53.6 kDa), A_2B_{1a} (52.4 kDa), and $A_{1b}B_2$ (52.2 kDa), and group II that consist of $A_5A_4B_3$ (61.2 kDa) and A_3B_4 (55.4 kDa) (Nielsen, 1985, Nielsen et al., 1989). The amino acid sequences of the subunits in each group have some commonality in which each subunits in group I has two cysteine and two cystine residues, while each subunit in group II has two cysteine and two cystine residues (Utsumi et al., 1997).

Various bioactive peptides have been isolated from soy protein hydrolysates and characterised. These peptides include antihypertensive peptides (Wu and Ding, 2001), antioxidant (Chen et al., 1995), anticariogenic (Kim et al., 2000), hypocholesterolemic (Yoshikawa et al., 2000), and anticarcinogenic (Sugano et al., 1988) peptides. These active peptides come from various soy protein subunits that indicate the potential benefits of soy proteins in maintaining human health. While many of these studies are conducted using soy protein hydrolysates, fermented soy bean products may also contain physiologically active peptides. Fermented soy bean products are common food ingredients consumed by many East Asian people hence an important protein sources required for growth. It is also important to note that soy bean is considered a healthier alternative to milk as it does not contain lactose, the milk sugar associated with intolerance in may people.

The production of bioactive peptides from food proteins through enzymatic hydrolysis or fermentation rely very much on the specificity of enzymes used and hydrolysis time. Enzyme specificity will determine the preferential peptide bond to cleave, hence determines both the N-terminal and the C-terminal residues of the resulting peptides, as well as the length of the peptides. The terminal amino acid residues of active peptides often determines the activity of the peptides as in the case of antihypertensive peptides, where the amino acid residues at the C-terminal and the N-terminal ends determine the strength of the inhibition of angiotensin I-converting enzyme (ACE) that is responsible for the production of vasopressor angiotensin II and the onset of hypertension.

Enzyme such as pure papain will tend to produce peptides with amino acid residue lysine, arginine, and phenylalanine at the C-terminal ends. The crude papain, however, has broader specificity hence will produce peptides with any possible amino acid residue at the C-terminal ends. Likewise, pure bromelain will produce peptides with tyrosine residue at the C-terminal ends in addition to lysine, arginine, and phenylalanine residues as the pure papain. The crude bromelain that has broad specificity will produce peptides with various amino acid residues at the C-terminal ends. Enzymes produced by *Aspergillus oryzea* such as Flavourzyme™, an endo and exopeptidase, also have broad specificity hence will produce peptides with various N- and C-terminal residues. Thermolysin, a metalloprotease from *Bacillus athermoproteoliticus*, will mainly produce peptides with isoleucine, leucine, valine, and phenylalanine at the N-terminal ends (Adler-Nissen, 1986).

The enzymes with narrow specificity will, therefore, produce peptides with certain amino acid residues at the N- and C-terminal ends and prolonged hydrolysis may not cleave the produced peptide further. Prolonged hydrolysis with enzymes of broader

specificity, on the other hand, will likely to cleave the existing peptides and produce smaller peptides and free amino acids. Therefore, hydrolysis times will play an important role in the production of bioactive peptides. Antihypertensive peptides derived from soy proteins, for instance, tend to decrease as the hydrolysis time, and so the degree of hydrolysis (DH), indicating further cleaving of the active peptides previously produced. In contrast, bioactivity of peptides produced through pepsin, trypsin, and chymotrypsin from the same soy bean proteins increased as the hydrolysis progressed (increased DH) (Chiang et al., 2006), indicating continual production of stable active peptides. These three enzymes are of animal origin and have narrow specificities (Adler-Nissen, 1986).

1.3. Bioactive peptides derived from food proteins

The bioactive peptides derived from food proteins can be classified into several groups as those that influence body regulating system, body defense system, body nervous system, and body nutrition system. The body regulating peptides consisted of antithrombotic and antihypertensive peptides; the body defense peptides consisted of antimicrobial, immunomodulating, and anti-oxidative peptides; the body nervous peptides consisted of opioid peptides; while the body nutrition peptides consisted of anticariogenic and mineral sequester peptides (Silva and Malcata, 2005). The opioid peptides can be grouped into agonistic and antagonistic peptides. In addition to the above, some peptides can also stimulate the growth of probiotic bacteria and lower cholesterol (Arihara, 2006).

Numerous studies have been carried out to investigate the bioactivity of peptides, ACE inhibitors in particular, derived from food proteins after the finding of ACE inhibitory

peptide from snake venom (Meisel et al., 2006). The term bioactivity refers to characteristic of food components that can alter biological processes or substrates and hence have an effect on the body function or condition and consequently the body health (Gerdes et al., 2001). The physiological functions of peptides are well reported which include anti-caries activity or anti-cariogenesis (Aimutis, 2004), anti-anaemia activity (Deng et al., 2004), opioid-like, anti-thrombotic and cholesterol-lowering activities (Gerdes et al., 2001), anti-microbial activity (Chan and Li-Chan, 2006), anti-oxidative activity (Kitts and Weiler 2003, Pihlanto, 2006) and anti-hypertensive activity (Gerdes et al., 2001; Kuono et al., 2005; Jung et al., 2006). Among all these physiological functions of peptides, anti-hypertensive and anti-microbial activity will be focused and discussed in a more detailed in the coming sections.

1.3.1. Anti-cariogenic peptides

Studies to investigate the effect of peptides derived from milk on prevention of cariogenicity, plague-forming bacteria, tooth enamel demineralisation and subsequent enamel remineralisation have shown positive results (Reynolds, 1987). Caseinophosphopeptides (CPP) and glycomacro-peptide (GMP), for instance, have been patented to be used in personal hygiene products to prevent dental caries. These two peptides can inhibit the growth of bacteria, and the CPP can form nanoclusters with amorphous calcium phosphate (AMP) at the surface of tooth to act as a reservoir of calcium and phosphate ions to maintain a state of supersaturation that will buffer the plague pH and provide ions for enamel remineralisation (Aimutis, 2004).

The effect of hydrolysed offal protein from *Harengula zunasi* (Bleeker), a sea water fish, on anaemia of mice injected with cyclophosphamide, a nitrogen mustard alkylating

agent used to treat various types of cancer and some autoimmune disorders, has also been studied (Deng et al., 2004). In this study, the mice injected with cyclophosphamide showed trend in decreasing their body weight while administration of hydrolysed offal proteins to these mice have shown to work against the effect of cyclophosphamide. The study also revealed the effect of hydrolysed offal protein in preventing the enlargement of the mice thymus, liver and spleen caused by cyclophosphamide injection. The enlargement of spleen is an indication of anaemia and the offal protein hydrolysate here may function as competitor to the alkylation of cell function group by cyclophosphamide by providing free functional group such as amido, mercapto, hydroxyl and carbonyl. This, however, remains unclear as the offal hydrolysate also contain minerals such as calcium, phosphorus and iron which are essential for the blood cells to divide and grow (Deng et al., 2004).

1.3.2. Opioid peptides

Several peptides derived from whey have been found to show an opioid-like activity. These opioid peptides are similar physiologically to opium (morphine); however, endogenous opioid peptides have shown positive sign as blood pressure modulator. The peptides derived from whey protein like α-lactalbumin and β-lactoglobulin exhibit this opioid-like activity. Peptide α-lactophorin, for instance, has shown to exhibit weak opioid activity by producing analgesic and sedative effect to smooth muscle (Gerdes et al., 2001).

Opioid peptides are peptides that have affinity for an opiate receptor, have opiate-like affects, and have naloxone-inhibitable (Höllt 1983; Meisel H. 1997). The majority of these exogenous opioid peptides such as β-casomorphins are fragments of β-casein

sequence 60-70 (Meisel Hans 1986), and has also been found in similar position in sheep, water buffalo and human β-casein (Schlimme and Meisel, 1995), while α_{S1}-casein fragments 90-96 also produce various opioid peptides α-casein exorphins. Other milk proteins such as κ-casein fragments 25-38, and whey proteins α-lactalbumin fragments 50-53, β-lactalbumin fragments 102-105, serum albumin fragments 399-404, and lactoferrin also produce opioid peptides. Some opioid peptides are listed in Table 1.2.

Opioid peptides can be classified into at least three types based on their binding to specific receptors. They are peptides that bind to μ-receptor for emotional behaviour and suppression of internal motility, those that bind to κ-receptor for sedation and food intake, and those that bind to δ-receptor for emotional behaviour (Corbett et al., 1982; Pihlanto-Leppälä, 2001; Pihlanto-Leppälä, 2002). Opioid peptides β-casomorphins are characterized as μ-type ligands, α-casein exorphins are δ-type ligands, while casoxins show preference for μ- and κ-receptors. Most opioid peptides have common amino acid sequence of YGGP at the N-terminal region. Opioid peptides derived from protein precursors, however, are atypical as they have different amino acids at their N-terminal sequences. The presence of tyrosine at the N-terminal residue remains mostly the same, while the presence of aromatic residue at the third or fourth position contributes to their structures that fit into the binding sites of the receptors (Pihlanto-Leppälä, 2002).

Some opioid peptides have shown antagonist properties. These include the casoxins derived from tryptic and peptic digest of bovine as well as human κ-casein, and the lactoferroxins from lactoferrin. Among the casoxins, casoxin 6 has the lowest IC_{50} value (15 μmol/L), though is of low potency as compared to naloxone. Casoxin 6 is characterised by an ester group attached to its C-terminal amino acid. Casoxin C is the only non esterified casoxins that show opioid antagonistic activity comparable to

casoxin 6. Lactoferroxins are also opioid peptides with antagonist properties (Pihlanto-Leppälä, 2002).

Opioid peptides are mostly produced through hydrolysis of milk protein precursor with trypsin or pepsin. These peptides, however, can also be produced from fermentation of milk. Recently, agonistic and antagonistic opioid peptides have been identified from five varieties of cheeses: three semi-hard rennet cheeses 'Edamski', 'Gouda', and 'Kasztelan', as well as two ripening mould cheeses 'Brie' and 'Rokpol' (Sienkiewickz-Szlapka et al., 2009). These cheeses had shown to contain β-casomorphin-5, β-casomorphin-7, casoxin C, casoxin 6, and lactoferroxin A. Opioid agonistic peptide β-casomorphins were found to be more abundant in ripening mould cheeses, while the antagonistic peptides were more abundant in semi-hard cheeses. This result suggests that longer ripening time will release more agonistic peptides from the β-casein fragments, while shorter ripening time will release opioid antagonistic peptides from κ-casein and lactoferrin.

Table 1.2. Opioid peptides derived from bovine milk proteins.

Name	Sequence	Protein precursor	IC_{50} Value[a]	Preparation	Reference
β-Casomorphin-5	YPFPG	β-Casein(f60-64)	1.1	Trypsin	Brantl et al., 1981.
β-Casomorphin-7	YPFPGPI	β-Casein(f60-66)	14	Trypsin	Brantl et al., 1981.
β-Casomorphin-11	YPFPGPIPNSL	β-Casein(f60-70)	10	Jejunum (minipigs)	Meisel, 1986.
α-Casein exorphin	YLGYLE	α_{S1}-Casein(f91-96)	45	Pepsin	Loukas et al., 1983.
α-Casein exorphin	RYLGYL	α_{S1}-Casein(f90-95)	12	Pepsin	Loukas et al., 1983.
α-Casein exorphin	RYLGYLE	α_{S1}-Casein(f90-96)	1.2	Pepsin	Loukas et al., 1983.
Casoxin C	YIPIQYVLSR	κ-Casein(F25-34)	50(↓)	Trypsin	Chiba et al., 1989.
Casoxin 6	SRYPSY.OCH_3	κ-Casein(f33-38)	15(↓)	Pepsin	Chiba et al., 1989.
α-Lactorphin	YGLF.NH_2	α-Lactalbumin(f50-53)	300	Pepsin	Mullally et al., 1996.
β-Lactorphin	YLLF.NH_2	β-Lactalbumin(102-105)	160	Pepsin + trypsin	Mulally et al., 1996.
Serorphin	YGFQNA	Serum albumin (f399-404)	85		Tani et al., 1994.
Lactoferroxin A	YLGSGY	Lactoferrin			Chiba et al., 1989.

Adapted from: Pihlanto-Leppala, 2002 and Meisel, 1997.
↓ Exert opioid antagonistic activity.
[a] Values are expressed as μmol/L

1.3.3. Anti-thrombotic peptides

The anti-thrombotic agents have also been derived from food protein derivatives. Thrombosis is a formation or presence of blood clot within the blood vessel and a risk factor for cardiovascular disease. This formation takes place when fibrinogen, a plasma protein, is converted into fibrins that form into platelets and aggregate them (Gerdes et al., 2001). These agents can be divided into two classes: anti-coagulants and anti-platelet agents and functions to prevent the formation and growth of thrombi. The anti-coagulant agents work by inhibiting thrombin production and fibrin formation, while anti-platelet agents work by blocking platelet aggregations (Hirsh and Weitz, 1999).

The established anti-thrombotic agents such as heparin and coumarins have various limitations such as narrow therapeutic windows and a highly variable dose-response relation (Hirsh, 1991). Heparin has a tendency to bind non-specifically to plasma proteins and proteins released from activated platelets and endothelial cells. The concentration of heparin-binding proteins is often variably high in patients with thromboembolic disease. This limits the amount of available heparin to interact with anti-thrombin. Heparin also unable to inactivate thrombin bound to fibrin and factor Xa (the activated form of Factor X or prothrombinase that function to cleave prothrombin to form active thrombus) bound to activated platelets trapped within the thrombus. The consequences of these limitations may lead to serious bleeding of the injured blood vessel once treatment with anti-thrombotic agents is stopped. Coumarins also have these limitations, although the mechanism is not well understood. Therefore, administration of these agents must be followed by close monitoring through laboratory tests (Hirsh and Weitz, 1999). Another major anti-thrombotic agent is aspirin which works by blocking the synthesis of thromboxane

A2 (Pratono, 1994), a member of a family of lipids known as eicosanoids and is produced by activated platelets and has pro-thrombotic properties that stimulates activation of new platelets as well as increases platelet aggregation, therefore has a very limited effectiveness. These discrepancies have provided the impetus for the developments of new anti-thrombotic agents.

New anti-thrombotic agents, therefore, need to exert wider therapeutic windows and less variable dose-response relation that can be safely administrated and required less laboratory monitoring. Various investigations of the anti-thrombotic effects of peptides derived from food proteins or blood proteins that function as blood coagulation factors have shown promising results. Nawroth et al., (1986) found that a 44 residue peptide derived from the first 44 N-terminal residues of bovine light chain blood coagulation factor X showed inhibition and blocking of thrombin formation. In another work, the effect of type III collagen-related octapeptide KOGEOGPK (where O is hydrolyproline) on the *in vitro* prevention of mouse platelet interaction/adhesion with sub-endothelial type III collagen was investigated. The administration of 80 mg/kg of KOGEOGPK reduced the induced-thrombi in mouse by 50% and could decrease the occurrence of arteriole occlusion by 76% 45 min after injection (Maurice et al. 2006). This interaction/adhesion is a normal hemostatis such as in the event of vascular injury, but can also contribute to the underlying pathologies associated with enhanced thrombosis such as myocardial infarction, stroke, unstable angina, and ischemia (Weiss, 1975a, b).

Anti-thrombotic peptides from food proteins have also been identified and characterised. These peptides are presumed to have the ability to inhibit the fixation of platelets (Gerdes et al., 2001). Peptides, called casoplatelins, derived from bovine κ-casein's C-terminal part (caseinoglycomacropeptide) can inhibit the aggregation of

platelets and binding the fibrinogen α-chain to a specific receptor site on the platelet surface. These κ-casein's anti-thrombotic peptides are from sequence 106-116 and are in various sizes (Mullally et al., 1997). In a recent study to evaluate the potential release of bioactive peptides from milk proteins using BIOPEP database of proteins and bioactive peptides (www.uwm.wdu.pl/biochemia), Dziuba et al. (2009) found that β-casein (genetic variant A_1) can produce anti-thrombotic peptides from sequences encrypted in various fragments of the protein. The anti-thrombotic peptides also can be found in κ-casein (generic variant A), and lactoferrin.

The release of anti-thrombotic peptides has also been reported from papain hydrolysate of defatted pork meat after oral administration to mice. The hydrolysate from 24-hour hydrolysis was subjected to a cation-exchange chromatography fractionation and all crude hydrolysate, absorbed fraction, and non-absorbed fraction were used for *in vitro* anti-thrombotic assays. The results showed that crude hydrolysate of 210 mg/kg body weight could inhibit the formation of thrombi, while purified non-absorbed peptide fraction at 70 mg/kg body weight could inhibit thrombin formation which is almost equal to the activity of aspirin at 50 mg/kg body weight (Shimizu et al., 2009). Although this study did not go further to elucidate the sequence of the peptides, it did indicate that pork meat proteins have encrypted anti-thrombotic peptides and hydrolyse the proteins may release those peptides. Other source that has been indicated to contain encrypted anti-thrombotic peptide is soy proteins. Lee (2004) found that fermentation of soy bean in the preparation of soy sauce produces bioactive peptides that can function as, among others, anti-hypertensive, anti-thrombotic, and anti-cancer agents.

1.3.4. Anti-oxidative peptides

Numerous anti-oxidative peptides derived from food proteins have been isolated and characterised. Anti-oxidants prevent the formation of free radicals or scavenge radicals such as hydrogen peroxide and other peroxides. The formation of excess radicals can overwhelm protective enzymes such as superoxide dimutase, catalase, and peroxidase and causes lethal cellular effects such as apoptosis by oxidising membrane lipids, cellular proteins, DNA, and enzymes, hence shutting down cellular respiration (Pihlanto, 2006). Table 1.3 lists some of the anti-oxidative peptides derived from food proteins. Fractions of fish protein hydrolysates from capelin (*Mallotus villosus*) have been found to delay bleaching at various rates of β-carotene in an emulsion system (Amarowicz and Shahidi, 1997) thus indicating their anti-oxidative activity. Mackerel (*Scomber australicus*) hydrolysates from autolytic process and enzymatic hydrolysis with commercial enzyme Protease N have also shown anti-oxidative activities related to the presence of free amino acids (FAAs) and dipeptides carnosine (β-alanylhistidine) and anserine (β-alanyl-1-methylhistidine). An anti-oxidative peptide with molecular weight of 1.4 kDa was isolated and characterised, and shown stronger anti-oxidative activity than other isolated peptide fractions of lower molecular weight (Wu et al., 2003).

Table 1.3. Anti-oxidative peptides derived from food proteins.

Peptides	Protein Source	Activity	Preparation	Reference
LPHSGY	Alaska Pollack frame proteins	35%[a]	MICE	Je et al., 2005.
FDSGPAGVL	Jumbo squid skin gelatine	90.90[b]	Trypsin	Mendis et al, 2005.
NGPLQAGQPGER	Jumbo squid skin gelatine	100.72[b]	Trypsin	Mendis et al., 2005.
GSTVPERTHPACPDFN	Hoki frame proteins	17.77[b]	Gastrointestinal digest	Kim et al., 2007.
VKAGFAWTANQQLS	Tuna backbone proteins	> α-Tocopherol[c]	Pepsin	Je et al., 2007.
PSKYEPFV	Grass carp muscle proteins	2.86[d]	Alcalase	Ren et al., 2008.
LHY	Sardinelle by-product proteins	63%[a]	SVCE	Bougatef et al., 2010.
DPALATEPDPM-PF	Nile tilapia scale gelatin	7.56[b]	Alcalase	Ngo et al., 2010.
YFYPEL	α_{S1}-Casein (f144-149)	79.2 μM[e]	Pepsin	Suetsuna et al., 2000.
VKEAMAPK	β-Casein (f9105)	0.95[f]	Trypsin, synthetic	Rival et al., 2000.
KVLPVPQK	β-Casein (f169-176)	0.99[f]	Trypsin, synthetic	Rival et al., 2000.
AVPYPQR	β-Casein (f177-183)	1.00[f]	Trypsin, synthetic	Rival et al., 2000.
WYSLAMAASDI	β-Lactoglobulin	2.6[g]	Corolase PP, synthetic	Hernández-Ledesma et al., 2005.
MHIRL	β-Lactoglobulin	0.31[g]	Corolase PP, synthetic	Hernández-Ledesma et al., 2005.
YVEEL	β-Lactoglobulin	0.80[g]	Corolase PP, synthetic	Hernández-Ledesma et al., 2005.

[a] Expressed as percent scavenging of hydroxyl radical in linoleic acid emulsion system.
[b] Expressed as IC_{50} value in μM of scavenging hydroxyl radical from 5,5-dimethyl-1-pyrroline-N-oxide (DMPO).
[c] Expressed as higher (>) or lower (<) in comparison to inhibition of lipid peroxidation by α-tocopherol in linoleic acid emulsion system.
[d] Expressed as IC_{50} value in mg/ml of scavenging hydroxyl radical from hydrogen proxide.
[e] Expressed as IC_{50} value in μM of superoxide anion scavenging activity (SOSA).
[f] Expressed as IC_{50} value in μM for scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.
[g] Expressed in μmol Trolox equivs/mg of peptide.
MICE : mackerel intestine crude enzyme
SVCE : sardine viscera crude enzyme

Hydrolysates and fermented products from other fish and seafood proteins have also been analysed for their anti-oxidative activities with positive results. These include hydrolysates from Alaska pollack (*Theragra chalcogramma*) frame proteins (Je et al., 2005a), fermented mussel sauce (Rajapakse et al., 2005), hydrolysates from umbo squid (*Dosidicus gigas*) skin gelatin (Mendis et al., 2005), hoki (Johnius belengerii) hydrolysates (Kim et al., 2007), tuna backbone protein hydrolysates (Je et al., 2007), oyster (*Crassostrea gigas*) protein hydrolysates (Qian et al., 2008), grass carp muscle protein hydrolysates (Ren et al., 2008), and from hydrolysates of numerous other species. Some of the peptides responsible for the anti-oxidative effects have been isolated and identified such as 672 Da LPHSGY from mackerel intestine crude enzyme (MICE) hydrolysates of Alaska Pollack frame proteins (Je et al., 2005a), 880.18 Da FDSGPAGVL and NGPLQAGQPGER from tryptic hydrolysates of jumbo squid (Mendis et al., 2005), 1801 Da GSTVPERTHPACPDFN from gastrointestinal digest of hoki frame proteins (Kim et al., 2007), 1519 Da VKAGFAWTANQQLS from peptic digest of tuna backbone proteins (Je et al., 2007), 966.3 Da PSKYEPFV from Alcalase grass carp muscle hydrolysates (Ren et al., 2008), GLY and six other peptides from sardine viscera crude enzyme (SVCE) digest of sardine (*Sardinella aurita*) by-product proteins (Bougatef et al., 2010), and 1382.57 Da DPALATEPDPMPF from Nile tilapia (*Oreochromis niloticus*) scale gelatin (Ngo et al., 2010).

Milk proteins have been the source of anti-oxidative peptides and their sequences positions within the milk protein sequences are well recognised. These peptides come from caseins and lactoglobulins and include 830.933 Da YFYPEL from peptic digest of α_{S1}-casein fragments 144-149 having IC_{50} value of 79.2 μM in superoxide anion scavenging activity (SOSA) system. Further characterisation using different

synthetic fragments of the peptide shown that EL (molecular weight 260.29 Da) has stronger anti-oxidative activity with IC_{50} value of 63.1 μM (Suetsuna et al., 2000), indicating the most active domain of the peptide. Tryptic digest of β-casein produced various anti-oxidative peptides such as 873.08 Da VKEAMAPK (f98-105), 908.15 Da KVLPVPQK (f169-176), and 829.95 Da AVPYPQR (f177-183) with IC_{50} values of 0.95, 0.99, and 1.00 μM for scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Rival et al., 2000). Anti-oxidative peptides have also been identified from whey protein β-lactoglobulin that includes WYSLAMAASDI, MHIRL, YVEEL derived through Corosale PP hydrolysis (Hernandez-Ledesma et al., 2005). Anti-oxidative peptides from milk proteins have also been produced through fermentation (Rossini et al., 2009). Apart from peptides derived from milk and fish proteins, anti-oxidative peptides have been produced from many other protein sources such as egg proteins (Sakanaka et al., 2004; You et al., 2010), porcine collagen and plasma proteins (Li et al., 2007; Liu et al. 2010), soy proteins (Moure et al., 2006; Wang et al., 2008), and from various other protein sources.

1.3.5. Prebiotic peptides

Peptides derived from food proteins have been reported to stimulate the growth of probiotic bacteria. These peptides, known as prebiotic peptides, have been reported to originate from milk and porcine proteins. According to Brody (2000), various studies have indicated that milk protein hydrolysates could stimulate the growth of lactic acid bacteria (LAB) and Bifidobacteria. Idota et al. (1994) assumed that the main growth factor was the sugar moieties such as N-acetylglucosamine and glucosamine of glycosilated peptides. However, non-glucosilated prebiotic peptides have also been isolated. These include peptides derived from pepsin digest of human

milk hydrolysate having 5.6 kDa and 5.8 kDa molecular weights identified as fragments of lactoferrin that selectively enhanced the growth of bifidobacteria. Based on this finding, a smaller peptide CAVGGCIAL was designed, characterised and labelled as probiotic lactoferrin-derived peptide-I (PRELP-I) (Lieple et al., 2002). Other prebiotic peptides have been isolated from papain hydrolysate of porcine skeletal muscle actomyosin. This peptide was able to enhance the growth of Bifidobacteria strains in media (Arihara, 2006).

1.3.6. Hypocholesterolemic peptides

It has been reported that the level of serum cholesterol could be lowered by dietary proteins such as milk and soy proteins (Nagaoka et al., 1992). This finding leads to the assumption that food proteins can produce cholesterol-lowering bioactive peptides. In 2001, a hypocholesterilemic peptide IIAEK was identified from enzymatic hydrolysis of β-lactoglobulin. This peptide demonstrated stronger cholesterol lowering activity in rats than β-sitosterol, a common cholesterol lowering drug. It was assumed that the peptide reduced the micellar solubility of cholesterol and inhibits cholesterol absorption (Nagaoka et al., 2001).

The well known anti-hypertensive peptides IPP and VPP derived from fermentation of milk have shown to be able to reduce the level of total cholesterol and LDL cholesterol in hypertensive and dyslipidemic subjects. The peptides were served to the above subjects in form of a spread mix with plant sterols. The HDL cholesterol level, high sensitivity C reactive protein (CRP), however, remained unchanged. This indicated that these two anti-hypertensive peptides together with plant sterols have health benefits in combating cardiovascular risk factors (Turpeinen et al., 2009). In another study, protein extract from Amaranth has shown cholesterol lowering effect

in male hamster. As in previous IPP and VPP study, the HDL cholesterol level was unaffected, but the total cholesterol and the LDL cholesterol levels were reduced (Mendonça et al., 2009).

1.3.7. Wound healing promoting peptides

Some bioactive peptides have also been reported to promote wound healing. Demidova-Rice et al. (2010) reported that *Clostridium histolyticum*'s collagenase limited hydrolysis of defined capillary-endothelial-derived extracellular matrices produced several bioactive peptides, which facilitate endothelial responses to injury, and accelerate microvascular remodeling in vitro. Fragments of collagen IV, fibrillin-1, tenascin X, and a novel peptide created by combining specific amino acids contained within fibrillin 1 and tenascin X each have profound proangiogenic properties. The peptides increases rates of microvascular endothelial cell proliferation by up to 47% at concentration between 10–100 nM and in vitro angiogenesis by 200% when compared with serum-stimulated controls. This study revealed that peptides could induce wound healing. Previously, Oudhoff et al. (2009) had also reported that histatin, naturally occuring peptide from human saliva, could promote wound healing by enhancing reepithelialization in a human full-skin wound model closely resembling normal skin. Although the peptide did not stimulate cell proliferation, it did induce cell spreading and migration, two key initiating steps in reepithelialization process.

1.3.8. Anti-hypertensive peptides

Peptides derived from food proteins have shown bioactivity towards angiotensin I-converting enzymes (ACE) (Gerdes et al., 2001). These peptides have been isolated

from various proteins such as buckwheat, corn, chickpeas, soy bean, milk, whey, and fish. Table 1.4 lists ACE inhibitory peptides derived from fish and other marine origins. Ma et al. (2006) have isolated and identified an ACE inhibitor from buckwheat. This tripeptide ACE inhibitor has an amino acid sequence of GPP and shown activity against ACE with an IC_{50} value of 6.25 μg protein/ml. In another study, Li et al. (2002) also reported the presence of ACE inhibitor peptides derived from buckwheat. These peptides consist of di- and tripeptides with IC_{50} values ranging from 4 μM to 628 μM. The *in vivo* ACE inhibition power of these peptides is, however, low compared to the peptides derived from sardine muscle hydrolysate.

Suh et al. (2003) had studied corn gluten hydrolysate of six commercial proteases viz., protamax, Flavourzyme™, proleather FG-F, protease A, aroase AP-10 and pescalase. The results showed that Flavourzyme™ produced hydrolysates with stronger ACE inhibitory activity of IC_{50} 0.18 mg solid. Legumin, the main protein in chickpea seeds, also produced six ACE inhibitory peptides when hydrolysed with alcalase (Yust et al., 2003). These peptides showed inhibitory activity with IC_{50} values ranging from 0.011 mg/ml to 0.021 mg/ml with a total IC_{50} value of 0.18 mg/ml; all these peptides contain methionine residues.

Soybean hydrolysate and fermented soybean products have been reported to produce ACE inhibitory peptides. Kuba et al. (2005) reported that acid proteinase hydrolysate of soybean proteins, β-conglycinin and glycinin, contained four bioactive peptides with ACE inhibitory IC_{50} values of 65 μM to 850 μM. These peptides have been identified as LAIPVNKP, LPHF, SPYP and WP. The synthesised versions of WL and LAIPVNKP showed high ACE inhibitory activities. This research also showed correlation between ACE inhibitory activity and the degree of hydrolysis.

According to Chiang et al. (2006), a six hour hydrolysis of soy protein with Alcalase at 50^{o}C, pH 9.0 and E/S ratio of 0.01 would produce hydrolysate with highest ACE inhibitory activity as compared to hydrolysates of Flavourzyme™, trypsin, chymotrypsin and pepsin. It was also evident that the higher the degree of hydrolysis (DH), the higher the ACE inhibitory activity. Hence, increase in the hydrolysis time indicated the significance of protease hydrolysis of proteins to produce bioactive peptides. In case of Flavourzyme™, however, the increase in degree of hydrolysis did not guarantee the increase in ACE inhibitor activity. This might be due to inactivation of active peptide sequence by exoproteases present in Flavourzyme™, through the splitting of one or more amino acids from the active N-terminal or C-terminal of the peptides. This is, however, in direct contrast to the finding of Suh et al. (2003) which showed that Flavourzyme™ hydrolysates of corn gluten had higher ACE inhibitory activity. This suggests that different proteins may produce different bioactive peptides when hydrolysed with the same enzyme.

Anti-hypertensive action of milk and milk products, and whey proteins has been studied extensively. Meisel et al. (2006) reported more than 100 peptides derived from bovine milk alone and consisted of two to eleven amino acids. Nakamura et al. (1995a) reported two ACE inhibitory peptides VPP and IPP from fermented sour milk. In this study unfermented milk was also a subject for ACE inhibitory assay and showed slight ACE inhibitory activity as compared to sour milk which indicate that the activity was markedly increased during fermentation with Calpis sour milk starter containing *Lactobacillus helveticus* and *Saccharomyces cereviceae*. A further study indicated that a relatively small quantity of these peptides could decrease the systolic blood pressure of spontaneous hypertensive male rats (SHR) but did not change the

systolic blood pressure of normotensive rats, indicating that these peptides worked only on hypertensive state (Nakamura et al., 1995b).

The same peptides, VPP and IPP were also produced in a similar hydrolysis of skimmed milk proteins using free-cell extract of *Lactobacillus helveticus* JCM1004 that contained proteinase, aminopeptidase and x-prolyl-dipeptidylaminopeptidase (Pan et al., 2005). The hydrolysis process reached its peak with respect to ACE inhibitory activity of the resulting peptides at pH 6.5-7.0 and 6-10 hours of hydrolysis. These two peptides have been claimed to have higher ACE inhibitory activities than other reported bioactive peptides derived from milk products (Mullally et al., 1997; Abubakar et al., 1998).

Gobbetti et al. (2000) reported that peptides derived from milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* SS1 and *Lactococcus lactis* subsp. *cremoris* FT4 also showed inhibitory activity towards ACE. The fermented milk gave nine bioactive peptides against ACE with IC_{50} values ranging from 8.0 mg/l to 11.2 mg/l. The synthesised forms of these peptides showed ACE inhibitory activities but were unstable and were strengthened to resist further proteolysis.

Bioactive peptides with ACE inhibitory properties from traditional and probiotic sheep milk yogurt have also been reported property (Papadimitriou et al., 2007). The traditional yogurt was prepared using normal yogurt culture (*Lactobacillus delbrueckii* subsp. *bulgaricus* Y10.13 and *Streptococcus thermophilus* Y10.7), while the probiotic yogurt was prepared using the same normal culture and *Lactobacillus paracasei* subsp. *paracasei* DC412. Comparing the bioactivity of the two yogurts with respect to their ACE inhibitory properties, probiotic yogurt produces major peptides YPVEPFTE from β-casein fragment 114-121 that was well known for its ACE inhibitory property (Papadimitriou et al., 2007).

Thermolysin hydrolysates of β-casein produced two potent ACE inhibitory peptides VYPFPGIPNSLPQNIPP and LVYPFPGIPNSLPQNIPP having IC_{50} values of 87 and 73 μM respectively. These two peptides are from fragments 58-76 and having similar –IPP at their C-terminal ends, the tripeptide that had shown potent ACE inhibitory properties in some other studies presented previously. In the same study, four potent ACE inhibitory peptides VSLPEW, GVSLPEW, YGGVSLPEW, and LKGYGGVSLPEW were isolated from α-lactalbumin, one of the major whey proteins, having IC_{50} values of 57, 30, 16, and 83 μM, respectively. These peptides are all from fragments 15-26 and having same –PEW residues at their C-terminal ends (Otte et al., 2007). Of notable interest is that the biggest peptide has lower activity, but as the hydrolysis continues smaller nonapeptide is released with higher activity but then the activity lowers as smaller hepta- and hexapeptides are released as the hydrolysis progresses further. Another major whey proteins, β-lactoglobulin, also produced ACE inhibitory peptide ALPMHIR through tryptic hydrolysis with IC_{50} value of 42.6 μmol/L (Ferreira et al., 2007), a value well comparable to many strong ACE inhibitory peptides derived from food proteins.

Fish and seafood protein hydrolysates and fermented fish and seafood protein products have also been studied for their production of ACE inhibitory bioactive peptides. Yokoyama et al. (1992) studied the effect of hydrolysing dried bonito (*Sarda spp*) (Katsuobusi) with pepsin, trypsin, chymotrypsin and thermolysin, and found that thermolysin hydrolysate showed the most potent inhibitory activity with an IC_{50} value of 29 μg/ml. There were eight bioactive peptides isolated, four of which have been identified with the fragment of the primary structure of actin (Table 1.4). By comparing the activity of these peptides to peptides recovered from boiling water extract of dried bonito and its hydrolysate, the peptides from dried bonito

hydrolysate were more potent. This indicates that these peptides were derived from insoluble fraction of the dried bonito which consisted of myofibrillar, stroma and denatured sarcoplasmic proteins. This suggests that washing fish mince prior to hydrolysis will wash out the soluble proteins and increase the possibility of recovering bioactive peptides from the insoluble protein fraction.

Sardine (*Sardinella spp*.) muscle protein has been linked with the production of potent ACE inhibitory peptides. Matsui et al. (1993) found that sardine muscle proteins hydrolysed by *Bacillus lichenifermis* alkaline protease produced crude bioactive hydrolysate with IC_{50} values ranging from 0.24 mg/ml to 0.26 mg/ml. An even more potent ACE inhibitor hydrolysate was produced from defatted sardine muscle protein hydrolysed with the same alkaline protease with an IC_{50} value of 0.18 mg/ml. Recently ACE inhibitory peptides have been grass carp (*Ctenopharyngodon idella*) scale neutral protease AS1396 hydrolysate and its fractions, although the sequence of the active peptides were not elucidated (Zhang et al., 2009). Tuna cooking juice also contains stable ACE inhibitory peptides and their activities were not affected by temperature, pressure, and pH (Hwang, 2010).

Table 1.4. Anti-hypertensive peptides derived from fish and seafood proteins.

Peptides	Protein Source	IC_{50}*	Preparation	Reference
IKPLNY	Dried Bonito (Myosin)	43	Thermolysin	Yokoyama et al., 1992
IVGRPRHQG	Dried Bonito – Actin	52	Thermolysin	Yokoyama et al., 1992
IWHHT	Dried Bonito – Actin	5.1	Thermolysin	Yokoyama et al., 1992
ALPHA	Dried Bonito – Actin	10	Thermolysin	Yokoyama et al., 1992
EQP	Dried Bonito – Actin	12	Thermolysin	Yokoyama et al., 1992
LKPNM	Dried Bonito	17	Thermolysin	Yokoyama et al., 1992
IY	Dried Bonito	3.7	Thermolysin	Yokoyama et al., 1992
DYGLYP	Dried Bonito (Fibronectin)	62	Thermolysin	Yokoyama et al., 1992
DMIPAQK	Dried Bonito – Creatine kinase	45	Boiling water extract	Yokoyama et al., 1992
IKP	Dried Bonito (Myosin)	1.7	Thermolysin	Yokoyama et al., 1992
LYP	Dried Bonito (Fibronectin)	6.6	Thermolysin	Yokoyama et al., 1992
IW	Dried Bonito – Actin	2.0	Chymotrypsin	Yokoyama et al., 1992
LKP		1.6	Synthetic	Fujita and Yoshikawa, 1999
LKPNM	Dried Bonito	2.4	Thermolysin	Fujita and Yoshikawa, 1999
LKP	Dried Bonito	0.32	ACE	Fujita and Yoshikawa, 1999
HERDPTHIKWGD	Bonito	8.0	Artificial gastric juice	Hasan et al., 2006
PTHIKWGD	Bonito	8.0	Artificial gastric juice	Hasan et al., 2006
PNRIKYGD	Bonito	4.0	Artificial gastric juice	Hasan et al., 2006
FHERDPTHIKWGD	Bonito	2.8	Artificial gastric juice	Hasan et al., 2006
TKTGRSAHVLSRYRPRA	Bonito	2.8	Artificial gastric juice	Hasan et al., 2006
FGASTRGA	Alaska Pollack	14.7	Pepsin	He, 2006

Adapted from: Meisel et al., 2006 and other sources as written in the reference column.

* Unless otherwise indicated, the concentration for IC_{50} values is μM.

[a] In μg/mL

[b] In mg/mL

[c] Synthetic products of the corresponding peptides derived from wakame seaweed.

Table 1.4. Anti-hypertensive peptides derived from fish and seafood proteins (Cont.).

Peptides	Protein Source	IC50*	Preparation	Reference
MIFPGAGGPEL	Yellowfin Sole	28.7[a]	α- Chymotrypsin	Jung et al., 2006
GY	Sea Bream Scale	265	Enzyme L	Fahmi et al., 2004
VY	Sea Bream Scale	16	Enzyme L	Fahmi et al., 2004
GF	Sea Bream Scale	708	Enzyme L	Fahmi et al., 2004
VIY	Sea Bream Scale	7.5	Enzyme L	Fahmi et al., 2004
VRK	Hard Clam	700	Protamex	Tsai et al., 2008
YN	Hard Clam	50	Protamex	Tsai et al., 2008
Unknown	Oyster (*Crassostrea gigas*)	0.087[b]	Fermentation	
Unknown	Chum Salmon Cartilage and skin	62.6[b]	Boiling Water Extract	Nagai et al., 2006
Unknown	Chum Salmon Cartilage	8.87[b]	Boiling water extract	Nagai et al., 2006
GPL	Alaska Pollack Skin	2.6	Alcalase, Pronase E and Collagenase	Byun and Kim, 2001
GPM	Alaska Pollack Skin	17.13	Alcalase, Pronase E and Collagenase	Byun and Kim, 2001
VKP	Freshwater Clam	3.7	Protamex	Tsai et al., 2006
VKK	Freshwater Clam	1045	Protames	Tsai et al., 2006
ENPGA	Sea Cucumber	0.0142[b]	Bromelain and Alcalase	Zhao et al., 2007
CF	Shark Meat	1.96	Protease SM98011	Wu et al., 2008
EY	Shark Meat	2.68	Protease SM98011	Wu et al., 2008
MF	Shark Meat	0.92	Protease SM98011	Wu et al., 2008

Adapted from: Meisel et al., 2006 and other sources as written in the reference column.

* Unless otherwise indicated, the concentration for IC_{50} values is μM.

[a] In μg/mL

[b] In mg/mL

[c] Synthetic products of the corresponding peptides derived from wakame seaweed.

Table 1.4. Anti-hypertensive peptides derived from fish and seafood proteins (Cont.)

Peptides	Protein Source	IC_{50}*	Preparation	Reference
FE	Shark Meat	1.45	Protease SM98011	Wu et al., 2008
FCVLRP	Shrimp *Acetes chinensis*	12.3	*Bacillus* sp Protease	He, 2006
IFVPAF	Shrimp *Acetes chinensis*	3.4	*Bacillus* sp Protease	He, 2006
KPPETV	Shrimp *Acetes chinensis*	24.1	*Bacillus* sp Protease	He, 2006
YLLF	Shrimp *Acetes chinensis*	172	*Bacillus* sp Protease	He, 2006
AFL	Shrimp *Acetes chinensis*	65.2	*Bacillus* sp Protease	He, 2006
VY	Wakame seaweed	35.2	Synthetic[c]	Sato, 2002
IY	Wakame seaweed	6.1	Synthetic[c]	Sato, 2002
AW	Wakame seaweed	18.8	Synthetic[c]	Sato, 2002
FY	Wakame seaweed	42.3	Synthetic[c]	Sato, 2002
VW	Wakame seaweed	3.3	Synthetic[c]	Sato, 2002
IW	Wakame seaweed	1.5	Synthetic[c]	Sato, 2002
LW	Wakame seaweed	23.6	Synthetic[c]	Sato, 2002

Adapted from: Meisel et al., 2006 and other sources as written in the reference column.

* Unless otherwise indicated, the concentration for IC_{50} values is μM.

[a] In μg/mL

[b] In mg/mL

[c] Synthetic products of the corresponding peptides derived from wakame seaweed.

In another work with fermented oyster sauce, Je et al. (2005) reported the finding of an ACE inhibitor peptide with IC_{50} value of 0.147 mM. Though this study did not go as far as to identify the bioactive peptides, assay on spontaneous hypertensive rats (SHRs) showed that the purified fraction was able to reduce the systolic blood pressure (SBP) three hours after oral administration and the activity was maintained for six hours. In a different work with peptic hydrolysate of oyster (*Crassostrea talienwhanensis*, Crosse) proteins, a nonapeptide VVYPWTQRF was isolated and characterised showing IC_{50} value of 66 µmol/L and was stable against temperature, pH, and gastrointestinal enzymes (Wang et al., 2008), indicating potential use as nutraceutical. Fermented blue mussel (*Mytilus edulis*) sauce also contained ACE inhibitory peptides EVMAGNLYPG with an IC_{50} value of 19.34 µg/ml (Je et al., 2005b), while Pramex hydrolysate of freshwater clam (*Corbicula fluminea*, Muller) produced two strong bioactive peptides VKP and VKK (IC_{50} = 3.7 and 1045 µM, respectively) (Tsai et al., 2006) and the same enzyme hydrolysate of hard clam (*Meretrix lusoria*) produce a dipeptide YN with an IC_{50} value of 51 µM (Tsai et al., 2008).

He et al. (2006) isolated three novel ACE inhibitory peptides from hydrolysates of shrimp *Acetes chinensis*, an underutilised shrimp of the Bo Hai Gulf, China. These peptides were identified as PCVLRP, IFVPAF and KPPETV. In addition to these peptides, two other peptides, YLLF and AFL were also identified. These peptides come from the ultrafiltered fraction with MWCOs of 3 kDa UF filter. This supports the idea that most on the ACE inhibitory peptides should have molecular weight of less than 3 kDa. A consecutive hydrolysis, first with 1% bromelain followed by alcalase (50:1 w/w substrate to enzyme ratio), of sea cucumber (*Acaudina molpadioidea*) body wall proteins produced an novel peptide of ACE inhibitory

property having a sequence of MEGAQEAQGD with IC_{50} value of 15.9 µM and further hydrolysis of this peptide with gastrointestinal protease decrease the IC_{50} value by 3.5 times (Zhao et al., 2009) indicating the release of an even more potent ACE inhibitory peptide.

ACE inhibitory peptides have also been found in other food proteins, some of which are not commonly consumed by people in general. These bioactive peptides include those from insect food such cotton leaf worm, bullfrog proteins, and many other proteins of animal origins. Vercruysse et al. (2008) reported the finding of a tripeptide AVF from a subsequent hydrolysis of cotton leaf worm (*Spodoptera littoralis*) using pepsin first then followed by trypsin and finally chymotrypsin, a process mimicking the gastrointestinal digestion of food proteins. The peptide has an IC50 value of 2123 µM. Bullfrog (*Rana catesbeiana*, Shaw) muscle proteins, a common food for some Asian people with an ever increasing popularity, hydrolysed with Alcalase produced a 1.3 kDa GAAELPCSAWW having an IC50 value of 0.95 µM, a non-competitive inhibitor that has shown *in vivo* activity in spontaneously hypertensive rats (SHRs) (Qian et al., 2007). Porcine muscle proteins (myosin B and actin) derived two ACE inhibitory peptides following a peptic hydrolysis namely KRVIQW (a novel ACE inhibitory peptide) and VKAGF (IC_{50} values 6.1 and 20.3 µM, respectively) (Mugurama et al., 2009).

Results from various studies have shown that some of ACE inhibitory peptides that showed strong activities when tested *in vitro* failed to show the same effect when tested *in vivo* in spontaneous hypertensive rats (SHRs) (Fujita and Yoshikawa, 1999). There are three possible explanations for this outcome:

1. The peptides were digested further by pepsin in the acidic environment of the stomach and lost their potency.

2. The peptides survived the peptic degradation in the stomach, but were digested by intestinal enzymes such as trypsin and chymotrypsin to release amino acids or smaller inactive peptides.
3. The peptides survived the acidic and intestinal enzymic degradations, but were further cleaved by the ACE itself to release smaller inactive peptides.

As most of the ACE inhibitory peptides are small peptides, therefore it is quite likely that these peptides may survive the acidic degradation in the stomach. Foltz et al. (2007) found that ACE inhibitory peptides derived from hydrolysis of food protein could be absorbed intact into human circulatory system. This finding also suggested that these peptides survived further degradation by intestinal enzymes. However, Yokoyama at al. (1992) reported the release of dipeptide IY by chymotrypsin digestion, *in vitro*, of IYHHT, a product thermolysin digest of dried bonito. While the product of this gastric enzyme hydrolysis showed stronger ACE inhibitory property than its parent peptide, the opposite might also be true for other peptides.

ACE can also cleave active ACE inhibitory peptides to produce either more active or less active smaller peptides. Fujita and Yoshikawa (1999) found that a tripeptide LKP was released after preincubation of LKPNM, an ACE inhibitory peptide produced from thermolysin digest of dried bonito, with ACE. This tripeptide is eight times stronger than its parent peptide. Based on the fate of the ACE inhibitory peptides after preincubation with ACE, they suggested the classification of ACE inhibitory peptides into three groups.

1. Inhibitor type. Preincubation with ACE does not affect the IC_{50} values of the peptides. These peptides still possess anti-hypertensive activity after oral administration.

2. Substrate type. Preincubation with ACE increases the IC_{50} values as ACE degrades these peptides.
3. Pro-drug type. Preincubation with ACE converts these peptides into smaller more active peptides as indicated by decreases in IC_{50} values. These peptides are expected to exert a long-lasting anti-hypertensive activity after oral administration.

Determination of enzyme activity is usually based on the rate of the appearance of products or the disappearance of the reactant or substrate. In the evaluation of ACE activity and inhibition, the use of Angiotensin I or [(^{125}I)Tyr^{8}]-bradykinin as a substrate was once employed. This method is complicated and suffered seriously from the interference by other peptidases that degrade both substrate and products. In recent decades, tripeptide substrate such as Z-FHL and Bz-GHL (Hip-His-Leu) are more commonly used. Z-FHL has about the same enzyme-binding affinity as Angiotensin I, as it is easily cleaved by ACE to form His-Leu which can then be quantified by fluorometric method (Ondetti and Cushman, 1982). In a rather similar way Bz-Gly-His-Leu (Hip-His-Leu or HHL) is used. It has a rather high enzyme-binding affinity than Z-Phe-His-Leu but the hippuric acid (Bz-Gly) release can be determined spectrophotometrically (Cushman and Cheung, 1971). A modification to this method was carried out successfully using 2,4,6-Trinitrobenzenesulfonic acid (TNBS) solution as colouring agent (Matsui et al., 1993).

In brief, procedure for ACE inhibitory peptide analysis is as follows. Testing peptide or inhibitor, ACE and HHL are mixed together and incubated at 37^{o}C for 30 to 60 min. The reaction is quenched with an addition of hydrochloric acid (HCl) followed by an addition of ethyl acetate to extract the hippuric acid liberated. The mixture is then centrifuged and the upper ethyl acetate layer is separated. Demineralised water

is then added to the ethyl acetate extract and the ethyl acetate is evaporated. The absorbance of the remaining aqueous fraction containing hippuric acid is measured at 228 nm (Cushman and Cheung, 1971). At present, there exists some modification to the above method, in which HPLC is used to measure the content of hippuric acid liberated without the need to extract it first with ethyl acetate (Wanasundara et al., 2002; Tsai et al., 2006; Yu et al., 2006). Another modification to the above method was also introduced with the use of capillary electrophoresis (CE) to determine the amount of hippuric acid liberated (He et al., 2007; Wu et al., 2008) and the use of 2,4,6-Trinitrobenzenesulfonic acid (TNBS) to react with His-Leu and the colour shifting was measured spectrophotometrically at 416 nm (Matsui et al., 1993).

Studies on the stability and classification of ACE inhibitory peptide were also based on the same principle as developed by (Cushman and Cheung, 1971) with a slight modification to the order of ACE addition. Here, the testing peptide or inhibitor was mixed with ACE and preincubated at 37^{o}C for 3 hours, followed by the addition of HHL and further incubation at 37^{o}C for 1 hour (Fujita and Yoshikawa, 1999). The hippuric acid or His-Leu produced are then determined.

1.3.9. Anti-microbial proteins and peptides

Since the finding of thionins, the first anti-microbial protein family, in early 1970s, more than 700 anti-microbial proteins and peptides have been discovered (Zasloff, 2002). The distribution of these broad-spectrum protein and peptide anti-microbials are widespread among multicellular organisms and are potent against bacteria, viruses, fungi and protozoa (Wilcox, 2004; Chan and Li-Chan, 2006). These findings bring about an understanding of the innate immune response to injury and infection (Janeway, 1998). The present increase in antibiotic resistant bacteria and the lack of

discovery of new antibiotic family means there is a need to look into these ancient weapons (Zasloff, 2002).

1.3.9.1. Anti-microbial Resistance

The discovery of penicillin in 1929 followed by the industrial-scale production in 1943 had allowed successful treatment of many infectious diseases as well as ability to perform major medical procedures including surgery and chemotherapy (Hancock, 2001). The two decades following the mass production of penicillin was known as the glory years of antibiotic discovery and many more new antibiotic classes were discovered and introduced one after another. These findings were, by and large, the product of screening of anti-microbial active compounds from natural products (Hancock and Knowles, 1998). Over the years, however, many key bacterial strains had evolved ways to adapt or become resistant to antibiotics (Wilcox, 2004).

Antibiotic resistance is defined as the ability of bacteria to acquire the capability to grow in the presence of antibiotics (Hancock and Knowles, 1998). Antibiotics are among very few drugs that cure, rather than just reduce the symptoms of diseases and many of the major medical procedures such as intensive care, advanced surgery, chemotherapy, and organ transplantation have been developed under the protective umbrella of antibiotics (Baquero and Blazquez, 1997). Therefore, the emergence of antibiotic resistance could have enormous effect on human health and the economics (Cassel, 1997).

There are several reasons for the emergence of antibiotic resistance. One of the reasons was the substantial increase in the antibiotic usage both in human and in animal feeds to prevent infection and promote growth (Cassel, 1997; Wilcox, 2004). The mechanisms by which bacteria evade antibiotics includes reduced drug uptake,

active pumping of drug out of the cell, enzymatically altering the antibiotics, modification of targets, drug sequestering by protein binding, overproduction of target and metabolic bypass of the targeted pathway (Nakaido, 1998). One approach to this problem is to consider a new class of antibiotic, 'nature's antibiotic' i.e. the cationic peptides (Hancock, 2001).

1.3.9.2. Nature's Antibiotics

Approximately two decades ago, it was discovered that the lymph of insects, the granules of human neutrophils, and the skin of frogs contained peptides that could kill bacteria in culture. Since then, more than 600 cationic peptides have been discovered. These cationic anti-microbial peptides are amphipathic, having both hydrophobic domain that interact with lipids and a positively charged hydrophilic domain that interact with water or negatively charged residues (Hancock, 2001; Wilcox, 2004). On the basis of their synthesis sites, these cationic peptides can be classified into two classes, non-ribosomally synthesised peptides and ribosomally (natural) synthesised peptides (Hancock and Chapple, 1999). The non-ribosomally synthesised peptides can be described as peptides elaborated in bacteria, fungi, and streptomycetes that contain two or more moieties derived from amino acids (Perlman and Bodanszey, 1971). Examples of nonribosomally synthesised peptides are gramicidins, polymixins, bacitracins and glycopeptides.

The best anti-microbial peptides kill susceptible bacteria *in vitro* at a concentration ranging from 0.25 to 4 μg/mL. Although these concentrations are higher than some more potent antibiotics that exist today, there are definite advantages of these anti-microbial peptides. These advantages includes an ability to kill target cells rapidly, having unusually broad spectrum activity, having activity against some of the more

serious antibiotic-resistant pathogenic bacteria and the relative difficulty in selecting resistant mutants *in vitro*.

Non-ribosomally synthesised peptides are produced mainly from bacteria and are often drastically modified. These anti-microbial peptides usually contain nonprotein constituents in the polypeptide chain which are either derived from modification of the chain or originated from non-ribosomal system. In such a system, the assembly of the chain is not directed by an RNA template, although aminoacyl-tRNA may participate. Rather, the assembly took place through enzymatic steps (Kleinkauf and Von Dohren, 1987).

The ribosomally (natural) synthesised peptides are recognised as part of innate immunity found throughout evolutionary tree but show little sequence homology which suggest that each peptide has evolved (probably convergently) to optimally act against local micro-organisms in the environment it is produced (Hancock and Chapple, 1999). Examples of ribosomally (natural) synthesised peptides are bombinin from frog skin, and melitin from bee venom. The ribosomally synthesised peptides are produced by all species (including bacteria) as a major component of the natural host defense molecule of the species (Kleinkauf and Von Dohren, 1987) and represent a new opportunity for the medical chemist for discovery of new antibiotics (Hancock and Chapple, 1999) in the face of the declining efficacy of conventional antibiotics as evidence of the rise of antibiotic-resistant pathogens (Hancock and Lehrer, 1998).

The ribosomally synthesised peptides are commonly isolated from various animals (including human), plants and bacteria. Since the isolation of bombinin from the skin of frog, more that a dozen of anti-microbial peptides have been discovered from the amphibian species. These peptides come from the granular glands of the skin, the

cells of gastric mucosa and intestinal tract (Hancock and Chapple, 1999). The frog *Phylomedusa sauvagii* has a family of five anti-microbial peptides, the dermaseptins, that show good anti-fungal activity (Mor and Nicolas, 1994). According to Kreil (1994), anti-microbial peptides from the amphibians tended to have little sequence homology. It was suggested that no two amphibians had homologous peptides, even among the same species there was a high degree of variation.

Anti-microbial peptides can be classified further into two major groups: anionic peptides and cationic peptides (Brogden et al., 2003). The anionic peptides are relatively new comers among the anti-microbial peptides. The discovery of three small anionic anti-microbial peptides from ovine pulmonary surfactant and was reported to posses anti-microbial activity against *Mannheimia haemolytica*, *Escherichia coli*, and *Klebsiella pneumoniae*. These peptides were hepatapeptides having amino acid sequences of GDDDDDD, DDDDDDD, and GADDDDD and their activity was maximalised in the presence of zinc (Brogden, 1992; Brogden et al., 1996). Another class of anionic anti-microbial peptides is the phosphorylated peptides such as peptide B and enkelytin derived from the processing of neuropeptide pre-cursors such as pro-enkepalin-A. These peptides have been found in cattle and human exudates and are mainly active against Gram-positive and concentrations as low as for the cationic peptides (Metz-Boutigue and Lugardon, 2000).

In addition, a peptide containing 47 amino acid residues called dermcidin has been isolated from human sweat showing activity against Gram-positive bacteria (Schittek et al., 2001). The skin of toad *Bombina maxima* also produces anionic peptide known as maximin H5 having amino acid sequence of ILGPVLGLVSDTLDDVLGIL-NH_2. This anionic aspartic acid (D) rich peptide showed activity only to Gram-positive

Staphylococcus aureus (Lai et al., 2002). More recently, a novel anionic peptide maximin S4 was identified and synthesised from the same toad skin consisted of 18 amino acid residues RSNKGFNFMVDMIQALSK-NH2. The peptide showed activity against two clinical strains of *Mycoplasma hominis* and two clinical strains of *Ureaplasma urealyticum* (Wang et al., 2005).

The cationic peptides, however, are found in a much larger group and are widely distributed among animals and plants (Vizioli and Salzet, 2002). This group can be structurally classified into three classes: linear peptides that form α-helical structure, cysteine rich open ended peptides having single or several disulfide bridges, and peptides rich in certain amino acids such as proline, glycine, histidine (Zasloff, 2002), arginine, phenylalanine, and tryptophane (Brogden et al., 2003).

Anti-microbial peptides of mamalian origin can come from within the granules of neutrophils, mucosal or skin secretions from ephitelial cells, or as degradation products of proteins (Boman, 1995). Most mamalian anti-microbial peptides share common chracteristics such as small size, cationic in nature and are amphipathic. These peptides are classified into two major families, the defensins and the cathelicidins (Chan and Li-Chan, 2006). Three types of defensins have been found among vertebrates viz., α-, β-, and θ-defensins (Lehrer and Ganz, 2002). The α-defensins are small peptides containing 29 to 35 amino acid residues with six-cysteine motif pairing to form three intramolecular disulfide bonds. The β-defensins are bigger peptides containing up to 45 amino acid residues with different pairings and relatively more lysine than arginine residues. The cyclic θ-defensins has only 18 amino acid residues, including six cysteines that forms three disulfide bond. This cyclic peptide is three times more potent than its open chained analogue and is able to protect human from HIV-1 infection (Chan and Li-Chan, 2006).

Anti-microbial peptides have also been isolated from marine origin. Protamins are a family of sperm nuclear basic proteins from fish milt. This family of anti-microbial proteins have been isolated from milt, contains about 30 amino acid residues that include about 66% of arginine which usually exist in clusters of four or five (Chan and Li-Chan, 2006). This family includes clupeinnes from Pacific herring (*Clupea pallasii*), salmine from chum salmon (*Onchorrhynchus keta*), and iridine from rainbow trout (*Salmo irideus*) (Ando et al., 1957). Protamines show anti-microbial activities against Gram-positive and Gram-negative bacteria, yeast and moulds probably due to many positively charged residues that interact with the negatively charged cell surfaces that cause permeabilisation of the cell envelopes (Chan and Li-Chan, 2006).

Many new anti-microbial peptides have been discovered from fish. These include pardaxins isolated from Pacific Peacock sole (*Pardachirus pavonius*) and the Red Sea Moses sole (*P. marmoratus*) (Thompson et al., 1986; Shai et al., 1988), pleurocidin isolated from the skin secretion of winter flounder (*Pleuronectes americanus*) (Cole et al., 1997). These two groups of anti-microbial peptides showed wide-range anti-microbial activities and contain 25 to 33 amino acid residues (Chan and Li-Chan, 2006). Misgurin is a highly polar peptide isolated from mudfish, rich in both basic and acidic residues and, while it is not quite amphipatic, quite potent in killing bacteria and yeast (Park et al., 1997). Parasin, a polar peptide containing 19 amino acid residues isolated from ephitelial mucosa of catfish (*Parasilurus asotus*), has a rod like α-helical structure, highly cationic and seems to be able to penetrate and inactivate bacteria without causing membrane permeabilisation (Park et al., 1998; Basañez et al., 2002). Two other peptide, moronnecidins, have also been isolated from skin and gill of white bass (*Morone chrysops*) and striped bass (*M.*

saxitilis). These peptides area isoforms with only one amino acid difference, with amidated C-terminal peptide and having 23 amino acid residues. A few other anti-microbial peptides have also been isolated from crustacean such as callinectin, tachyplesin, big defensin tachicitin and tachystatin and from molluscs such as mytilins and myticins (Chan and Li-Chan, 2006).

Most if not all of those anti-microbial peptides mentioned in the previous paragraphs are from naturally occurring peptides. There are, however, some anti-microbial peptides that have been derived from digestion of food proteins such as milk and egg proteins (Chan and Li-Chan, 2006). Zucht et al. (1995) reported that Casosidin-I, a 39-amino-acid fragment derived from acid treatment of α_{s2}-casein, could inhibit the growth of *Escherichia coli* and *Staphylococcus carnosus*. It was also found that pepsin digestion of α_{s2}-casein released cationic fragments with potent anti-microbial inhibitory activity. Many other anti-microbial peptides have been derived from milk protein, casein.

Pellegrini et al. (1997) reported the finding of a pentadecapeptide derived from clostripain digestion of egg lysozyme with anti-bacterial activity against Gram-positive and Gram-negative bacteria. Lysozyme is an enzyme that can cause enzymatic action against microbial cell material of Gram-positive bacteria. Inactive lysozyme, however, can still cause damage to microbial cells which indicates that this is due to structural factors rather than enzymatic lysis. Ovotransferrin, or conalbumin, of hen egg white has shown anti-microbial activities against *Pseudomonas sp.*, *Escherichia coli*, *Streptoccocus mutans* and *Candida albicans*. A technique called trypsin-nicking has then been employed to analyse the active domain of ovotransferrin and a 92-residues cationic peptide (OTAP-92) has been indicated as the active domain (Chan and Li-Chan, 2006).

1.4. Structure and activity characteristics of bioactive peptides.

The activity of bioactive peptides can be due to the combination of various factors such as sequence of the amino acid residues in the peptides, conformation of the peptides, hydrophobicity, and overall net charges of the peptides. For small peptides, the activity is likely to be related to their sequence, hydrophobicity, and net charges, while for large peptides structures may play important roles in addition to the other factors. Proteins and peptides are defined by their amino acid constituents and their sequences. The amino acid sequence of proteins and peptides is known as the *primary structure* of the proteins or peptides. The *secondary structure* of proteins/peptides refers to the folding of local regions into minimal-energy conformations that are stabilised by non-covalent bonds such as hydrogen bonds between non-adjacent amino acid residues. There are two major motifs of secondary structure, the α-helix and the β-sheet. A third motif is the turn that indicates a connection commonly found between different stretches of α-helix or β-sheet (Axley, 1998).

The α-helix structures are characterised by the right-handed helix formed by the peptide chain with about 3.6 amino acids per turn of the helix. In this, an amino hydrogen from an amino acid residue forms a hydrogen bond with the carbonyl oxygen of another amino acid residue in an adjacent turn of the helix. This hydrogen bond formed by an amino acid to residues three amino acids away in both direction and the cross-linking results in a stable α-helical structure with the side groups (R) facing outside the main peptide backbone. Some proteins have hydrophobic groups along one side of the helix and charged groups on the other side resulting in an amphipathic property. The β-sheet structures are formed when certain linear stretches of amino acids align with other linear stretches of the polypeptide. The hydrogen

bonds can be formed between an amino acid residue in one stretch with another amino acid residue in a different stretch of the peptide chain. Hence the R groups will be arranged above or below the plane of the sheet (Axley, 1998).

The secondary structures of proteins fold into a three dimensional conformation, the *tertiary structure*, that is essential for the function of proteins. This structure is a minimum energy structure that is held together by multiple hydrogen bonds as well as ionic and hydrophobic interactions. One or two covalent bonds such as disulfide bonds may form oxidative cross-linking between two cysteine sulfhydryl groups (Axley, 1998; Carter, 1998). The R groups from various distant parts of a polypeptide may be juxtaposed in certain orientation providing chemical sites necessary for the functional activity of a protein. Proteins can also contain more than one polypeptide chain, or subunits, to form a *quaternary structure*. The subunits are held together by non-covalent bonds as in the tertiary structure (Axley, 1998).

The secondary polypeptide structure of proteins, as mentioned earlier, has two major motifs (the α-helix and β-sheet) folded into a thermodynamically stable arrangement for the successive residues along the chain to positions at minimal energy. This folding has two important consequences: it buries a considerable number of backbone carbonyl and amide groups, joining them in hydrogen bonding configuration, and expose a highly textured surface composed almost entirely of the R groups. The folding of polypeptide chain in proteins assumed fundamental property of the proteins for being compact and in globular shape, instead of fibrous. However, protein structure has the ability to change their direction abruptly, of which is the change of directions within the span of several residues. These are known as 'hairpin', 'reverse', or 'β-' turns and are specific to secondary structure that effect

chain reversal without changing the protein conformation (Carter, 1998), and may play important role in explaining the activity of proteins or peptides.

1.4.1. Activity of ACE inhibitory peptides

ACE is a key enzyme in rennin angiotensin system that converts angiotensin I into angiotensin II. It is also a multi functional enzyme that plays an important role in regulating physiological action of local levels of some endogenous bioactive peptides such as encephalin, substance P and bradykinin that are inhibitors and competitive substrates for ACE (Ondetti and Cushman, 1982). Angiotensin I is an inactive hormone, but angiotensin II is a potent molecule that directly constrict vascular smooth muscle resulting in increasing blood pressure (Gerdes et al., 2001). ACE affects the blood pressure by inactivating the vasodilatory peptide bradykinin, in the kinnin-kallikrein system, and converts angiotensin I into the vasoconstrictory (hypertensive) agent angiotensin II (Erdos et al., 1999), by removing a carbonyl-terminal dipeptide (Fiordan, 2003). The role of ACE inhibitory agents are, therefore, to strengthen the vasodilatory (hypotensive) properties of bradykinin and decrease blood pressure, hence they are used widely in therapy of hypertension, heart failure, myocardial infarction and diabetic nephropathy (Meisel et al., 2006).

ACE had been shown to resemble zinc metallopeptidases such as neurolysin, but there was no detectable sequence similar with ACE (Natesh et al., 2003). It was identified that ACE has three different forms, i.e., somatic form of ACE (sACE) consisting of two homologous domains (N- and C-domains) and both contain an active site that catalyses the hydrolysis of angiotensin I (Lei et al., 1992), testicular form (gACE) that contains C-terminal active site as of somatic ACE (Meisel et al., 2006) and ACE homologue (ACEH) or also known as ACE2 which contains N-

terminal active site as of the somatic ACE (Turner and Hooper, 2002; Oudit et al., 2003). Both these two domains contain a high affinity inhibitor binding site. ACE is an unusual zinc-metallopeptidase that is activated by chloride and lacks a narrow *in vitro* substrate specificity (Lei et al., 1992) enabling it to cleave bradykinin as well. The active sites of sACE, ACE2, and to a lesser extent gACE are activated by high concentration of chloride. The effect is primarily an enhancement of substrate binding, but its physiological significance is not well understood (Fiordan, 2003). Figure 1.1 shows the schematic representation of human ACEs and its structure in a complex with an ACE inhibitor.

Human somatic ACE (sACE) is type-I membrane-bound protein comprising of a 28-residue C-terminal cytosolic domain, a 22-residue hydrophobic transmembrane domain, and a 1227-residue extracellular domain that is heavily glycosylated (30% by weight). This extracellular domain consists of two homologous domains, a 612-residue N domain linked by a 15 residue sequence into a 600-residue C-domain. Each of the extracellular domains contains a HEXXH sequence (Figure 1.1) in which two histidine residues serve as zinc-binding ligands, together with a glutamine residue located 23-24 residues toward the C-terminus, as well as a water molecule (Jaspard et al., 1993). The gACE is similar to C-domain of sACE with 28-residue cytosolic and 22-residue transmembrane domains, and 615-residue extracellular domains differ only in the first 36-residues (Natesh et al., 2003).

The relationship of structure and activity of bioactive ACE inhibitory peptides derived from food proteins remain unclear. However, there seems to be some similarity in the way these peptides correlate to ACE. According to Meisel et al. (2006), it appears that ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate and prefers substrate or competitive inhibitors containing

hydrophobic (either aromatic or branched side chains) amino acid residues at each of the three C-terminal positions. In addition, many naturally occurring peptide inhibitors have proline at the C-terminus. This seems to be in agreement with other short chain active peptides. Most of the bioactive di- and tripeptide inhibitors contain a Tyr, Phe, Trp or Pro residue at the C-terminal end, with Tyr appears to be more effective in increasing ACE inhibitory potential. For amino acids at the N-terminal end, Ile and Val are more dominant as highly effective inhibitors, while for the long-chain peptides it is expected that the peptide conformation, or the structure adopted in a specific environment of the binding site, should contribute to the bioactivity.

ACE inhibitory peptides also come with sequences that contain many other amino acid residues at their N- and C-terminal positions. ACE inhibitory peptides with lysine and arginine at the C-terminal position have positive charge at their respective ε-amino and guanidine groups that may contribute to their inhibitory potency (Ariyoshi, 1993), while peptides with glutamic acid at the C-terminal end the activity may be due to the chelating effect on the zinc ion which is bound to the active site of ACE (Wei et al., 1992). Most of this account is related to study of structure-activity relationship using di- and tripeptide ACE inhibitors. Hence, the primary structure of peptides is more related to the activity of the peptides, while for longer peptides more comprehensive analysis will have to be carried out to identify the contribution of other factors.

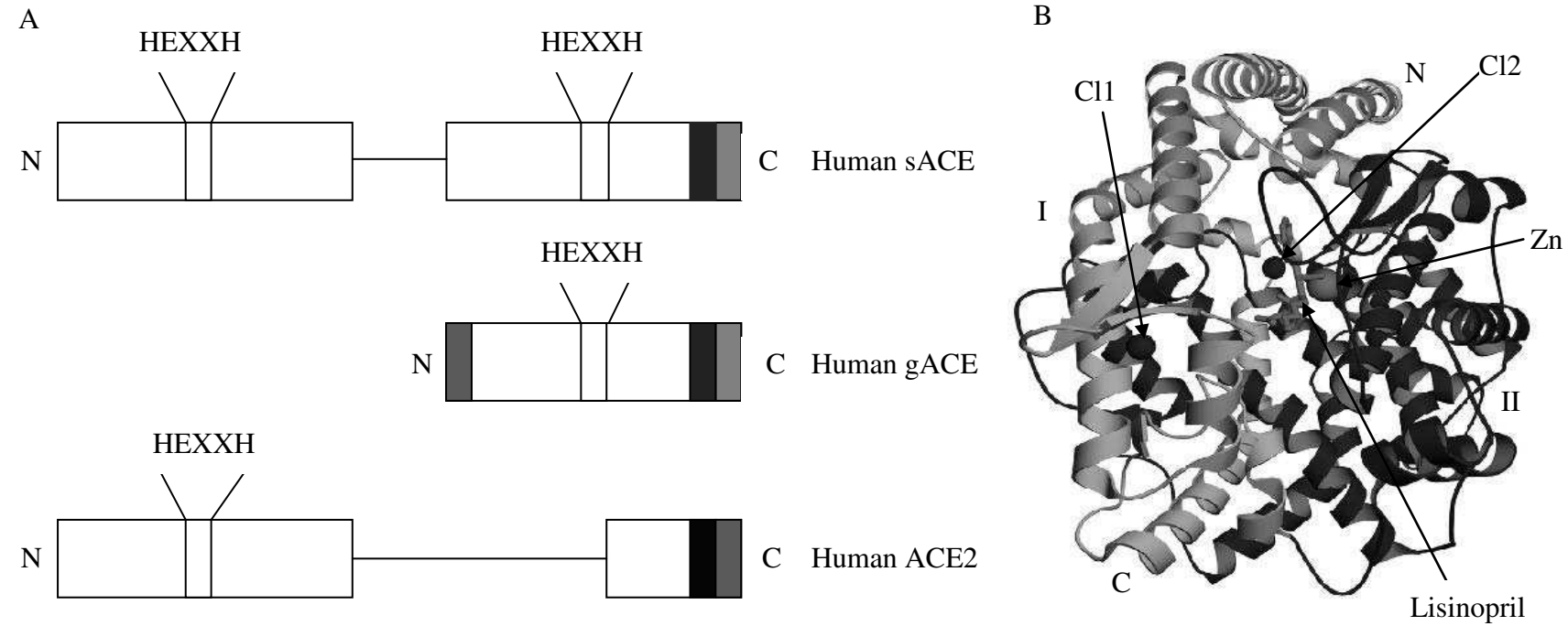


Figure 1.1. Schematic presentation of three members of human ACE: somatic ACE (sACE), testicular ACE (gACE), and ACE homologue (ACE2) (A). The active zinc-binding site is represented by HEXXH; the transmembrane domain is in black. Human g ACE is identical to sACE C-domain except for the first 36 residues of the N-domain. Human sACE and gACE have the same C-terminal transmembrane, while ACE2 has distinct transmembrane and cytosolic sequence. The schematic presentation of the structure of truncated, deglycosylated human gACE in a complex with inhibitor lisinopril (B). Human gACE has twosubdomains: I (light grey), and II (dark grey), that enclosed the active binding site. The zinc atom is shown associated with lisinopril (in stick presentaion), and two bound chloride ions (Cl1 and Cl2). N and C are the terminal ends of gACE.
Adapted from: Fiordan, 2003.

The role of inhibitory peptides derived from food proteins are expected to be similar to the role of anti-hypertensive drugs in that they provide competitive substrate to bind to the active sites of ACE, or non-competitive substrate that can bind to a site other than the active site of ACE. Lei et al. (1992) found that once reaction between substrate and ACE occurs, chloride would stabilise the inhibitor-ACE complex for both C- and N- domains of ACE by slowing their dissociation rate. At a high concentration of chloride, the C-domain was more affected than that of the N-domain resulting in a higher affinity of this inhibitor for the C-domain.

1.4.2. Activity of anti-microbial peptides

The activity of anti-microbial peptides has been linked to their structures, net charges, and their ability to fold into amphipathic or amphiphilic conformation (Powers and Hancock, 2003). According to Hancock and Lehrer (1998), peptides are classified into four major classes based on their structures. They are β-sheet, α-helical, loop, and extended peptides. In nature, however, the first two classes of peptides are the most common. The β-sheet class of peptides is characterised by the presence of anti-parallel β-sheet that is stabilised by disulfide bonds. The larger peptides within this family may also contain minor helical segments (Powers and Hancock, 2003).

Most of the structures of cationic peptides identified to date are of β-sheet class. An example of the β-sheet peptides that has been well studied is tachyplesin I isolated from Japanese horse crab *Tachypleus tridentatus* (Nakamura et al., 1988). This peptide is rather small with anti-parallel β-sheet conformation (residues 3-8 and 11-16) that is connected by a type I β-turn (residues 8-11) stabilised by two disulfide bonds (residues 3-16 and 7-12) and with an amidated C-terminus (Kawano et al.,

1990). Studies to determine the requirement of the disulfide bond for the anti-microbial activity indicated that although the disulfide bonds in tachyplesin are not absolutely required for anti-microbial activity, they are necessary to permit membrane translocation. Proton nuclear magnetic resonance (^{1}H NMR) studies indicate that association between tachyplesin with micelles (a membrane-like environment) results in a conformational change leading to the bending of the molecule around the central arginine residue along with an associated exposure of specific hydrophobic side chain (Powers and Hancock, 2003).

The α-helical class of cationic peptides is the second most abundant structurally determined anti-microbial peptides. This class of peptides are characterised by their α-helical conformation, and often contain a slight bend at the centre of the molecule (Powers and Hancock, 2003). The α-helical class of cationic peptides are very common in nature. The leukocytes of many mammals contains cathecidins, an example of a group of α-helical cationic peptides, polypeptides with a preserve N-terminal precursor (chatelin) domain comprised of around 100 amino acid residues and is followed by an anti-microbial peptide domain. Chatelin-associated α-helical peptides are found in the blood cells of many animals such as cattle, pigs, mice, rabbits, and sheep as well as in human (Hancock and Lehrer, 1998).

1.4.3. Determination of the structure and activity of peptides

Determination of the structure and activity of peptides can be carried out in various ways. These include kinetic determination of the mode of action, amino acid sequence with Edman degradation technique, molecular mass and amino acid sequence with mass spectrometry, and detailed structural studies of peptides with nuclear magnetic resonance (NMR) spectroscopy.

1.4.3.1. Study of the mode of action

The mode of actions of ACE inhibitory peptides is usually assessed kinetically to determine their binding preference toward ACE. This is carried out by reacting ACE with different concentrations of substrate hippuric-histidyl-leucine (HHL) and peptide inhibitors, the reaction mixture is incubated at 37^{o}C, and the hippuric acid liberated is quantified at different time intervals. The activity was transferred into velocity or speed of ACE (µmol/min of HA released) catalysis. The data collected were plotted into a Lineweaver-Burk plot. The modes of inhibition of ACE by peptide inhibitors were estimated by comparing their ability to release hippuric acid at a given time interval with the hippuric acid released from a similar reaction but without the presence of peptides. The intercept on the horizontal axis is the value of the inhibition constant (K_i) (Wu and Ding, 2002; Tsai et al., 2006; Atkins and Paula, 2006, 2009). The results from this determination will classify the inhibitors into three major inhibitions that give rise to distinctly different kinetic behaviours. They are competitive inhibition, uncompetitive inhibition, and non-competitive or mixed inhibition. The competitive inhibition occurs when the inhibitor binds only to the active site of an enzyme and inhibits the attachment of the substrate. The uncompetitive inhibition occurs when the inhibitor binds to a site of an enzyme remote from the active site, but only if the substrate is already present. In the non-competitive inhibition, the inhibitor binds to a site other than the active site, and its presence reduce the ability of the substrate to bind to the active site (Atkins and Paula, 2006).

1.4.3.2. Edman degradation

The primary structures of peptides are usually determined using Edman degradation technique employing protein sequencer to identify the peptides sequence chemically from the N-terminal end. In Edman sequencing, the amino acid residue at the N-terminal end is removed one at a time and determined by high performance liquid chromatography (HPLC). This is carried out using a fixed cycle of standard chemical reaction to facilitate the removal of the N-terminal amino acid in a stepwise manner (Edman and Begg, 1967). The chemical reactions take places during the stepwise degradation are shown in Figure 5.2. At first the target peptide is bound to a membrane support followed by a reaction between the amine group of the N-terminal amino acid and phenylisothiocyanate (C_6H_5-N=C=S) to form a phenylthiocarbamyl-peptide (PTC-peptide). This is then cleaved by an anhydrous acid to yield thiazolinone intermediate which is then hydrolysed further to form phenylthiocarbamyl derivate of the N-terminal amino acid, which then cycles to form the phenylthiohydantoin derivative (PTH-amino acid). The PTH-amino acid is extracted into organic solvents and identified by chromatography, while the resulting new N-terminal residue is continually subjected for the next cycle of reaction.

This is a very sensitive method that can afford measurement in picomolar concentration of peptide samples. In addition, recent technology enables automatic continuation of reaction cycles allowing convenient analysis (Gevaert and Vandekerkhove, 2000). There are, however, pitfalls in the application of Edman degradation techniques for amino acid sequences of peptides. The presence of glutamine at the N-terminal end, for instance, tends to cycle to form pyroglutamate residue that is not reactive to N-terminal sequencing. Another obstacle in doing the sequence analysis is that the cleaved N-terminal amino acid derivative is identified

based on the known retention times; therefore, modified or uncommon amino acid residues may be overlooked. Furthermore, incomplete degradation reaction may cause contamination hence conclusive identification may be at stake. Then, as the remaining peptides become smaller in sizes, they may be washed out and may miss the N-terminal reaction.

1.4.3.3. Mass spectrometry for peptide analysis

The problems associated with Edman degradation, as mentioned earlier, can be overcome with the use of mass spectrometry for peptide mass and sequence analysis. Mass spectrometry is a sensitive (zeptomolar range), accurate, rapid, and probably the most versatile method for the characterisation of molecules. In addition, mass spectrometry allows chemists or biochemists to study wide ranges of analysis from reaction dynamics and chemistry of ions (Dass, 2007) to characterise large biomolecules such as proteins.

1.4.3.4. Protein and peptide structural studies employing NMR spectroscopy

The nuclear magnetic resonance (NMR) spectroscopy is an important technique to determine secondary and tertiary structures of protein/peptides. This technique involves subjecting protein/peptide molecules to a steady magnetic field and the RF pulses. The resulting resonance of specific isotope presence in the molecules, usually ^{1}H (hydrogen atom isotope) or ^{13}Carbon (carbon atom isotope) are detected and quantified. Since different protons in the protein molecule will have different chemical and magnetic environment, specific structural properties of the molecule under investigation can be identified and the structure determined. These include protein backbone protons, side chain amide protons, aromatic side chain protons, α-

protons in regions of β-sheet structure, aliphatic side chain protons (Reid et al., 1997). In this section, the principles of NMR spectroscopy will be outlined with emphasis given to its application in peptide/protein structure determination.

1.4.3.4.1. One- and Two-Dimensional NMR Spectroscopy

The NMR spectroscopy discussed earlier is known as one dimensional NMR spectroscopy (1D NMR). This technique is useful to analyse small molecules. For large molecules such as peptides and proteins that posses numerous magnetically non-equivalent nuclei, extending the basic 1D NMR into multiple dimensions is of great significance. The basic idea behind two-dimension NMR (2D NMR) is that intensity is plotted against two frequency axes; each peak in a two-dimensional spectrum thus has an intensity and two frequency co-ordinates (Keeler, 2005). One of the most useful 2D-NMR experiments is the homonuclear correlation spectroscopy in which the cross peaks give chemical shift correlation of J-coupled spins. This technique is known as correlation spectroscopy (COSY).

Correlated Spectroscopy (COSY)

Correlated spectroscopy (COSY) produces NMR signals based on the homonuclear through bond interaction (J coupling). It allows the investigation of the connectivity of a molecule through determination of protons that are J-coupled to each other by either two or three bonds (Aue et al., 1976). A simple COSY (commonly carried out with 90^{o} pulses) experiment consists of a radio-frequency (RF) pulse (p_1) followed by an evolution time (t_1), followed by the second RF pulse (p_2), and finally measurement time (t_2). The first pulse forms magnetisation in the transverse plane that evolves over t_1 time interval. The second RF pulse rotates the evolved

magnetisation through 90^{o} angle. This leads to a situation in which the magnetisation is redistributed among the scalar coupled nuclei by a process known as coherence transfer (Evans, 1995). The precession frequency of the coupled spins is then recorded during t_2. Double Fourier transformation in both time domains results in a cross-peak between two spins (i and j) that occur at positions (δ_i, δ_j) and (δ_j, δ_i) in the spectrum indicating that spins i and j are directly coupled to one another (Clore and Gronenborn, 1989). The scalar coupling between the two protons responsible for the signal causes the cross-peak to appear as an anti-phase multiplet that can be of importance in further structure analysis.

COSY is a standard experiment for studies of scalar spin-spin coupling connectivities. The COSY spectrum can provide information to identify the chemical shifts of spins that are scalar couple to one another, hence enabling to trace out the J-coupling network in the molecule. A typical COSY spectrum contains two kinds of peaks, cross peaks and diagonal peaks. A peak in a COSY spectra appears at frequency $\omega_1 = \Omega_A$, $\omega_2 = \Omega_B$ shows that a spin at offset (chemical shift) Ω_A is coupled to another spin at offset (chemical shift) Ω_B (Wuthrich, 1986; Keeler, 2005). The diagonal peaks have the same frequency co-ordinate in ω_1 and ω_2, and are centred at the offset (chemical shift) of each spin. These spins do not convey any particular information about the connectivity of the spins, but serve to locate the shifts in the spectrum (Keeler, 2005). The cross peaks manifesting the connectivities with a particular diagonal peak lie on the cross section parallel to ω_1 and ω_2 axes through this diagonal peak (Wuthrich, 1986).

Total Correlation Spectroscopy (TOSCY)

TOCSY is also a homonuclear 2D-correlation NMR experiment, generally used for protons, that gives a spectrum in which coupling between two spins is indicated by the presence of a cross-peak multiplet. The TOCSY, in addition, will give correlations between all protons in a given coupled spin system. The key to producing a TOCSY spectrum is to have a period during the pulse sequence (mixing period) during which only scalar coupling is acting, while chemical shift is suppressed. During the mixing period, magnetization is exchanged among all coupled spins in a network (Keeler, 2005). TOCSY magnetisation transfer can be achieved by employing a variety of mixing schemes, but the most effective- and therefore now universally used methods involve composite pulse spin locking.

TOCSY is a very useful experiment especially for identifying spins that belong to an extended network of coupled spins. This is particularly true in complex overlapping spectra that are not always possible to unambiguously identify series of related cross peaks (Keeler, 2005). This experiment is applicable to peptide studies as the correlation peaks enable researchers to identify protons belonging to the individual amino acids (Friebolin, 2005). This feature is of great importance for assigning proton signals as in a protein spectrum, signal overlap tends to increase when moving from N_H and H_α protons to the side chain protons (Clore and Gronenborn, 1989).

Nuclear Overhauser Effect Spectroscopy (NOESY)

NOESY is a 2D-NMR method that aims to identify spins undergoing cross-relaxation and to measure the cross-relaxation rates. In NOESY, direct dipolar couplings provide the primary means of cross-relaxation, and so spins undergoing cross-relaxation are those which are close to one another in space. Thus, the cross

peaks in a homonuclear NOESY spectrum indicates which protons are close to which other protons in space (Wuthrich, 1986). The NOESY experiment can identify all the nuclear Overhauser effects (NOEs) present in a molecule during an experiment, hence enable identification of protons that are close together in space (Neuhaus and Williamson, 1989), which are essential for sequential assignment of resonances and form the basis for three-dimensional structure investigation of peptides and proteins.

The pulse sequence for a general NOESY experiment consists of three 90° pulses. The first pulse creates the magnetization into the xy-plane that precesses during the evolution time t_1. The second pulse transforms some of the magnetisation to the z axis and during the following mixing period, the non-equilibrium z component of magnetisation will exchange among spin in close vicinity through intra- or intermolecular dipolar relaxation (dipole-dipole mechanism) (Wuthrich, 1986; Neuhaus and Williamson, 1989). This exchange of magnetization is known as NOE (Nuclear Overhauser Effect). Thus, the basic idea is to produce an initial situation for the mixing period τ_m (the time during which cross relaxation occurs) where the longitudinal polarization of each spin is labelled by its resonance frequency. The longitudinal magnetization is allowed to relax during the mixing time τ_m. In the basic NOESY experiment, τ_m is kept constant throughout the 2D experiment. The third pulse creates transverse magnetization from the remaining longitudinal magnetization. Acquisition begins immediately following the third pulse, and the transverse magnetization is observed as a function of the time t_2. The NOESY spectrum therefore gives cross-peaks that are generated from the magnetisation transfer, hence are due to proton pairs that are close together in space (Evans, 1995).

Rotating-frame Overhauser Effect Spectroscopy (ROESY)

Rotating-frame Overhauser Effect Spectroscopy (ROESY) is an experiment in which homonuclear NOE effects are measured under spin-locked condition. ROESY experiment is similar to NOESY except that instead of generating cross-peaks by cross relaxation between the z-magnetisation of different spins, the cross peaks in ROESY arise from cross relaxation between spin-locked transverse magnetisation. The pulse sequence for 2D ROESY experiment is quite similar to the NOESY pulse sequence in that frequency-labelled magnetisation is prepared during t_1. The mixing time, however, is different. In NOESY the frequency-labelled magnetisation is rotated onto the z-axis, where cross relaxation takes place. In ROESY, it is the x-magnetisation present at the end of t_1 which is spin-locked so that transverse cross relaxation can take place (Keeler, 2005).

The application of ROESY in structural studies of molecules depends on the size of the investigated molecule. For small molecules within the extreme narrowing limit, there is no theoretical difference between NOEs and ROEs, so there seems little to be gained from undertaking the more complex ROE investigations. However, the use of ROESY is unavoidable when conventional experiments give negligible enhancements. More often this happens when studying relatively large molecules such as peptides (Claridge, 2009).

NMR assignments in peptides

The NMR spectroscopy does not produce an image of a molecule. It does, however, supply an abundant stream of structural data that can be extensively analysed employing computer calculation to obtain the peptide structure in solution. The first step in this direction is to obtain NMR assignments for the proton resonances in the

molecule. These assignments usually involve a combination of 2D NMR experiments (Wuthrich 1986) to correlate the signals in individual spin system; hence the resonance of all amino acids can be assigned. Identification of amino acids to their position in the sequence is carried out by sequence-specific assignment procedure.

The signals in individual spin systems are usually assigned through TOCSY and COSY spectra. Results from TOCSY experiments, and complimentary COSY experiment in the event of overlapping signals, provide correlations of all resonances in the same spin system and hence cross-sections of these spectra show cross-peaks from the H_N to H_α, H_β, H_γ, H_δ, and H_ω (if appropriate) (Jacobsen, 2007). The resonances of the spin systems are then compared with published chemical shifts of random coil (Wuthrich, 1986; Reid et al., 1997). Identification of amino acids into their position in the sequence is most commonly achieved through NOESY or ROESY experiments by identifying protons of adjacent amino acids that are close together in space. The distances between protons in two adjacent amino acids are called sequential distances. In a perfectly rigid macromolecule, selective NOE's can detect a pair of protons that are at a distance of approximately 3.0 Å or less (Wuthrich, 1986).

Proteins are linear polymers of amino acids that are joined together through peptide bonds. There are 20 natural amino acids that compose protein molecules that share many things in common to one another and only differ in the side chains. The spin system of amino acid residue, for the purpose of NMR study, starts with proton on the backbone nitrogen (H_N) and moving to the proton on the α-carbon (H_α) and out to the side chain (H_β, Hγ, H_δ, H_ε, etc.). The typical regions of proton chemical shifts are backbone amide H_N (peptide linkage, 7-11 ppm), side chain amide H_N (Asn and Gln, 6-7.5 ppm), aromatic protons (6.5-8 ppm), alpha protons (3.5-5.5 ppm), side chain

protons (-0.5-3.3 ppm, unless close to oxygen), and methyl group (-0.3-1.3 ppm, unless connected to S) (Jacobsen, 2007). The chemical shifts of protons on non-terminal amino acid residues in random coil peptides and proteins are shown in Table 1.5.

The chemical shift of a specific nucleus depends on its covalent chemistry and, to a lesser extent, its non-bonded environment. The biggest deviations of chemical shifts from the value they would display in an unstructured (i.e. random coil) peptide can be caused by involvement in hydrogen bonding, and proximity to aromatic and carbonyl groups. The effects associated with the non-bonded environment can count for the protein's secondary and tertiary structure, and can provide useful information about the folded state. For examples, internuclear through-bond (scalar or J) coupling can be analysed in term of torsion angels, thus provide information about peptide backbone and side chain conformation, while the molecular mobility can be obtained by assessing the relaxation times or their reciprocal relaxation rates (Reid and MacLachlan, 1997).

The random-coil chemical shifts of peptides and proteins have very little variation in H_N or H_α chemical shifts among the 20 amino acids. This will help in distinguishing the chemical shifts of amino acids present in the studied peptides. However, if there is more than one of a particular amino acid in the unstructured proteins, they will be undistinguishable by chemical shifts. If all amino acid residues show random coil chemical shift values, then all H_N and H_α cross-peaks, no more than 20 cross-peaks, will appear in a small region between 8-9 ppm (H_N) and 4-4.8 ppm (H_α). Using the COSY and TOCSY experiments the link of all the protons within a single spin system that correspond to a single amino acid residue can be established. The sequence-specific assignment of a peptide, however, can be obtained by correlating

proton in one residue to proton in the next residue in the sequence. This is done with NOE interaction, the sequential interaction, which indicates certain protons in one residue are constrained by the peptide bond to be close to certain protons in the next residue. Therefore, it is expected that there is NOE correlation between H_α of the first residue (*i*) to H_N of the next residue (*i* + 1) and sometimes between H_β of residue *i* and H_N of residue *i+1* (Jacobsen, 2007).

Table 1.5: ^{1}H Chemical shifts of protons on non-terminal amino acid residues of Random Coil peptides and proteins.

Residue	Abbreviation	H_N	H_α	H_β	H_γ	H_δ	H_ε	H_ζ
Ala	A	8.25	4.35	1.40				
Arg	R	8.27	4.40	1.80 1.92	1.72	3.31		
Asn	N	8.75	4.76	2.76 2.83				
Asp	D	8.41	4.77	2.75 2.84				
Cys	C	8.31	4.69	2.96 3.28				
Gln	Q	8.41	4.37	2.01 2.13	2.38			
Gly	G	8.39	3.96					
His	H	8.41	4.63	3.20 3.26		7.14 (4)	8.12 (H2)	
Ile	I	8.19	4.22	1.89	1.19 1.48 0.94	0.89		
Leu	L	8.42	4.39	1.65	1.65	0.94 0.90		
Lys	K	8.41	4.36	1.75 1.87	1.47	1.71	3.02	
Met	M	8.42	4.51	2.00 2.16	2.63		2.13	
Phe	F	8.23	4.66	2.99 3.22		7.34	7.34	7.34
Pro	P		4.71	1.98 2.30	2.30	3.65		
Ser	S	8.38	4.50	3.89				
Thr	T	8.24	4.35	4.22	1.23			
Trp	W	8.09	4.70	3.20 3.22		7.24 (H2) 7.65 (H4)	7.17 (H4) 7.24 (H6)	7.50 (H7)
Tyr	Y	8.18	4.60	2.92 3.13		7.15 (H2,6)	6.86 (H3,5)	
Val	V	8.44	4.18	2.13	0.94 0.97			

Adopted from Wuthrich (1986) and (Reid et al. (1997).

1.5. Drawbacks in bioactive peptide production

Discussions in the previous sections do indicate the promising physiological benefits of peptides derived from food protein degradations either through fermentation or enzymatic hydrolysis. However, productions of peptides of specific physiological properties and their efficacy rely on many factors that may not be easily achieved. These factors include amino acid sequences of the parent proteins, appropriate enzymes to cleave the proteins, quantity of the active peptides produced, isolation of the active peptides, efficacy of the peptides, possible allergic reactions, and production cost.

The amino acid sequences of parent proteins are a determining factor in the release of bioactive peptides as these sequences may have variations between same proteins from different species or even between variant of a same species. The limited knowledge of the amino acid sequence of many food proteins means that the production of bioactive peptides from food proteins is largely a trial-and-error experiment using various food proteins and enzymes. The well-studied milk protein is an exception, where the well documented sequence can be used to guide the choice of enzymes needed to cleave the peptide bond and release desired peptides. The knowledge of enzyme specificity, therefore, plays an important role in achieving optimum condition for hydrolysis.

The amount of certain active peptides produced from hydrolysis of food proteins depends on the quantity of occurrence of the peptide sequence within the parent proteins and the specificity of the enzyme used in hydrolysis. The yield of a particular active peptide is usually low. In addition to the low yield, purification of active peptides is a major challenge and costly. At laboratory level, chromatographic separation is the most common method of choice. However, this method is costly and

time consuming, and since the hydrolysates from hydrolysis contain numerous peptides with relatively close in sizes and hydrophobicity, contaminations are unavoidable. Various studies indicated that most bioactive peptides, in particular anti-hypertensive peptides, are small peptides hence fractionation of hydrolysates with molecular weight cut off membrane to produce hydrolysate fraction containing active peptides that can be used in product development.

The efficacy of the active peptides and their potential cause of allergic reaction are other issues associated with bioactive peptide production and use. Reports from various studies indicated that there exists bioactive peptide that showed *in vitro* activity but failed when tested *in vivo*. These are believed to be the inability of these peptides to resist degradation by gastrointestinal peptides as well as pancreatic enzymes. Other reports, however, showed that bioactive peptides can be absorbed intact into the blood circulation following an oral administration. This raise the stake of possible allergic reaction due to consumption of bioactive peptides, although, as far as recent information are concerned, there is no report of allergic reaction experienced during oral administration of bioactive peptides, anti-hypertensive peptides in particular, among volunteer subjects involved in bioactive peptide research.

1.6. Application of bioactive Peptides

Studies on bioactive peptides derived from food proteins indicate that there are many possible uses of these peptides. Two of these are as anti-hypertensive and anti-microbial agents. As an anti-hypertensive agent, the use of bioactive peptides as a food ingredient is advisable (Matsui, et al., 1993). Therefore, the functional properties of these peptides assume importance.

According to Kinsella (1976), properties such as aroma and taste are sometimes also regarded as functional. This is particularly important when these properties are exploited in the formulation of foods. In the case of enzymic-modified food protein, Adler-Nissen (1986) said that it is the off-flavour which dominates and proposed, among others, an application in small quantity as highly functional ingredient in complex food items.

Various proteolytic enzymes have been used successfully to hydrolyse food proteins yielding different bioactive peptides with potential use as food ingredients. However certain protease of bacterial origin (e.g., alkaline protease) needs further investigation into antigen-antibody reaction (food allergy) and to digestive resistance (Matsui et al., 1993). This therefore indicates the need for *in vivo* investigation with laboratory animals and human to observe possible allergic reaction upon administration of these peptides.

Numerous *in vitro* studies have confirmed the activities of those active ACE inhibitory peptides. *In vivo* studies with both animal and human subjects have also been carried out and promising results have been reported. Peptides IPP and VPP derived by *Lactobacillus helveticus* fermentation of milk, such as Calpis sour milk, are well characterised for their activities against ACE both *in vitro* and *in vivo* with animal and human subjects. A variety of products are available commercially either as functional food supplement or as bacterial-based functional foods. Among these are three milk-based functional foods with claimed potency for reducing blood pressure. They are Ameal S from Calpis (Kanagawa, Japan), Evolus from Valio (Finland), and Cardi-04^{TM} from Chr. Hansen (Denmark). These first two products contain bioactive peptides IPP and VPP and some other active peptides. Cardi-04^{TM}

is a fermented milk product produced by *Lactobacillus helveticus* strain Cardi-04TM (Flambard and Johansen, 2007).

The use of bioactive peptides as an anti-microbial agent may come in many different types such as for clinical use and as food preservative. According to Wilcox (2004) to become a good therapeutic agent, a drug has to show good activity, appropriate function, low toxicity, and have stability *in vivo*. So far only two bioactive (cationic) peptides are used in topical creams and solutions. As a food preservative Chan and Li-Chan (2006) suggested that apart from having good *in vivo* activity, synergistic effect of an anti-microbial peptide with other preservative agent may be important for their successful use in food products.

1.7. Summary

The information presented and discussed in the previous sections does indicate the promising physiological roles of bioactive peptides derived from food proteins in human health. These physiological functions include anti-hypertensive, anti-microbial, anti-thrombotic, anti-oxidative, anti-cariogenic, anti-cancer, opioid, immunomodulatory, cholesterol lowering, and prebiotic activities. Anti-hypertensive and anti-microbial peptides are by far the most studied bioactive peptides. These bioactive peptides are derived from their main protein precursors by the action of fermentative cultures, isolated proteolytic enzymes, and to a much lesser extent through acid and heat treatments. The most commonly studied protein precursors are milk proteins. Other proteins sources such as fish and soya beans were also studied. Unlike milk and soya proteins that have little variation due to common breed, species, and cultivar available commercially, fish proteins vary greatly due to numerous species available. Therefore, proteins from different species of fish may

produce different bioactive peptides even when produce under same production conditions.

Various enzymes as well as enzyme producing microbial cultures have been used successfully to hydrolyse food proteins. Thermolysin, Flavourzyme™, and Alcalase are some of the enzymes commonly used in various bioactive peptide productions. Other enzymes such as papain and bromelain have also been used; however thermolysin and Flavourzyme™ seemed to be the more productive enzymes in term of bioactive peptide productions. Papain and bromelain are commonly used in food production and are active around pH 5-9, hence good enzymes to use without pH adjustment as most foods have pH within this range that suit the characteristic of papain and bromelain. Flavourzyme™ also actives around pH 4-8 and can extensively hydrolyse proteins, indicating its ability to produce high quality food protein hydrolysates.

Characterisations of these two classes of bioactive peptides are carried out using various approaches such as *in vitro* and *in vivo* activity, mode of inhibition, structural, and stability studies. These studies aimed at understanding the strength and nature of inhibition as well as *in vivo* activity and stability that leads to efficacy of the active peptides. Structure-activity relationship studies of anti-hypertensive peptides involved mainly primary structure analysis with amino acid replacement technique often employed to investigate the contribution of certain amino acid residues on the overall activity of anti-hypertensive peptides. These procedures are quite useful as most anti-hypertensive peptides are small peptides.

Nuclear magnetic resonance (NMR) technique is one of the most commonly used techniques to investigate the structure-activity relationship activity of bigger peptides such as those with anti-microbial activities. Bigger anti-hypertensive peptides can

also be studied with this technique, although only one such study has been reported so far.

Anti-hypertensive and anti-microbial peptides may play important roles in the future. This is due to the promising prospect these two classes of bioactive peptides may offer in preparation of functional foods and clinical agents. Anti-hypertensive peptides offer great prospect in formulation of functional foods for mild hypertensive subjects, while anti-microbial peptides have attracted great interest from scientists due to the advent of antibiotic resistance some pathogenic bacteria shown against some antibiotics in use today. Development of anti-hypertensive foods are associated with the link between hypertension and cardiovascular diseases, hence consumption of anti-hypertensive functional foods can lower the risk of getting a cardiovascular disease. Anti-microbial peptides also offer good prospect for developments of new anti-microbial agents for clinical uses as well as food preservatives. Developments and characterisation of these bioactive peptides will, therefore, provide knowledge based information on the activity and efficacy of these peptides that can lead to utilisation of these peptides in formulation of functional foods and anti-microbial agents.

Chapter 2

Materials and Methods

2.1. Sources of raw materials and treatments

2.1.1. Raw materials

Two species of fish namely leatherjacket (*Meuchenia* sp.) and trevally (*Pseudocaranx* sp.) were purchased from Sydney Fish Market in Pyrmont, Sydney. Three enzymes viz., papain, bromelain (Sigma-Aldrich Chemical Company, Castle Hill, NSW, Australia), and Flavourzyme (Novozymes, North Rocks, NSW, Australia) were used to hydrolyse the fish proteins.

2.1.2. Preparation of fish hydrolysate

Fresh trevally and leatherjacket were filleted, deboned, skinned, and then minced. The mince, in 50 g portions, was then mixed with ice-cold Milli-Q water in 1:2 ratio and shaken at 5^{o}C and 200 rpm for 60 min. The mixture was centrifuged at 5^{o}C, at 5580 g for 30 min. The supernatant, containing water-soluble proteins, was collected and subjected to enzymatic hydrolysis. The pellets that contain the insoluble proteins were resuspended in Milli-Q water in 1:2 ratio prior to hydrolysis with the enzymes. Crude protein content of the supernatant and the pellet suspension was determined at this stage using BioRad protein assay kit (BioRad Laboratories, Gladesville, NSW, Australia) and modified Micro Kjeldahl method (AOAC, 2005), respectively. The pH of the supernatant and the pellet suspension was measured before the addition of individual enzymes (papain, bromelain and Flavourzyme™), but was not adjusted to the specific optimum pH of the individual enzymes to avoid the need for the removal of the salt from the final products (hydrolysates) (Aspmo et al., 2005). Enzymes,

0.5% (w/v wet basis) papain and bromelain and 1.25% (v/v) for Flavourzyme were added into the supernatant and 1% (w/v wet basis) papain and bromelain and 2.5% (v/v) for Flavourzyme™ were added into the pellet suspension, and were hydrolysed at 50^{o}C with shaking at 170 rpm for up to 10 h using a C24KC Refrigerated Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA). The hydrolysis was stopped at various time intervals (every two hours, see treatments) by withdrawing 30 ml of supernatant followed by immersion of the sample flask into a boiling water bath for 15 min to deactivate the enzymes. The hydrolysates were cooled to room temperature, then the pH and degree of hydrolysis were measured. The hydrolysates were then centrifuged at 5^{o}C, 5580 g for 30 min using Sigma Laboratory Centrifuge 6K15 (B.Braun Biotech International, Melsungen, Germany) to remove the solid undigested material, then were frozen at -85^{o}C and used for further analysis. Figure 2.1 shows the flow process chart of the production of fish protein hydrolysate.

2.1.3. Methods

The fish protein hydrolysates were prepared by treating the water soluble and insoluble protein fractions with proteolytic enzymes and hydrolysis for up to 10 h. In detail, the treatments were organised as follows (Table 2.1):

Table 2.1. Design of the treatments given to each fraction of fish protein for the preparation of fish hydrolysates.*

Protein fractions	Enzymes	Hydrolysis Time
Leatherjacket: - Soluble-protein - Insoluble-protein	- papain - bromelain - Flavourzyme™	- 2 h - 4 h - 6 h - 8 h - 10 h
Trevally: - Soluble-protein - Insoluble-protein	- papain - bromelain - Flavourzyme™	- 2 h - 4 h - 6 h - 8 h - 10 h

* Each protein fraction was mixed individually with each enzyme and hydrolysed for up to 10-hour. The soluble protein was mixed with 0.5% papain and bromelain, and 1.25% of Flavourzyme™ (wet basis). The insoluble fraction was mixed with 1.0% papain and bromelain, and 2.5% Flavourzyme™ (wet basis).

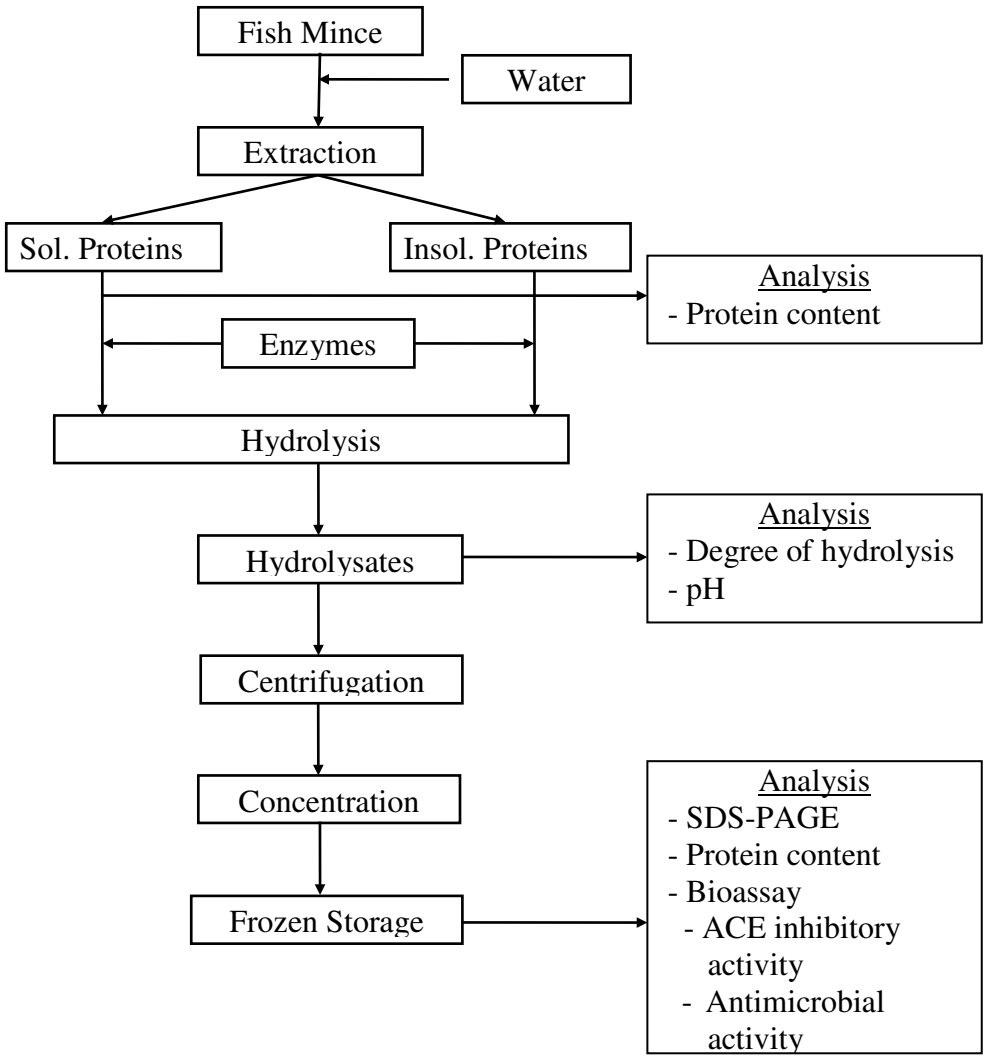


Figure 2.1. Flow process chart of the preparation of hydrolysates from fish muscle protein.

2.2. Chemicals, reagents, microbial cultures and media

2.2.1. Chemicals

All chemicals were of reagent grade and obtained from Sigma-Aldrich Chemical Company (Castle Hill, New South Wales, Australia), BioRad Laboratories (Gladesville, NSW, Australia)), APS Ajax Finechem (Taren Point, NSW, Australia), Lab-Scan Analytical Science (Bangkok, Thailand), and Merck Pty Ltd (Kilsyth, Vic, Australia). All solvents were of analytical grade except HPLC grade solvent for HPLC. Deionised water was prepared using Milli-Q® reagent water system (Millipore, Bedford, MA, USA).

2.2.2. Microbial strains

Microbial strains used in this study were *Staphylococcus aureus* 184, *Escherichia coli* 185, *Bacillus cereus* 106 and *Candida albicans* X26 from culture collection of the University of Western Sydney, Hawkesbury Campus, Richmond, NSW, Australia.

2.2.3. Microbiological Media

All media used in this study was supplied by Oxoid (Oxoid Australia Pty. Ltd., Thebarton, SA, Australia) and Difco Laboratories (Sparks, NJ, USA). Prepared media was sterilised by autoclaving at 121^{o}C for 15 min using Siltex HC2 (MK1-94) steriliser (Siltex Australia Pty Ltd, East Bentleigh, Vic, Australia). Sterilised media was stored at 4^{o}C until used.

2.2.4. Antibiotics and medium constituents

Antibiotics tetracycline and polymyxin B sulphate (Sigma-Aldrich, Castle Hill, NSW, Australia), were used. The antibiotics were prepared and stored following the manufactures' instructions unless otherwise stated. Medium additives (glucose and NaCl) were also used during media preparation.

2.3. Stock solutions, buffers, media, reagents, and solvents

All solutions, reagents, and buffers were prepared using Milli-Q water. Analytical balance AND HR-200 (AND Co. Ltd., Tokyo, Japan) or AA-200 (Denver Instrument, Bohemia, NY, USA) was used for weighing fine quantity of chemicals, and OHAUS Precision Standard (Crown Scientific Pty Ltd, Minto, NSW, Australia) or AND HF-3000G (AND, Japan) balance was used for large quantity chemicals. Inolab pH Level 1 (WTW, Weilheim, Germany) pH meter was used to measure pH of buffers and or mixtures of reactants.

2.3.1. Stock solutions

Unless otherwise stated, all stock solutions and media were prepared using Milli-Q water, sterilised at 121^{o}C in an autoclave for 15 min as needed using a Siltex HC2 (MK1-94) steriliser (Siltex Australia Pty Ltd, East Bentleigh, Vic, Australia) and kept at room temperature.

Bovine serum albumin (BSA)

BSA stock solution (0.5 mg/ml) was prepared by diluting lyophilised BSA solution (1.47 mg/ml) with deionised water and stored at -20^{o}C. The BSA stock solution was

used to prepare standard solutions, containing 0-100 μg/ml proteins, for assay of protein content.

Trinitrobenzenesulphonic acid (TNBS, 0.1%) solution

The 0.1% TNBS solution was made by diluting 200 μl 5% TNBS (2,4,6-Trinitrobenzenesulphonic acid) solution with Milli-Q water to make 10 ml final volume and stored at 4^{o}C in dark bottles. This solution was used in the determination of degree of hydrolysis (DH) of fish proteins.

Tetrazolium Solution 0.5 mg/ml

The tetrazolium solution 0.5 mg/ml was prepared by dissolving 10 mg tetrazolium salt (2,3-bis-(2-methyloxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT)) in 10 ml phosphate-buffered saline, filtered, sterilised and stored at -20^{o}C until used.

L-Leucine (1500 mM) solution

L-Leucine, 1500 mM, stock solution was prepared by dissolving 1.97 g L-Leucine (MW 131.17) in Milli-Q water to 10 ml final volume and stored at 4^{o}C. This stock solution was used to prepare standard solutions for the determination of degree of hydrolysis.

Angiotensin I-converting cnzyme (ACE, 1 U/ml)

The 1 U/ml ACE stock solutions was made by dissolving 2 U ACE in 2 mL Milli-Q water. The stock solution was stored at -20^{o}C in separate vials of 100 μl volume until used.

Hippuric acid (0.5 mg/mL)

The hippuric acid 0.5 mg/mL stock solution was made by dissolving 0.005 g hippuric acid in 10 mL 50% methanol/Milli-Q water. The stock solution was used to prepare standard hippuric acid solutions for the ACE inhibition assay.

2.3.2. Buffers

HEPES (50 mM containing 300 mM NaCl)

HEPES (50 mM containing 300 mM NaCl, pH 8.3) was prepared by dissolving 13.01 g HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt) and 17.53 g NaCl in Milli-Q water to make 1000 ml final volume. The pH was adjusted to 8.3 by addition of 1 M HCl. This buffer was used to dissolve HHL for ACE inhibition assay.

Phosphates (50 mM, pH 7.2) containing 5% glycerol

The phosphate buffer (50 mM, pH 7.2) containing 5% glycerol was prepared by mixing 50 mM Na_2HPO_4 containing 5% glycerol and 50 mM NaH_2PO_4 containing 5% glycerol until the pH reached 7.2.

Phosphate buffer (0.2125 M, pH 8.2)

Phosphate buffer (0.2125 M, pH 8.2) was made by mixing 0.2125 M Na_2HPO_4 solution with 0.2125 M NaH_2PO_4 solution until the desired pH was achieved. The buffer was used in the determination of degree of hydrolysis.

Staining and destaining buffers

The destaining solution was made by mixing 400 ml methanol, 100 ml acetic acid glacial and Milli-Q water to a final volume of 1000 ml. The staining buffer was made by dissolving 1.25 g Coomasie brilliant blue in the destaining buffer to make 500 ml final volume. The solution was filtered through Whatman No 1 filter paper.

10X Electrode running buffer (pH 8.3)

Glycine (144.0 g), 30.0 g tris[Hydroxymethyl] aminomethane (trizma base) and 10.0 g sodium dedocyl sulphate (SDS) were dissolved and made up to 1000 ml with Milli-Q water. The pH was adjusted to 8.3 with concentrated HCl.

2.3.3. Microbiological Media

Luria broth (LB)

The Luria broth was prepared by dissolving 7.75 g Luria media powder (Difco, Sparks, NJ, USA) in distilled water to make 500 ml media solution. The media was sterilised at 121^{o}C for 15 min and stored at 4^{o}C until used.

Brain heart infusion (BHI) broth

The BHI broth was prepared by dissolving 18.5 g BHI media powder (Difco, Sparks, NJ, USA) in distilled water to make 500 ml media solution. The media was sterilised at 121^{o}C for 15 min and stored at 4^{o}C until used.

Preservation media

The media as prepared by mixing 50 ml glycine and 50 ml double strength Luria or BHI broth. The media was used to preserve stock bacteria used in this study.

Sabouraud liquid medium

The Sabouraud liquid medium was prepared by dissolving 2.5 g trypton as pancreatic digest of casein, 2.5 g Lab-Lemco powder in place of peptic digest of fresh meat, and 10.0 g glucose in distilled water to make 500 ml final volume, sterilised at 121^{o}C for 15 min and stored at 4^{o}C until used.

Tryptone (30 mg/ml)

Tryptone (2.40 g) was dissolved in distilled water to make 80 ml final volume, sterilised and stored at 4^{o}C. The tryptone solution was used to replace hydrolysate or peptide solution in anti-microbial susceptibility test and minimum inhibition concentration (MIC) assay.

0.5 McFarland standard

The standard was prepared by adding in 0.5 ml 0.048 M $BaCl_2$ into 99.5 ml 1% H_2SO_4 with constant stirring and stored at 4^{o}C until used.

2.3.4. Reagents and solvents

Ammonium persulphate (10%)

Ammonium persulphate (10% w/v) was made by dissolving 20 g ammonium persulphate in Milli-Q water to make 200 ml final volume. The solution was transferred into 1 ml vials and stored at -20^{o}C until used.

Bromophenol blue (0.1%)

Bromophenol blue (0.1%) solution was made by dissolving 0.1 g bromophenol blue in Milli-Q water to make 100 ml final volume, filtered through Whatman No 1 filter

paper and stored at 4^{o}C. This solution was used in sample preparation for SDS-PAGE.

Hippuryl-histidyl-leucine (HHL, 5 mM)

HHL, 5 mM, solution was made freshly by dissolving 0.0215 g HHL (MW 429.47 anhydrous basis) in 50 mM HEPES buffer containing 300 mM NaCl, pH 8.3, to make a final 10 mL. This solution was used as substrate for ACE in ACE inhibition assay.

HPLC solvent (50% methanol containing 0.1% (v/v) TFA)

Methanol 50% in Milli-Q water containing 0.1% (v/v) trifluoroacetic acid (TFA) was prepared, degassed, and used as isocratic column eluent in analysis of ACE inhibitory activity.

HPLC solvent (19% methanol and 1% tetrahydrofuran containing 0.05M sodium acetate, pH 5.9)

Methanol 19%, 1% tetrahydrofuran (THF) in Milli-Q water containing 0.05M sodium acetate (NaAc) was prepared, degassed, and used as isocratic column eluent in analysis of ACE inhibitory peptide's mode of inhibition. The pH was adjusted to 5.9 by addition of 1M acetic acid solution.

HPLC solvent (acetonitrile containing 0.1% (v/v) TFA)

Solvent was prepared by mixing 1.02 ml TFA and acetonitrile to make 1000 ml final volume and degassed in an ultrasonic tank. The solvent was used as gradient eluent in fractionation of active hydrolysates and peptide purification.

HPLC solvent (Milli-Q water containing 0.1% (v/v) TFA)

Solvent was prepared by mixing 1.02 ml TFA and Milli-Q water to make 1000 ml final volume and degassed in an ultrasonic tank. The solvent was used as eluent in fractionation of active hydrolysates and peptide purification.

2.4. Analytical instrumentation

2.4.1. High performance liquid chromatography (HPLC)

A Shimadzu VP class HPLC instrument consisting of a DGU-20A5 Prominence degasser, SIL-20A Prominence auto sampler unit, LC-20AT Prominence liquid chromatography solvent delivery system, a RID-10A refractive index detector, an SPC-M20A diode array detector equipped with a temperature controller, RF-10AxL fluorescence detector and an FRC-10A fraction collector (Shimadzu Scientific Instrument (Oceania) Lty. Ltd, Mt. Waveley, Vic, Australia) was used. A C-18 Shim-Pack PREP-ODS column (20x250 mm, 15µ, Shimadzu Scientific) and Altima C-18 analytical column (Altech, 5µ, 4.6x250mm) coupling with a guard column were also used.

2.4.2. Multiwell plate reader

Benchmark Plus multiwell plate reader (BioRad Laboratories, Gladesville, NSW, Australia) was used for determination of protein content, degree of hydrolysis and anti-microbial assays.

2.4.3. Electrophoresis unit

Vertical slabs BioRad Mini Protean 3 System electrophoresis apparatus coupled with Power PAC 300 (BioRad Laboratories, Gladesville, NSW, Australia) was used in electrophoresis of fish protein and hydrolysates.

2.4.4. Protein sequencer

Applied Biosystems 494 Procise Protein Sequensing System (Applied Biosystems, Carlsbad, CA, USA) at Australia Proteomic Analysis Facility, Macquarie University, (Sydney, Australia) was used for automated Edman N-terminal sequencing of selected active peptides. Procise™ Protein Sequencer System software was used to analyse the sequence. The performance of the sequencer was assessed routinely with 10 pmol β-Lactoglobulin standard.

2.4.5. Liquid chromatography/mass spectrometry (LC/MS)

An LC/MS system consisting of Varian ProStar HPLC that include 330 Photodiode array detector, 240 Quaternary solvent delivery module, 410 AutoSampler with variable volume, and Altima C18 column (3µm, 3.0x150 mm). The LC unit was coupled with Varian 1200L Quadrupole MS/MS System and Varian 1200LCMS (Varian Inc., Palo Alto, CA, USA) data acquisition software.

2.4.7. Nuclear magnetic resonance (NMR) spectrometer

A Bruker Avance 500 MHz nuclear magnetic resonance (NMR) spectrometer (Bruker BioSpin, Alexandria, NSW, Australia) was used for the NMR studies.

2.5. Raw Material and hydrolysate analyses

2.5.1. pH

The pH of the protein suspensions and hydrolysates of different time intervals was measured. Freshly calibrated inoLAB pH Level 1 pH meter (WTW, Weilheim, Germany) was used for this measurement.

2.5.2. Protein content analysis

The protein concentration of fish soluble and insoluble fractions was measured according dye binding method (Bradford, 1976) for liquid samples and Micro Kjeldhal method for the solid samples (AOAC, 2005).

2.5.2.1. Protein dye-binding procedure

The determination of protein content was carried out using Bovine serum albumin (BSA) stock and dye reagent (Bradford, 1976) supplied by Bio-Rad Laboratories. The standard solutions of BSA with protein concentration ranging from 10–90 µg/ml were made by mixing stock BSA with Milli-Q water. Soluble protein sample was mixed with Milli-Q water in 1:7 ratio, while the dye reagent was diluted with Milli-Q water in 1:2 ratio. BSA standard solutions (120 µl) of different concentrations were mixed with dye reagent (40 µl) in adjacent wells in the order of ascending concentrations. Samples of soluble fish protein (120 µl) were also mixed with dye reagent (40 µl). The mixtures were incubated at room temperature for 10 min and the absorbance was read at 595 nm using Benchmark Plus plate reader (BioRad Laboratories, CA, USA). The protein concentration of the samples was extrapolated against the standard curve of the BSA standard concentrations.

2.5.2.2. Protein modified spectrophotometric procedure

The protein content of the insoluble protein was measured following the modified micro Kjeldahl procedure as follows. Solid fish insoluble protein (50 mg) was mixed with 100 µl 60% H_2SO_4 and 3-4 drops of H_2O_2 in a glass tube. As control, standard nitrogen solution (135 µl) was mixed with 100 µl 60% H_2SO_4 and 3-4 drops of H_2O_2 in glass tubes. The mixtures were digested in a heating block for 225 min. The temperature of the heating block was increased from 140°C to 260°C at every 45 min intervals, during which 3-4 drops of H_2O_2 were also added to each tube. After digestion, 4.95 ml Milli-Q water was added into each tube followed by centrifugation for 15 min at 3000 rpm and 25°C. Supernatant, 250 µl, of each sample and standards were transferred into cuvettes followed by additions of 25 µl 2.5 N NaOH, 50 µl 10% K-Na tartrate and 2.125 ml Milli-Q water and mixed. Nessler's reagent, 50 µl, was added and the mixtures were vortexed for 10 min. The absorbance of the mixtures was read at 420 nm using Helios γ spectrophotometer (Thermo Electron Corporation, Auchtermuchty Fife, UK).

2.5.3. Degree of hydrolysis (DH)

The determination of the degree of hydrolysis (DH) was performed following the TBBS method (Adler-Nissen, 1979) with some modification to fit the use of microplate reader.

2.5.3.1. L-Leucine standard solutions and samples

Both L-leucine standard solutions and samples were dissolved/dispersed in 0.44% SDS. L-Leucine standard solutions were prepared by serial dilutions with Milli-Q water. The concentrations of L-Leucine in the final mixtures (200 µl) ranged from

0.02-0.9 mM. Samples of hydrolysates were mixed with Milli-Q water to adjust the concentration of free amino groups to within the range of the L-Leucine standard concentrations.

2.5.3.2. Preparation for total amino groups

Samples for total amino group were prepared by mixing 1 ml of soluble protein fraction or 1 g of insoluble protein fraction of fish mince with 10 ml 6 M HCl and hydrolysed at 110^{o}C for 18 h. The hydrolysates were diluted with Milli-Q water to adjust the free amino acid concentration to within the L-Leucine standard concentration.

2.5.3.3. Procedure

L-Leucine standard solutions, 20 µl of each, were placed into adjacent wells in a multiwell plate in the order of increasing concentrations. Then, 20 µl 0.44 % SDS and 95 µl 0.2125 M pH 8.2 phosphate buffer were added. The same procedure was also done for the samples. For blank, 40 µl 0.22 % SDS was placed in separate wells and mixed with 95 µl 0.2125 M pH 8.2 phosphate buffers. To all wells, 15 µl 0.1 % TNBS solution was added. The TNBS solution was added to standard solutions and samples first then to the blank. The plate was covered with plate lid and with aluminium foil then incubated for 60 min at 50^{o}C. The reaction was stopped by adding 50 µl 0.2 N HCl, and then cooled down at room temperature. The absorbance was read at 340 nm. Summary of the analytical procedure is shown in Table 2.2.

Table 2.2. Summary of the procedure for the determination of degree of hydrolysis of fish proteins.

Reagents	Volumes (µl)		
	Samples	Blank	Standard*
L-Leucine	-	-	20
0.44% SDS	20	-	20
0.22% SDS	-	40	-
Peptide/sample	20	-	-
Phosphate buffer	95	95	95
TNBS	15	15	15
Incubation at 50^{o}C for 60 min			
0.2 N HCl	50	50	50
Cool down at room temperature for 30 min			
Read absorbance at 340 nm			
Total	200	200	200

* Various concentration of L-Leucine is used to obtained standard curve.

2.5.4. SDS-PAGE

The SDS-PAGE was performed following the method of Laemmli (1970) to observe the extent of the hydrolysis of the proteins.

2.5.4.1. Sample buffer (pH 6.75)

The sample buffer containing 0.125 M Trizma-HCl, 10% sodium dedocyl sulphate (SDS), 10% 2-mercaptoethanol, 20% glycerol and 0.004% bromophenol blue. This solution was made by mixing 8.33 mL 1.5 M tris[Hydroxymethyl]aminomethane-HCl pH 8.8, 20 mL glycerol, and 50 ml 20% SDS. The pH was adjusted to 6.75 with concentrated HCl followed by addition of 0.04 ml 0.1% bromophenol blue. Aliquot of each 900 µl sample buffer was transferred into 1 mL vials and frozen storage until

used. Prior to use, the sample buffer was defrosted and 100 µl of 2-mercaptoethanol was added and mixed.

2.5.4.2. Sample preparation

Water soluble, insoluble protein fractions and hydrolysates from hydrolysis process were used. The water-soluble and peptide fractions were concentrated by a freeze-dryer and reconstituted in 1 mL Milli-Q water. The insoluble protein fraction, 1 g, was quickly frozen by the addition of liquid nitrogen and ground. Five ml phosphate 50 mM buffer pH 7.2 was added while grinding, followed by 20 µl Triton 100X and continued grinding until a right liquid consistency was formed. The mixture was transferred to 1 ml sample vials and was centrifuged at 1300 rpm, at 5^{o}C and 15 min. The supernatant containing insoluble protein was separated and stored at –20^{o}C. Sample (100 µl) was mixed with 100 µl sample buffer and incubated in a boiling water bath for 1.5 min followed by immersion in an ice-bath. The final sample contained 0.031 M Tris-HCl (pH 6.8), 1.0% (w/v) sodium dedocyl sulphate, 5% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, and 0.0025% (w/v) bromophenol blue.

2.5.4.3. Preparation of standard proteins

Peptide and protein molecular weight mixtures for SDS-PAGE were purchased from Sigma Chemicals Co., Sydney, Australia and BioRad Laboratories, respectively. The peptide standard consisted of myoglobin (1-153) mol wt 16,950 Da, myoglobin I (56-131) mol wt 8,160 Da, myoglobin I + II (1-131) mol wt 14,440 Da, myoglobin I + III (56-153) mol wt 10,600 Da, myoglobin II (1-55) mol wt 6,210 Da, and myoglobin III (132-153) mol wt 2,510 Da. A set of protein standard consisting of ten sharp bands of the following molecular weights: 10, 15, 20, 25, 37, 50, 75, 100, 150,

and 250 kDa were also used. The standard mixture was prepared by adding 1.5 mL sample buffer solution and mixed by inverting and vortexing the tube. Standard peptide solution (100 µl) was transferred to a small test tube, mixed with 100 µl sample buffer, and incubated in a boiling water bath for 1.5 min while the rest was stored at –20^{o}C.

2.5.4.4. Gel preparation and casting

Two 12.5% acrylamide gels were prepared by mixing 4.25 ml 40% (w/v) acrylamide, 2.31 ml 2.0 % (w/v) N-N'-methylene-bis-acrylamide, 3.5 ml 1.5 M Tris-HCl pH 8.8, 3.73 ml Milli-Q water, 140 µl 10% SDS, 140 µL ammonium persulphate and 14 µl TEMED. Each gel had the capacity to take 10 samples. A 4% stacking gel with the following composition: 6.1 mL of nanopure water, 2.5 ml of 0.5M Tris-HCl, 100 µl of 10% SDS, 1.3 mL acrylamide/bis stock; 100 µl of 10% ammonium persulphate and 10 µl of TEMED was poured about 1.5 cm above the resolving gel. A comb was inserted about 0.3 cm above the stacking gel immediately after pouring. After polymerisation the comb was gently removed and wells were washed with running buffer to remove traces of unpolymerised gel mixture.

2.5.4.5. Electrophoresis conditions

Electrophoresis on vertical slabs was carried out using BioRad Mini Protean 3 System electrophoresis apparatus. Tris-HCl buffer with a composition described in 2.3.2 was used as the running buffer. A total of 450 mL of the buffer was required to fill both the inner and outer chambers. Sample (25 µl) was applied to the wells. Electrophoresis was carried out for 3 hours until the tracking dye reached the bottom

of the gel. The power pack, Bio-Rad Power PAC 300, was set at 108 volts and 15 mA.

2.5.4.6. Staining and destaining of gels

After electrophoresis, the gels were removed from the plates and transferred into a staining tray and left overnight in the tray with the staining solution. The staining solution consisted of 0.25% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad Chemicals) dissolved in destaining solution as described in section 2.3.2. After overnight staining, the gels were transferred to a destaining tray and left in the tray for up to three hours until sharp bands were visualised.

2.6. Determination of ACE activity inhibition

ACE inhibitory activity was determined following combined method of Nakamura et al. (1995a) and Tsai et al. (2008) with some modifications.

2.6.1. Procedure

Hippuryl-Histidyl-Leucine (HHL), 200 µl 5 mM in 50 mM in HEPES buffer (pH 8.3) containing 300 mM NaCl, was mixed with with 80 µl of hydrolysate or peptide fraction of different concentrations (A) in reaction tubes, and preincubated for 10 min at 37^{o}C. As blank (B), 80 µl Milli-Q water was used in place of the peptide or inhibitor, while for control (C), 20 µl of Milli-Q water was added in place of ACE. After preincubation, 20 µl of ACE (100 mU/ml) was added to all reaction tubes except for the control (C) groups. The mixtures were incubated for 30 mins at 37^{o}C, and then the reaction was stopped by an addition of 250 µl of 1 N HCl, and mixed. Table 2.3 gives the summary of the reaction mixture. The reaction mixture was

injected into HPLC for analysis of the concentration of hippuric acid liberated by ACE.

2.6.2. HPLC conditions

Analysis on HPLC was carried out using C-18 analytical column as described in section 2.4.1 above. The column was eluted with isocratic 50% (v/v) methanol in Milli-Q water containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 450 µl/min for 19 min, and the absorbance was detected at 228 nm.

2.6.3. Calculation of inhibition activity

The inhibition (%) was calculated with the following formula.

$$\text{Inhibition (\%)} = (B - A)/(B - C) \times 100$$

Where: A = optical density in the presence of both ACE and peptide fraction or synthetic peptide

B = optical density without peptide fraction or inhibitor

C = optical density without ACE

The extent of the inhibitory activity is expressed as the concentration of the peptide that inhibits 50% of ACE activity (IC_{50}).

Table 2.3. Summary of reaction mixture of ACE inhibition assay.

Reagents	Volume (μL)		
	A	B	C
MQ-Water	-	-	20
Inhibitor/peptide	80	-	80
HHL 5 mM	200	200	200
HEPES Buffer	-	80	-
Preincubate at 37°C for 10 min.			
ACE, 100 mU	20	20	-
Incubate at 37°C for 1 hour.			
HCl, 1 N.	250	250	250
Total	550	550	550

2.6.4. Stability assay ACE inhibitory peptide

Assay of the stability of ACE inhibitory peptides followed the same method as for the determination of ACE inhibition activity except that the peptides or inhibitor were mixed with ACE first then preincubated at 37°C for 3 h (Fujita and Yoshikawa, 1999) followed by the addition of HHL and further incubation at 37°C for 1 h. The reaction was stopped by addition of 250 μl 1 N HCl and the hippuric acid liberated was measured with HPLC as described in Section 2.6.2.

2.7. Anti-microbial activity assays

Anti-microbial assays were carried out using *Staphylococcus aureus* 184, *Escherichia coli* 185, *Bacillus cereus* 106 and *Candida albicans* X26 bacteria on a multiwell plate using tetrazolium salt as colouring agent (Tunney et al., 2004). Standard microdilution method (Andrews 2001) was also carried out to confirm the results from the XTT method. The minimal inhibitory concentration (MIC) was observed and quantified spectrophotometrically on a microplate reader (BioRad

Benchmark Plus). Anti-fungal activity was performed against *Candida albicans* (Hazen and Howell 2004) with some modifications.

2.7.1. Bacterial strain preservation

Bacterial strains supplied by the University of Western Sydney culture collection were grown on nutrient agar plates for 48 h at 37^{o}C. A healthy colony from each strain was then transferred to its respective broth and grown for 24 hours. Stock culture was prepared by aseptically transferred 0.5 ml culture of *E. coli* into 2.5 ml sterile mixture of 50% glycerol and double strength Luria broth, while *S. aureus* and *B. cereus* into 2.5 ml sterile mixture of 50% glycerol and double strength BHI broth and stored at -40^{o}C, while working stock was stored at -20^{o}C for immediate use. Prior to use, 250 µl of the working stock was transferred into 9.75 ml sterile respective broth and grown overnight at 37^{o}C.

2.7.2. Gram staining

Gram staining was carried out using crystal violet (50% in distilled water), iodine solution, decolouring agent containing 50% acetone and 50% ethanol, and safranin solution. Bacterial smears were prepared by using clean glass slide, dried and heat fixed. The slide was flooded with crystal violet solution for 1.5 min, washed gently with tap water followed by addition of iodine solution for 1.5 min. The slide was washed again with tap water followed by decolourising using decolouring agent for about 10 sec and counter stained with safranin for 1 min. After washing with tap water, the slide was air-dried. The morphology of the Gram stained bacteria strain was observed using light microscope (Nikon, Japan).

2.7.3. Preparation of inoculum

Staphylococcus aureus 184 and *Bacillus cereus* 106 bacteria, were grown up from a frozen stock by incubation overnight at 37^{o}C in BHI broth, while *Escherichia coli* 185 was grown in Luria broth. After overnight incubation, the culture was diluted with the respective broths and the turbidity was compared to equal that of 0.5 McFarland standards that correspond to an optical density of about 0.1 at 595 nm. The *Candida albicans* culture was prepared by growing the stock fungal species on Sabouraud's broth at 37^{o}C overnight. The culture was diluted in the same broth to attain final concentration of 10^{7} CFU/ml that correspond to an optical density reading of about 1.0 at 450 nm.

2.7.4. Determination of minimum inhibitory concentration (MIC) microdilution method with tetrazolium (XTT) for bacteria

In a multiwell plate, 50 µl inoculum and 50 µl mixture of broth with hydrolysate, peptide solution or antibiotic solution (control for positive inhibition) of different concentrations were mixed. For negative control, tryptone solution was added instead of peptide or antibiotic. The initial optical density of the mixture was read at 595 nm using Benchmark Plus plate reader (BioRad Laboratories, CA, USA). The mixture was incubated at 37^{o}C for 4 h. After incubation, the optical density was again measured at 595 nm, then 100 µl XTT solution was added to each well. The optical density was again measured at 492 nm followed by additional incubation at 37^{o}C for 1 h. After incubation the optical density at 492 nm was again measured. Summary of the procedure is listed in Table 2.4.

Table 2.4. Summary of minimum inhibition concentration (MIC) assay with XTT.

Solutions	Volume (µl)		
	Sample	Negative Control	Positive Control
Hydrolysate/Peptide solution	50	-	-
Tryptone solution	-	50	-
Antibiotic	-	-	50
Inoculum	50	50	50
Initial absorbance reading at 595 nm			
Incubation at 37^{o}C for 4 h			
Absorbance reading at 595 nm			
Tetrazolium solution	100	100	100
Initial absorbance reading at 492 nm			
Incubation at 37^{o}C for 1 h			
Absorbance reading at 492 nm			
Total volume	200	200	200

2.7.5. Determination of minimum inhibitory concentration (MIC) standard microdilution method for bacteria

MIC assay with standard microdilution method (Andrews, 2001) was also carried out to verify the results of MIC assay with XTT method. In a multiwell plate, 75 µl inoculum and 75 µl mixture of broth with hydrolysate, peptide solution or antibiotic solution (control for positive inhibition) of different concentrations were mixed. For negative control, tryptone solution was added instead of peptide or antibiotic. The initial optical density of the mixture was read at 595 nm using Benchmark Plus plate reader (BioRad Laboratories, CA, USA). The mixture was incubated at 37^{o}C for 18-20 h. After incubation, the optical density was again measured at 595 nm. Summary of the procedure is listed in Table 2.5.

Table 2.5. Summary of minimum inhibition concentration (MIC) assay (standard method).

Solutions	Volume (µl)		
	Sample	Negative Control	Positive Control
Hydrolysate/Peptide solution	75	-	-
Tryptone solution	-	75	-
Antibiotic	-	-	75
Inoculum	75	75	75
Initial absorbance reading at 595 nm			
Incubation at 37^{o}C for 18-20 h			
Absorbance reading at 595 nm			
Total volume	150	150	150

2.7.6. Anti-fungal activity

The assessment of anti-fungal activity was carried out by microdilution method (Hazen and Howell 2004) with some modifications using microplate reader at 590 nm. *Candida albicans* culture solution, 100 µl, was placed in a microwell plate followed by an addition of 75 µl of tested hydrolysate, peptide or antibiotic solutions (control for positive inhibition). For negative control, tryptone solution was added instead of hydrolysate of peptide solution. Initial optical density was measured at 590 nm followed by incubation at 37^{o}C overnight. After incubation, the optical density was again read at 590 nm. Summery of the assay is shown in Table 2.6.

Table 2.6. Summary of anti-fungal assay microdilution method.

Solutions	Volume (µl)		
	Sample	Negative Control	Positive Control
Hydrolysate/Peptide solution	75	-	-
Tryptone solution	-	75	-
Antibiotic	-	-	75
Inoculum	100	100	100
Initial absorbance reading at 595 nm			
Incubation at 37^{o}C for 24 h			
Absorbance reading at 595 nm			
Total volume	175	175	175

2.8. Purification and identification of bioactive peptides

Active hydrolysates found from ACE inhibition and anti-microbial assays were subjected to filtration through 0.20 μm filters. The filtrates were fractionated by means of molecular weight cut off membrane (Dandenong South, Vic, Australia) and RP-HPLC on Shim-Pack PREP-ODS column coupled with Shimadzu fraction collector. The column was eluted with gradient acetonitrile and Milli-Q water containing 0.1% TFA. The acetonitrile concentration was increased overtime and reached 70% at 70 min. Peptide fractions collected were lyophilised and resuspended in Milli-Q water prior to be used in further assays. Fractions that showed activity against ACE and pathogenic micro-organism were subjected for further purification as in all cases the fractions still consisted of more than one peptide as indicated by the number of peaks in the chromatograms.

2.9. Statistical Analysis

The data collected for pH and degree of hydrolysis were analysed with one-way or two-way ANOVA, whenever appropriate, with two replications and linear regression, whenever applicable. The mathematical model of the two-way ANOVA is the following (Weber and Skillings, 2000).

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta_{ij}) + \varepsilon_{ij}$$

where : Y_{ij} = response to the experimental unit at which a levels of A, b levels of B have been applied. A and B are the treatments namely enzymes (a = 3 levels) and hydrolysis times (b = 5 levels), respectively.

μ = overall mean observed

α_i = the effect of i^{th} treatment (i=1, 2,3)

β_j = the effect of j^{th} treatment (j=1,2,3,…5)

$\alpha\beta_{ij}$ = the effect of interaction between i^{th} level of A and j^{th} level of B

ε_{ij} = uncorrelated random error associated with the l^{th} experimental unit of the i^{th} and j^{th}.

Chapter 3

Fish Protein Hydrolysis: Optimising Conditions

3.1. Abstract

Optimising conditions for hydrolysis of fish muscle proteins with papain, bromelain and Flavourzyme™ showed that the three enzymes were suitable for the production of fish protein hydrolysates without pH adjustment. The use of 0.5% (w/v) of papain and bromelain, and 1.25% (v/v) of Flavourzyme™ for hydrolysis of soluble protein fractions and 1% (w/v) of papain and bromelain and 2.5% (v/v) of Flavourzyme™ for both trevally and leatherjacket were appropriate. The respective degrees of hydrolysis (DH) were 24.8, 25.4, and 21.6% for trevally, and 22.1, 22.7, and 23.0% for leathrjacket soluble proteins. The DH of both trevally and leatherjacket soluble proteins were significantly affected ($\alpha = 0.05$) by hydrolysis time but not affected by enzymes or interaction between enzymes and hydrolysis time. The respective DH of trevally and leatherjacket papain, bromelain, and Flavourzyme™ insoluble protein hydrolysates were 30.5, 31.0, 32.1, 30.0, 32.8, and 33.4%. The DH of trevally insoluble protein hydrolysate were significantly ($p < 0.05$) affected by type of enzymes, hydrolysis time, and interaction between enzymes and hydrolysis time. The DH of leatherjacket insoluble protein hydrolysates was significantly ($p < 0.05$) affected by hydrolysis times, but were not affected by enzymes and interaction between enzyme and hydrolysis time. The pH of all trevally and leatherjacket hydrolysates, except papain and Flavourzyme™ hydrolysates of trevally soluble proteins, changed significantly during the course of hydrolysis but remained within the optimum pH of all enzymes used. Electrophoresis results indicated that all hydrolysates consist of mostly peptides with molecular weight of less than 10 kDa.

3.2. Introduction

The process of protein hydrolysis is relatively simple. However, there are factors that have to be carefully taken into consideration before, during, and after hydolysis. These factors include the nature and quality of the raw materials, the choice of enzymes and pH condition (Kristinsson, 2006). The nature and quality of the raw materials are of great importance as it gives insight into the proximate composition and sensory properties of the raw material that can determine the functional quality of the hydrolysate produced. The choice of enzymes plays a very important role as each enzyme has its own specificity that will produce product with specific make-up, functional preperties and to some extent, if any, bioactivity. The choice of enzyme will also determine the pH condition and the optimum temperature for the enzyme to give an optimum impact on the hydrolysis process.

In this study, fresh trevally (*Pseudocaranx sp.*) and leatherjacket (*Meuchenia sp.*) were used as raw materials. Also, three different eznzymes: papain, bromelain, and Flavourzyme™ were chosen to hydrolyse the proteins. Therefore, the hydrolysis condition should be optimised to meet the characteristics of both the raw materials and the enzyme employed, and in order to produce the hydrolysates with desired degree of hydrolysis for the purpose of producing bioactive peptides.

This chapter includes the evaluation of the nature of the raw materials, the specificity of the enzymes, the optimised conditions for hydrolysis and the degree of hydrolysis (DH) as the indicator of the extent of the hydrolysis. These studies were carried using preliminary experiments. Also this chapter will cover preparation of fish protein hydrolysates under optimised condition which includes pH changes during hydrolysis, degree of hydrolysis and SDS-PAGE pattern of the hydrolysates.

3.3. Materials and methods

3.3.1. Raw materials and hydrolysis

Two species of fish, leatherjacket and trevally were purchased and used in this study. The fish was prepared and treated as outlined in section 2.1.2 and 2.1.3. The fish protein fractions were hydrolysed with papain, bromelain (Sigma-Aldrich, Castle Hill, NSW, Australia), and Flavourzyme™ (Novozyme, North Rocks, NSW, Australia) for two to ten hours. The hydrolysis process was terminated by immersing the reaction flasks and their contents in a boiling water bath for 15 min. The hydrolysates were then centrifuged to separate the undigested materials and then stored for analyses.

3.3.2. Analysis of raw materials

Analysis of the raw materials included protein content of fish muscle, water soluble and insoluble fractions, and pH as discribed in sections 2.5.1 and 2.5.2.

3.3.3. Analysis of the hydrolysates

The fish protein hydrolysates were analysed for their pH, degree of hydrolysis (DH), and electrophoresis pattern following the methods described in sections 2.5.1, 2.5.3, and 2.5.4. These analyses were aimed to ensure that the hydrolytic process progressed within the optimum pH of the enzymes employed, to evaluate the extent of the hydrolysis, and to observe the molecular weight distribution of peptides in the hydrolysates.

3.3.4. Statistical analysis

The protein contents and pH of the fish proteins fractions were analysed over two replications and five observations. The data were calculated and expressed as mean ± s.d. (standard deviation). Data obtained from the obsevations of pH and degree hydrolysis (DH) of the hydrolysates were analysed with analysis of variance (ANOVA) based on one-way or two-way design with two replication as described in section 2.10. MINITAB 15 software was used for this analysis. Tukey pairwise comparison test was carried out to investigate the difference between means (Weber and Skillings, 2000).

3.4. Results and discussions

3.4.1. The nature of the raw materials

As mentioned earlier, two species of fish were used in this study namely trevally and leatherjacket. Trevally is a pelagic fish with a wide zonal migration pattern covering the coastal areas as well as estuaries and deep into the demersal zone. As an active fish, trevally consume variety of foods include small fish, moluscs, and crustaceans. The proximate compostion of whole trevally is 19.4% protein, 4.5% fat, 72.3% moisture, and 3.9% ash (FAO, 2007). Therefore, trevally is considered a fatty fish and, as a typical pelagic fish, has dark muscle and high in blood. This characteristic will affect the quality of the hydrolysate due to lipid oxidation creating unpleasant flavour and odour as well as colour problem. Separating the soluble proteins prior to hydrolysis will also separate the other water soluble components such blood and pigments as well as reduce the fat content of the remaining insoluble protein pellet.

Leatherjacket (a filefish), on the other hand, is a demersal fish with sedentary migration pattern. As a sedentary fish that lives in rocky weedy reef areas,

leatherjacket consumes mainly encrusting animals (such as hydroids and barnacles), seaweeds, and jellyfish. The proximate composition of whole leatherjacket is 17.2% protein, 1.7% fat, 75.0% moisture and 6.2% ash (FAO, 2007). Therefore, leatherjacket is considered a lean fish and, as typical demersal fish, has white smooth flesh. Separation of water soluble proteins fraction will separate other water soluble components such as blood and pigment as well as fat, although these components will not cause a major problem to the quality of the hydrolysate.

Results from the analysis of the protein contents of trevally and leatherjacket flesh and their water soluble and insoluble fractions are shown in Table 3.1. The protein contents of both trevally and leatherjacket flesh are within the range of 19.4-21.1 g/100g and 18.4-19.2 g/100 g flesh (fillet) respectively (FAO, 2007). The water soluble fraction of both fish flesh is 27.1% and 25.5%, while the insoluble protein contents are 71.9% and 73.2% for the respective fish. The values of the water soluble fractions of both fish are lower than average 30% of the total protein (Sikorski et al., 1990). This is possible as the sample was minced rather than homogenised, therefore it is expected that not all sarcoplasmic proteins were extracted. Therefore, the remaining pellet, the insoluble fraction, contained some sarcoplasmic, myofibrillar and stromal proteins. These protein contents, however, indicate significant amount of substrate for the hydrolysis process.

Table 3.1. Protein contents of trevally and leatherjacket flesh and their water soluble and insoluble fractions*.

Fractions	Fish species	
	Trevally	Leatherjacket
Flesh[a]	19.6 ± 0.60	18.6 ± 0.43
Water Soluble	5.3 ± 0.28	4.7 ± 0.25
Insoluble[b]	14.1 ± 0.22	13.6 ± 0.31

* Expressed as g protein per 100 g flesh ± s.d. (n= 10).

[a] Sample was deboned, skinned, and minced. Extraction of water soluble proteins was carried out by means of constant shaking at 200 rpm, 5°C, and for one hour followed by centrifugation for 30 min at 5°C and 5580g.

[b] Fraction included myofibrillar and stromal proteins, as well as some entrapped and/or denatured soluble proteins.

3.4. 2. Enzymes specificity and hydrolysis condition

As mentioned earlier in section 2.1.3, three enzymes were employed in this study namely papain, bromelain and Flavourzyme™. The challenge was, therefore, to obtain an optimised condition to hydrolyse water soluble and insoluble protein fractions of two different species of fish. This included finding optimum concentration of each enzyme for each fraction and optimum hydrolysis temperature so the hydrolysis of all fractions could be carried out at the same temperature and at an optimum pace.

The optimum temperature of the three enzymes have been reported to be around 55°C for papain (Gildberg, 1994) while Kristinsson (2006) reported a higher temperature of 65°C-80°C. The optimum temperature for bromelain is between 45°C-65°C (Adler-Nissen, 1986; Kristinsson, 2006), while Flavourzyme™ has been sucessfully used at 50°C to hydrolyse food proteins (Klompong et al., 2007). Small scale hydrolysis at 45°C, 50°C, 55°C, 60°C and 65°C of trevally and leatherjacket proteins with the three enzymes during preliminary study showed that there was little

variation in the degree of hydrolysis at the hydrolysis temperature above 50^{o}C after the digested material was separated by means of centrifugation at 5580 x g. Therefore, 50^{o}C was chosen as the hydrolysis temperature for the three enzymes.

The concentrations of each enzyme used was determined following hydrolysis trials of each fraction with different concentrations of each enzyme for 10 h, a period at which most of the fish muscle proteins was digested as observed by the amount of undigested material left after centrifugation. The degree of hydrolysis (DH) of the hydrolysates was measured after all unhydrolysed material was precipitated and separated through centrifugation and filtration. The summary of the effect of different concetrations of enzymes on protein contents of hydrolysates is shown in Table 3.2.

Results from the one-way analysis of variance (ANOVA) for papain hydrolysate of trevally soluble protein showed that there was significant difference between means of different concentrations of the enzyme used. Post hoc pairwise comparison analysis between means by Tukey ($\alpha = 0.05$) test indicated that the mean of papain concentration of 0.25% (w/v) was different significantly ($p < 0.05$) from means of the other three concentrations while no significant differences were observed between papain concentration of 0.50% from 0.75%, and 0.50% from 1.00%. There was also no significant difference between papain concentrations of 0.75% from 1.00%. For leatherjacket soluble protein hydrolysates of 10-hour hydrolysis, the degree of hydrolysis for papain concentration of 0.25% was significantly ($p < 0.05$) different from the other concentrations, however there was no significant differences observed between the means of 0.5% and 0.75% papain, and between 7.5% and 1.0% papain, while a significant ($p < 0.05$) difference was observed between hydrolysates of 0.5% and 1.0% papain. Data and post hoc results (Table 3.2) indicated that there

were differences between means of the degree of papain hydrolysis of soluble protein fraction of both trevally and leatherjacket muscle proteins. These differences were significant ($p < 0.05$) between hydrolysates of 0.25% papain and those of other concentrations for hydrolysates of both fish species and between hydrolysates of 0.5% and 1.0% of leatherjacket soluble protein fraction. However, as there was rather little margin of differences between the degree of hydrolysis the papain hydrolysates from 0.5%, 0.75%, and 1.0% concentrations, then 0.5% (w/v) papain could be considered as an optimum concentration to hydrolyse soluble protein fraction of both fish species.

ANOVA of degree of hydrolysis of bromelain hydrolysate of trevally water soluble protein also indicated significant difference ($p < 0.05$) between treatments. Results from Tukey analysis showed that there is defference between means of 0.25% bromelain and the other concentrations, concentration of 0.50% was significantly different ($p < 0.05$) from 0.75% and 1-0%, while there was no difference observed between 0.75% and 1.00%. Bromelain hydrolysis of leatherjacket soluble protein also showed significant difference ($p < 0.05$) between concentratation of 0.25% and the other concentration and between 0-5% and 1-0%, but no difference was observed between 0.50% and 0.75%, and between 0.75% and 1.00%. These results indicated that 0.5% bromelain can give optimum hydrolysis condition for soluble protein of both fish species.

Table 3.2. Degree of hydrolysis of water soluble and insoluble fish protein hydrolysates at different concentrations of enzyme*.

Soluble Protein Fraction			Insoluble Protein Fraction		
Enzyme concetration[#]	Trevally	Leatherjacket	Enzyme concetration[#]	Trevally	Leatherjacket
Papain			Papain		
0.25	18.5a	17.1a	0.50	22.3a	23.4a
0.50	23.4b	21.9b	0.75	27.3b	28.0b
0.75	24.1b	22.2bc	1.00	30.3b	29.8c
1.00	23.9b	22.8c	1.25	30.4b	30.1c
Bromelain			Bromelain		
0.25	19.9a	19.2a	0.50	24.0a	25.4a
0.50	24.7b[a]	22.0b	0.75	29.6b	31.5b
0.75	25.1c[a]	22.2bc	1.00	30.9bc	32.2c
1.00	25.c	22.8c	1.25	31.1c	33.0c
Falvourzyme™			Falvourzyme™		
1.00	17.4a	18.6a	1.50	26.5a	25.7a
1.25	21.2b	23.3b	2.00	31.8b	29.3b
1.50	21.7bc	23.4b	2.50	32.5c	33.9b
1.75	22.0c	23.5b	3.00	32.8c	33.7b

* Number followed by different letters indicate significant difference ($p < 0.05$), $n = 6$.

[#] Expressed in % (w/v) for papain and bromelain and % (v/v) for Flavourzyme™.

[a] A very little margin of difference was observed between these two means.

ANOVA results from Flavourzyme™ hydrolysis of both trevally and leatherjacket soluble proteins showed that Flavourzyme™ concentration of 1.00% (v/v) was siginificanly different from the other concentrations. There was no difference, however, between Flavouzyme™ concentration of 1.25%, 1.50%, and 1.75% for leatherjacket soluble protein, while significant difference was observed between Flavourzyme™ concentration of 1.25% and 1.75% of trevally hydrolysates. The concentration of 1.25% (v/v) will then be used for further preparation of fish protein hydrolysates.

Based on these results, the concentration of 0.50% (w/v) of papain and bromelain, and 1.25% (v/v) Flavourzyme™ were chosen for the preparation of fish protein hydrolysates. The concentration of enzymes used in hydrolysis of fish proteins can vary greatly depending on individual preference, hydrolysis time, and on the purpose of the studies. The main objective of this study is to isolate anti-microbial and anti-hypertensive peptides. Most of the anti-microbial peptides had up to 50 amino acids residues, while most of the anti-hypertensive peptides consisted of less than 10 amino acid residues. The hydrolysis process, therefore, was focused on obtaining peptides from hydrolysis of fish protein with degree of hydrolysis between 10% and 35%. Various studies have shown that most of the anti-hypertensive peptides are of small peptides (Yokoyama et al., 1992; Fujita and Yoshikawa, 1999; Theodore and Kristinsson, 2007) that can be obtained from hydrolysis of food proteins with degree of hydrolysis (DH) of about 27% (Yust et al., 2003) to 30% (Theodore and Kristinsson, 2007). However, there are also reports of the findings of anti-hypertensive peptides from hydrolysates with DH values as low as 2-3% (Mullally et al., 1997) and as high as 67-81% (Qian et al., 2007). Therefore, it was concluded that enzyme specificity rather that DH values mainly determines the development of ACE

inhibitory peptides (FitzGerald and Meisel, 1999). However, as most findings were from hydrolysates with DH values around 30% or lower, hydrolysis of fish proteins up to 10 hours was considered sufficient for recovering bioactive peptides.

Studies on hydrolysis of insoluble proteins with papain, bromelain and Flzvourzyme™ of different concentrations were also carried out to obtain optimum concentration of each enzyme. Results from ANOVA followed by Tukey test for difference between means ($\alpha < 0.05$) are shown in Table 3.2. The concentrations of papain and bromelain used here were between 0.50% to 1.25%. For trevally protein hydrolysis, papain concentration of 0.50% was significantly different ($p < 0.05$) from the other concentrations, while there was no difference observed between 0.75%, 1.00% and 1.25%. A slightly different result was observed for leatherjacket protein hydrolysis, where 0.5% papain concentration was significantly different ($p < 0.05$) from the other three concentrations, 0.75% papain was significantly differerent ($p < 0.05$) from 1.0% and 1.25%, while there was no difference observed between 1.0% and 1.25%.

The results from bromelain hydrolysis of insoluble protein showed that there was no difference observed between 0.75% and 1.00%, and between 1.00% and 1.25% for trevally, while significant difference ($p < 0.05$) was observed between 0.5% bromelain hydrolysis and the other three concentrations, and between 0.75% and 1.25% bromelain. The effects of bromelain concentrations on leatherjacket insoluble proteins showed significant difference between 0.5% and the other three concentrations and between 0.75% with 1.0% and 1.25%, but there was no difference observed between 1.0% and 1.25% bromelain hydrolysis.

Results from Flavourzyme™ hydrolysis of trevally insoluble protein showed significant difference between 1.5% and the other three concentrations and between

2.0% with 2.5% and 3.0%, but there was no difference observed between 2.5% and 3.0% of Flavourzyme™ used to hydrolyse the proteins. For leatherjacket insoluble protein hydrolysis, significant difference was observed between 1.50% (v/v) from the other concentrations, while there was no difference observed between 2.0% Flavourzyme™ hydrolysis and those of 2.50% and 3.00%.

It has been reported that hydrolysis of food protein to around 30% degree of hydrolysis will produce peptides with molecular weights of mostly around 10 kDa or less. This is in line with the purpose of this study which was to obtain anti-hypertensive peptides having molecular weight mostly smaller than 10 kDa, while anti-microbial peptides can vary in sizes from less than 1000 Da to about 50 kDa. The concentration of 1.00% (w/v) for papain and bromelain, and 2.5% (v/v) for Flavourzyme™ was chosen for preparation of fish protein hydrolysates for future studies. This, albeit some differences the the degree of hydrolysis between 1.0% and either 0.75% or 1-25% for papain and bromelain, or between 2.5% Flavourzyme™ and either 2.0% or 3.0%, was mainly based on the fact that these concentration were able to produce hydrolysates with degree of hydrolysis as desired. The hydrolysis of fish protein was kept below 35% degree of hydrolysis as extended hydrolysis may reduce the bioactivity of the peptides produced (Chiang et al., 2006).

3.4.3. Preparation of fish protein hydrolysate

In this preparation, 0.50% (w/v) of papain and bromelain, and 1.25 (v/v) were used to hydolyse trevally and leatherjacket water soluble protein fractions, while 1.00% (w/v) of papain and bromelain, and 2.5% (v/v) of Flavourzyme™ were used to hydrolyse the insoluble fractions. This section will cover changes of the pH of

hydrolysates under optimised condition, degree of hydrolysis (DH) and SDS-PAGE pattern of hydrolysates produced during hydrolysis for up to 10 hours.

As mentioned in Chapter 2 section 2.1.2., water soluble and insoluble protein suspensions were hydrolysed with papain, bromelain and Flavourzyme™. Prior to hydrolysis, the temperature of the suspension was increased to 75^{o}C for 15 min to inactivate most of the endogenous enzymes, then the temperature was reduced to 50^{o}C, followed by addiditon of enzymes and hydrolysis for up to 10 h. An amount of sample was taken every two hour intervals, and the hydrolysis was terminated by immersing the flask in boiling water bath for 15 min, cooled to room temperature, then the pH and degree of hydrolysis were measured. Centrigugation of the hydrolysates was carried out at 5580 x g for 30 min and 5^{o}C and the supernatant was separated from the undigested material and frozen until analysis.

3.4.3.1. Changes in pH during hydrolysis

The changes in pH during hydrolysis was monitored in order to ensure that the hydrolysis process would not proceed beyond the optimum pH of enzymes employed. This was of significant inportance as the hydrolysis was carried out without pH adjustment. Results from ANOVA analysis showed that pH of trevally water soluble protein bromelain hydrolysates changed during the course of 10 h hydrolysis, while no significant ($p < 0.05$) changes were observed for papain and Flavourzyme™ hydrolysates. Enzyme bromelain and hydrolysis time as well as their interaction significantly ($p < 0.05$) affected the pH (Table 3.3, Figure 3.1) of trevally water soluble hydrolysates. However, the pH of the hydrolysates remained within the optimum pH of the enzyme. The pH of papain hydrolysate of trevally soluble proteins increased steadily as the hydrolysis progressed. The pH of the bromelain

hydrolysates increased from two to six hour hydrolysis then decreased as the hydrolysis continued. The pH of the Flavourzyme™ hydrolysates showed a decrease then increase pattern during hydrolysis. The increase or decrease of pH may be due to the release of basic or acidic peptides during hydrolysis.

Enzymes, hydrolysis time, and their interaction significantly ($p < 0.05$) affected the pH of letherjacket water soluble protein hydrolysates (Table 3.3, Figure 3.1). However, these changes did not go beyond the optimum pH of the three enzymes. These results suggest that hydrolysis of trevally and leatherjacket water soluble protein can be comfortably carried out without pH adjustment. The pH of leatherjacket soluble protein hydrolysates increased with little variation, as compared to trevally soluble protein hydrolysates, as hydrolysis progressed regardless of the type of enzymes involved. These differences may be due to different amino acid sequences of the soluble proteins of these two species that released peptides with different charges upon hydrolysis with the same enzymes.

Results from two-way ANOVA followed by Tukey test suggested that papain, bromelain and Flavourzyme™ significantly affected ($p < 0.05$) the pH of trevally insoluble hydrolysates (Table 3.4, Figure 3.3). The pH of papain hydrolysates increased as the hydrolysis proceeded, while the pH of bromelain hydrolysates showed a trend of increasing up to six hour hydrolysis then decreased slightly. The pH of Flavourzyme™ hydrolysates showed decreasing pattern during hydrolysis. A similar pattern took place at six hour hydrolysis in which all hydrolysates showed increased pH pattern that indicates the release of negatively charged peptides.

Enzymes papain, bromelain and Flavourzyme™ did give significant changes ($p < 0.05$) in the pH of leatherjacket insoluble hydrolysates (Table 3.4, Figure 3.2) during hydrolysis. In general, the pH of all hydrolysates increased as the hydrolysis

progressed after four hours. The change of pH was observed at 6 h hydrolysis as all hydrolysates showed a rather sharp increase in pH, while the pH of Flavourzyme™ hydrolysates of 4 h and 8 h hydrolysis showed a decreasing trend during which acidic peptides may have been released.

The pH changes in both trevally and leatherjacket insoluble protein hydrolysates remained within the optimum pH of all enzymes used in this study, therefore, hydrolysis of these protein fractions up to 10 h without pH adjustment did not affect the hydrolysis. In general, the pH of Flavourzyme™ hydrolysates of both soluble and insoluble trevally and leatherjacket proteins showed various increase and decrease pattern as compared to both papain and bromelain hydrolysates. Flavorzyme™ is an endo and exopeptidase that can extensively cleave protein molecules into peptides and amino acids that may account for the dynamic pH changes of the hydrolysates. Papain and bromelain used in this study are the crude extract which usually have broad specificity hence are expected to release various peptides with different amino acid residues at their C-terminal ends as compared to their pure enzymes counterparts.

Table 3.3. Changes in pH of trevally and leatherjacket soluble protein hydrolysates during hydrolysis.*

Hydrolysis Time (h)	Trevally			Leatherjacket		
	Papain	Bromelain	Flavourzyme™	Papain	Bromelain	Flavourzyme™
2	6.23a	6.22a	6.28a	6.21a	6.21a	6.30a
4	6.25a	6.23a	6.26a	6.22a	6.22a	6.31a
6	6.27a	6.28b	6.28a	6.23a	6.26ab	6.34b
8	6.28a	6.27b	6.26a	6.29bc	6.29bc	6.35b
10	6.28a	6.26b	6.27a	6.30c	6.34c	6.41c

* Number followed by different letters in the column indicate significant difference ($p < 0.05$), $n = 6$.

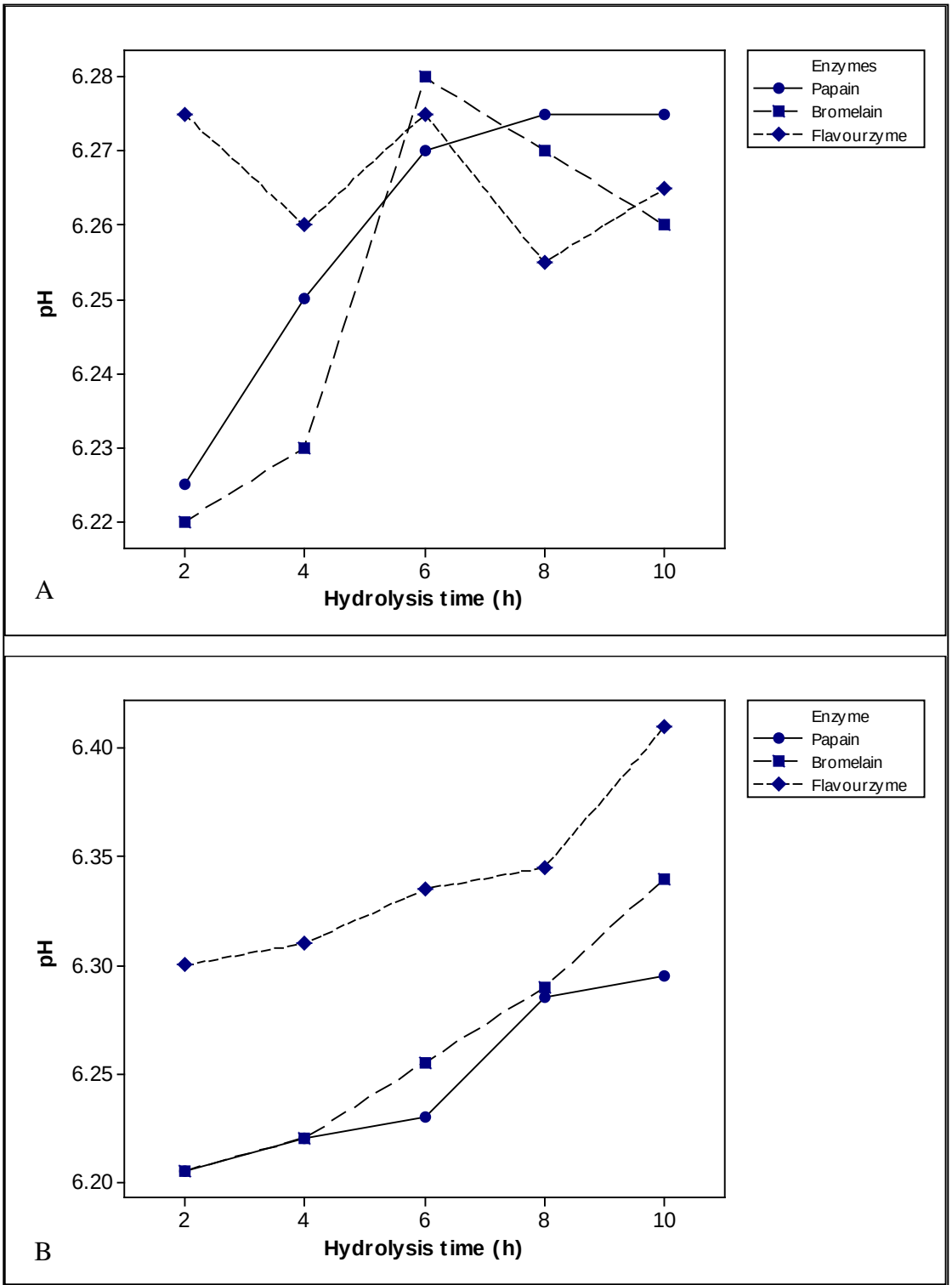
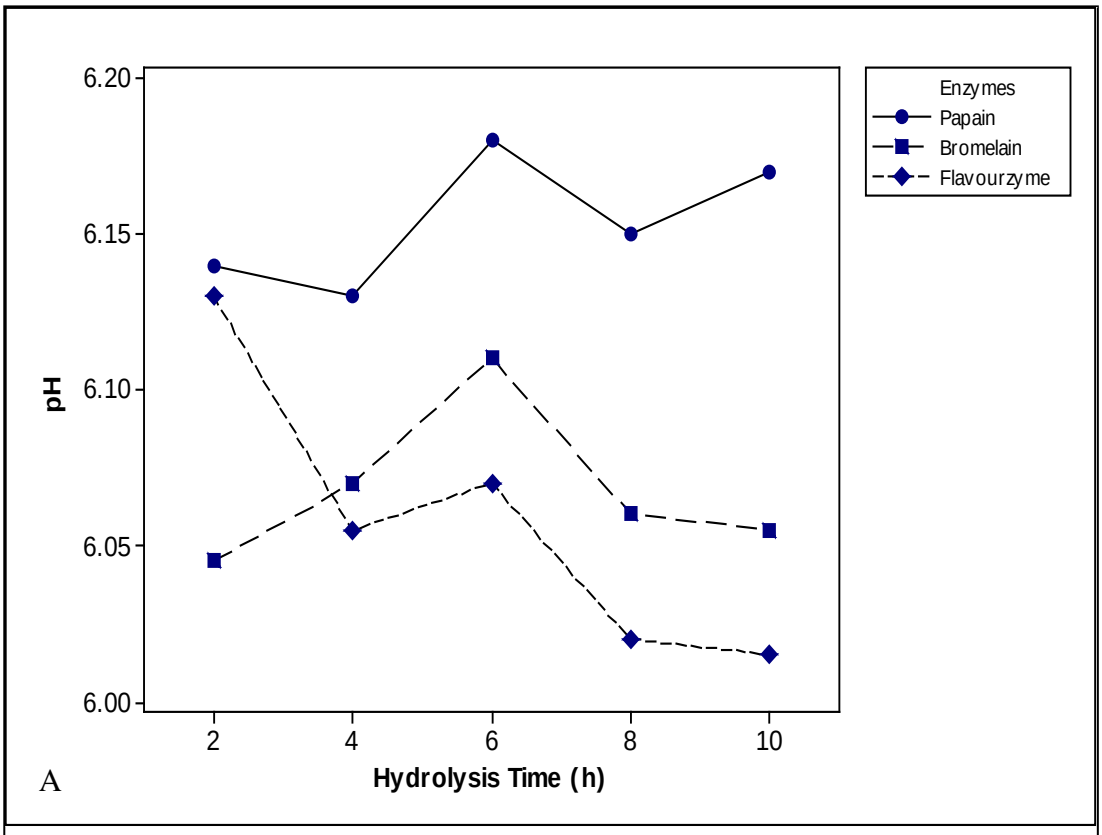


Figure 3.1. Interaction plots of pH vs hydrolysis times of trevally (A) and leatherjacket (B) water soluble protein hydrolysates (n = 6).

Table 3.4. Changes in pH of trevally and leatherjacket insoluble protein hydrolysates during hydrolysis.

Hydrolysis Time (h)	Trevally			Leatherjacket		
	Papain	Bromelain	Flavourzyme™	Papain	Bromelain	Flavourzyme™
2	6.14a	6.05a	6.13a	5.99a	5.96a	6.00a
4	6.13a	6.07a	6.06a	5.99a	5.96a	5.99a
6	6.18b	6.11b	6.07a	6.08b	6.04b	6.05b
8	6.15b	6.06a	6.02b	6.09b	6.05b	6.04b
10	6.17b	6.06a	6.02b	6.11b	6.07b	6.07b

* Number followed by different letters in a column indicate significant difference ($p < 0.05$), $n = 6$.



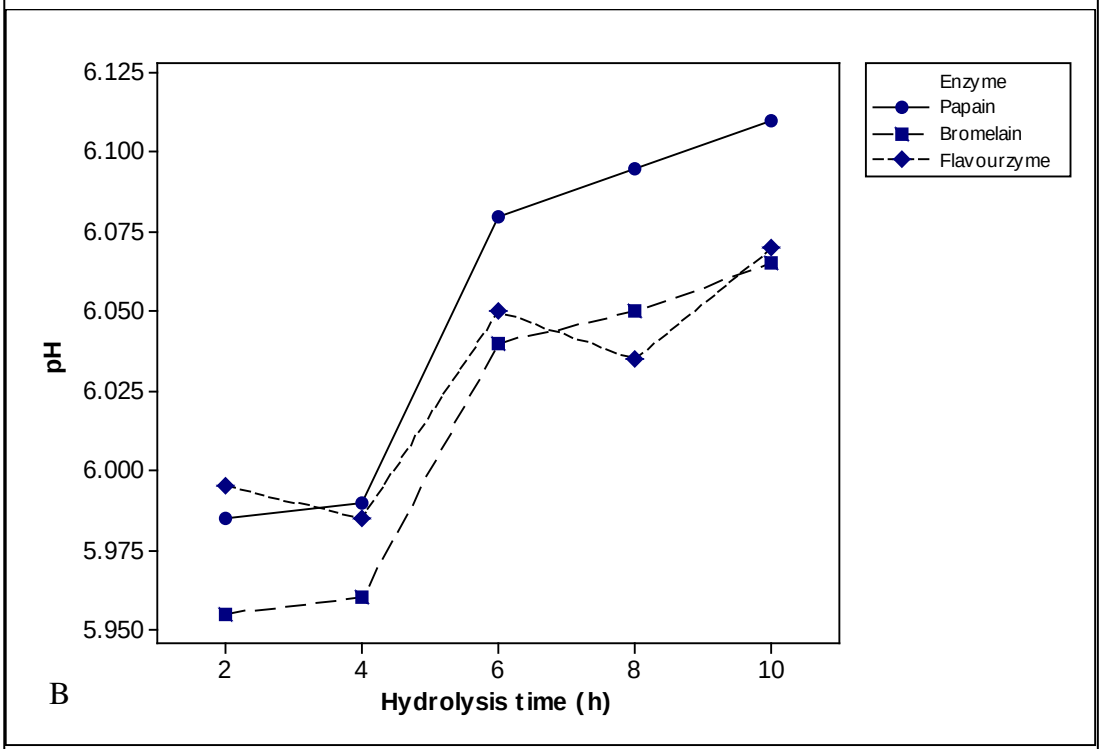


Figure 3.2. Interaction plots of pH vs hydrolysis times of trevally (A) and leatherjacket (B) water insoluble protein hydrolysates (n = 6).

3.4.3.2. Degree of hydrolysis (DH)

The degree of hydrolysis was determined following a spectrometric method (Adler-Nissen, 1979) with modifications to fit the use of a microplate reader. While there are a few other methods of choice for this purpose, this method was chosen due to the method used for the sample preparation in which the pH of the hydrolysis condition was not adjusted during the course of hydrolysis. Under pH adjusted condition, the degree of hydrolysis can be calculated directly from the amount of NaOH consumed to keep the pH at a certain point. However, this method is more applicable in the situation in which the hydrolytic process is carried out in a fermentor and the pH is continuously monitored and adjusted.

The method used in this study is more of an endpoint application and can be used for any hydrolysis and fermentation products under any hydrolytic conditions. In principle, this method is based on the reaction between primary amino acids with 2,4,6-Trinitrobenzenesulphonic acid (TNBS) under basic conditions and the absorbance is measured at 340 nm. The concentration of the primary amino acids is determined by means of a standard curve prepared from L-leucine of different concentrations.

Results from statistical analysis of the water soluble protein hydrolysates' degree of hydrolysis for both trevally and leatherjacket are shown in Table 3.5 and Figures 3.3 (A and B). This result showed that hydrolysis time gave significant effect ($p < 0.05$) on the degree of hydrolysis of trevally soluble protein, while enzymes or interaction between enzymes and hydrolysis time did not affect the degree of hydrolysis. The DH values of all hydrolysates increased steadily up to 25% as hydrolysis progressed.

The degree of hydrolysis of lethaerjacket soluble protein hydrolysates was also significantly ($p < 0.05$) affected solely by hydrolysis time, while enzymes or interaction between enzymes and hydrolysis time did not affect the degree of hydrolysis. The DH values of trevally and leatherjacket water soluble protein hydrolysates indicated different affinity of the proteins toward different enzymes. Papain and bromelain seemed to have more affinity toward water soluble proteins of trevally, while bromelain and Flavourzyme™ showed more affinity toward leatherjacket water soluble proteins. These differences may be due to the amino acid sequence of the proteins that related to the specificity of the enzymes used. The degree of hydrolysis of trevally and leatherjacket water soluble protein hydrolysates ranged from 12-25% during hydrolysis from 2-10 h. These values indicated that the peptides produced from hydrolysis will be small enough to exert any bioactivity should the enzymes specivity and amino acid sequence of the native protein do match each other.

Table 3.5. Degree of hydrolysis (DH) of trevally and leatherjacket soluble protein hydrolysates during hydrolysis.

Hydrolysis Time (h)	Trevally			Leatherjacket		
	Papain	Bromelain	Flavourzyme™	Papain	Bromelain	Flavourzyme™
2	13.37a	12.38a	14.59a	12.86a	12.40a	12.31a
4	15.21ab	14.66a	14.59ab	16.80b	16.64b	17.10b
6	19.32bc	18.83b	17.76bc	18.42bc	17.15bc	19.37b
8	20.75cd	22.04bc	19.15cd	20.90cd	19.61cd	21.00bc
10	24.81d	25.43c	21.60d	22.01d	22.67d	22.99c

* Number followed by different letters in the column indicates significant difference ($p < 0.05$), $n = 6$.

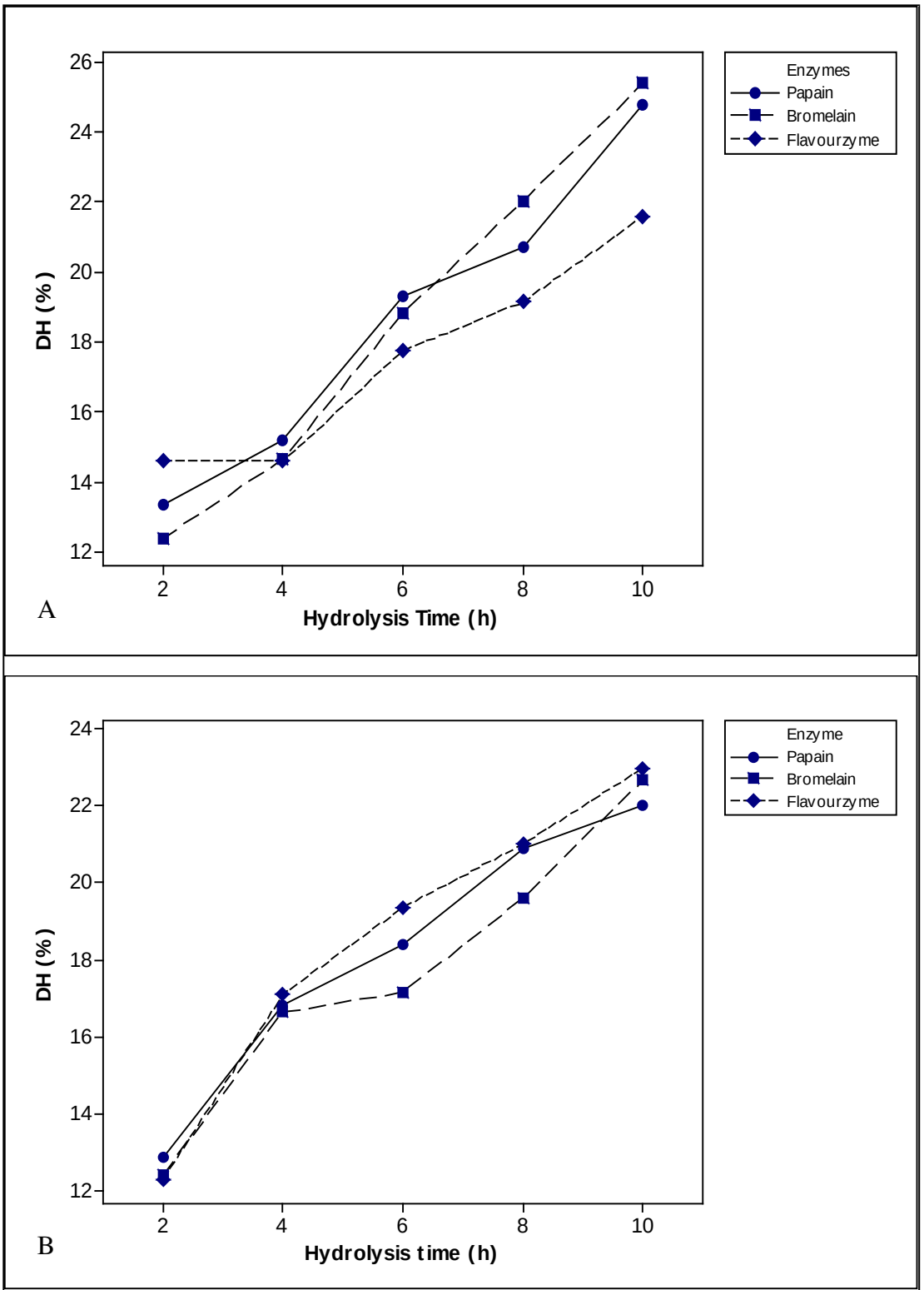


Figure 3.3. Interaction plots of the degree of hydrolysis vs hydrolysis times of trevally (A) and leatherjacket (B) soluble hydrolysate at different hydrolysis time (n = 6).

The degree of hydrolysis of trevally and leatherjacket insoluble protein hydrolysates are shown in Table 3.6, while Figure 3.4 (A and B) show the interaction plot between hydrolysis time and enzymes. The degree of hydrolysis of trevally insoluble protein hydrolysate was significantly affected ($p < 0.05$) by enzymes, hydrolysis time, and interaction between enzymes and hydrolysis time. The degree of hydrolysis of trevally insoluble protein increased to over 30% after 10 h hydrolysis. With this level of degree of hydrolysis, the hydrolysates are expected to contain mostly peptides with molecular weight of about 5 kDa to 10 kDa, and preferable in terms of the possibility to produce bioactive peptides (especially anti-hypertensive peptides that are usually small peptides).

The degree of hydrolysis of leatherjacket insoluble protein hydrolysates was significantly affected by hydrolysis times, while enzymes and interaction between eanzyme and hydrolysis time did not affect the degree of hydrolysis. The degree of hydrolysis of leatherjacket insoluble proteins also increased to over 30% as the hydrolysis time progressed up to 10 h. Likewise, it is expected that the hydrolysate with these level of degree of hydrolysis will produce bioactive peptides.

Table 3.6. Degree of hydrolysis (DH) of trevally and leatherjacket insoluble protein hydrolysates during hydrolysis.

Hydrolysis Time (h)	Trevally			Leatherjacket		
	Papain	Bromelain	Flavourzyme™	Papain	Bromelain	Flavourzyme™
2	17.14a	13.85a	17.12a	14.04a	14.79a	13.76a
4	18.47a	16.51b	17.12a	18.26a	17.52b	17.86ab
6	19.26a	21.91c	24.38b	22.80bc	22.60c	22.35bc
8	24.19b	26.51d	26.99b	24.65c	28.47d	25.38cd
10	30.49c	30.99e	32.12c	30.04d	32.81e	33.36d

* Number followed by different letters indicate significant difference ($p = 0.05$), $n = 6$.

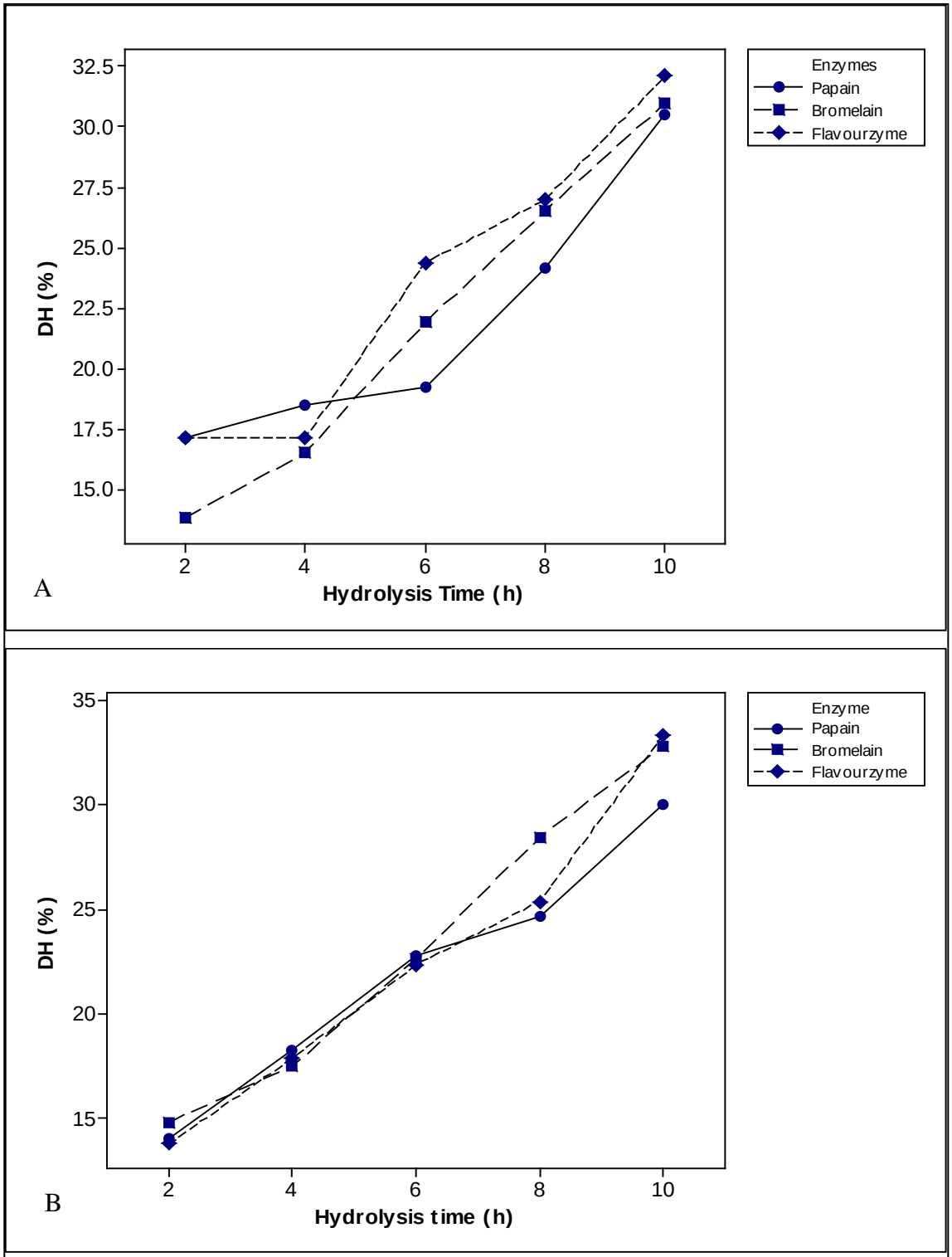


Figure 3.4. Interaction plots of the degree of hydrolysis vs hydrolysis times of trevally (A) and leatherjacket (B) insoluble hydrolysate at different hydrolysis time (n = 6).

3.4.3.3. SDS-PAGE of fish protein hydrolysates

The SDS-PAGE electrophoresis was performed on 15% gel (Laemmli, 1970) to observe the degree of hydrolysis and molecular weight of the peptides produced as compared to peptide markers. While this procedure was only qualitative in nature without incorporating the use of a densitometer, it can be a useful guide to predict the extent of hydrolysis.

The SDS-PAGE pattern of papain hydrolysates of trevally soluble and insoluble proteins is shown in Figure 3.5 (A and B). As compared to the peptide marker, the soluble protein hydrolysates were of molecular weight of about 8 kDa after hydrolysis for 10 h. The insoluble protein hydrolysates showed a band of aboud 6 kDa. It can be seen that the bands of the insoluble protein hydrolysates are lighter than the the hydrolysates of soluble proteins. This is an indication of the extent of hydrolysis, as the higher the degree of hydrolysis the smaller the peptides produced or even to the extent producing amino acid that will not conjugate with coomasie brilliant blue, hence can not be visualised.

The electrophoretogram of bromelain hydrolysates of soluble trevally proteins (Figure 3.6) showed that the peptides produced at two hour hydrolysis were of molecular weight of about 10 kDa and the size decreased to around 3 kDa as the hydrolysis time proceeded to 10 h. The hydrolysates of trevally insoluble proteins also showed molecular weight of about 10 kDa after two hour hydrolysis. As the hydrolysis progressed, smaller peptides were formed and the bands tended to fade.

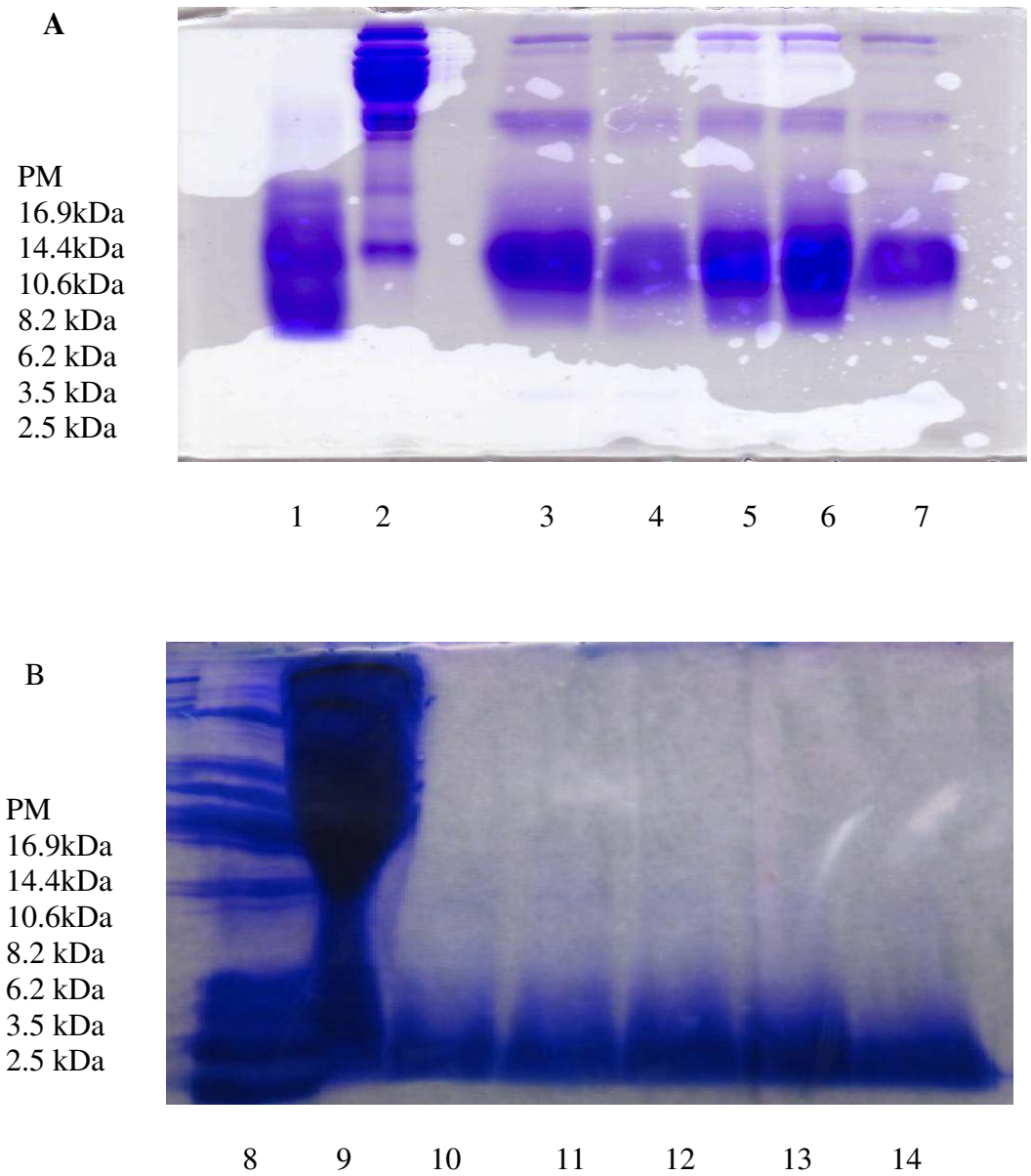


Figure 3.5. Electrophoretogram of papain hydrolysate of trevally soluble proteins (A) and insoluble proteins (B). Lanes: 1 = peptide marker, 2 = soluble protein fraction, protein fraction, 3-7 = 2-10 h hydrolysates of soluble protein fraction. Lanes: 8 = peptide marker, 9 = insoluble protein fraction, 10 = insoluble protein fraction, 10-14 = 2-10 h hydrolysates of soluble protein fraction. Hydrolysate samples were loaded at different amount (20 µl for soluble protein hydrolysates and 40 µl concentrated insoluble hydrolysates) due the coloration problem associated with insoluble hydrolysate samples.

Papain and bromelain are endopeptidases. As the concentration of these enzymes was quite low (0.50% and 1.00% w/v), the degree of hydrolysis was also rather low especially for soluble protein fraction. The protein content of soluble protein fraction was also somewhat lower than that of insoluble fraction; therefore the chance for enzyme and the proteins to collide and initiate a hydrolysis reaction was also lower. This is, however, not a major concern as the intention of this study is to obtain bioactive peptides that usually produced at degree of hydrolysis between 20% and 35%.

The electrophoregram of Flavourzyme™ hydrolysates of trevally soluble and insoluble proteins are shown in Figure 3.7 (A and B). The electrphoregram indicated that the hydrolysates of soluble protein are of molecular weight of about 10 kDA after two hour hydrolysis and becoming smaller as the hydrolysis time progressed. The molecular weight of insoluble hydrolysates were smaller than that of the soluble fraction. The degree of hydrolysis of Flavourzyme™ hydrolysates were slightly higher than both papain and bromelain hydrolysates as this was a rather stronger enzymes that is normally used for extensive hydrolysis of food proteins. Flavourzyme™ is a mixture of endopepidase and exopeptidase and has been reported to produce ACE inhibitory peptides, but the activity of this peptides decreased as hydrolysis time increased probably due to further cleavage of the active site by this enzyme (Chiang et al., 2006). Hence, for the interest of producing bioactive peptides, the degree of hydrolysis must be controlled by limiting the concentration of the enzyme and the hydrolysis time.

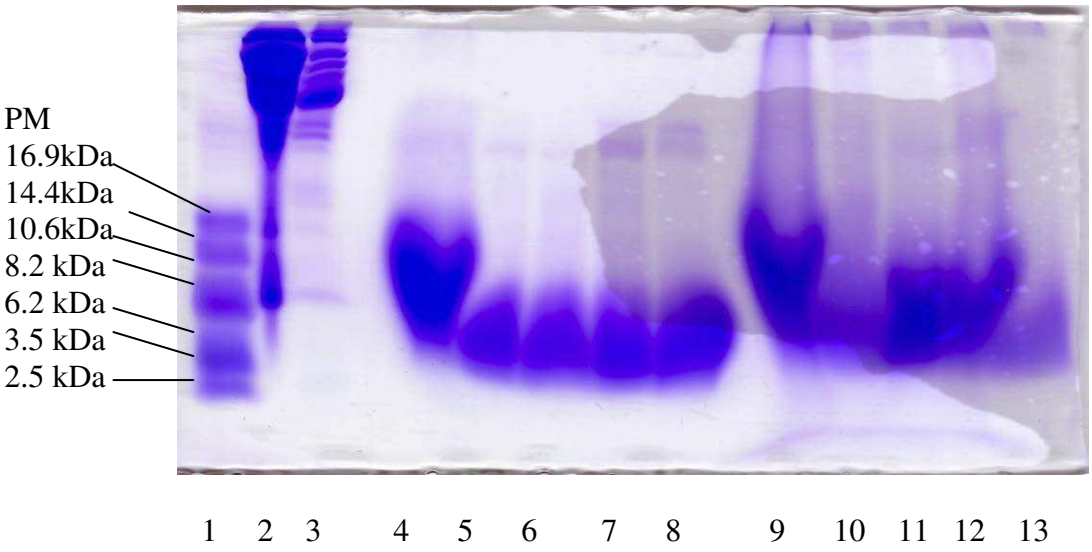


Figure 3.6. Electrophoretogram of bromelain hydrolysate of trevally soluble and insoluble proteins. Lanes: 1 = peptide marker, 2 = soluble protein fraction, 3 = insoluble protein fraction, 4-8 = 2-10 h hydrolysates of soluble protein fraction, 9-13 = 2-10 h hydroysates of insoluble protein fractions.

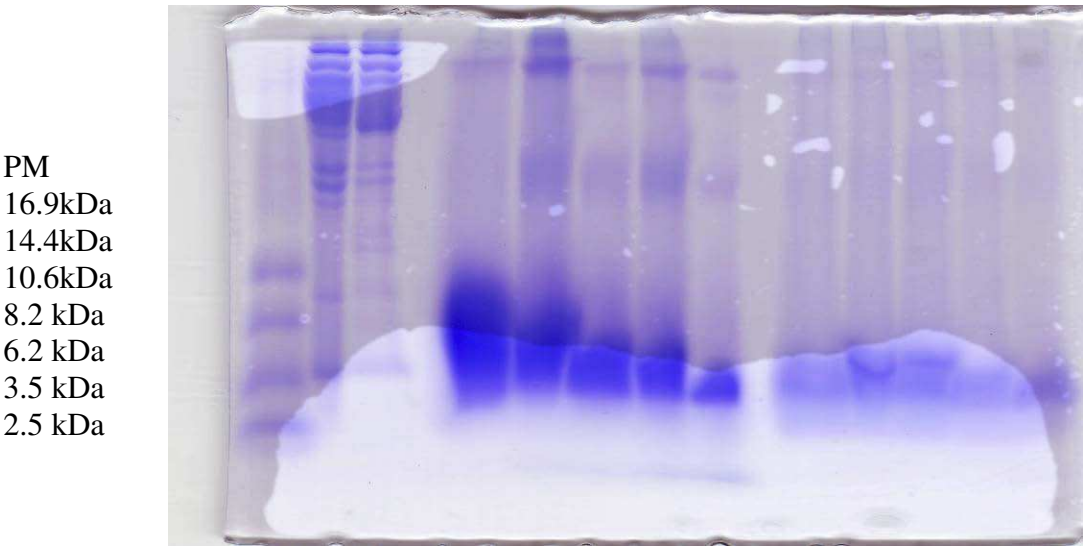


Figure 3.7. Electrophoretogram of Flavourzyme™ hydrolysate of trevally soluble and insoluble proteins. Lanes: 1 = peptide marker, 2 = soluble protein fraction, 3 = insoluble protein fraction, 4-8 = 2-10 h hydrolysates of soluble protein fraction.

Electrophoretogram of papain hydrolysates of leatherjacket soluble and insoluble proteins is shown in Figure 3.8. The papain hydrolysates of soluble protein showed strong bands of about 8 kDa as compared to peptide marker. The insoluble protein hydrolysates were having a more fading band that indicate a slightly higher degree of hydrolysis. Results from DH analysis showed that hydrolysate of insoluble leatherjacket protein was having up to 32% degree of hydrolysis as compared to 22% for the water soluble fraction. This difference was well visualised in the intensity of the electrophoresis lanes. Bromelain hydrolysates of leatherjacket soluble and insoluble protein (Figure 3.9) also showed similar pattern as those of the papain hydrolysates. The hydrolysates of insoluble protein fraction of 6 to 10 h hydrolysis were less intense than the two previous bands and indication of further hydrolysis of proteins into smaller peptides. Data from degree of hydrolysis showed that the DH value for bromelain hydrolysate was up to 32% after 10 h hydrolysis, hence the reason for fading bands. As in bromelain hydrolysates, Flavourzyme™ hydrolysates of leatherjacket soluble and insoluble protein (Figure 3.10) also tended to show intense bands for soluble protein hydrolysates and faded band for insoluble hydrolysates.

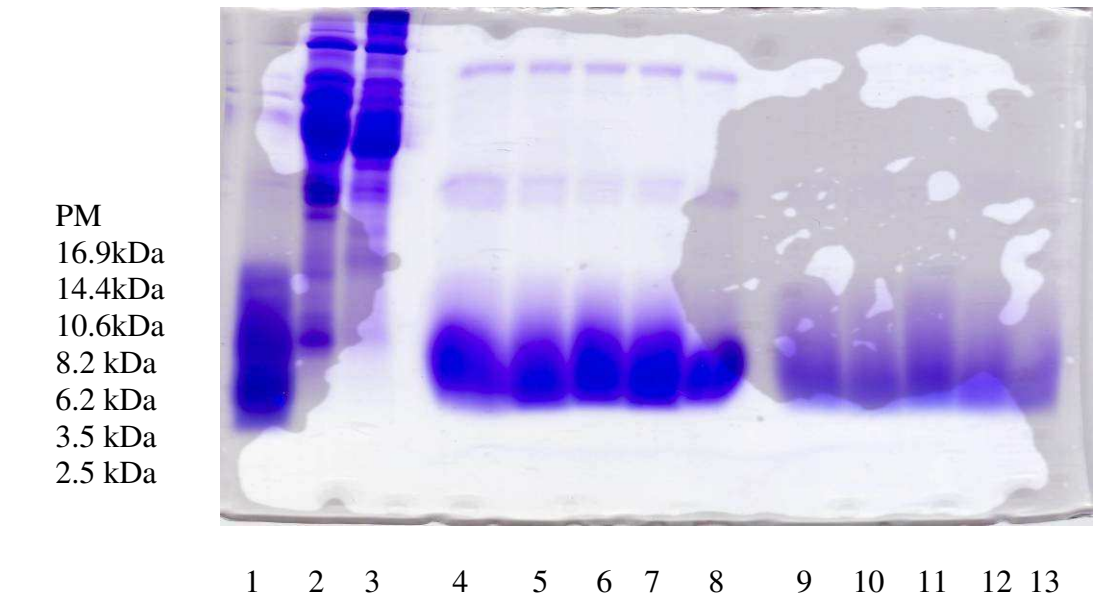


Figure 3.8. Electrophoretogram of papain hydrolysate of leatherjacket soluble and insoluble proteins. Lanes: 1 = peptide marker, 2 = soluble protein fraction, 3 = insoluble protein fraction, 4-8 = 2-10 h hydrolysates of soluble protein fraction, 9-13 = 2-10 h hydroysates of insoluble protein fractions.

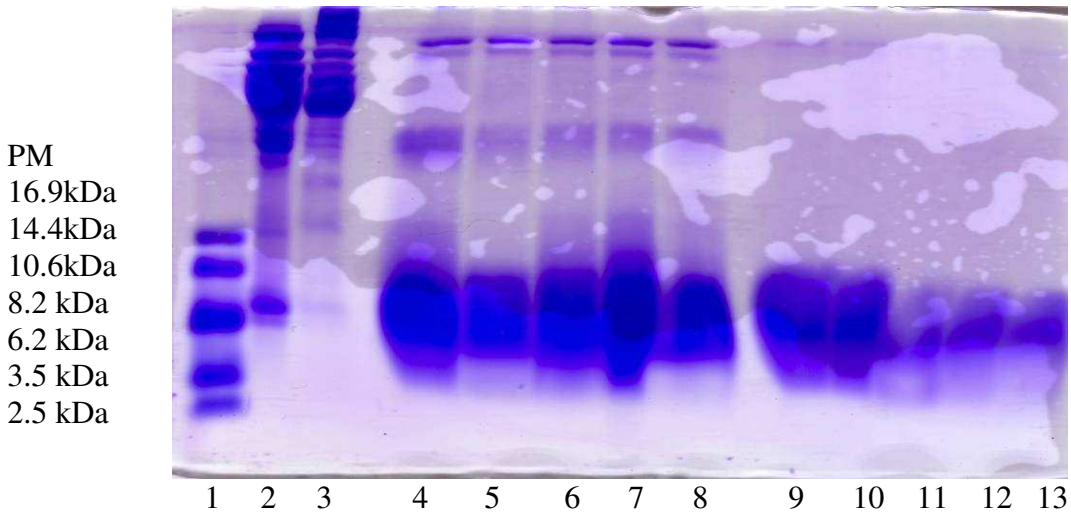


Figure 3.9. Electrophoretogram of bromelain hydrolysate of leatherjacket soluble and insoluble proteins. Lanes: 1 = peptide marker, 2 = soluble protein fraction, 3 = insoluble protein fraction, 4-8 = 2-10 h hydrolysates of soluble protein fraction, 9-13 = 2-10 h hydroysates of insoluble protein fractions.

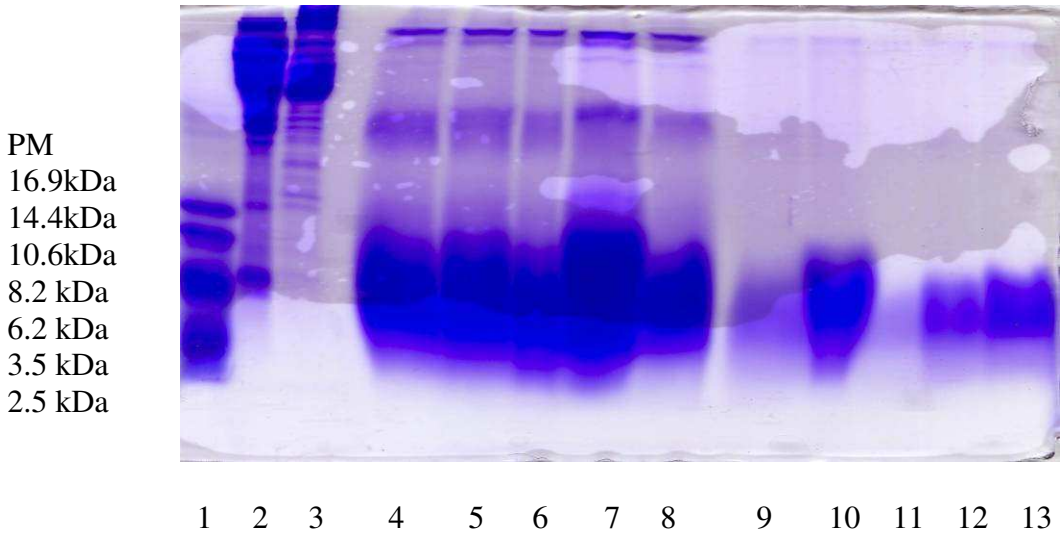


Figure 3.10. Electrophoretogram of Flavourzyme™ hydrolysate of leatherjacket soluble and insoluble proteins. Lanes: 1 = peptide marker, 2 = soluble protein fraction, 3 = insoluble protein fraction, 4-8 = 2-10 h hydrolysates of soluble protein fraction, 9-13 = 2-10 h hydroysates of insoluble protein fractions.

Visualisation of the proteins and peptides with Coomassie briliant blue is the most commonly used method in gel electrophoresis such SPS-PAGE. The colouration is based on its binding to proteins via physical absorption to arginine, the aromatic amino acids and histidine, hence the presence of these amino acids in protein samples determine good colouration. As indicative from the electrophoregrams, Coomassie briliant blue gave good colouration with proteins/peptides to a certain degree and as the hydrolysis continued and smaller peptides are released faded band appeared indicating the lack of the above amino acid residues in peptides released. Therefore, while this dye is useful for proteins, it is not quite useful to determine degree of hydrolysis by using Coomassie briliant blue in SDS-PAGE of peptides especially if the degree of hydrolysis is quite high. The analysis of the degree of hydrolysis with colorimetric TNBS method is therefore sufficient, while the use of SDS-PAGE can give further supportive information of the molecular weight distribution of the peptides produced.

3.5. Conclusion

Results from the preliminary studies showed that hydrolysis of trevally and leatherjacket soluble and insoluble proteins can be carried out at 50^{o}C with papain, bromelain , and Flavourzyme™ without pH adjustment. The hydrolysis process up to 10 h could reach degree of hydrolysis of around 30% which was suitable as the intention of this study is to find bioactive peptides for anti-microbial and anti-hypertensive uses. There is supporting report to suggest that hydrolysis of food protein up to 30% degree of hydrolysis is likely to produce bioactive peptides, while extentive hydrolysis will reduce the likelihood of finding them.

Hydrolysis of fish protein with papain, bromelain, Flavourzyme™ up to 10 h did change the pH significantly ($p < 0.05$) but did not cause the pH of the hydrolysates to fall beyond the optimum pH of all enzymes. Data from analysis of degree of hydrolysis showed that hydrolysis time significantly affected the degree of hydrolysis of trevally and leatherjacket soluble and insoluble protein, and hydrolysis up to 10 h did not increase the degree of hydrolysis value beyond 35% as the amount of enzyme used were quite low. SDS-PAGE patterns of all hydrolysates did indicate that most the peptides produced were having molecular weight of around 3 – 10 kDa as compared to peptide merker. However, visualisation of peptides with Coomassie briliant blue did not seem to be appropriate indicating lack of certain amino acid residues in the released peptides that usually bind the dye.

The production of fish muscle protein hydrolysates using papain, bromelain, and Falavourzyme™ without pH adjustment with the aim of producing bioactive peptides can be achieved and the products are subjected to screening for their activities against angiotensin I-converting enzymes (ACE) (Chapter 5) and some pathogenic microbial

strains (Chapter 4). The use of adjusted pH procedure in the preparation of fish hydrolysates and other protein hydrolysates are common in order to control the hydrolysis as well as to limit hydrolysis time. However, this process is costly as basic solution such as sodium hydroxyde (NaOH) is usually added to control the pH. As NaOH is accumulated in the product, certain follow up procedures have to be carried out to remove it from the products, hence increases production cost. The result from this study is, therefore, an indication of an alternative way of producing protein hydrolysates at a lower production cost.

Chapter 4

Anti-microbial Peptides derived from Fish Protein Hydrolysates

4.1. Abstract

Anti-microbial peptides have been reported to exhibit a broad range of activities against various microorganisms including Gram-positive and Gram-negative bacteria, virus, fungi, and protozoa. In this study, papain, bromelain, and Flavourzyme™ hydrolysates of leatherjacket (*Meuchenia* sp.) and trevally (*Pseudocaranx* sp.) were used for screening the anti-microbial peptides. Results from anti-microbial assays showed that 8 h bromelain hydrolysate of leatherjacket insoluble protein fraction (labelled as LBI8H) showed strongest activity. Two active fractions, fraction 9 (labelled as LBI9) and 12 (labelled as LBI12), showed activity against *Staphylococcus aureus* and *Bacillus cereus*, but no activity against *Escherichia coli* and *Candida albicans*. Fraction LBI9 exhibited some activity against *B. cereus* without a MIC being reached at 5.35 mg/ml peptide concentration. Peptide fraction LBI12, on the other hand, showed activity against both *B. cereus* and *S. aureus* with an MIC value of 4.3 mg/ml. Edman N-terminal sequence analysis revealed that the purified active fraction LBI12 consisted of mainly heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ; MW = 858.9 Da; net charge = -2) and another minor amount of peptide Ala-Pro-Ile-Asp-Asn-Leu-Gln (APIDNLQ; MW = 769.85 Da; net charge = -1). Anti-microbial activity assays using synthetic EQIDNLQ revealed the heptapeptide is active against both testing pathogenic bacteria having MIC values of 5.3 mg/ml and 7.96 mg/ml against *B. cereus* and *S. aureus*,

respectively. This active peptide is an anionic peptide that is active against Gram-positive bacteria.

4.2. Introduction

Anti-microbial peptides also known as nature's antibiotics are a group of bioactive peptides that exert anti-microbial activities and are naturally occurring or derived from hydrolysis of proteins. Numerous anti-microbial peptides have been isolated and characterised (Chan and Li-Chan, 2006; Dziuba et al., 2009) having four to more than 50 amino acid residues. The presence of these peptides enhances the immunity of the hosts and their resistance against pathogenic microorganisms and diseases in their natural environments. Recent studies indicated that anti-microbial peptides played multiple roles in their hosts defence, inflammation, and tissue regeneration (Chan and Li-Chan, 2006).

Anti-microbial peptides have been isolated from various organisms including mammalians, plants, fish, crustaceans, molluscs, microorganisms, dairy, and egg proteins. Most of these peptides are naturally occurring, while some are derived from food proteins. The naturally occurring anti-microbial peptides comprised of ribosomally and non-ribosomally synthesised peptides (Hancock and Chapple, 1999). The ribosomally synthesised peptides are recognised as part of innate immunity found throughout evolutionary tree but shows little sequence homology which suggests that each peptide has evolved (probably convergently) to optimally act against local micro-organisms in the environment in which it is produced (Hancock and Chapple, 1999) and are found in parts such as skin and venom indicating their readiness to face risks from the environment. The non-ribosomally synthesised peptides are produced mainly from bacteria and usually contain non-protein constituents in the polypeptide chain which are

either derived from modification of the chain or originated from non-ribosomal system (Kleinkauf and Von Dohren, 1987).

A limited number of anti-microbial peptides have been derived from food proteins through hydrolysis. Milk proteins such as α-lactalbumin, β-lactoglobulin, and especially lactoferrin have been indicated as the sources of anti-microbial peptides (Dziuba et al., 2009). Egg lysozyme, an enzyme commonly used as a preservative agent, exerts anti-microbial action through enzymic lyses of Gram-positive bacteria cell membrane. However, as heat and genetically inactivated lysozyme also showed the same anti-microbial activity, it was assumed that lysozyme contains structurally active fragments within its primary structure. This leads to the discovery of the active sequence after clostripain hydrolysis of inactive lysozyme (Pellegrini et al., 1997).

As mentioned earlier, there is little homology of the anti-microbial peptides that occur naturally, and with the limited number of anti-microbial peptides derived from food proteins meant little similarities would be expected from the primary sequences of the existing active peptides. These peptides also differ in length, amino acid composition, charge, hydrophobicity, and secondary structure. Production of anti-microbial peptides through hydrolysis of food proteins has been fruitful in the case of milk and egg white proteins. This may be due to the role of foods in human health that provide not only nutrients for growth but also source of beneficial compounds as natural agents to promote health. This chapter will, therefore, cover various methodologies for screening, analysis, purification, and characterisation of anti-microbial peptides from hydrolysates of fish muscle proteins.

4.3. Materials and methods

4.3.1. Materials

The hydrolysates (120 samples in total) obtained from the hydrolysis of fish muscle proteins (as detailed in section 2.1.2) and peptide fractions from fractionations of active hydrolysates were used for screening of anti-microbial peptides. The concentrations of the hydrolysates and the peptide fractions were adjusted to 30 mg/ml and 10 mg/ml, respectively, prior to assays. Three strains of pathogenic bacteria i.e. *Escherichia coli* 185, *Bacillus cereus* 106, and *Staphylococcus aureus* 184, and fungi *Candida albicans* X26 were used for the screening. Commercial antibiotics tetracycline and polymyxin B sulphate were used as positive inhibition controls (see section 2.2.4). Microbiological media used included Luria broth for *E. coli*, brain heart infusion (BHI) broth for *B. cereus* and *S. aureus*, and Sabouraud liquid medium for *C. albicans*. Methods for the preparation of these media are described in section 2.3.4. Some other additives such as glucose, NaCl, and tryptone were also used in this assay.

4.3.2. Anti-microbial activity assay

Anti-microbial assay was carried out using the above mentioned pathogenic bacteria in a multiwell plate with tetrazolium (XTT) salt as colouring agent (Tunney et al., 2004). Standard microdilution method (Andrews, 2001) was also carried out to verify the results from the XTT method. The extent of inhibition was observed and quantified spectrophotometrically on a BioRad Benchmark Plus microplate reader (BioRad Laboratories, Gladesville, NSW, Australia). Anti-fungal activity was performed against *C. albicans* (Hazen and Howell, 2004) with some modification to use Sabouraud broth rather than RPMI 1640. The *C. albicans* culture suspension was mixed directly with

hydrolysate or peptide without prior harvesting of the fungi. The methods for the assays are detailed in section 2.7 and all the sub-sections therein. These methods were used for both screening of anti-microbial active samples and for determination of minimum inhibition concentration (MIC) (Figure 4.1).

4.3.3. Fractionation and purification of active peptides

The active hydrolysates were purified by means of high performance liquid chromatography (HPLC) using Shimadzu VP class instrument as detailed in Section 2.4 and Section 2.9. Crude hydrolysate was first filtered through 0.20 µm MiniSart membrane filter (Sartorius-Stendim, Dandenong South, Victoria, Australia) and 400 µl sample was injected into Alltech Altima C-18 reverse phase Preparative column (20 x 250 mm, 5 µm, 100 Å) (Grace Division Discovery Science, Baulkham Hills, NSW, Australia) and eluted with gradient acetonitrile containing 0.1% trifluoroacetic acid (TFA) (Solvent B) initially at 2.0% and increased over time to 70% at 70 min then reduced to 2.0% at 71 min and maintained isocratically for another 9 min. Fractions collected were freezed-dried in an Alpha-1-4 freeze dryer with controller LDC-1M (CRIST® Gefriertrocknungsanlagen, Söse, Ostorede, Germany). Observed active fractions were subjected for purification with HPLC using Altima C-18 analytical column (4.6 x 250 mm, 5 µm, 100 Å) (Grace Division Discovery Science, Baulkham Hills, NSW, Australia) and eluted isocratically with 30% acetonitrile containing 0.1% TFA.

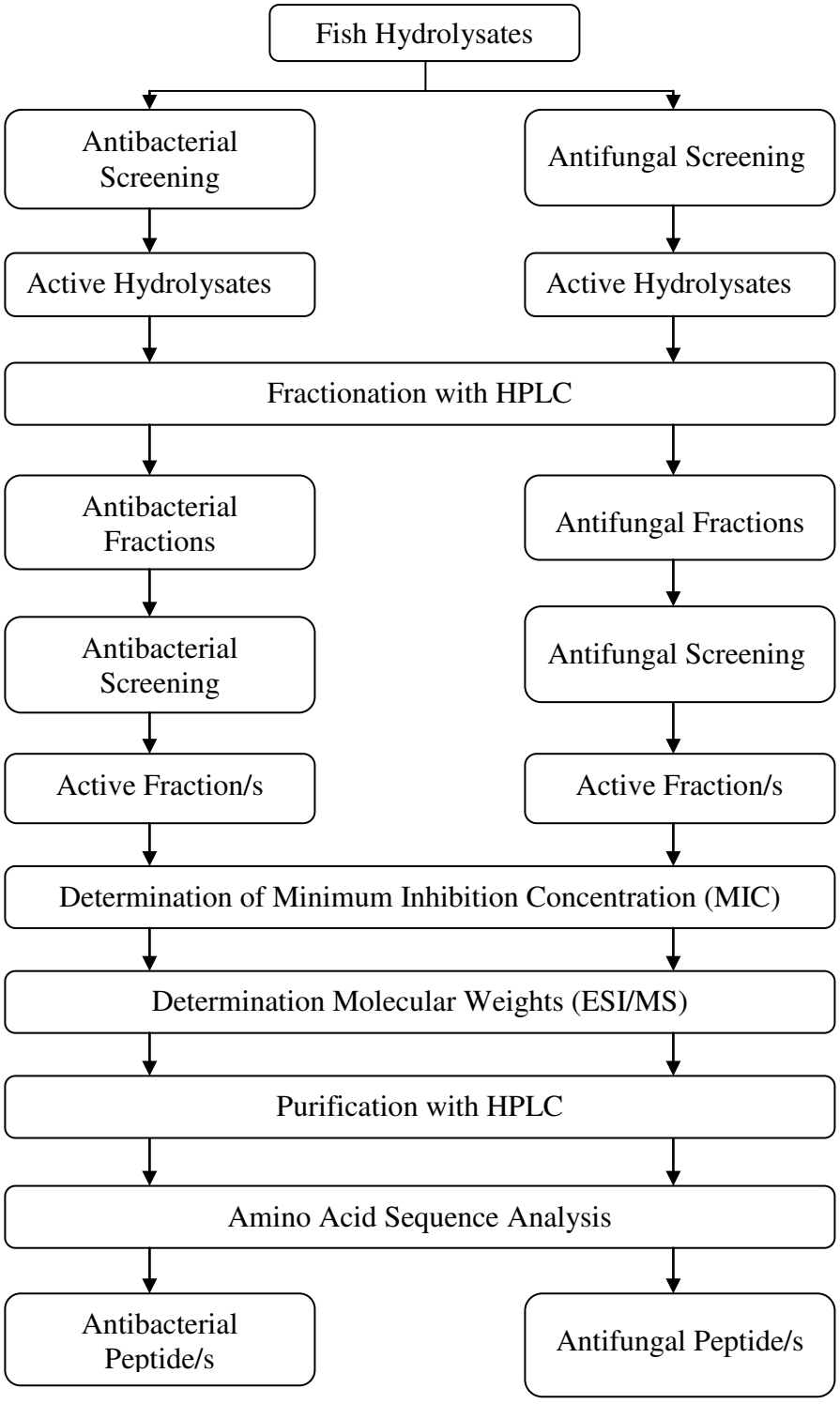


Figure. 4.1. Flow chart of antimicrobial peptide screenings. Screenings were based on spectrophotometric method with tetrazolium (XTT) salt for coloration of antibacterial assay samples.
HPLC = high performance liquid chromatography.
ESI/MS = Electronspray ionisation/mass spectroscopy.

4.3.4. Amino acid sequence analysis

The amino acid sequence of for the active peptide was carried out based on Edman N-terminal sequencing method using Applied Biosystems 494 Procise Protein Sequencing System (Perkin Elmer, Waltham, MA, USA) at Australia Proteomic Analysis Facility, Macquarie University, Sydney, Australia (Section 2.4.2). Procise™ Protein Sequencer System software was used to analyse the sequence. Prior to analysis, peptide fraction was concentrated by means of vacuum centrifuge, and 100 µl of sample was used for analysis.

4.3.5. Liquid chromatography/mass spectrometry (LC/MS)

The molecular weight of the peptide sample was determined using an LC/MS system consisting of Varian ProStar HPLC and Varian 1200L Quadrupole MS/MS System (Varian Inc., Palo Alto, CA, USA) as detailed in Section 2.4.5.

4.3.6. Peptide synthesis

Custom made active peptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) from fraction LBI12 was synthesised by GenScript USA Inc. (GenScript USA Inc., Piscataway, NJ, USA). The synthesised peptide was used to confirm the activity and for structure-activity study with nuclear magnetic resonance (NMR).

4.3.7. Statistical analysis

All values are reported as mean of at least three observations from three replications. One Way ANOVA was used to analyse data collected using Minitab 15.1 (Minitab Inc., State College, PA, USA), followed by Tukey pairwise test ($\alpha < 0.05$) to evaluate

significant difference between means.

4.4. Results and discussion

Assays of anti-microbial peptides derived from hydrolysis of trevally and leatherjacket fish proteins were carried out with the following steps: screening for active hydrolysates, fractionations of active hydrolysates, screening for active fractions, purification of active fractions, determination of minimum inhibition concentration (MIC), and amino acid sequence analysis (Fig. 4.1). Screening for active hydrolysates, fractions, peptides, and MIC were carried out using *E. coli*, *B. cereus*, *S. aureus*, and *C. albicans*.

Several methods do exsist for susceptibility and MIC assays. These include the amended BSAC Guide to Sensitivity testing (Andrews, 2001) and the colorimetric method with XTT (Tunney et al., 2004). The former is a standard method for routine susceptibility test with an overnight incubation, and using more samples. The latter method is a rapid method with a total of five hour incubation with a culture suspension ten times as high as the culture suspension used in the former method, using less sample, but needing an additional dye reagent (XTT). It has been used successfully for MIC test against *Pseudomonas aeruginosa*, but no report for MICs against other bacteria. The latter method, with some modification, was used in this study mainly due to the limited amount of available sample. However, the former method was also used to verify the accuracy of the latter method with the bacterial strains used in this experiment. Screening against *C. albicans* was carried out using a microdilution method (Hazen and Howell, 2004) with some modification to use microplate reader and the absorbance was read at 590 nm.

4.4.1. Method verification

Verification of the colorimetric method with XTT was carried out to ensure that there is no significant difference from the result obtained from the amended BSAC Guide to Sensitivity testing (Andrews, 2001) as described in section 2.7.4. Tetracycline and polymyxin dilutions were used as the antibiotic solution (Table 4.1). The results showed that the colorimetric method is well comparable to the BSAC Guide to Sensitivity Testing method. The MIC values of polymyxin against both *B. cereus* and *S. aureus* could not be observed based on the antibiotic dilution ranges and are expressed as more that 64.0 mg/l (64.0+ mg/l). This is due to the fact that polymyxin is not strongly active against Gram positive bacteria. The MIC values of polymyxin are 25-150 mg/ml against various strains of *S. aureus* and more than 200 mg/ml against various strains of *B. cereus* (Meyers et al., 1973).

Table 4.1. Comparison of MIC from the amended BSAC Guide to Sensitivity Testing method and XTT colorimetric method[a].

Antibiotic and culture suspension	BSAC Method	Colorimetric Method
Polymixin - *E. coli*	1.00	1.00
Polymixin - *B. cereus*	64.0+	64.0+
Polymixin - *S. aureus*	64.0+	64.0+
Tetracycline - *E. coli*	8.06	8.06
Tetracycline - *B. cereus*	1.02	1.02
Tetracycline - *S. aureus*	1.02	1.02

[a] Concentrations are expressed in mg/l.

The method used for the anti-fungal assay was also tested before used in real assay against *C. albicans*. Results from the trial tests indicated that this modified method could support optimum growth of *C. albicans*, thus used for the rest of the anti-fungal experiments.

4.4.2. Screening for anti-microbial peptides

Preliminary assay for anti-microbial activity was carried out using crude hydrolysates from all enzymic digestions and hydrolysis times against *E. coli*, *B. cereus*, *S. aureus* and *C. albicans*. Samples were incubated with culture suspension for 4 h followed by additional 1 h incubation after the addition of XTT to facilitate colouration as an indication of the presence of bacterial growth. The culture suspension used in this method was 10 times higher than the suspension used in the amended BSAC Guide to Sensitivity Testing (Andrews, 2001) as the incubation time was shorter. Comparative study of these methods showed no difference in the results (Table 4.1).

Hydrolysates that showed dominant anti-microbial activity were subjected to purification and characterisation. Plate 4.1 shows an example of the microplate sample used in the anti-microbial assays. The results from anti-microbial screening are shown in Table 4.2 and Table 4.3. The results showed that various hydrolysates from trevally and leatherjacket proteins possess various degree of inhibition towards *B. cereus* and *S. aureus*; however there was no activity against *E. coli* and *C. albicans* (complete results are shown in Appendix 1).

These results seemed to indicate that the active hydrolysates or peptides work only against Gram-positive bacteria. Anti-microbial peptides have been isolated from several food proteins such as milk and chicken egg. These peptides have shown activities

against various Gram-positive and Gram-negative bacteria as well as fungi like *C. albicans* (Chan and Li-Chan, 2006). However, there are also anti-microbial peptides that work only on specific groups of bacteria such as polymyxin that is active particularly against Gram-negative bacteria such as *E. coli*.

Table 4.2. Inhibition (%) of microbial growth by trevally soluble and insoluble protein hydrolysates.*

	Microorganisms	
Hydrolysates	*B. cereus*	*S. aureus*
Soluble Papain hydrolysates		
4 h hydrolysis	3	5
Insoluble Papain hydrolysates		
10 h hydrolysis	0	15
Soluble Bromelain hydrolysates		
4 h hydrolysis	14	0
Insoluble Bromelain hydrolysates		
4 h hydrolysis	0	6
8 h hydrolysis	23	35
10 h hydrolysis	0	15

* Only active hydrolysates against any of the tested microorganism are shown. Complete screeing results are presented in Appendix 1.

Table 4.3. Inhibition (%) of microbial growth by leatherjacket soluble and insoluble protein hydrolysates.*

Hydrolysates	Microorganisms	
	B. cereus	*S. aureus*
Soluble Papain hydrolysates		
2 h hydrolysis	3	0
Insoluble Papain hydrolysates		
2 h hydrolysis	2	0
4 h hydrolysis	3	0
10 h hydrolysis	0	7
Soluble Bromelain hydrolysates		
2 h hydrolysis	3	0
6 h hydrolysis	0	42
8 h hydrolysis	0	36
Insoluble Bromelain hydrolysates		
2 h hydrolysis	3	11
4 h hydrolysis	4	0
6 h hydrolysis	0	23
8 h hydrolysis	23	43
10 h hydrolysis	22	13
Soluble Flavourzyme hydrolysates		
2 h hydrolysis	0	17

* Only active hydrolysates against any microorganism are shown. Complete screeing results are presented in Appendix 1.

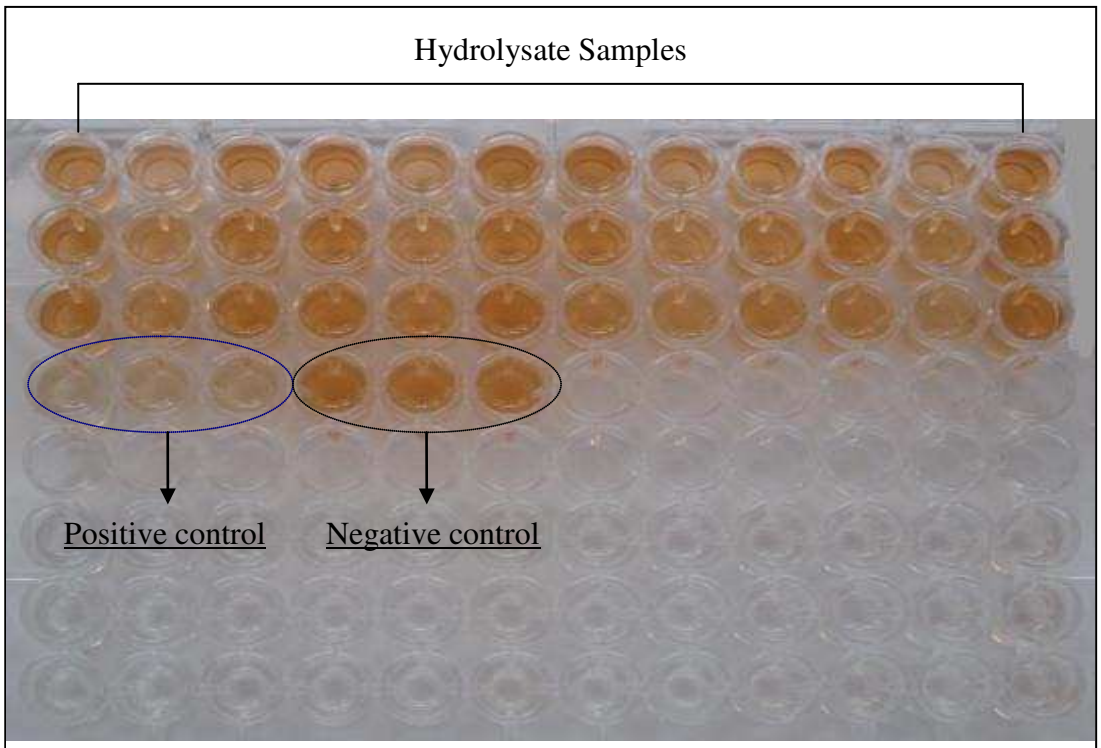


Plate 4.1. Microplate profile of six leatherjacket soluble and insoluble protein hydrolysates against *S. aureus*. Various degrees of inhibition are observable through different colour intensity. Positive control showed the effect of tetracycline inhibition, while negative control showed growth of *S. aureus* without inhibition.

4.4.3. Purification of active hydrolysates

Results from anti-microbial screening of crude hydrolysates indicated that there are indeed a number of active hydrolysates that showed activities of various degrees. Hydrolysates that showed stronger inhibition were selected for fractionations on C-18 RP-HPLC preparative column and further analysis. Anti-microbial analysis for the collected fractions resulted in decreasing activities as compared to the activity of their parent hydrolysates. This might be due to cumulative effect of weak anti-microbial peptides present in the hydrolysates, and these peptides might be fractionated into different fractions based on their hydrophobicities, thus the resulting effects were minimised. The only hydrolysate fractions that showed significant inhibition were peptide fractions from 8 h bromelain hydrolysis of leatherjacket insoluble protein (labelled as LIB8H). These hydrolysate fractions showed activity against *B. cereus* and *S. aureus* but not against *E. coli* and *C. albicans*. Results from the fractionation of LIB8H on preparative C-18 RP-HPLC column gave only about 13 major peaks (Figure 4.2). This is not unusual, as most of the peptides are of similar molecular weight and separation in such a big column became increasingly difficult. Further anti-microbial assays of the 13 fractions showed that fraction 9 (labelled as LBI9) and fraction 12 (labelled as LBI12) were the most active fractions indicating that the elution of most of the quantity of the active peptides into these fractions. Fraction 11 and fraction 13 also showed some activity against both *B. cereus* and *S. aureus*. However, it was presumed that these two fractions contained the same active peptide that mostly eluted at fraction 12. Further separation of these two peptide fractions with analytical C-18 column (Figure 4.3) showed that these fractions consisted of many peptides and further purification is needed to identify the peptide that possess the activity.

Increasing the resolution of fractionation and purification is, therefore, imperative in order to get pure fractions of the peptides that possess the anti-microbial activities, to elucidate their structures, and to further characterise them. This was done by a combination of gradient fractionation followed by isocratic purification for several times as the two fractions collected were impure. These difficulties in obtaining pure selected fractions lied on the fact that the extent of hydrolysis produced peptides with similar molecular weights (see Chapter 3, Figure 3.9) and possibly same charges hence purification on C18 column that is based on hydrophobicity did not give an optimum separation.

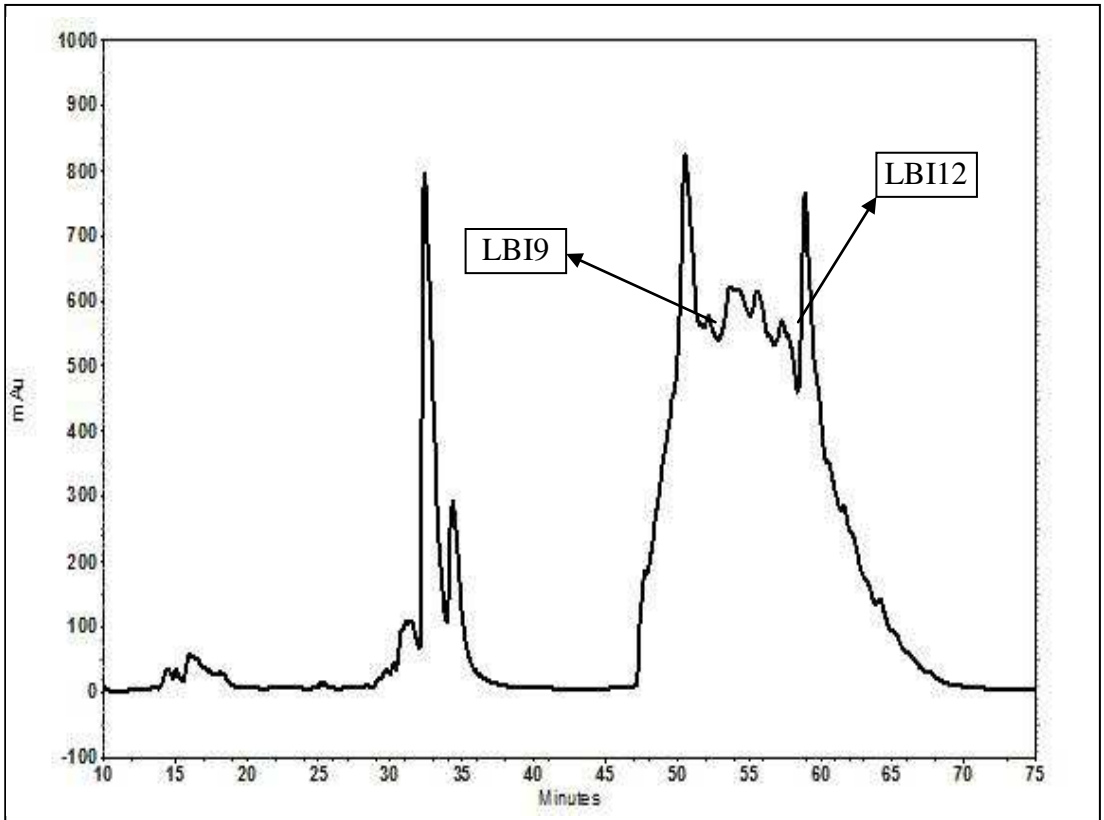


Figure 4.2. Chromatogram of 8 h bromelain hydrolysis (LIB8H) of leatherjacket insoluble proteins.

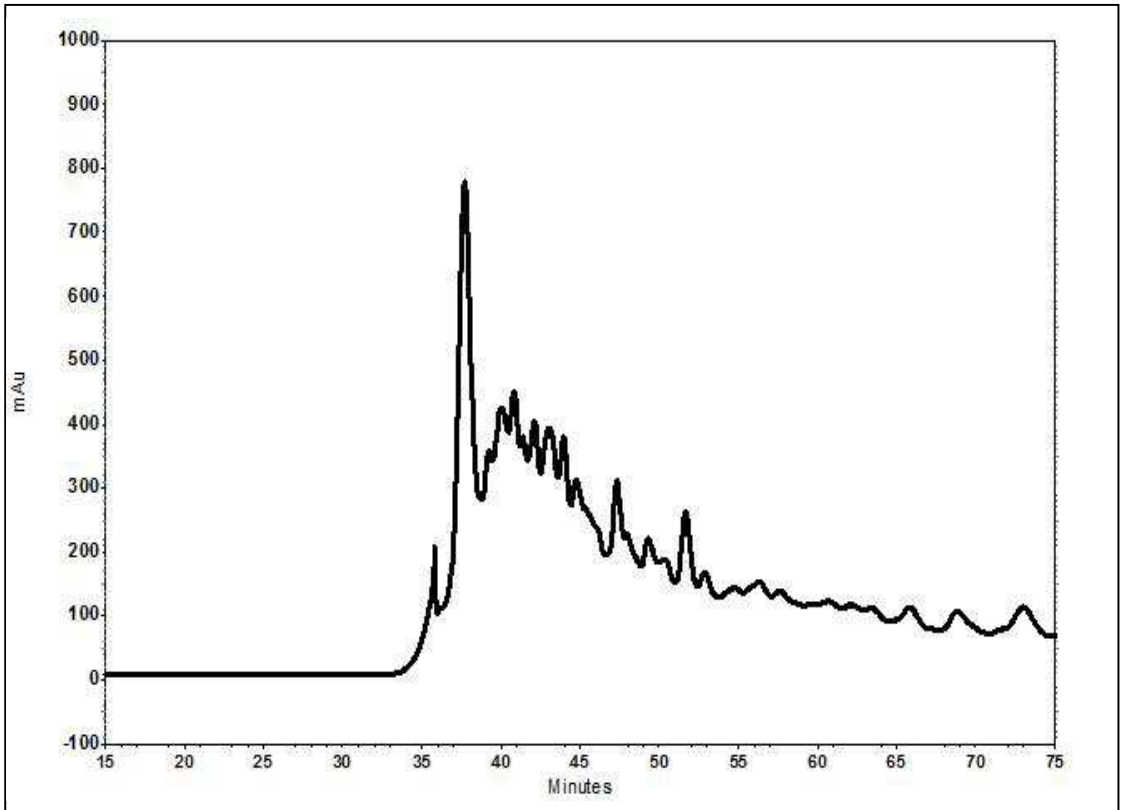


Figure 4.3. Chromatogram of further separation of fraction LBI12 of 8 h bromelain hydrolysis (LIB8H) of leatherjacket insoluble proteins. The fraction was impure and contained many other peptides.

4.4.4. Determination of minimum inhibitory concentration (MIC)

Further anti-microbial assays of the 13 fractions collected from the preparative fractionation showed that fraction LBI9 and fraction LBI12 exhibited stronger activities (Table 4.4) than other fractions and were, therefore, subjected to determination of their minimum inhibition concentration (MIC). The determinations of minimum inhibitory concentration of fractions LBI 9 and LBI12 were performed in triplicates. The method used was the microdilution colorimetric procedure (Tunney et al., 2004) in the presence of 2,3-bis (2-methyloxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5- carboxanilide (XTT). The results are summarised in Plate 4.2 and Plate 4.3.

Results from the analysis showed that fraction LBI12 exhibited anti-microbial activity against *B. cereus* and *S. aureus* with MIC values of 4.3 mg/ml, while fraction LBI9 only showed some activity against *B. cereus* without a MIC being reached at 5.35 mg/ml peptide concentration. These values are much higher than that of polymyxin MIC values of 0.025-0.15 mg/ml against various strains of *S. aureus* and more than 0.2 mg/ml against various strains of *B. cereus* (Meyers et al., 1973). Interestingly, these active fractions are found only in hydrolysate of 8 h hydrolysis. Electrophoretogram of this hydrolysate showed band of peptides with molecular sizes of around 6 kDa (Figure 3.9). The degree of hydrolysis of this hydrolysate is 28.2% which indicates that the active peptide was released when the hydrolysis approached around 28% and was further hydrolysed as the hydrolysis progressed. Bromelain is a proteolytic enzyme mainly found in the stem of pineapple fruits and is commonly used to enhance hydrolysis or fermentation process such as in the preparation of soy and fish sauces, but there was no report on the release of anti-microbial peptide by this enzyme. However, bromelain is mentioned to be able to cleave milk proteins and release anti-microbial peptides (Dziuba

et al., 2009). Therefore, this may be the first finding of anti-microbial peptide released from the hydrolysis of fish or food proteins by bromelain. This finding also extends the list of the few anti-microbial peptides that have been released through hydrolysis of food proteins.

As the amount of active peptide fraction LBI12 was very low, synthetic peptide was made available to confirm the previous finding and to perform structure-activity relationship study with NMR. This was done after Edman N-terminal sequence of the active fraction LBI12 had been completed and the sequence of the peptide was established as Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ). Further study using custom made synthetic Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) showed that the peptide was active mostly against *Bacillus cereus* and to a lesser extent against *S. aureus*. The MIC value against *B. cereus is* 5.3 mg/ml and 7.96 mg/ml for *S. aureus* (Plate 4.4). This somewhat different results from the crude fraction LBI12 activity against the two testing microorganisms as previously reported may be due to the presence of another active peptide in the fraction that is active against either one or both *B. cereus* and *S. aureus* either solely or in a sum up and/or in synergy with Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ). The absorbance-concentration plots in Plate 4.2, Plate 4.3, and Plate 4.4 also show that the MIC points were somewhat higher than the initial absorbance of each peptide and culture suspension mixture. As the initial absorbance was taken before the incubation, the mixture might develop colour over the period of incubation in addition to increasing turbidity due to the bacterial growth. Result from observation of the colour of sterile media showed that the colour intensity increased between 1 – 4% over a period of 24 h.

Table 4.4. Anti-microbial activity of fractions of 8 h bromelain hydrolysis (LIB8H) of leatherjacket insoluble proteins.

Fractions	Inhibition (%)	
	S. aureus	*B. cereus*
F1 (LBI1)	-2.48	-4.74
F2 (LBI2)	5.94	7.34
F3 (LBI3)	-2.91	-5.59
F4 (LBI4)	4.98	4.17
F5 (LBI5)	-2.52	0.69
F6 (LBI6)	4.49	5.21
F7 (LBI7)	5.34	5.15
F8 (LBI8)	9.63	13.92
F9 (LBI9)	12.75	15.61
F10 (LBI10)	11.40	8.87
F11 (LBI11)	5.65	14.49
F12 (LBI12)	40.23	48.93
F13 (LBI13)	20.24	17.31

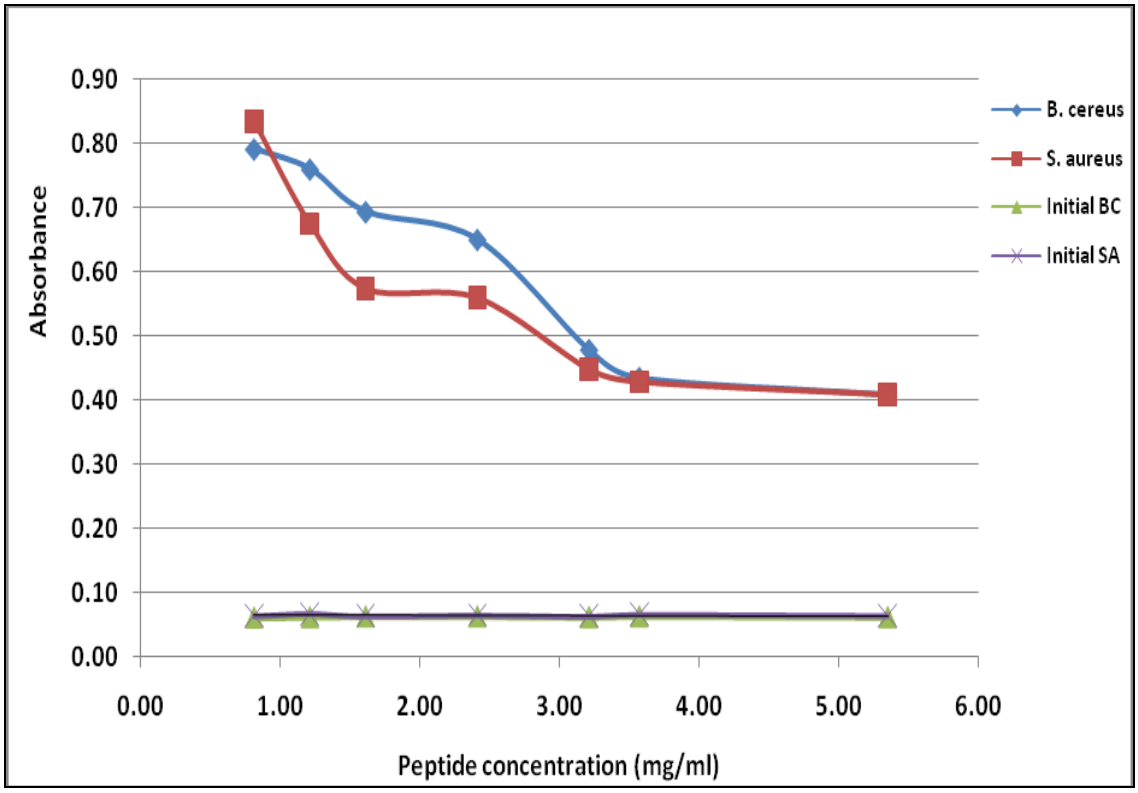


Plate 4.2. Absorbance-peptide concentration plot (mean ± sd, n = 9) from anti-microbial assay of fraction LBI9 of LIB8H against *B. cereus* and *S. aureus*. Absorbance readings were carried out before incubation, after 4 h incubation, and after the addition of 2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and additional 1-hour incubation (total 5 h incubation).

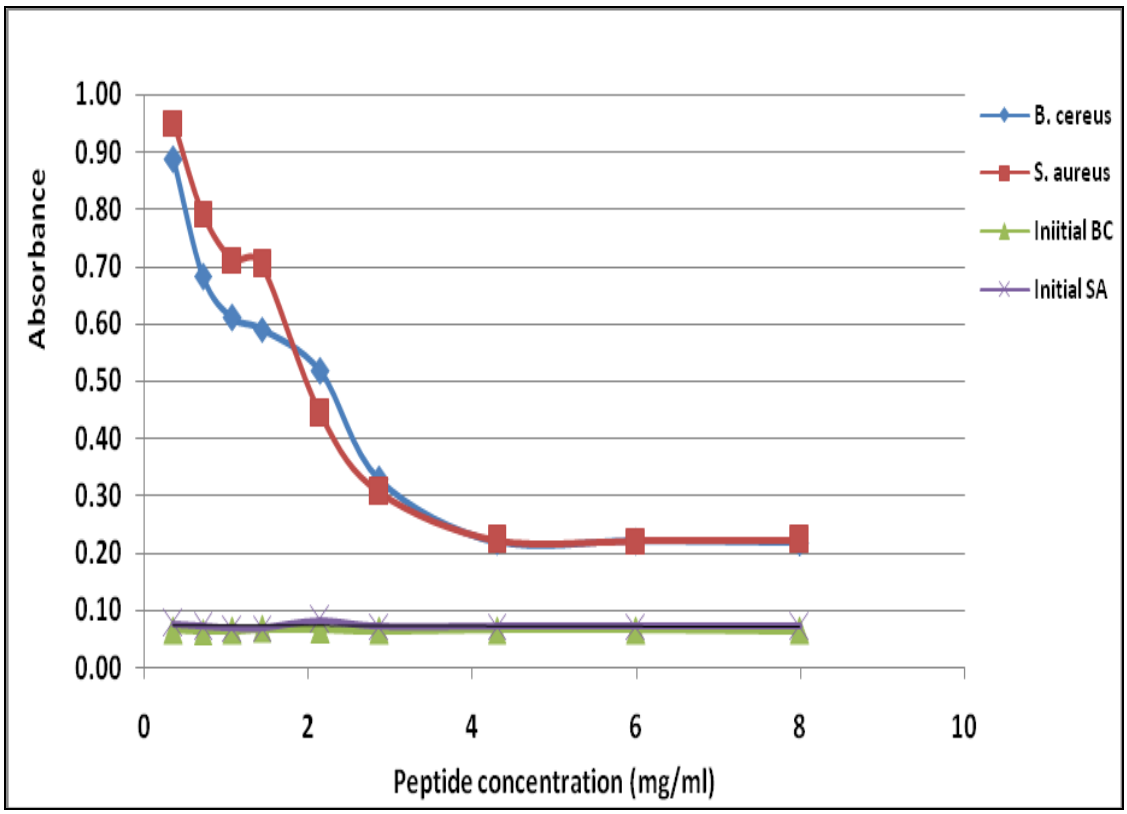


Plate 4.3. Absorbance-peptide concentration plot (mean ± sd, n = 9) from anti-microbial assay of fraction LBI12 of LIB8H against *B. cereus* and *S. aureus*. Absorbance readings were carried out before incubation, after 4 h incubation, and after the addition of 2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and additional 1 h incubation (total 5 h incubation).

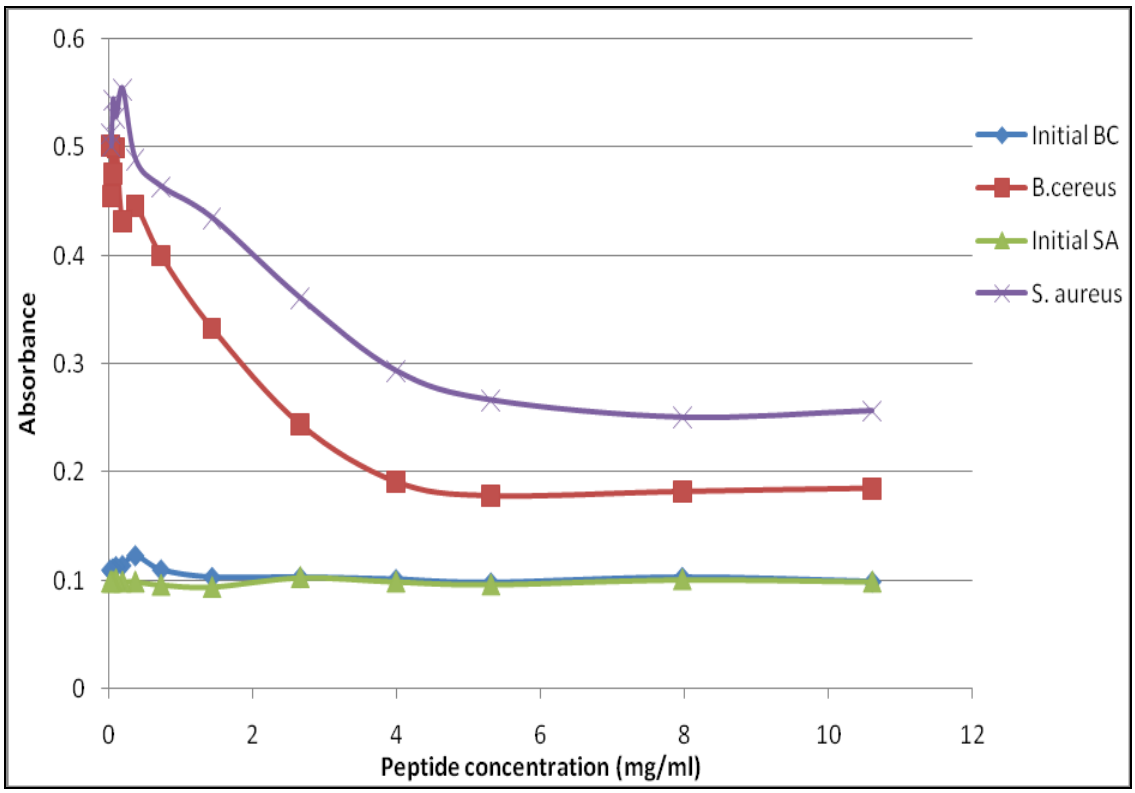


Plate 4.4. Absorbance-peptide concentration plot (mean ± sd, n = 6) of anti-microbial synthetic peptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) against *B. cereus* and *S. aureus*. Absorbance readings were carried out before incubation and after 18 h incubation based of Standard microdilution method (Andrews, 2001).

4.4.5. Structure elucidation of active peptides

The amino acid sequence and the molecular weight of the active peptide fraction LBI12 were determined in order to elucidate the primary structure of the active peptides. The sequences were determined based on Edman N-terminal degradation of the peptide, while the molecular weights were determined complimentarily using electronspray ionisation/mass spectrometry (ESI/MS) technique. Crude active peptide fraction was ionised by electronspray technique and the total ion charge and specific ion charge were identified.

Results from Edman N-terminal sequence analysis showed that the purified active fractions consisted of mainly heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ; MW = 858.9 Da, net charge = -2) and another minor peptide Ala-Pro-Ile-Asp-Asn-Leu-Gln (APIDNLQ; MW = 769.85 Da, net charge = -1). The presences of the peptides were confirmed with the results from mass spectroscopy analysis (Figure 4.4). The use of crude active fraction for molecular weight determination and identification was to avoid the trifluoroacetic acid (TFA) suppressing effect on the peptide signals and to increase the peptide concentrations. The heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) showed a peak of m/z point at 859.8, while Ala-Pro-Ile-Asp-Asn-Leu-Gln (APIDNLQ) showed a peak of m/z at 770.8. These peptides are, therefore, anionic peptides designated by rich in glutamic acid (Glu, E) and aspartic acid (Asp, D) and having negative net charge. Most of the anti-microbial peptides are cationic peptides characterised by rich in certain amino acid residues such as proline, arginine, phenylalanine, glycine, and tryptophane. However, anionic anti-microbial peptide is also present naturally (Vizoli and Salzet, 2002). Maximin H5, for instance, is an anionic anti-microbial peptides found in the skin of toad Bombina maxima having as sequence of Ile-

Leu-Gly-Pro-Val-Leu-Gly-Leu-Val-Ser-Asp-Thr-Leu-Asp-Asp-Val-Leu-Gly-Ile-Leu-NH2 (LGPVLGLVSDTLDDVLGIL-NH_2) which is rich in aspartic acid and no basic amino residues (Lai et al., 2002). There is no structural similarity between maxima H5 and the anti-microbial peptide found in this research. However, both peptides showed positive inhibition of the growth of S. aureus and therefore can be a potential anti-bacterial agent to control the negative impact of this pathogenic bacteria.

The results from anti-microbial assays of both fraction LBI12 and its synthetic heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) showed MIC values were quite higher than any known anti-microbial peptides such as polymyxin, 0.025-0.15 mg/ml against *S. aureus* strains and more than 0.2 mg/ml against *B. cereus* strains (Meyers et al., 1973), indicating a weaker anti-microbial peptide. Further study to evaluate or increase its potency can be carried out by either synthetic modification to increase its potency such as increasing its negative charge, and synergism with other anti-microbial agents and/or with metal ion such as zinc. Referring to Figure 4.5, the negative charge of the heptapeptide can be increased to -4 if the peptide size is increased to become pentadecapeptide *Ala-Asp-Ser-Thr-Ala-Asp-Glu-Leu-Gly-Asp*-Glu-Gln-Ile-Asp-Asn-Leu-Gln (*ADSTAELGE*QIDNLQ). This combination of increased size and net charge may alter its activity as the peptide may have better structural conformation that allow it to interact with bacterial membrane and perturb it. Anionic anti-microbial peptide AP1 (Gly-Glu-Gln-Gly-Ala-Leu-Ala-Gln-Phe-Gly-Asp-Trp-Leu, GEQGALAQFGEWL), for instance, is a longer peptide and had been reported to kill Gram-positive bacteria such as *S. aureus* through interaction with the membrane lipid headgroup region of the bacteria (Dennison et al. 2006), hence the anionic anti-microbial heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) found in this study may

behave in a manner similar to AP1. The trend in which the inhibition of the growth of *S. aureus* by Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) increased as function of the peptide concentration may indicate its mode of inhibition through membrane disruption.

Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) has been identified in the sequence of chicken skeletal myosin heavy chain fragments 1207-1213 (Figure 4.5, complete sequence can be seen in Appendix 2), while Ala-Pro-Ile-Asp-Asn-Leu-Gln (APIDNLQ) is a homologue that may occur at a relatively the same fragments of myosin heavy chain. This parent protein was used as reference protein because the complete sequence of actin, myosin, and other myofibrillar proteins from leatherjacket are unknown. Myosin is considered as less preserved proteins as compared to actin, but can serve as a good reference for peptide identification of fish origin. The use of chicken skeletal myosin heavy chain as reference was at least once employed by Yokoyama et al. (1992) when referring to angiotensin I-converting enzyme (ACE) inhibitory peptides derived from thermolysin digest of dried bonito. This is due to the fact that chicken muscle proteins are closer in to fish proteins in their characteristics hence a better option than other muscle proteins.

As mentioned earlier, the complexity of this cocktail of peptides made it extremely difficult to purify the active peptide in a quantity that will enable further analysis such as for the determination of minimum bactericidal concentration (MBC) and for structure-activity relationship study with nuclear magnetic resonance (NMR) spectroscopy. This issue was addressed by utilisation of a custom made peptide. These two peptides were, as far as size is concerned, quite small. However, they may be able to produce certain structural conformation in aqueous solution to indicate their activity as functions of their structures. However, under real-time condition upon contact with bacterial cells, these

small peptides may interact in such a way that prompts them to be able to function as an active peptide. This is studied and presented in Chapter 6.

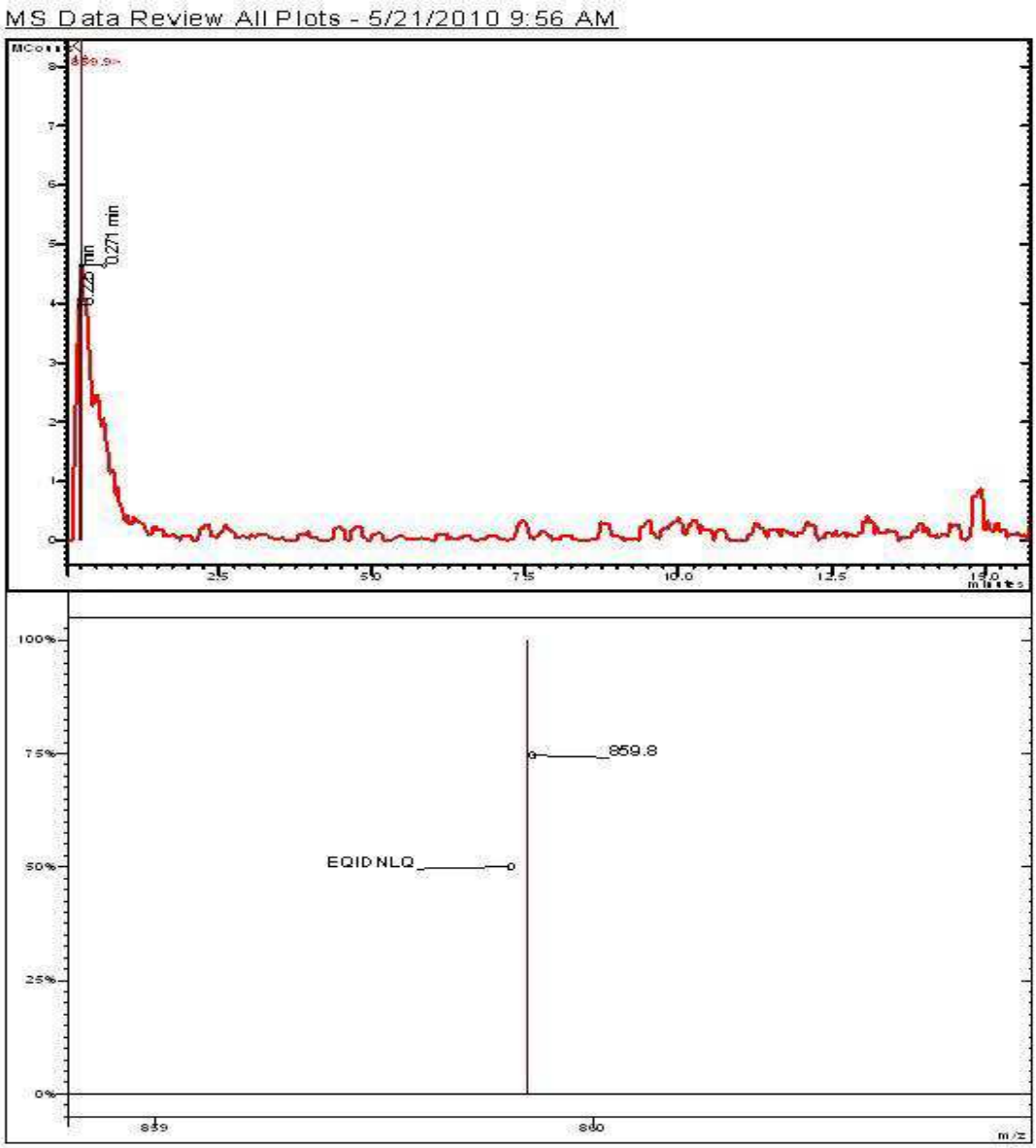


Figure 4.4. ESI/MS chromatogram and target specific MS analysis of fraction 12 of LIB8H. The presence of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) is confirmed at m/z point 859.8

LQEAHQQTLDDLQVEEDKVNTLTKAKTKLEQQVDDLEGSLEQEKKLRMD
1050
LERAKRKLEGDLKLAHDSIMDLENDKQQLDEKLKKKDFEISQIQSKIEDEQ
1101
ALGMQLQKKIKELQARIEELEEEIEAERTSRAKAEKHRADLSRELEEISERL
1153
EEAGGATAAQIEMNKKREAEFQKMRRDLEEATLQHEATAAALRKKHAD
1201
STAELG***EQIDNLQ***RVKQKLEKEKSELKMEIDDLASNMESVSKAKANLEK
1250
MCRTLEDQLSEIKTKEEQNQRMINDLNTQRARLQTETGEYSRQAEEKDALI
1301
SQLSRGKQGFTQQIEELKRHLEEEIKAKNALAHALQSARHDCDLLREQYE
1351
EEQEAKGELQRALSKANSEVAQWRTKYETDAIQRTEELEEAKKKLAQRLQ
1401
DAEEHVEAVNAKCASLEKTKQRLQNEVEDLMVDVERSNAACAALDKKQ
1453
KNFDKILAEWKQKYEETQTELEASQKESRSLSTELFKMKNAYEESLDHLETL

Figure 4.5. Partial amino acid sequence of chicken skeletal myosin heavy chain. Peptide of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) is in fragments 1207-1213 indicated by bold italic.
Adapted from http://prowl.rockefeller.edu.

The smallest anti-microbial peptide has ever been indicated is tetrapeptide CIRA of milk lactoferrin fragments 45-48 (Dziuba et al., 2009). It is understood that milk, as the first food for the neonates, contain unique amino acid sequences that are of health benefits. This is particularly true for the neonates where lack of immune system may cause serious illness due to the foreign materials surround them. The reasons for the present of anti-microbial peptides in the parent leatherjacket muscle proteins as well as why they can be released by bromelain hydrolysis are unknown. Leatherjacket is a vile fish that eat mainly seaweed and encrusted animals, while bromelain is an enzyme of plant (pineapple) origin. Bromelain is a collective name for proteolytic enzymes or proteases found in tissues including stem, fruit, and leaves of the pineapple plant. Bromelain from pineapple stem is a glycosilated cystein protease and is immunologically different from

the non-glycosilated cysteine protease of the fruit (Rowan et al., 1990). The functions of bromelain in pineapple plant remains unclear. It was postulated that carnivorous plants derived their supply of nitrogen and phosphorus from degradation of organic material such as foliage, insects, and microbes by means of highly active proteases (Bogre et al., 1996). This is where the stem and leave bromelain may play their role to kill and degrade the protein of the entrapped organisms. The nitrogen supply is then used in the synthesis of proteins which content increases rapidly within a few weeks of the cessation of flowering then decreases steadily during maturation until the pineapple fruit is ripe. At this stage the fruit bromelain may play its role to hydrolyse the protein as during the depletion of the protein, the free amino acid and total-soluble nitrogen content increased (Gortner and Singleton, 1965). Bromelain has been postulated as a potential enzyme to release anti-microbial peptides from milk proteins (Dziuba et al., 2009). Therefore, it is possible that leatherjacket has a peptidase with specificity similar to bromelain that can hydrolyse its muscle proteins to release anti-microbial peptide in the event such as injury thus protect the fish from further pain while the wound is healing.

The heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) has not been anywhere found and characterised as an active anti-microbial peptide. There is little structural similarity between known anti-microbial peptides and the anti-microbial heptapeptide found in this study. The two N-terminal residues of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ), however, are similar to pentapeptide Glu-Gln-Leu-Thr-Lys (EQLTK) anti-microbial peptide from milk α-lactalbumin fragments 1-5 (Dziuba et al., 2009). Although these two peptides are small, they may be able to conform an α-helix conformation, as this conformation may occur on an average of 3.6 amino acids in a sequence (Axley, 1998). The active peptide found in this study is quite likely a linear

peptide that may form an extended structure as it is too small to form other classic and more complicated structures such helix, β-strand, or loop.

As mentioned earlier, the active anti-microbial peptide found in this research is active against *S. aureus* and *B. cereus* that can be of beneficial application. Although only these two Gram-positive bacteria were used in this investigation, it is expected that these active heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) could also inhibit any other Gram-positive coccus and bacilli (rod-shaped) bacteria such as pathogenic *Streptococcus* and *Enterococcus* bacteria. *B. cereus* is a Gram-positive bacteria well accepted as a potential foodborne pathogen of significant consequence. The cells of *B. cereus* are motile by means of peritrichous flagella. *B. cereus* grows rapidly in foods held in the 30-40^{o}C range over pH range 4.9-9.3, and can form heat resistant spores that mimic other mesophilic spore forming bacteria (Bennett, 2001a). Ingestion of contaminated foods may cause illness characterised by abdominal cramps with profuse watery diarrhoea, rectal fenesmus, and occasionally nausea that seldom results in vomiting. The incubation period is within the range of 8-16 h. Another type of illness described as the 'emetic type' of intoxication caused by *B. cereus* is characterised by an acute attack of vomiting occurs between 1-5 h after ingestion of contaminated foods. *B. cereus* strains that cause diarrhoea produce an extracellular mature toxic protein of 55-60 kDa, while the strains that cause the 'emetic type' intoxication are reported to produce a dodecadepsipeptide (a low molecular weight peptide) that is heat resistant (withstanding 120^{o}C for more than one hour). The principal food vehicles for the diarrheal strains are meat products, soups, vegetables, and pudding, while cooked rice and pasta are the principal carries for the 'emetic type' (Bennett, 2001a). The use of

active anti-microbial peptide to inhibit the growth of *B. cereus* in the above foods could, therefore, reduce the potential illness due to ingestion of toxin produced by the bacteria.

S. aureus is the common species in the *Staphylococcus* genus that primarily colonised mucous membranes such as nose, mouth, and throat, mostly an extraintestinal dwellers and the most common cause of suppurating infections such as boils and pimples (Martin et al., 2001). *S. aureus* is of great concern in food industry as it is the second most prevalent cause of foodborne illness. This is due to the fact that *S. aureus* has the ability to grow at a a_w of 0.83, a water activity level that is too low for the growth of many competing bacteria. Most strains of *S. aureus* are also highly tolerant to salts and sugars, although their enterotoxin production decreased at low a_w. *S. aureus* is capable to produce at least 34 extrecellular proteins, although no single strain can produce all of the proteins. These proteins include coagulase, the haemolysins, exfoliatin, leucocidin, and pyrogenic exotoxin. The last three proteins caused scalded skin syndrome, lyses of white blood cells, and fever, respectively. Staphylococcal enterotoxins (SE) are produced by *S. aureus* and are the predominant causative agent of staphylococcal food poisoning. Some strains of *S. aureus* produce SE that is known to be the emetic cause of food poisoning (gastroenteritis) (Martin et al., 2001). The enterotoxins are heat-stable, hygroscopic, water soluble proteins secreted from the cells (Dack et al., 1930) that can cause symptoms of gastroenteritis even at very low concentration (1.0 µg) of ingested SE (Raiser et al., 1974). In addition to heat resistance, SEs is also resistant toward gamma irradiation (Read and Bradshaw, 1967) as well as towards some proteolytic enzymes such as trypsin, chymotrypsin, rennin, and papain (Bergdoll 1967).

The onset of staphylococcal food poisoning is usually rapid (2-6 h) and in many cases acute depending on the individual susceptibility to SEs and the amount of the

contaminated food consumed, the amount of SEs in the food, and the general health of the victim (Bennett, 2001b). The most common symptoms of staphylococcal food poisoning include nausea, vomiting, retching, abdominal cramping, and prostration. Headache, muscle cramping, and transient changes in blood pressure and pulse rate may also occur in more severe cases. A wide range of staphylococcal food poisoning vehicles that include almost every kind of foods make it reasonably fair to expect that an anti-microbial agent including anti-microbial peptide to be used to inhibit the growth of *S. aureus* in foods. Peptide antibiotic may play this role and may also be used as ointment for treatments of boils and pimples due to *S. aureus* infection.

4.5. Conclusion

Anti-microbial peptides or nature antibiotics have been isolated and characterised from many different sources, most of which are naturally occurring, while some are derived from hydrolysis of food proteins. In this study, anti-microbial peptides have been found from 8-hour bromelain hydrolysis of leatherjacket fish insoluble muscle protein with primary structure not similar to any other anti-microbial peptides identified and characterised so far. Results from anti-microbial assays of fractions LBI9 and LBI12 of the hydrolysate against *S. aureus* and *B. cereus* showed that fraction LBI9 has some activity against *B. cereus* without an MIC being reached at 5.35 mg/ml peptide concentration. Peptide fraction LBI12, on the other hand, showed activity against both *B. cereus* and *S. aureus* with an MIC value of 4.3 mg/ml.

Edman N-terminal sequence analysis revealed that the purified active fraction LBI12 consisted of mainly heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ; MW = 858.9 Da; net charge = -2) and another minor peptide Ala-Pro-Ile-Asp-Asn-Leu-Gln

(APIDNLQ; MW = 769.85 Da; net charge = -1). The presence of these peptides were confirmed with the results from mass spectroscopy analysis where the heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) showed a peak of m/z point at 859.8, while Ala-Pro-Ile-Asp-Asn-Leu-Gln (APIDNLQ) showed a peak of m/z point at 770.8. Anti-microbial activity assays using synthetic Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) revealed that the heptapeptide is active against both testing pathogenic bacteria having MIC values of 5.3 mg/ml and 7.96 mg/ml for against *B. cereus* and *S. aureus*, respectively. This indicates that the heptapeptide is a weaker peptide and further study to evaluate its synergism with other anti-microbial agents and/or metal ions or to synthetically modify its structure may be needed to improve its potency.

Chapter 5

ACE Inhibitory Peptides Derived from Fish Protein Hydrolysates

5.1. Abstract

Food proteins have been the subject of research focussing on the bioactive peptides derived through enzymatic hydrolysis. These bioactive peptides showed various activities such as anti-microbial, anti-hypertensive, anti-cancer, anti-thrombotic, anti-cariogenic, and anti-oxidative activities, as well as opioid, immunomodulating, and prebiotic peptides. In this study, soluble and insoluble fish protein fractions from leatherjacket (*Meuchenia* sp.) and trevally (*Pseudocaranx* sp.) were hydrolysed with papain, bromelain and Flavourzyme™, and the hydrolysates were used in screening for angiotensin I-converting enzyme (ACE) inhibitory (anti-hypertensive) peptides. The strength of the inhibition is expressed as IC_{50} values. The IC_{50} values of leatherjacket soluble protein fraction ranged from 1.35 mg/ml to 1.89 mg/ml, while the IC_{50} values of the leatherjacket insoluble protein fractions ranged from 0.77 mg/ml to 6.78 mg/ml. The IC_{50} values of trevally soluble hydrolysates ranged from 1.99 mg/ml to 3.34 mg/ml, while the IC_{50} values of the trevally insoluble protein fractions ranged from 2.45 mg/ml to 4.74 mg/ml. Following fractionation with molecular weight cut off (MWCO) membrane and C18 RP-HPLC fractionation and purification, 12 fractions were collected and labelled as LPI5, LPI6, LBI2, LBI5, and LFI5 from leatherjacket hydrolysates, and TPI3, TPI4, TBS1, TBS2, TBS6, TBI2, and TBI4 from trevally hydrolysates. The fractions were subjected for ACE inhibition and stability assays. The active stable

fractions that are classified as inhibitor and pro-drug types were analysed for their amino acid sequences. The primary structures of the four leatherjacket peptide fractions LPI5, LPI6, LBI5, and LFI5 are Glu-Pro-Leu-Tyr-Val (EPLYV), Asp-Pro-His-Ile (DPHI), Ala-Glu-Arg (AER), and Trp-Asp-Asp-Met-Glu (WDDME), having molecular weights of 619.71, 480.52, 374.40, and 694.71 Da, and IC_{50} values of 0.05,0.02,0.11, and 0.01 mg/ml respectively. The primary structure of three trevally peptide fractions TBS1, TBS2, and TBI2 are Ala-Arg (AR), Ala-Val (AV), and Ala-Pro-Glu-Arg (APER), having molecular weight of 245.28, 188.23, and 471.51 Da, and IC50 values of 0.11, 0.07, and 0.09 mg/ml, respectively. In addition, leatherjacket fraction LBI12 with sequence Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ), an anionic anti-microbial peptide found in this research, also showed ACE inhibitory activity with an IC_{50} value of 0.24 mg/ml. The MWCO and peptides fractions were also subjected into simulated gastrointestinal enzyme degradation and showed decreased IC_{50} values that can be an indication of their efficacy to be used in formulation of therapeutic food or supplement for mild hypertensive subjects. The IC_{50} values of the selected active peptide fractions, however, showed mixed trends of increased or decreased activity, or remained unchanged after incubation with pepsin or trypsin/chymotrypsin mixture, indicating the ability or inability of these gastrointestinal enzymes to cleave the peptides. Peptides that are not resistant towards the gastrointestinal enzymes produced smaller peptides that exert either weaker or stronger activity against ACE depending on their primary structures.

5.2. Introduction.

In hydrolysis and fermentation of food proteins, active isolated enzymes and enzymes produced by starter cultures cleave the protein to produce peptides of different sizes. As different proteolytic enzymes cleave proteins based on their specificity, peptides with different sizes, sequences, and characteristics are produced and different functional properties are expected to arise.

Since the discovery of bioactive peptide from snake venom (Meisel et al., 2006), many bioactive peptides against angiotensin I-converting enzyme (ACE) have been derived from hydrolysis or fermentation of food proteins. These active peptides are usually small consisting between two to around 10 amino acid residues, and to a much lesser extent bigger peptides with more than 10 amino acid residues in the sequences. Various studies to characterise and utilise these peptide have been performed and positive results from both *in vitro* and *in vivo* assays are forthcoming indicating possible use of these peptides to lower the blood pressure of mild hypertensive subjects. Most of the studies on ACE inhibitory peptides come from research with milk protein, and to a lesser extent come from fish and soy proteins. Many other proteins have been used to produce ACE inhibitory peptides with positive results, indicating that all food proteins can release ACE inhibitory peptides when appropriate enzymes are employed.

Fish is one of the major protein sources, consisting of thousands of species, and are consumed all over the world. However, not all species of fish are commercially important. In fact, volumes of fish consisting of hundreds of species are caught as by-catch in shrimp fishery, most of it is dumped back into the sea while some, including leatherjacked and trevally, are frozen and sold cheaply in many local markets. The intention of this study is to produce, isolate, identify, and characterise ACE inhibitory

peptides with the aim to use them in formulation of functional foods for mildly hypertensive people.

5.3. Materials and methods

5.3.1. Materials

All chemicals were of reagent grade and obtained from Sigma-Aldrich Chemical Company (Castle Hill, NSW, Australia), Bio-Rad Laboratories (Gladesville, NSW, Australia), APS Ajax Finechem (Taren Point, NSW, Australia), Lab-Scan Analytical Science (Bangkok, Thailand), and Merck Pty Ltd (Kilsyth, Victoria, Australia). All solvents were of analytical grade except HPLC grade solvents. Deionised water was prepared using Milli-Q® reagent water system (Millipore, Bedford, MA, USA). Angiotensin I-Converting Enzyme (ACE, 0.1 U/mL) in Milli-Q water, hippuric acid (0.44 mg/mL) in 50% methanol, and hippuryl-histidyl-leucine (HHL, 5 mM in HEPES buffer pH 9.3 containing 300 mM NaCl) were specifically used for the ACE inhibitory analysis.

5.3.2. Screening for ACE inhibitory peptides

Screening for ACE inhibitory peptides was carried out using crude hydrolysates, hydrolysates fractions of different molecular weight ranges, and isolated peptides fractions. Figure 5.1 below shows the flow process of ACE inhibitory peptides screening. The hydrolysates, hydrolysate fractions, and peptides fractions were analysed for their ACE inhibitory activity following the procedure described in section 5.3.3 below.

5.3.3. Analytical procedures

ACE inhibitory activity was determined following a combined method of Nakamura et al. 1995 and Tsai et al. 2008 with some modification to use HPLC for analysing the hippuric acid released. Hip-His-Leu, 200 µl 5 mM in 50 mM in HEPES buffer (pH 8.3) containing 300 mM NaCl, was mixed with 80 µl of hydrolysate, peptide fraction, or isolated single peptide of different concentrations (A) in reaction tubes, and preincubated for 10 min at 37^{o}C. As blank (B), 80 µl HEPES buffer was used in place of the peptide or inhibitor, while for control (C), 10 µl of Milli-Q water was added in place of ACE. After preincubation, 10 µl of ACE (100 mU/ml) was added to all reaction tubes except for the control (C) groups. The mixtures were incubated for 30 min at 37^{o}C, and then the reaction was stopped by an addition of 250 µl of 1 N HCl. Summary of the reaction mixture is presented in Table 2.3 section 2.6.3. The reaction mixture was filtered through a 0.20 µm filter (Sartorius Stedim Biotech S.A., Aubagne, France) and injected into HPLC for analysis of the concentration of hippuric acid liberated by ACE.

5.3.4. HPLC conditions

Analysis on HPLC was carried out using C-18 analytical column as described in section 5.3.3 above. The column was eluted with 50% (v/v) methanol in Milli-Q water containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 400 µl/min and the absorbance was detected at 228 nm. For fractionation of active hydrolysates or hydrolysate fraction, acetonitrile (solvent B) and Milli-Q water (solvent A) both containing 0.1% TFA was used. The hydrolysates or hydrolysate fractions were eluted with increasing gradient of solvent B to 15% over a period of 95 min. The peptides fractions were collected in a fraction collector.

5.3.5. Calculation of activity inhibition

The percentage of ACE inhibition was calculated according the formula mentioned in section 2.6.3. The extent of inhibition is expressed as IC_{50} value that is the concentration of peptide to inhibit 50% of the activity of ACE.

5.3.6. Fractionation and purification of active peptides

Hydrolysates that showed strong ACE inhibitory activity were selected and fractionated using molecular weight cut off (MWCO) membranes. The active hydrolysate was first filtered through a 0.20 μm filter before loading into molecular weight cut off (MWCO) membranes (5 kDa and 10 kDa) for fractionation. Three fractions were collected from this MWCO fractionation: fractions that contained less than 5 kDa peptides (labelled as <5 kDa fractions), fractions that contained 5-10 kDa peptides (labelled as 10 kDa fractions), and fractions that contained bigger than 10 kDa peptides (labelled as >10 kDa fractions). Active MWCO fractions were purified on C-18 reverse phase column HPLC coupled with a fraction collector (Shimadzu Oceania, Rydelmere, NSW, Australia). Purification was carried out using linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (0-15% in 80 min) at a flow rate of 0.8 ml/min. The fractions were pooled, lyophilised, and used for ACE inhibitory analysis. Fractions with high activity were further purified and were used for further ACE inhibitory analysis and stability assays.

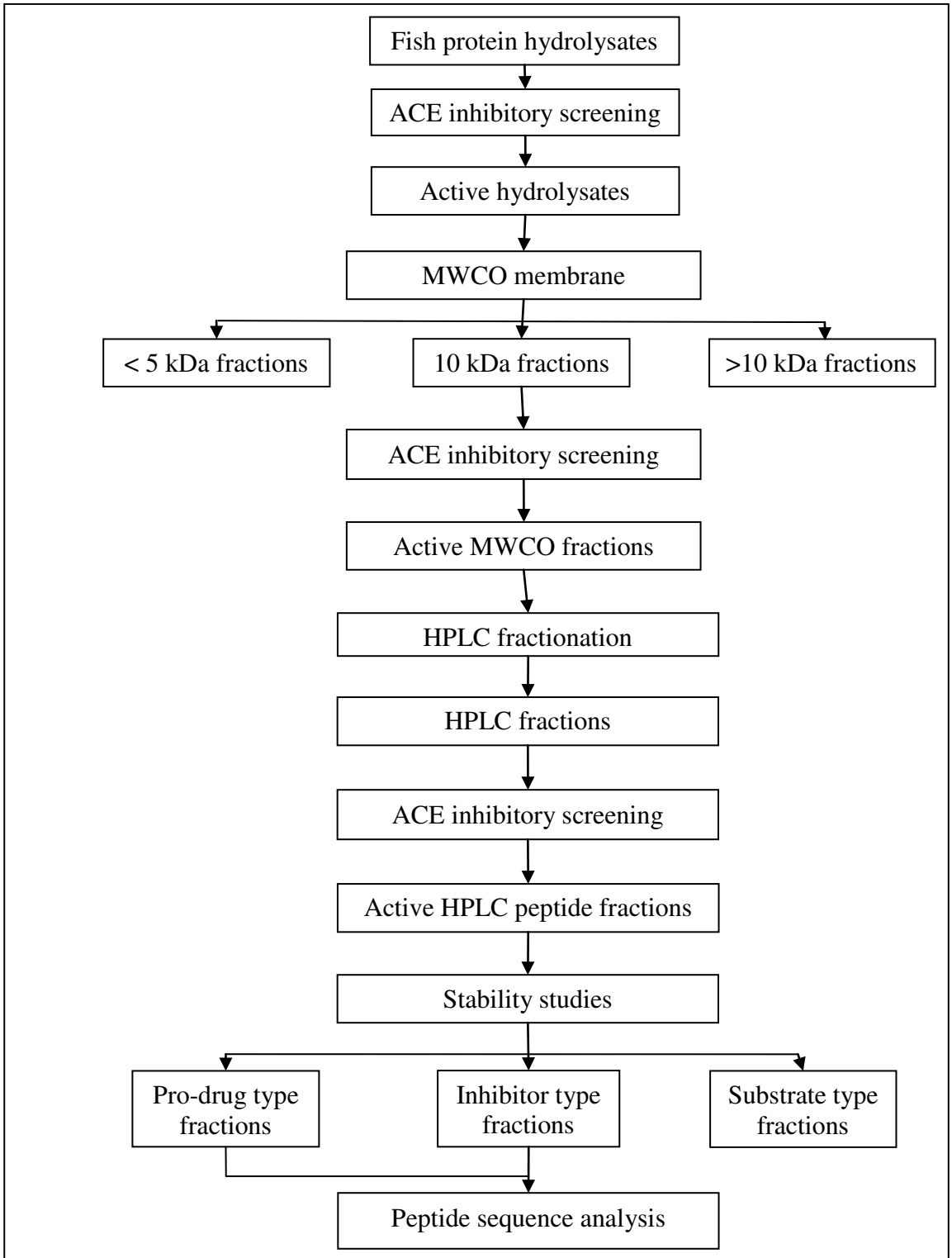


Figure 5.1. Flow chart of angiotensin I-converting enzyme (ACE) inhibitory peptides screenings. Screening is based on IC_{50} values of hydrolysates, MWCO fractions or HPLC peptide fractions analysed with methods described in sections 5.3.3 – 5.3.5.
MWCO = molecular weight cut off

5.3.7. Stability assay against ACE

Assay of the stability of ACE inhibitory peptides followed the same method as for the determination of ACE inhibition activity except that the peptides or inhibitor were mixed with ACE first then preincubated at 37^{o}C for 3-h (Fujita and Yoshikawa, 1999; Fujita et al., 2000) followed by the addition of HHL and further incubation at 37^{o}C for 1 h. The reaction was stopped by addition of 250 µl 1 N and the hippuric acid (HA) liberated was measured with HPLC as described above.

5.3.8. Stability assay against gastrointestinal enzymes

Assay of the stability of ACE inhibitory peptides against gastrointestinal enzymes followed the same method as for the determination of ACE inhibition activity except that the peptides or inhibitor were mixed with pepsin first then incubated at 37^{o}C for 1.5-hour (Ruiz et al., 2004; Quirós, et al. 2009). The reaction was stopped by means of boiling water bath for 15 min and the supernatant was used for ACE inhibition assay as detailed in section 5.3.3. by the addition of HHL and further incubation at 37^{o}C for 30 min. The remaining sample was used for simulated stability assay by mixing with a mixture of trypsin and chymotrypsin after pH adjustment to 7.6 and incubated for another 4 h. The reaction was stopped in a similar manner and the reaction mixture was used for ACE inhibition assay as above.

5.3.9. Amino acid sequence analysis

The amino acid residues of selected active peptide fraction were sequenced at Australian Proteome Analysis Facility (APAF), University of Macquarie. Peptide fractions were concentrated under vacuum centrifugation. The samples were loaded onto precycled,

biobrene-treated disc and were subjected to 12 cycles of Edman N-terminal sequencing using the pulse liquid cycle. Automated Edman degradation was carried out using Applied Biosystems 494 Procise Protein Sequencer System (Perkin Elmer, Waltham, MA, USA).

5.3.10. Liquid Chromatography/Mass Spectrometry

The molecular weights of peptides contained in crude peptide fractions were determined using an electronspray ionisation/mass spectrometry (ESI/MS) technique on an LC/MS system consisting of Varian ProStar HPLC and Varian 1200L Quadrupole MS/MS System as detailed in Section 2.4.5.

5.3.11. Statistical Analysis

All values are reported as mean of at least three observations from two replications ± standard deviation. One Way ANOVA was used to analyse data collected using Minitab 15.1 (Minitab Inc., USA), followed by Tukey pairwise test ($\alpha < 0.05$) to evaluate significant difference between means.

5.4. Results

5.4.1. Screening of ACE inhibitory hydrolysates

The effects of enzymes and hydrolysis times on the ACE inhibitory activities of leatherjacket and trevally protein hydrolysates were screened initially to isolate, purify, and identify any anti-hypertensive peptides. Results from the screening of ACE inhibitory activity of the hydrolysates, crude fractions of different molecular weight ranges (MWCO fractions), and peptide fractions are presented in various graphs below.

The IC_{50} values of leatherjacket soluble protein hydrolysates ranged from 1.35 mg/ml to 1.89 mg/ml (Figure 5.2a). Bromelain 8 h and 10 h hydrolysis of leatherjacket soluble proteins gave the lowest IC_{50} values (1.35 mg/ml) as compared to the IC_{50} values of 6 h papain and 6 h Flavourzyme™ hydrolysates (1.42 and 1.63 mg/ml). The IC_{50} values of leatherjacket bromelain soluble protein hydrolysates tended to increase during the first 6 h hydrolysis then decrease to its lowest at 8 h hydrolysis and 10 h hydrolysis. The IC_{50} values of papain hydrolysates decrease to its lowest (1.42 mg/ml) at 4 h hydrolysis then increase slightly thereafter. The IC_{50} values of Flavourzyme™ hydrolysates increased at 4 h hydrolysis, decreased to its lowest (1.63 mg/ml) at 6 h hydrolysis, and then increased again thereafter. The IC_{50} values of leatherjacket soluble proteins hydrolysates are significantly affected ($p < 0.05$) by both enzymes and hydrolysis times.

The IC_{50} values of trevally soluble protein hydrolysates ranged from 1.99 mg/ml to 3.34 mg/ml (Figure 5.2b) and were significantly affected ($p < 0.05$) by the enzymes and hydrolysis times. The IC_{50} values vary during hydrolysis and tended to decrease then increase as the hydrolysis progressed. Bromelain hydrolysate of 4 h hydrolysis showed the lowest IC_{50} value (1.99 mg/ml) as compared to other hydrolysates. In general, the IC_{50} value of bromelain hydrolysate at any hydrolysis time is lower than the papain and Flavourzyme™ hydrolysate of the same hydrolysis time. The activity of papain hydrolysates decreased as the hydrolysis time increased up to 8 h, then increased slightly at 10 h hydrolysis. Flavourzyme™ hydrolysates showed weaker activity against ACE compared to the other hydrolysates namely papain and bromelain.

The ACE inhibitory activity of leatherjacket insoluble hydrolysates varied and were significantly affected ($p < 0.05$) by the enzymes used and hydrolysis times. Papain hydrolysate at 4 h showed the lowest IC_{50} value (0.77 mg/ml) compared with other

hydrolysates. The papain hydrolysates tended to show weaker activity as hydrolysis progressed further than 4 h. The IC_{50} values of bromelain hydrolysates remained the same during hydrolysis, while the IC_{50} values of Flavourzyme™ hydrolysates increased significantly as hydrolysis progressed. The IC_{50} values of trevally insoluble hydrolysates vary greatly with the lowest value (2.45 mg/ml) obtained from 4 h Flavourzyme™ hydrolysis, and the value increased thereafter. Papain hydrolysates showed highest activity at 6 h hydrolysis and decreased thereafter, while bromelain hydrolysates showed the lowest activity at 2 h hydrolysis.

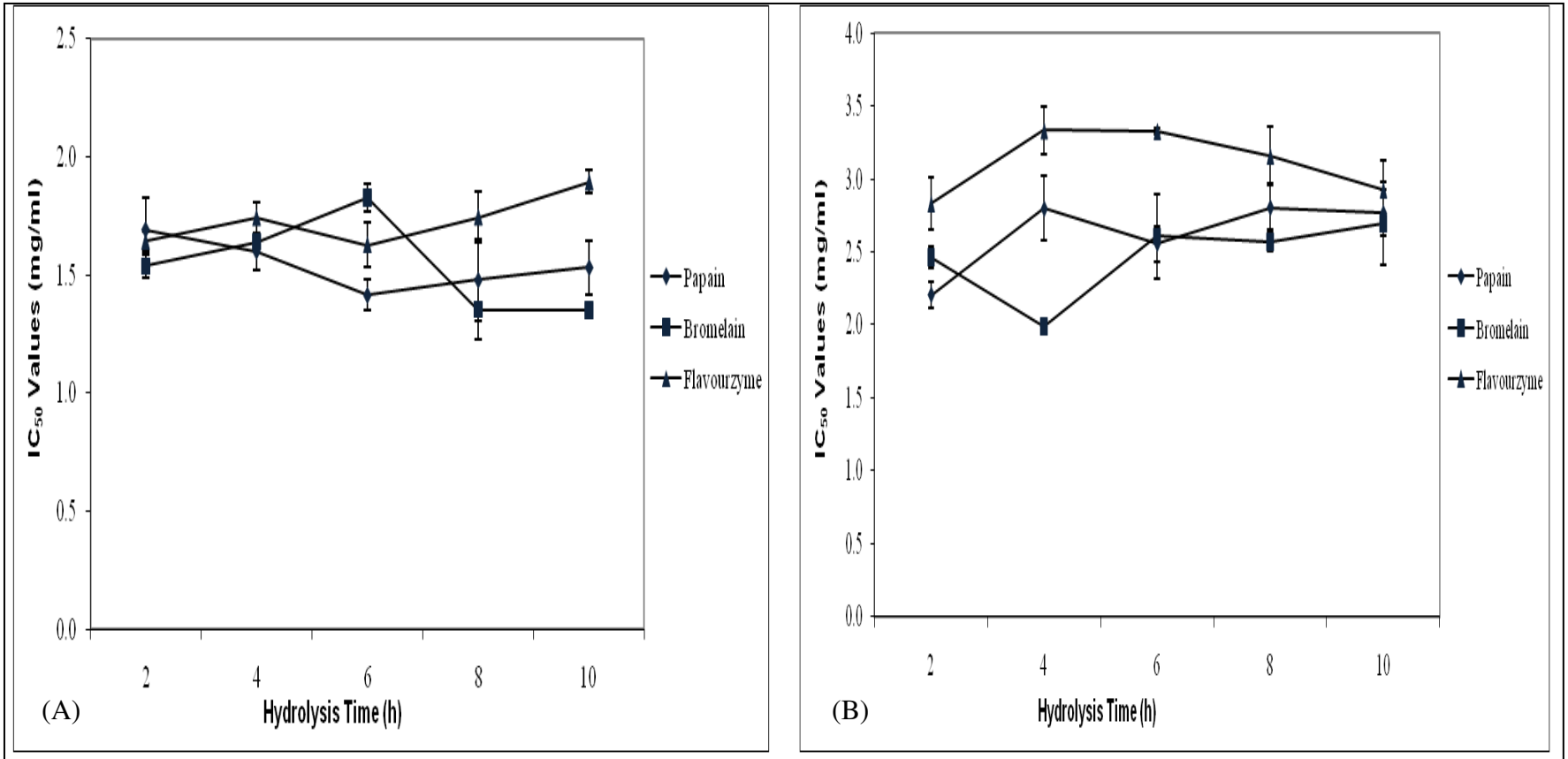


Figure 5.2. ACE inhibitory activity (IC_{50} values) of leatherjacket (A) and trevally (B) soluble protein hydrolysates at different hydrolysis times of 2, 4, 6, 8, and 10 hour (Mean ± s.d., n = 8). Lower IC_{50}'s indicate higher ACE inhibitory activity.

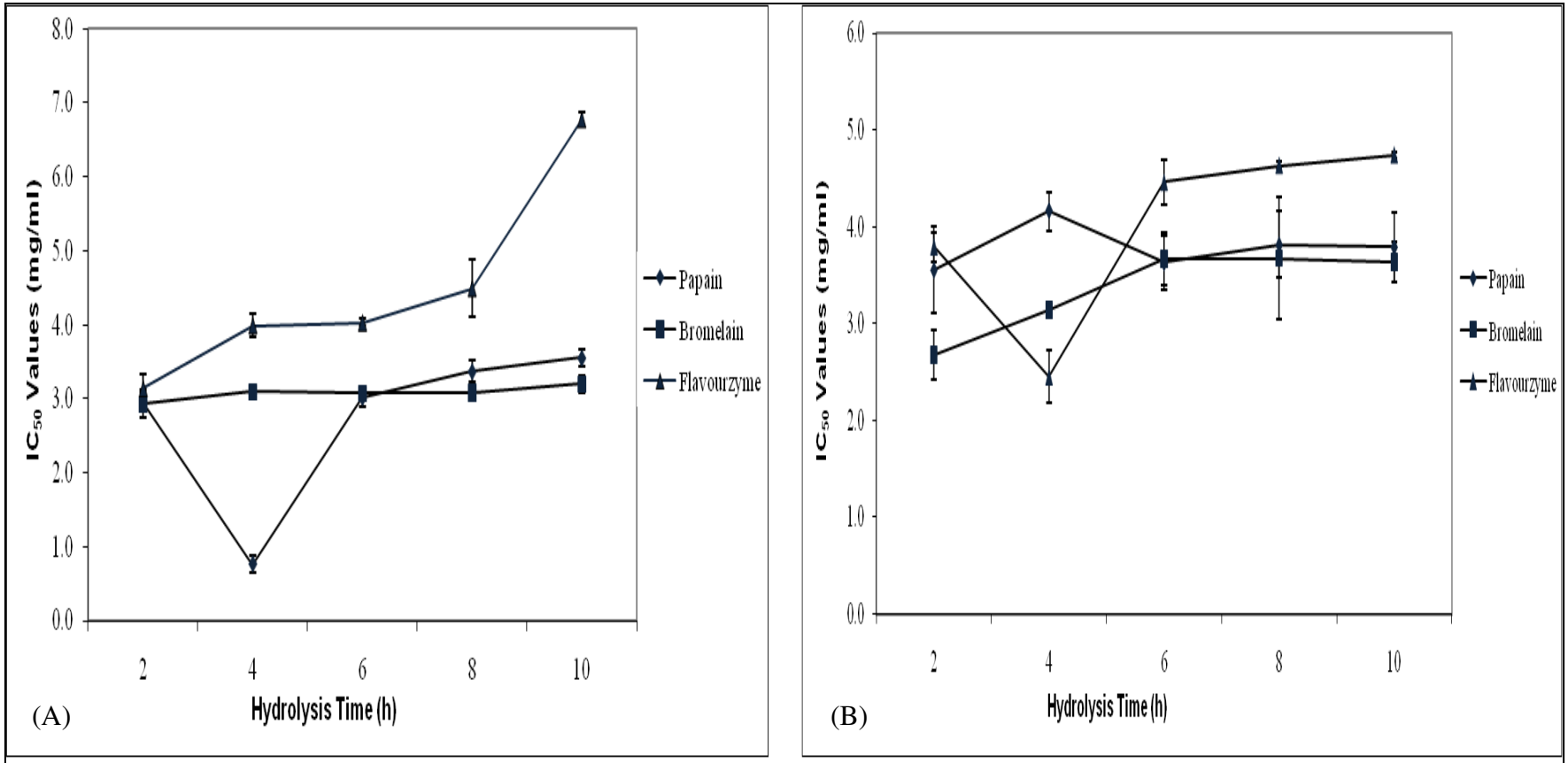


Figure 5.3. ACE inhibitory activity (IC_{50} values) of leatherjacket (A) and trevally (B) insoluble protein hydrolysates at different hydrolysis times (Mean ± s.d., n = 8). Lower IC_{50}'s indicate higher ACE inhibitory activity.

5.4.2. Molecular weight fractionation of selected active ACE inhibitory hydrolysates

Selected hydrolysates with high activity against ACE were subjected to fractionation using molecular weight cut off (MWCO) membranes of <5 kDa and 10 kDa giving out three fractions of <5 kDa (containing peptides less than 5 kDa molecular weight), 10 kDa (containing between 5-10 kDa peptides) >10 kDa (containing peptides more than 10 kDa). This is to evaluate the possibility of grouping potential active fractions in a molecular weight range that can easily be obtained and used as a rather crude fraction in the formulation of functional foods and to further guide the selection of hydrolysates for peptide purification purposes. Eight leatherjacket hydrolysates and 13 trevally hydrolysates were selected for fractionation followed by *in vitro* analysis of their ACE inhibitory activities.

Results from ACE inhibition activity analysis showed that an 8 h bromelain hydrolysate of soluble leatherjacket protein gave the lowest IC_{50} values for the MWCO fractions as compared to the other fractions of the selected leatherjacket hydrolysates (Plate 5.1). In general, <5 kDa fractions of the selected hydrolysates showed stronger activity against ACE with the exception of <5 kDa fraction of 10-hour papain hydrolysate of soluble proteins where the IC_{50} values was much higher than those of fraction 10 kDa and >10 kDa. There was no consistent pattern observed between the IC_{50} values of fraction 10 kDa and >10 kDa as their IC_{50} values did not show similar trends compared to <5 kDa fractions most of the selected hydrolysates. Significant difference ($p < 0.05$) was observed between fractions of different molecular weight ranges from the same hydrolysates.

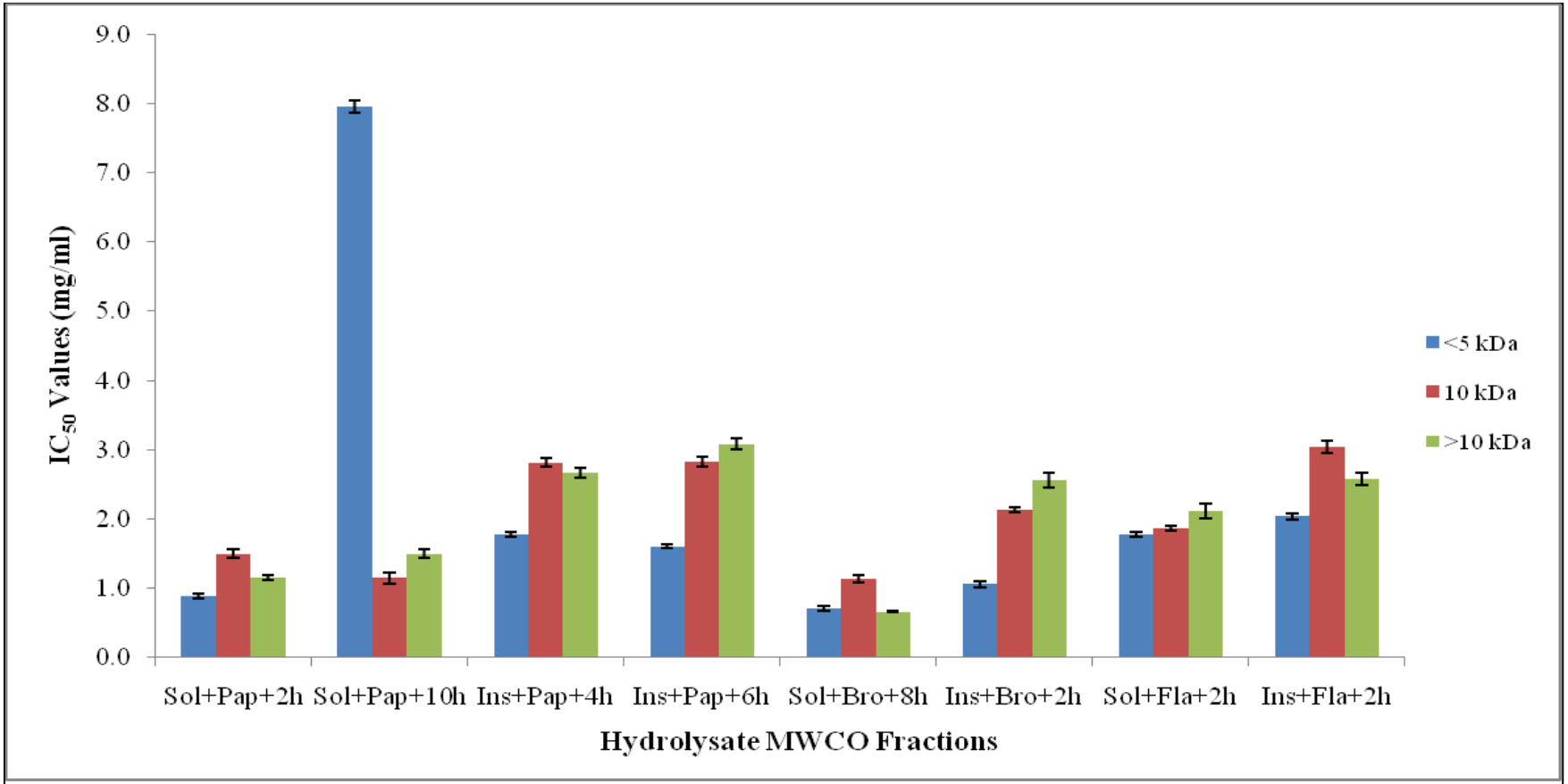


Plate 5.1. ACE inhibition activity (presented based on their IC_{50}^{*} values) of fraction <5 kDa, 10 kDa, and >10 kDa of selected leatherjacket hydrolysates (Mean ± s.d., n = 8).
*Lower IC_{50} value indicate stronger activity and more inhibition
Pap – papain, bro – bromelain, Fla – Flavourzyme™.

Results from ACE inhibition analysis of fractions of selected trevally soluble protein hydrolysates showed that fraction >10 kDa of 6 h Flavourzyme™ hydrolysate had the lowest IC_{50} value (0.27 mg/ml) (Plate 5.2). The IC_{50} values of <5 kDa fraction also showed a similar pattern having lower values compared to 10 kDa and >10 kDa fractions of the same hydrolysates. However, some exceptional pattern were observed where the 10 kDa and >10 kDa fractions showed stronger activity than the <5 kDa fractions. Significant differences ($p < 0.05$) were observed among the IC_{50} values of fractions from the selected hydrolysates.

The IC_{50} values of fractions of selected trevally insoluble protein hydrolysates also showed similar pattern as their soluble hydrolysates in which the 5 kDa fractions tend to have lower IC_{50} values as compared to 10 kDa and 10+ kDa fractions of the same hydrolysates with some exception (Plate 5.3). It is also observed that the IC_{50} values of fractions 10 kDa are usually lower than those of 10+ kDa fractions. Therefore, the IC_{50} values of MWCO fractions follow an increasing order from 5 kDa, 10 kDa, then 10+ kDa. The lowest IC_{50} value is 1.37 mg/ml of 2 h bromelain hydrolysis. Results from statistical analysis indicate significant difference ($p < 0.05$) among means of different fractions.

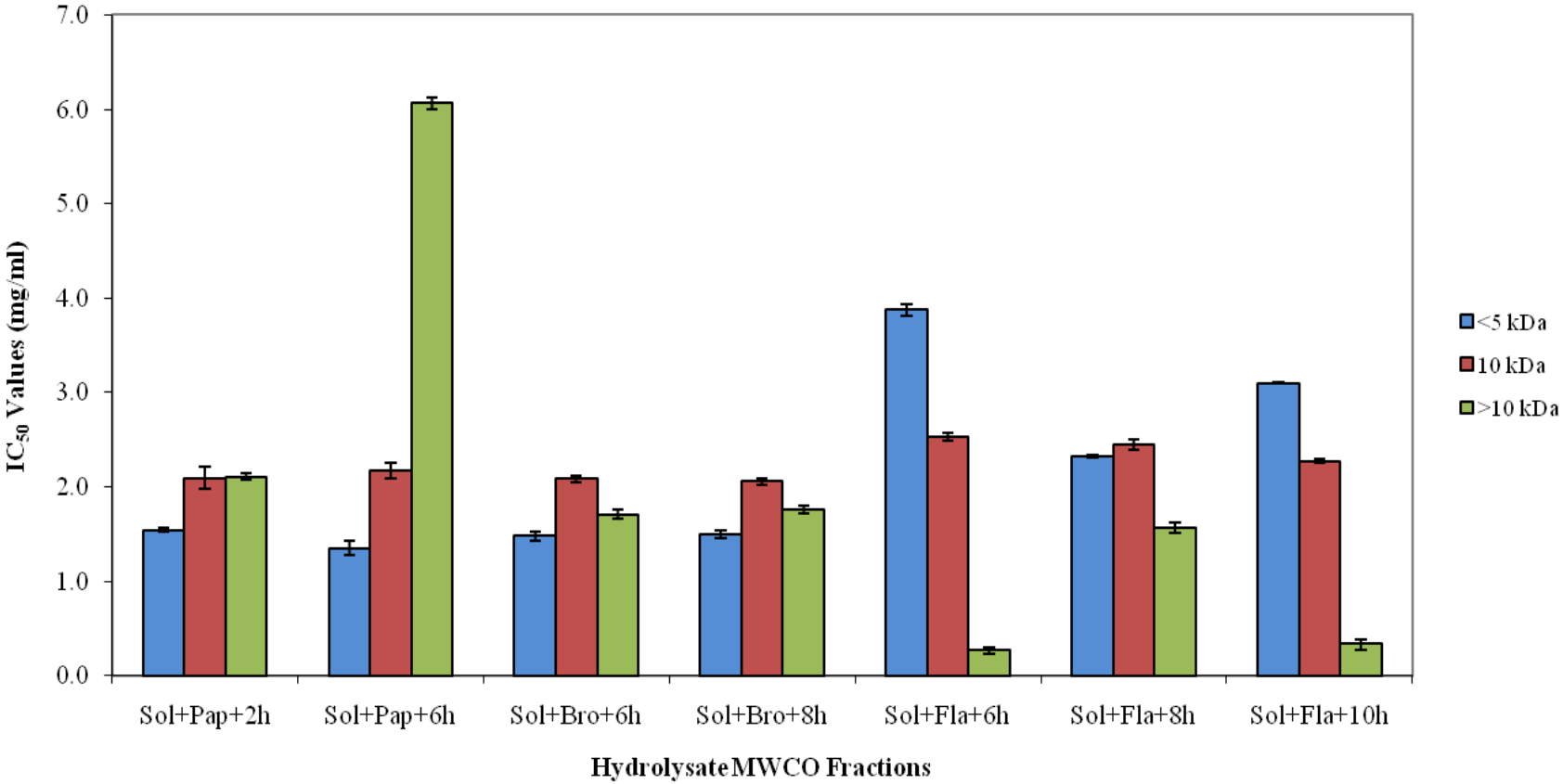


Plate 5.2. ACE inhibition activity (presented based on their IC_{50}^{*} values) of fraction 5 kDa, 5-10 kDa, and 10+ kDa of selected soluble trevally hydrolysates (Mean ± s.d., n = 8).
*Lower IC_{50} value indicate stronger activity and more inhibition
Pap – papain, Bro – bromelain, Fla – Flavourzyme™.

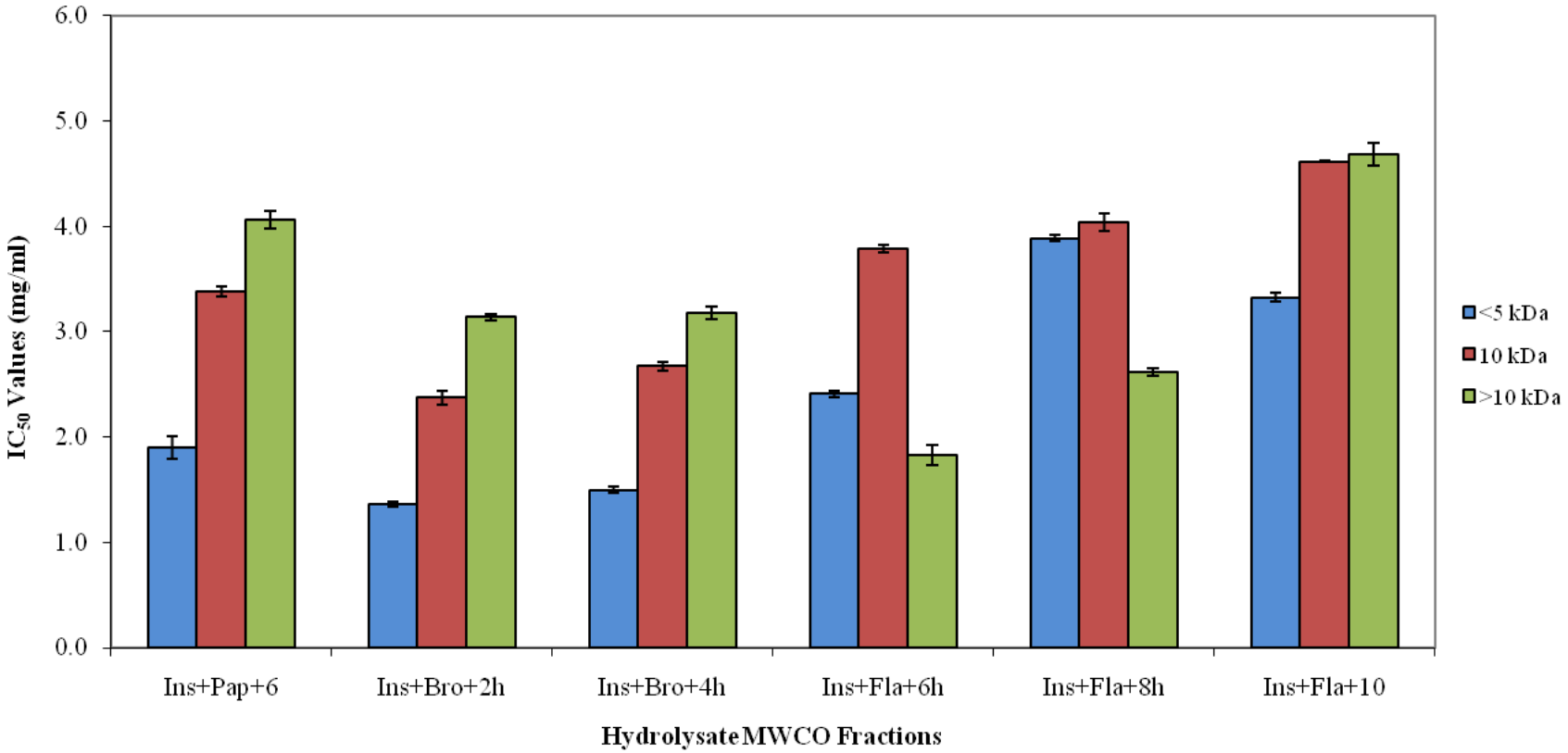


Plate 5.3. ACE inhibition activity (presented based on their IC_{50}^{*} values) of fraction 5 kDa, 5-10 kDa, and 10+ kDa of selected insoluble trevally hydrolysates (Mean ± s.d., n = 8).
*Lower IC_{50} value indicate stronger activity and more inhibition
Pap – papain, Bro – bromelain, Fla – Flavourzyme™.

5.4.3. ACE inhibitory activities of selected peptide fractions and their stability.

Further to MWCO fractionation of selected hydrolysates, the MWCO fractions that showed strong ACE inhibition were subjected to fractionation and purification using C-18 RP-HPLC, followed by ACE inhibition assays to evaluate potential active peptides in the purified fractions. Three leatherjacket hydrolysates and three trevally hydrolysates were fractionated to obtain isolated peptide fractions for this purpose (Figure 5.4 and Figure 5.5). The choice of these selections was based on the IC_{50} values of their MWCO fractions and possibly the finding of new ACE inhibitory peptides from a specific enzyme used to hydrolyse fish proteins.

These further fractionations were carried out based on earlier assessments on IC_{50} values of rather limited fractions that include few peaks representing peptides. In these fractionations, some single peaks were identified and collected for further investigations of their ACE inhibition strength and stability. The stability study was carried out to observe the fate of the ACE inhibitory peptides after mixing with ACE in the absence of substrate hippuryl-histidyl-leucine (HHL). Results from these analyses are shown in Table 5.1. Complete ACE inhibitory results of the peptide fraction can be seen in Appendix 5. Five selected peptide fractions from leatherjacket hydrolysates were analysed and showed significant ACE inhibitory activities, of which fractions FS5 and FS6 of 6 h papain hydrolysate (referred to as LPI5 and LPI6, respectively), FS5 of 2 h bromelain hydrolysate of insoluble protein (LBI5) and FS5 of 2 h Flavourzyme™ hydrolysates of insoluble proteins (LFI5) showed inhibitor characteristics, where ACE could not hydrolyse the peptides, hence retained their IC_{50} values. One peptide fraction FS2 of 2 h bromelain hydrolysis of insoluble proteins (LBI2) showed substrate characteristics, where its IC_{50} value increased after incubation with ACE prior to the addition of HHL indicating the ability of ACE to

cleave this peptide into smaller peptide/s with weaker activity against ACE. The anti-microbial fraction LBI8H described in Chapter 4 was also shown ACE inhibitory activity with an IC_{50} value of 0.24, a rather weaker ACE inhibitory peptide.

Seven peptide fractions from trevally protein hydrolysates were also subjected for further characterisation. Peptide fractions FS2 of 6 h bromelain hydrolysate of soluble protein and FS2 of 2 h bromelain hydrolysate of insoluble proteins (referred to as TBS1 and TBI2, respectively) showed pro-drug characteristics, where its IC_{50} value decreased after incubation with ACE prior to the addition of HHL, indicating the ability of ACE to cleave these peptides and produce peptide/s with stronger activity against ACE. Peptide fractions FS5 and FS6 of 6 h bromelain hydrolysate of soluble proteins (TBS2 and TBS6) and FS4 of 2 h bromelain hydrolysate of insoluble proteins (TBI4) showed inhibitor-type characteristics, where the IC_{50} value did not change after incubation with ACE. This indicates their stability to resist further cleaving by ACE. Peptide fractions FS3 and FS4 of 6 h papain hydrolysate of insoluble proteins (TPI3 and TPI4) showed substrate type characteristics with higher IC_{50} values after incubation with ACE prior to the addition of HHL. This indicates the ability of ACE to cleave these peptides into smaller less active peptide/s.

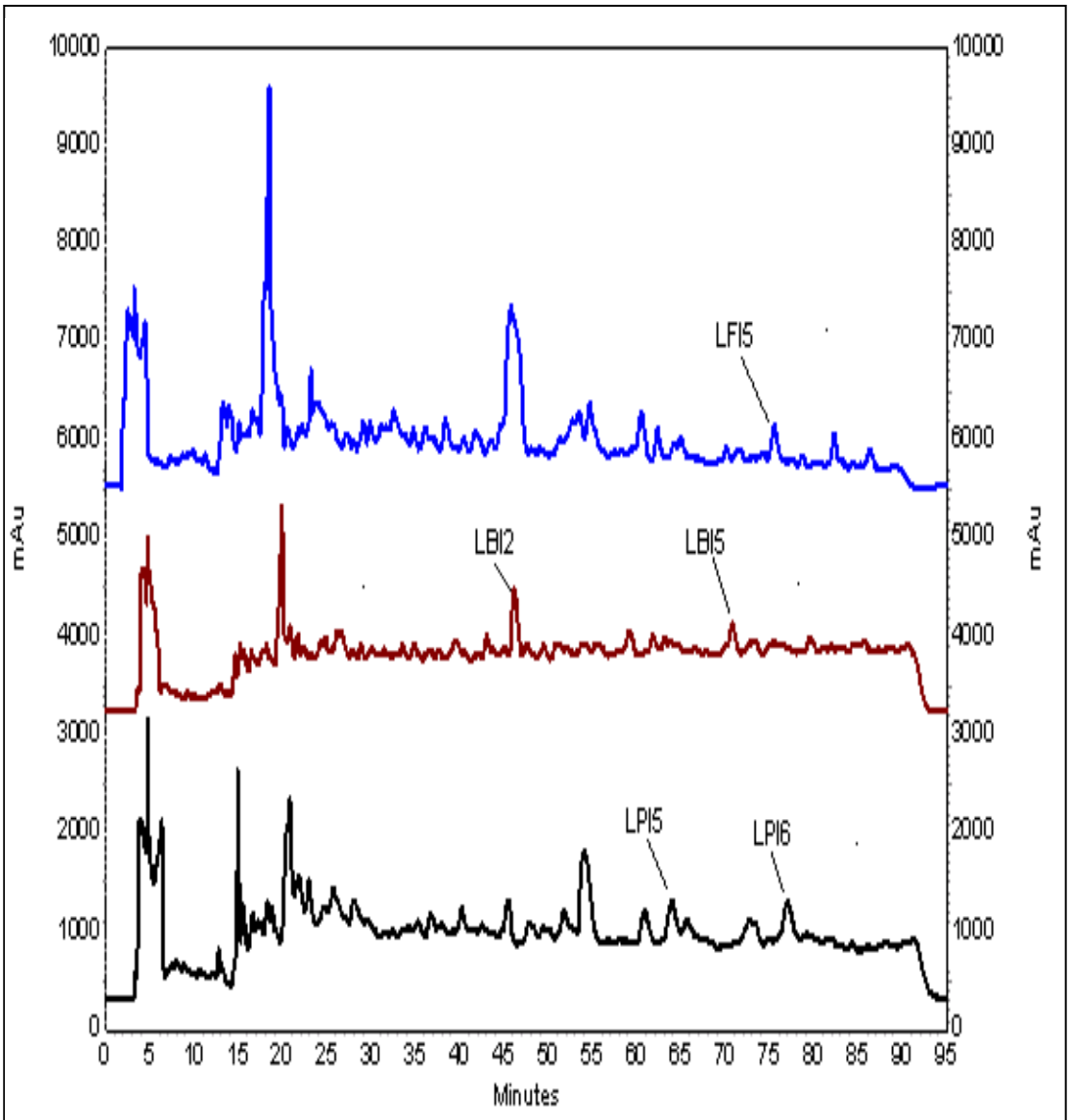


Figure 5.4. Chromatograms of selected leatherjacket (hydrolysates for isolation of active peptides. Selected peptide fraction are indicated and used for stability study.
LPI5 and LPI6 are from 6 h papain hydrolysate of insoluble proteins.
LBI2 and LBI5 are from 2 h bromelain hydrolysate of insoluble proteins.
LFI5 is from 2 h Flavourzyme hydrolysate of insoluble proteins.
L = leatherjacket proteins.

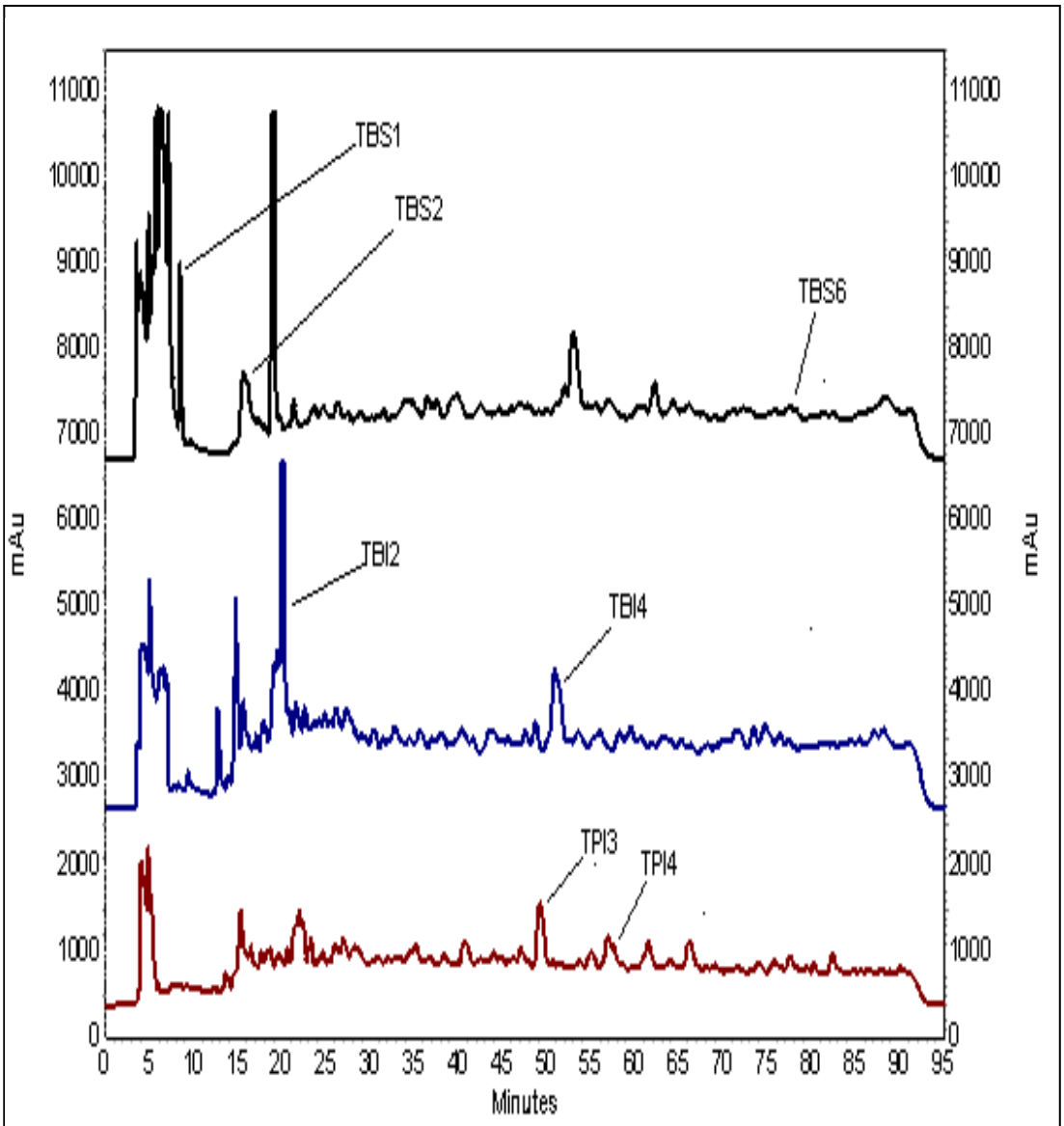


Figure 5.5. Chromatograms of selected trevally (hydrolysates for isolation of active peptides. Selected peptide fraction are indicated and used for stability study.
TPI3 and TPI4 are from 6 h papain hydrolysate of insoluble proteins
TBS1, TBS2, and TBS6 are from 6 h bromelain hydrolysates of soluble proteins
TBI2 and TBI4 are from 2 h bromelain hydrolysate of insoluble proteins
T = trevally proteins.

Table 5.1 Percent inhibition and IC_{50} values of selected isolated peptide fractions and their stability (Mean ± s.d., n = 8).

Sample Fractions	ACE Inhibition Activity		Stability[a]		Classification
	Percent Inhibition	IC_{50} Values	Percent Inhibition	IC_{50} Values	
LPI5	79.77	0.05	82.19	0.04	Inhibitor
LPI6	85.39	0.02	81.57	0.02	Inhibitor
LBI2	26.45	0.03	7.49	0.10	Substrate
LBI5	19.90	0.11	21.64	0.10	Inhibitor
LBI12[b]	74.87	0.24	53.39	0.14	Pro-drug
LFI5	58.38	0.01	58.77	0.01	Inhibitor
TPI3	74.25	0.06	67.35	0.07	Substrate
TPI4	65.03	0.04	54.05	0.05	Substrate
TBS1	34.64	0.11	58.26	0.06	Pro-drug
TBS2	51.52	0.07	47.44	0.07	Inhibitor
TBS6	58.86	0.08	56.23	0.08	Inhibitor
TBI2	47.70	0.09	82.18	0.05	Pro-drug
TBI4	56.10	0.12	60.54	0.11	Inhibitor

[a] Stability of the selected peptide fraction is a value presented both as percent inhibition and IC_{50} value and serve as an indication of the strength of the peptide to stand against ACE before the introduction of hippuryl-histidyl-leucine (HHL) as substrate for ACE.

[b] LBI12 is from 8 h bromelain hydrolysis of insoluble proteins and is an anionic anti-microbial peptide (see Chapter 4) found to be able to inhibit the action of ACE.

LPI5 and LPI6 are from 6 h papain hydrolysate of insoluble proteins.

LBI2 and LBI5 are from 2 h bromelain hydrolysate of insoluble proteins.

LFI5 is from 2 h Flavourzyme™ hydrolysate of insoluble proteins.

TPI3 and TPI4 are from 6 h papain hydrolysate of insoluble proteins

TBS1, TBS2, and TBS6 are from 6 h bromelain hydrolysates of soluble proteins

TBI2 and TBI4 are from 2 h bromelain hydrolysate of insoluble proteins

L = leatherjacket proteins, T = trevally proteins.

5.5. Stability against gastrointestinal enzymes

Results from gastrointestinal enzyme degradation studies revealed that the peptides under investigation were resistant toward gastrointestinal enzymes pepsin, trypsin, and chymotrypsin degradation. Results from gastrointestinal hydrolysis of three leatherjacket and two trevally <5 kDa fractions, and eight selected active fractions are presented in Plate 5.4 and Plate 5.5, respectively. In general, the IC_{50} values of the <5 kDa fractions decreases sharply after incubation with pepsin and further decreased after incubation with trypsin and chymotrypsin. These indicated breakdowns of peptides and produced smaller peptides with stronger inhibition.

The IC_{50} values of selected active peptide fractions, however, showed mixed patterns decreasing or increasing after incubation with pepsin and mixture of trypsin and chymotrypsin, compared to the initial IC_{50} values of their parent peptide fractions. Comparison of the IC_{50} values of the peptide fractions before and after incubation with pepsin and trypsin/chymotrypsin were carried out to study the fate of the peptides under *in vitro* gastrointestinal conditions. Fractions LPI5 and TBI2 have had their IC_{50} values decreased by 80% and 22% indicating the ability of pepsin and trypsin/chymotrypsin to digest the peptides. The IC_{50} values of LPI6 increased by 67% and 75% after incubation with pepsin and trypsin/chymotrypsin, respectively. The IC_{50} values of LBI5 and LBI12 decreased by 18% and 75% after incubation with pepsin, and then increased by 18% and 33% after further incubation with trypsin/chymotrypsin. The IC_{50} values of LFI5 remained unchanged after incubation with pepsin, and then increased by 50% after further incubation with trypsin/chymotrypsin. The IC_{50} values of TBS1 and TBS2 fraction remained almost unchanged after incubation with both pepsin and trypsin/chymotrypsin.

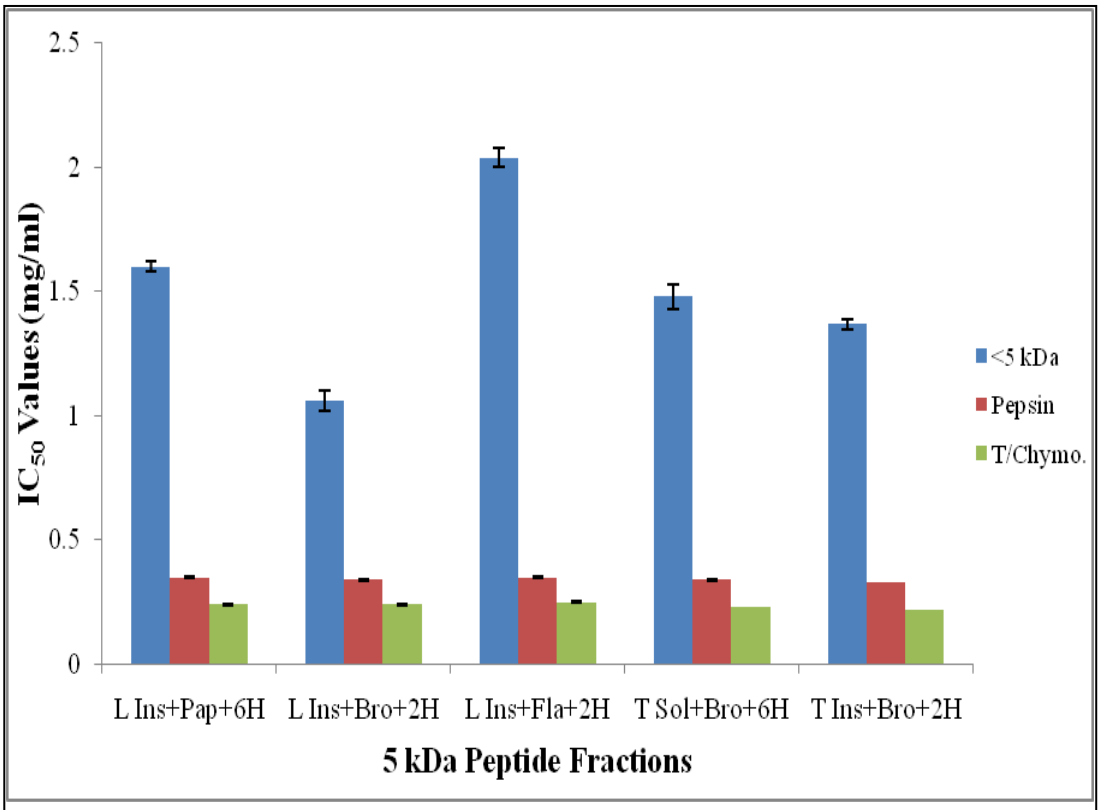


Plate 5.4. Results from gastrointestinal enzyme hydrolysis of selected 5 kDa hydrolysate fractions (Mean ± s.d., n = 8).

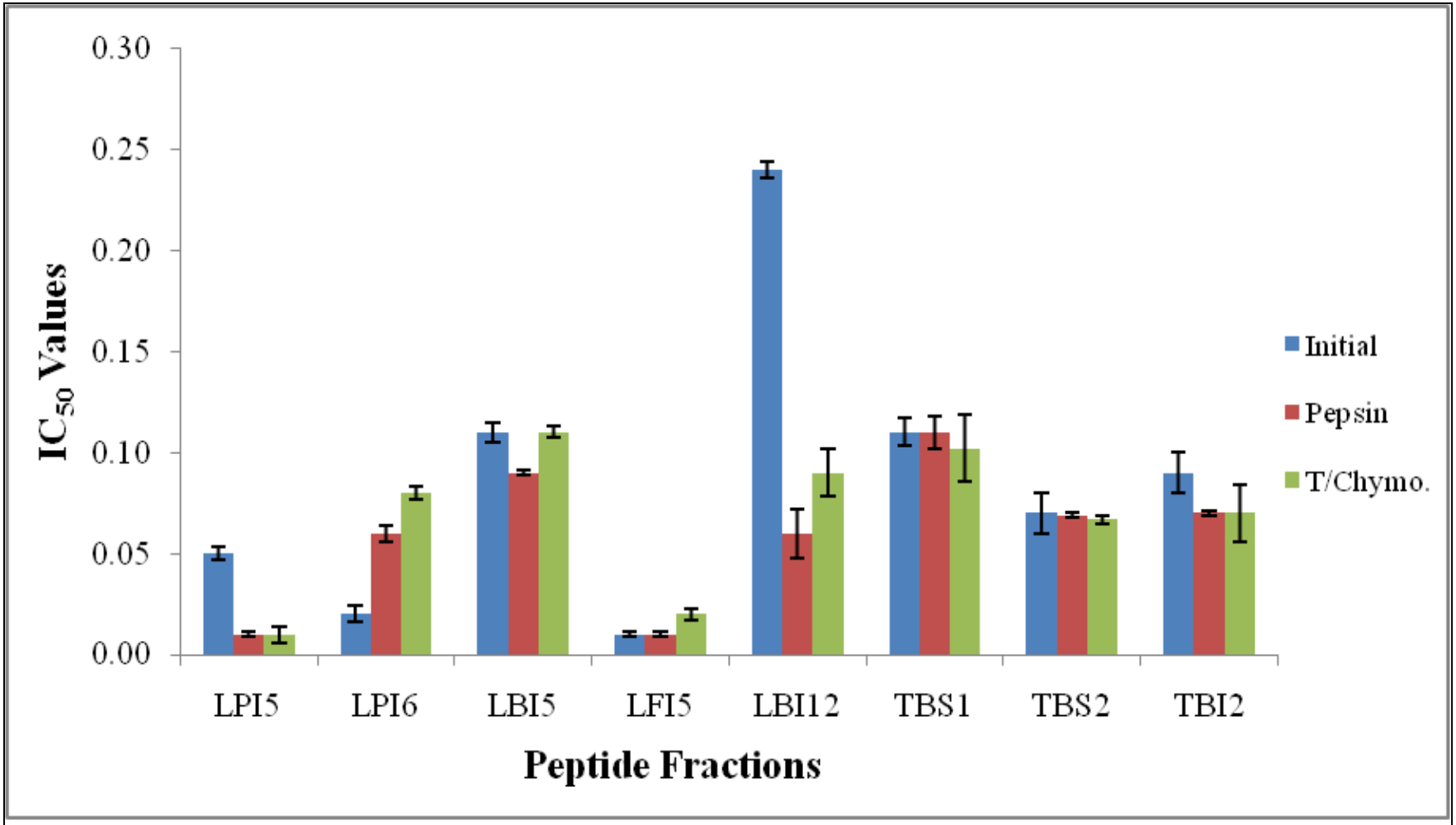


Plate 5.5. Results from simulated gastrointestinal enzyme degradation of selected active peptide fractions (Mean ± s.d., n = 10).

5.6. Structure elucidation of ACE inhibitory peptides

The primary structures of active peptides contained in seven active peptide fractions classified as pro-drug and inhibitor types were determined by Edman degradation method followed by complimentary ESI/MS analysis to verify the presence of the peptides. The amino acid sequences of the active peptides and their activities expressed in molar concentration are presented in Table 5.2, while the ESI/MS spectra are presented in Figure 5.6 to Figure 5.9. Results from Edman degradation showed that fraction LPI5 contained pentapeptide Glu-Pro-Leu-Tyr-Val (EPLYV; 619.71 Da) as the main peptide and its presence was confirmed by complimentary ESI/MS showing a peak at an m/z point of 620.6. Glu-Pro-Leu-Tyr-Val (EPLYV) may have originated from fish myosin alkali light chain fragment 86-90 as a homologue Phe-Glu-Asp-Tyr-Val (FEDYV) has been identified in chicken myosin alkali light chain (http://prowl.rockefeller.edu). Complete sequences of proteins that are used to aid the identification of the active peptides are shown in Appendix 2, Appendix 3, and Appendix 4. Fraction LPI6 consisted of mainly tetrapeptide Asp-Pro-His-Ile (DPHI; 480.52 Da), the presence of which had been confirmed by ESI/MS at 481.5 m/z. Homologues of Asp-Pro-His-ile (DPHI) have been identified in various fragments of chicken skeletal myosin heavy chain and myosin alkali light chain (http://prowl.rockefeller.edu).

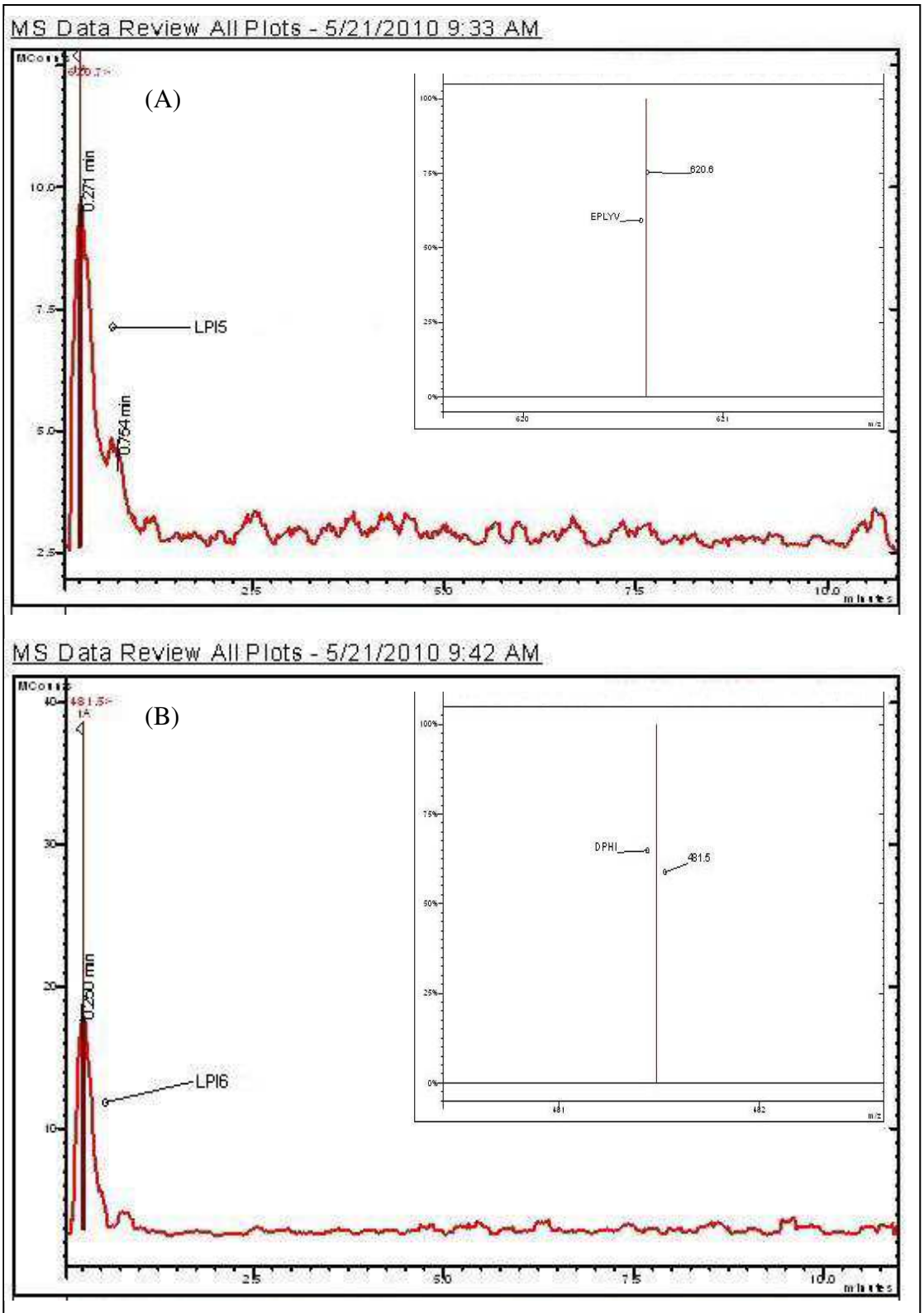


Figure 5.6. Mass spectra of peptide fractions LPI5 (A) and LPI6 (B) as measured by ESI/MS. Peptide fraction LPI5 contains pentapeptide Glu-Pro-Leu-Tyr-Val (EPLYV) having molecular weight as appears at 620.6 m/z. Peptide fraction LPI6 contains tetrapeptide Asp-Pro-His-ile (DPHI) having molecular weight of 481.5 m/z.

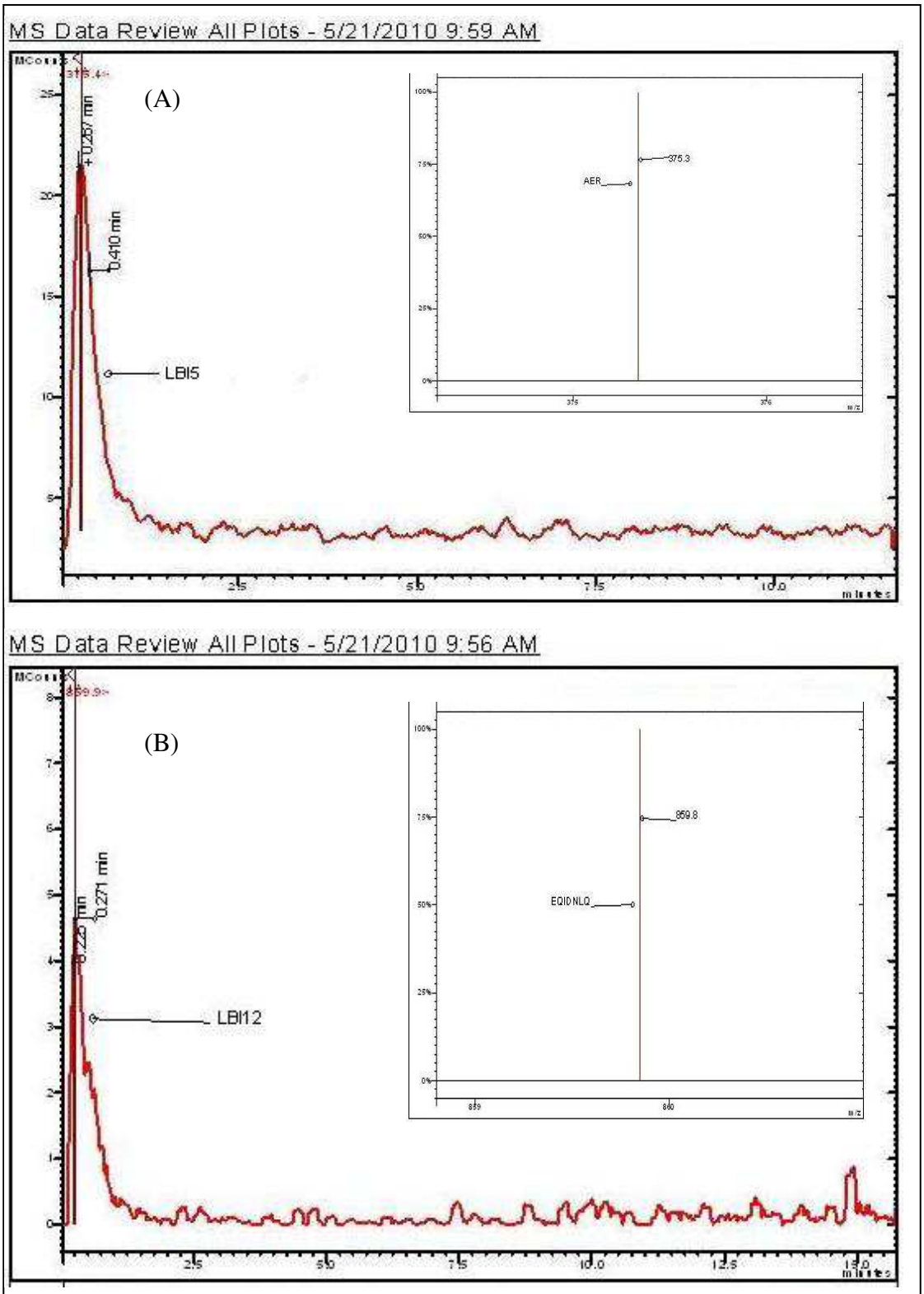


Figure 5.7. Mass spectra of peptide fractions LBI5 (A) and LBI12 (B) as measured by ESI/MS. Peptide fraction LBI5 contains tripeptide Ala-Glu-Arg (AER) having molecular weight as appears at 375.3 m/z. Peptide fraction LBI12 contains heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) having molecular weight of 859.8 m/z. Heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) is an anionic antimicrobial peptide that also shows ACE inhibitory activity.

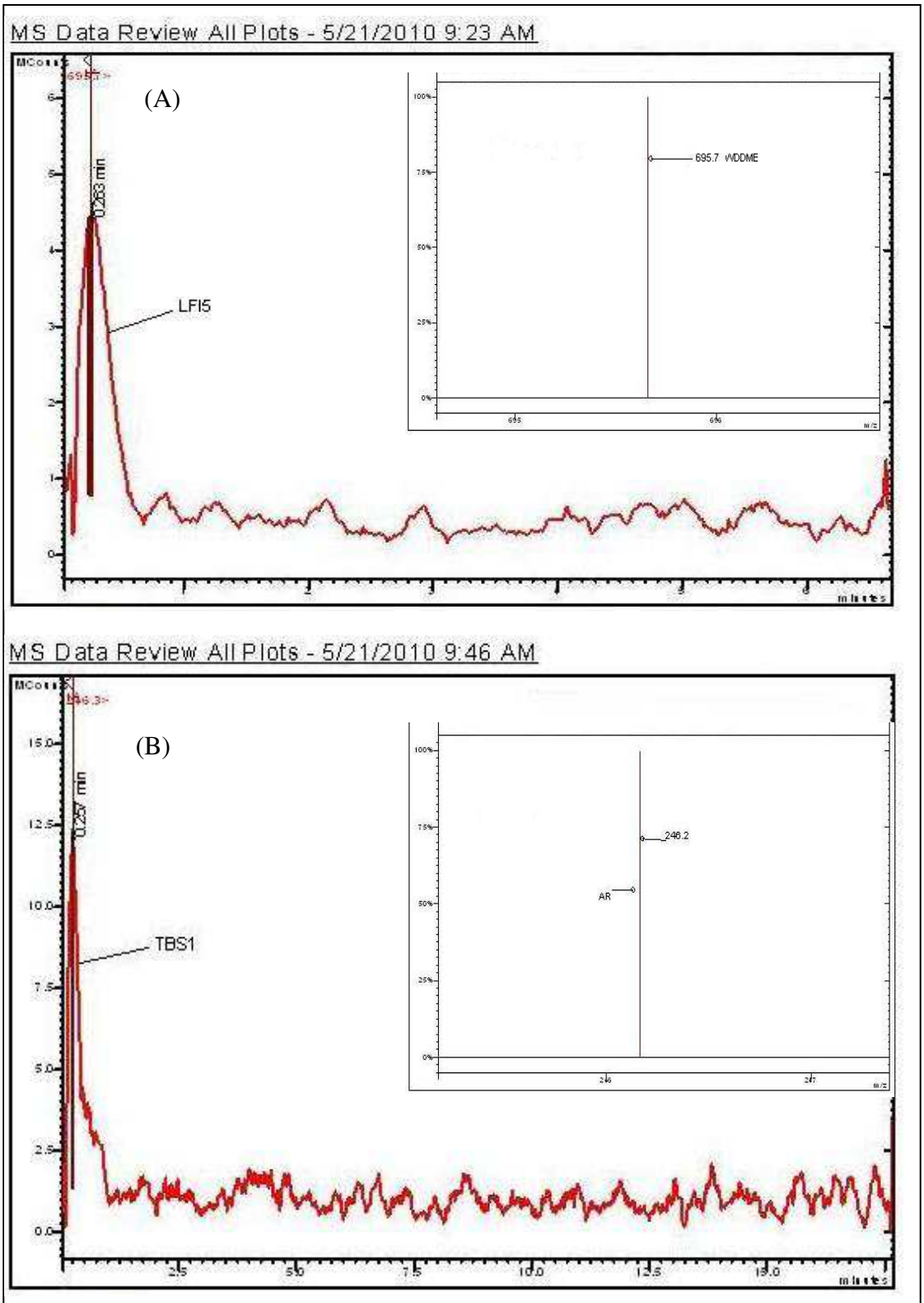


Figure 5.8. Mass spectra of peptide fractions LFI5 (A) and TBS1 (B) as measured by ESI/MS. Peptide fraction LFI5 contains pentapeptide Trp-Asp-Asp-Met-Glu (WDDME) having molecular weight as appears at 695.7 m/z. Peptide fraction TBS1 contains dipeptide Ala-Arg (AR) having molecular weight of 246.2 m/z.

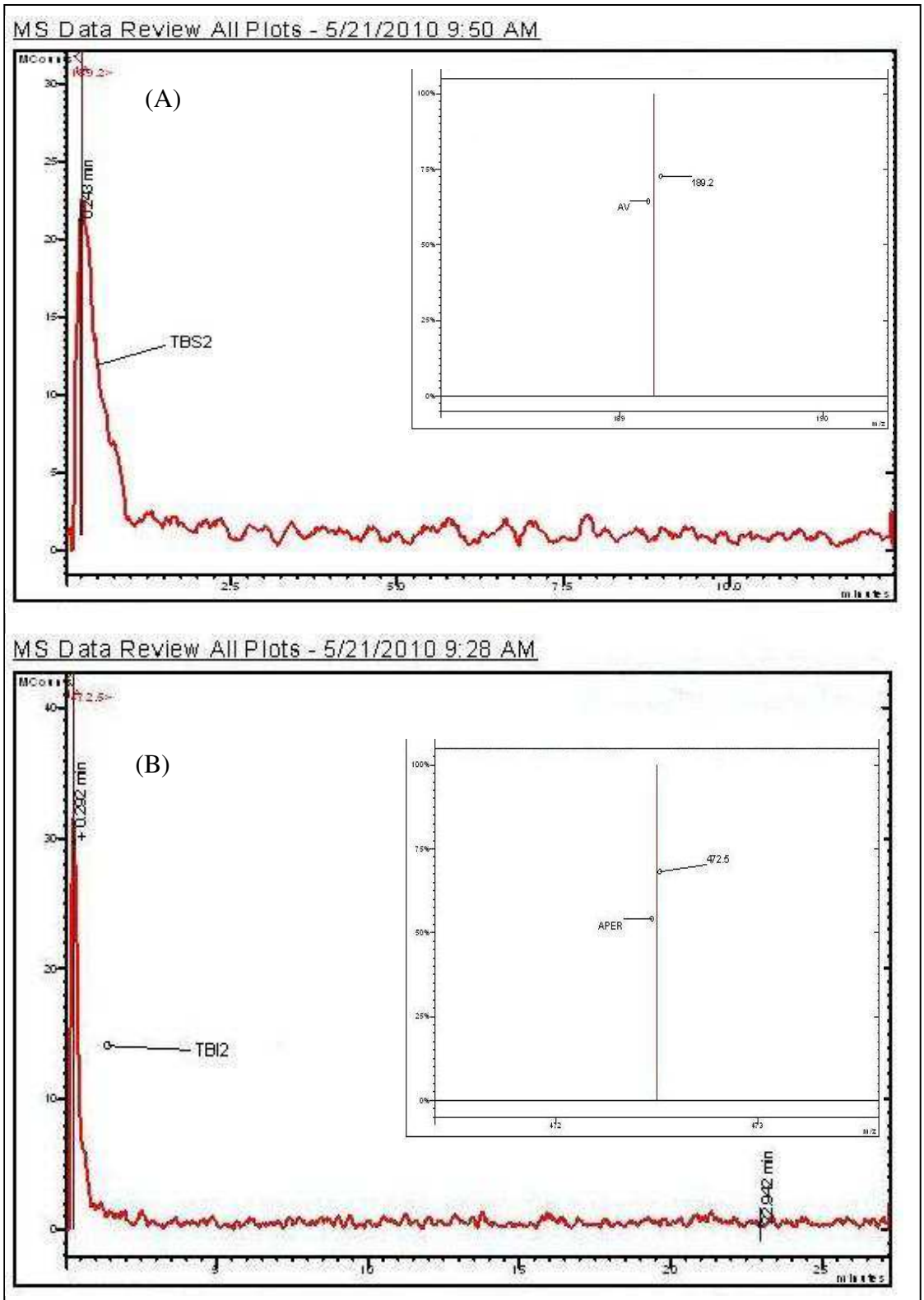


Figure 5.9. Mass spectra of peptide fractions TBS2 (A) and TBI2 (B) as measured by ESI/MS. Peptide fraction TBS2 contains dipeptide Ala-Val (AV) having molecular weight as appears at 189.2 m/z. Peptide fraction TBI2 contains tetrapeptide Ala-Pro-Glu-Arg (APER) having molecular weight of 472.5 m/z.

Table 5.2. Amino acid sequences and molecular weights of active peptide fractions*.

Sample Fractions	Amino acid sequences	Empirical molecular weights	m/z values	IC_{50}[a] (mM)
LPI5	Glu-Pro-Leu-Tyr-Val (EPLYV)	619.71	620.6	80.68
LPI6	Asp-Pro-His-Ile (DPHI)	480.52	481.5	41.62
LBI5	Ala-Glu-Arg (AER)	374.40	375.3	293.80
LBI12[b]	Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ)	858.90	859.8	279.43
LFI5	Trp-Asp-Asp-Met-Glu (WDDME)	694.71	695.7	14.39
TBS1	Ala-Arg (AR)	245.28	246.2	448.47
TBS2	Ala-Val (AV)	188.23	189.2	371.89
TBI2	Ala-Pro-Glu-Arg (APER)	471.51	472.5	190.88

* Only selected active peptides that are classified as inhibitor or pro-drug types were subjected for amino acid sequence and ESI/MS analyses.

[a] Calculated based on the IC_{50} values expressed in mg/ml as presented in Table 5.1.

[b] LBI12 is an anionic anti-microbial peptide fraction (see Chapter 4) found to be able to inhibit the action of ACE.

Peptide fraction LBI5 contained a small tripetide Ala-Glu-Arg (AER; 473.40 Da) with ESI/MS peak appeared at 275.3 m/z and has been identified in various fragments of both actin homolog protein of red swamp crayfish and chicken skeletal muscle myosin heavy chain. A pentapeptide Trp-Asp-Asp-Met-Glu (WDDME, 694.71 Da) was identified as the main peptide in fraction LFI5. Result from ESI/MS analysis showed peak at 695.7 m/z. The peptide may originate from actin as a homolog Trp-Asp-Asp-Met-Glu (WDLME) has been identified at fragment 85-89 of Japanese puffer fish actin related protein (http://prowl.rockefeller.edu) and fragment 81-85 of carp actin (NCBI, http://www.ncbi.nlm.nih.gov). The peptides identified from leatherjacket hydrolysates are small peptides and can be isolated into MWCO fraction of less than 5 kDa.

Similar to leatherjacket active ACE inhibitory peptides, active peptides derived from hydrolysis of trevally proteins are also small peptides that can be isolated in fraction less than 5 kDa. Peptide fraction TBS1 contains a dipeptide Ala-Arg (AR; 245.28 Da) and its presence was confirmed at 246.2 m/z by ESI/MS analysis. This peptide originated from both actin and myosin heavy chain. This has been identified in Japanese puffer fish actin related protein and chicken skeletal myosin heavy chain (http://prowl.rockefeller.edu). Another dipeptide was identified in fraction TBS2 having Ala-Val (AV; 188.23 Da) sequence and 189.2 m/z by ESI/MS. Likewise, this peptide has been identified in various fragments of Japanese puffer fish actin related protein and chicken skeletal myosin heavy chain (http://prowl.rockefeller.edu). Fraction TBI2 contained a bigger tetrapeptide Ala-Pro-Glu-Arg (APER; 471.51 Da) and 472.5 m/z as confirmed by ESI/MS. Various homologues of Ala-Pro-Glu-Arg (APER) have been identified from various fragments in Japanese puffer fish actin

related protein and chicken skeletal myosin heavy chain (http://prowl.rockefeller.edu).

5.7. Discussion

Results from ACE inhibitory screening of fish protein hydrolysates showed that all hydrolysates exhibits ACE inhibitory activities of different extent. These results agreed with results from other research on the ACE inhibitory activity of food protein hydrolysates that showed active inhibition of various extents from very weak inhibition to very strong inhibition that merits further investigations. The IC_{50} values of fish protein hydrolysates were quite low ranging from 1.35 mg/ml to 1.89 mg/ml for leatherjacket soluble protein hydrolysates and from 1.99 mg/ml to 3.34 mg/ml for trevally soluble protein hydrolysates. The IC_{50} values for insoluble protein hydrolysates ranged from 0.77 mg/ml to 6.78 mg/ml for leatherjacket insoluble protein hydrolysates and from 2.45 mg/ml to 4.74 mg/ml for trevally insoluble protein hydrolysates. The low IC_{50} values are understood as a cumulative effect of various active peptides present in each hydrolysate as evidenced from the results from ACE inhibitory activity values of various peptide fractions of selected hydrolysates that have been subjected for HPLC fractionations to obtain the active peptides.

The three enzymes used in preparation of fish protein hydrolysates were able to produce ACE inhibitory peptides. Papain hydrolysates of leatherjacket soluble proteins showed decreasing trend of IC_{50} values after 2 h hydrolysis then increased after 4-hour hydrolysis, while trevally soluble protein hydrolysates showed increased trend after 2 h hydrolysis then decreased slightly after 8 h hydrolysis. The effects of papain hydrolysis on IC_{50} values of leatherjacket insoluble protein hydrolysates

showed similar trends as its soluble protein hydrolysates, while papain hydrolysates of trevally insoluble proteins showed a slightly different trend from its soluble hydrolysates with a significant increase in its IC_{50} value at 4 h hydrolysis, decreasing slightly at 6 h hydrolysis, then a slight increase thereafter. Bromelain hydrolysates of leatherjacket soluble fraction showed an increasing trend up to 6 h hydrolysis then decreased thereafter, while the insoluble protein hydrolysates showed only slight increase in their IC_{50} values during hydrolysis. The bromelain hydrolysates of trevally soluble proteins showed similar trend as leatherjacket soluble protein hydrolysates, while the insoluble protein hydrolysates showed increased trend of IC_{50} values during hydrolysis with a sharp increase observed at 8 h hydrolysis. Flavourzyme™ hydrolysates of both leatherjacket and trevally soluble and insoluble protein fractions showed a generally increasing trend as hydrolysis progressed with an uncharacteristic drop of the IC_{50} value at 2 h hydrolysis of trevally insoluble protein hydrolysate. The increasing trend in the IC_{50} values of hydrolysates is generally expected as the enzymes employed continued to cleave the peptides as hydrolysis time progressed. The continuing cleaving may result in either a decrease or an increase of ACE inhibitory activity as certain amino acids at the C- or N-terminus, that usually have strong affinity toward the active site of ACE may be cleaved, resulting in smaller peptides with weaker activity against ACE or produce new more active peptides having N- or C-terminal amino acid residues that have a strong affinity toward ACE. Another reason may be due to the endopeptidase (such as papain and bromelain) cleavage from within the peptides and producing different smaller peptides with weaker activity. Among the enzymes, Flavourzyme™ (an endo and exopeptidase) has been shown to decrease the activity of ACE inhibitory peptides due to its broad range specificity that will cleave the active peptides from

either C-terminal or N-terminal ends of the peptides (Chiang et al., 2006). Another report, however, showed increase in ACE inhibitory activity of Flavourzyme™ hydrolysates of corn gluten (Suh et al., 2003). These contradictive reports may be due to different types of proteins used that may contribute to different characteristics of amino acid residues in their sequences.

The results from ACE inhibitory screening of fish hydrolysates showed that all samples, 120 all together, exhibit activities of different strengths. Hence, some hydrolysates that showed strong inhibition toward ACE were selected for fractionation with molecular weight cut off (MWCO) membranes to produce fraction with different molecular weight ranges (<5 kDa, 10 kDa, and >10 kDa). The selection was based on the assumption that hydrolysates with strong activity against ACE may contain individual peptides with strong activity as well. There is, however, possibility that strong individually active peptides may not be able to be identified. However, as far as time and cost of analysis are concerned, this decision is inevitable. Hence, four soluble protein and four insoluble protein hydrolysates from leatherjacket were selected together with seven soluble protein and six insoluble protein hydrolysates from trevally were selected for MWCO fractionations (Plate 5.1 – Plate 5.3).

Analysis for ACE inhibitory activities of MWCO fractions of the selected hydrolysates are shown in Plate 5.1 – Plate 5.3. In general, hydrolysate fractions of <5 kDa showed lower IC_{50} values compared to the other two fractions of the same hydrolysates. These results are in line with most reports of ACE inhibitory peptides that are usually small peptides below 5 kDa. There are, however, some irregularities of which the IC_{50} values of 10 kDa and >10 kDa fractions are lower than that of their <5 kDa fractions. In all of these cases the hydrolysates in question are from

hydrolysis time of 6 h, 8 h or 10 h. These seem to correlate with the previous assessment that as the hydrolysis time increase smaller peptides are formed. These small peptides may come from further degradation of bigger active peptides which then lose their activities. The IC_{50} values of the selected <5 kDa leatherjacket hydrolysate fractions ranged from 0.72 mg/ml to 7.95 mg/ml, while for selected <5 kDa trevally hydrolysate fractions ranged from 1.35 mg/ml to 3.89 mg/ml. These values are slightly higher than the IC_{50} values of numerous <5 kDa fractions of food protein hydrolysates such as blood plasma hydrolysate (IC_{50} as low as 0.12 mg/ml) (Hyun and Shin, 2000), whey hydrolysate (IC_{50} values 0.07 – 0.14 mg/ml) (Vermeirssen et al., 2003), and sea cucumber gelatin hydrolysate (IC_{50} values 0.35 – 0.47 mg/ml) (Zhao et al., 2007). However, they are well comparable to the IC_{50} values of sea cucumber *Acaudina molpadioidea* hydrolysate (IC_{50} 0.62 – 1.98 mg/ml) (Zhao et al., 2009).

Further fractionations of selected strong hydrolysate fractions of different molecular weight ranges were performed on C18 RP-HPLC and described in section 5.3.4. Peptides fractions were collected and subjected for another round of ACE inhibitory activity assay as well as stability assays. The IC_{50} values of some selected peptide fractions before (initial IC_{50} values) and after stability assays are shown in Table 5.2. By comparing the initial IC_{50} values of the peptide fractions with the IC_{50} values of the same fractions after preincubation with ACE in the stability assay, these peptides are then classified into three different groups: pro-drug, inhibitor, and substrate types ACE inhibitory peptides. The pro-drug type ACE inhibitory peptides are those that when preincubated with ACE produces smaller more active peptides with decreased IC_{50} values. The inhibitor type peptides are those that when preincubated with ACE

do not change their IC_{50} values, while the substrate types are marked by increased IC_{50} values after preincubation with ACE (Fujita and Yoshikawa, 1999).

The IC_{50} values of these active peptide fractions ranged from 0.01 mg/ml to 0.24 mg/ml. These values are higher than many ACE inhibitory peptides derived from various food proteins, hence indicating weaker peptides. However, they are quite comparable to some other ACE inhibitory peptides such as 840 Da ACE inhibitory peptide derived from sea cucumber gelatin hydrolysate (IC_{50} = 0.0142 mg/ml) (Zhao et al., 2007), ACE inhibitory peptides fraction derived from fish scale (IC_{50} = 0.13 mg/ml) (Zhang et al., 2009), ACE inhibitory peptide fractions derived from pepsin hydrolysis of globin (IC_{50} = 1.19, 0.67, and 0.10 mg/ml), ACE inhibitory peptide Val-Lys-Lys (VKK; IC_{50} = 1045 μM or 0.39 mg/ml) derived from fresh water clam protein (Tsai et al., 2006), ACE inhibitory peptide Ala-Val-Phe (AVF; IC_{50} = 2123 μM or 0.71 mg/ml) derived from cotton leaveworm hydrolysate (Vercruysse et al., 2008), and various other ACE inhibitory peptides derived from food proteins.

The pro-drug and inhibitor type peptides fractions were then used for amino acid sequence and ESI/MS to elucidate their structures, molecular weights, and amino acid residues, and the results are presented in Table 5.3. Selected leatherjacket fraction LPI5, LPI6, LBI5, and LFI5 contained peptides with sequences Glu-Pro-Leu-Tyr-Val (EPLYV), Asp-Pro-His-Arg (DPHI), Ala-Glu-Arg (AER), and Trp-Asp-Asp-Met-Glu (WDDME), respectively. At present, numerous ACE inhibitory peptides have been identified and isolated from almost every food protein. These vast numbers of ACE inhibitory peptides have little commonality in terms of their amino acid sequence and their structure-activity relationship has not been established. However, it is indicated that the structure-activity relationship is strongly influenced by the C-terminal tripeptide sequence of the substrate (in this case ACE inhibitory

peptides), and ACE seemed to prefer substrates or competitive inhibitors containing hydrophobic (aromatic or branched side chains) amino acid residues at each of the three C-terminal positions. For the N-terminal positions, branched-chain aliphatic amino acids are more preferable (Meisel et al., 2006).

In light of the above assertion, peptide Glu-Pro-Leu-Tyr-Val (EPLYV) very much agrees with the principle. Although Glu-Pro-Leu-Tyr-Val (EPLYV) is not similar to any of the known ACE inhibitory peptide discovered so far, its C-terminal tripeptide amino acid residues are all hydrophobic amino acid of which tyrosine (Tyr, Y) has been indicated as one of the most common aromatic hydrophobic amino acid residues that are associated with strong affinity towards the active site of ACE when it is in one of the C-terminal tripeptide residue of the active peptides. Valine as the C-terminal residue has been listed in association with strong affinity towards ACE based on the application of Artificial Neutral Network (ANN) for dipeptide ACE inhibitory peptides (Meisel et al., 2006). The N-terminal dipeptide EP has been identified in sequence of synthetic ACE inhibitory peptide Glu-Pro-Lys-Ala-Ile-Pro (EPKAIP) (Kohmura et al. 1990) and may play supporting role in the strength of the inhibition, while proline (Pro, P) is expected to be the main contributor to the strength of the activity.

Tetrapeptide Asp-Pro-His-Ile (DPHI) also has not been found so far, but as with Glu-Pro-Leu-Tyr-Val (EPLYV), two of the C-terminal tripeptide, Proline (Pro, P) and Isoleucine (Ile, I) are hydrophobic residues. The presence of isoleucine at the C-terminal residue is quite uncommon and only a few ACE inhibitory peptides such as synthetic Tyr-Pro-Phe-Pro-Gly-Pro-Ile (YPFPGPI) (Meisel and Schlimme, 1994). These two peptides, however, have proline (Pro, P) in their C-terminal tripeptide residue that may be the contributing factor to their activity. The tripeptide Ala-Asp-

Arg (AER) has a quite common C-terminal residue of arginine (Arg, R). It is suggested that the positive charge on the guanidino group of arginine side chain contributed to the activity of ACE inhibitory peptides that have arginine at the C-terminal residue (Cheung et al., 1980; Ariyoshi, 1993). The use of ANN to analyse strength of amino acid residue of di- and tripeptides indicated that arginine did not particularly have strong affinity towards ACE, so the presence of other residues in Ala-Glu-Arg (AER), glutamic acid (Glu, E) in particular, may also contribute to the activity of this peptide. The pentapeptide Trp-Asp-Asp-Met-Glu (WDDME) is an uncommon ACE inhibirory peptide with glutamic acid (Glu, E) at the C-terminal residue, a feature that is not in accordance with the structure-activity relationship principle given earlier. The negative charge of glutamic acid (Glu, E) residue probably has the chelating effect on the zinc ion that is bound at the active site of ACE as an important catalytic component (Wei et al., 1992).

In addition to the above, leatherjacket ACE inhibitory peptides, the anionic anti-microbial peptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) found in this study (see Chapter 4) has also shown ACE inhibitory activity with an IC_{50} value of 0.24 mg/ml. Various ACE inhibitory peptides contained C-terminal glutamine (Gln, Q) residue. These include Leu-Ile-Val-Thr-Gln (LIVTQ) from enzymatic hydrolysis of bovine β-lactoglobulin (Meisel et al., 2006), Leu-Phe-Arg-Gln (LFRQ) from enzymatic hydrolysis of bovine α_{S2}-casein (Meisel et al., 2006), Gly-Lys-Lys-Val-Leu-Gln (GKKVLQ) from Alcalase hydrolysis of porcine haemoglobin (Mito et al., 1996), Leu-Gln-Gln (LQQ) and Ile-Arg-Ala-Gln-Gln (IRAQQ) from thermolysin digest of maize endosperm α-zein (Miyoshi, 1991). Many other ACE inhibitory peptides contained glutamine (Gln, Q) at their C-terminal di- and tripeptides residues (Meisel et al., 2006). This is in accordance with the structure-activity relationship

principle mentioned earlier. Glutamine is the neutral form of glutamic acid (Glu, E), so possibly glutamine may also has a chelating effect on the zinc ion attached to the active site of ACE, especially in a basic environment. Another possibility is that the glutamic acid (Glu, E) residue at the N-terminal position contributes to the activity of the peptide through the chelating effect on the zinc ion in the similar manner when it is at the C-terminal position.

Three ACE inhibitory peptides, Ala-Arg (AR), Ala-Val (AV), and Ala-pro-Glu-Arg (APER), were also identified from trevally soluble and insoluble protein hydrolysates. The dipeptide Ala-Arg (AR) and Ala-Val (AV) are from bromelain hydrolysis of soluble proteins, while the tetrapeptide Ala-Pro-Glu-Arg (APER) is from bromelain insoluble protein hydrolysate. The peptides Ala-Arg (AR) and Ala-Pro-Glu-Arg (APER) have arginine (Arg, R) at the C-terminal residues. As mentioned earlier, the positive charge on the guanidino group of arginine side chain is the main contributor to their activities. Researchers have proved that replacement of the arginine residue from its C-terminal position resulted in a sharp decrease in the activity of ACE inhibitory peptide as in the case of bradykinin (Arg-Pro-Pro-Gly-Ser-Phe-Pro-Phe-Arg, RPPGSFPFR) (Meisel et al., 2006). The dipeptides Ala-Arg (AR) and Ala-Val (AV) are quite similar to dipeptide Ala-Phe (AF), a pea vicilin synthetic peptide, a strong ACE inhibitory peptide (IC_{50} = 15.2 µM or 3.59 x 10^{-6} mg/ml) (Vermeirssen, 2003). Results from di- and tripeptides comparative study using ANN indicated that phenylalanine (Phe, F) at the C-terminal residue responsible for the activity (Meisel et al. 2006). As valine (Val, V) and then arginine (Arg, R) have weaker affinity towards ACE as compared to phenylalanine then Ala-Arg (AR) is expected to be a weaker peptide than Ala-Val (AV), and Ala-Val (AV) is weaker than Ala-Phe (AF). Ala-Val (AV) has been identified as C-terminal

dipeptide residues in some ACE inhibitory peptides such as in Val-Thr-Ala-Thr-Ala-Val (VTATAV), from enzymatic hydrolysis of bovine milk κ-casein (Meisel et al., 2006) and synthetic Val-Ala-Val (VAV) of human κ-casein (Kohmura et al., 1990). Similarly, Ala-Arg (AR) has been identified as C-terminal dipeptide residues of ACE inhibitory peptides such as in synthetic peptide Ala-Leu-Lys-Ala-Trp-Ser-Val-Ala-Arg (ALKAWSVAR) from fragments of bovine serum albumin (Chiba and Yoshikawa, 1991) and Tyr-Leu-Tyr-Glu-Ile-Ala-Arg (YLYEIAR) of human plasma trypsin hydrolysate (Nakagomi et al., 1998). The C-terminal dipeptide Glu-Arg (ER) has also been identified in Gly-His-Lys-Ile-Ala-Thr-Phe-Gln-Glu-Arg (GHKIATFQER) and Lys-Lys-Ile-Ala-Thr-Tyr-Gln-Glu-Arg (KKIATYQER), ACE inhibitory peptides derived from autolysis of yeast enzyme glyceraldehyde 3-phosphate dehydrogenase (GADPH) (Ariyoshi, 1993).

Numerous researches have been done to evaluate the ACE inhibitory activities of various protein hydrolysates. The hydrolysates include the use Flavourzyme™ for hydrolysis of soy proteins (Chiang et al., 2006) and corn gluten (Suh et al., 2003), bromelain hydrolysis of sea cucumber gelatin (Zhao et al., 2007; Zhao et al., 2009), beef sarcoplasmic proteins (Jang et al., 2008) and papain for hydrolysis of beef sarcoplasmic protein (Jang et al., 2008), tuna frame proteins (Lee et al., 2009b), bullfrog muscle proteins (Qian et al., 2007), algae protein waste (Sheih et al., 2009), and rotifer proteins (Lee et al., 2009a). The IC_{50} values of the hydrolysates of the same enzymes and of different proteins vary, hence indicates the unique nature of the enzyme specificity and amino acid sequence of each protein.

The active ACE inhibitory peptides found in this study are very small, having two to seven amino acid residues in their primary structures. These small peptides are mostly resistant towards further ACE degradation and are classified as either

inhibitor or pro-drug type ACE inhibitors (Table 5.2). Results from simulated gastrointestinal degradation indicated that these peptides are mostly not stable against the breakdown of gastrointestinal enzymes pepsin, trypsin, and chymotrypsin. The <5 kDa fractions and the selected active peptide fractions were first subjected for pepsin hydrolysis at pH 1.2 and incubated at 37^{o}C for one hour with constant shaking. The IC_{50} values of the fractions were then determined and compared to the initial IC_{50} values of their respective fractions. Significant decrease in the IC_{50} values of the <5 kDa fractions was observed indicating their inability to resist peptic degradation and produced peptides with stronger ACE inhibitory activities. These IC_{50} values were decreased further after incubation with trypsin/chymotrypsin mixture. The <5 kDa hydrolysates fraction could contain peptides consisting of 2 to 40 amino acid residues of different primary structures, hence a rich substrate on their own right. The reaction mixtures from pepsin degradation were then subjected to trypsin and chymotrypsin hydrolysis at pH 7.5, 37^{o}C, and for three hours with constant shaking.

A rather different trend between pepsin and trypsin/chymotrypsin hydrolysis trials of peptide fractions occurred indicating their stability or instability against intestinal enzyme degradation. The IC_{50} values of peptide fractions TBS1 and TBS2 were not significantly affected by pepsin and trypsin/chymotrypsin. These two fractions contain small peptides Ala-Arg (AR) and Ala-Val (AV) that are possibly too small to be effective target substrates for the enzymes, hence were unaffected. Peptide fractions LPI5 and TBI2 have their initial IC_{50} values decreased after incubation with pepsin, but further incubation with trypsin/chymotrypsin did not affect their activities. The two peptide fractions contain active peptides Glu-Pro-Leu-Tyr-Val (EPLYV) and Ala-Pro-Glu-Arg (APER). These results indicate that pepsin could

cleave these two peptides and release smaller peptides with stronger activity. Pepsin is an enzyme with a rather broad specificity and could cleave proteins and produce peptides with aromatic residues such as histidine (His, H), phenylalanine (Phe, F), tryptophane (Trp, W), and tyrosine (Tyr, Y) at either terminal end, as well as leucine (Leu, L), aspartic acid (Asp, D), and glutamic acid (Glu, E) at the C-terminal residue (Adler-Nissen, 1986). Therefore Glu-Pro-Leu-Tyr-Val (EPLYV) might be cleaved to produce a combination of Glu-Pro-Leu (EPL) and Tyr-Val (YV), while Lal-Pro-Glu-Arg (APER) might be cleaved and produce Ala-Pro (AP) and Glu-Pro (ER), and was not affected by further incubation with trypsin/chymotrypsin.

Peptide fractions LPI6 and LFI5 have their initial IC_{50} values increased and unchanged, respectively, after hydrolysis with pepsin and further increased after additional hydrolysis with trypsin/chymotrypsin. These fractions contained active peptides Asp-Pro-His-Ile (DPHI) and Trp-Asp-Asp-Met-Glu (WDDME), which are susceptible for peptic degradation. Pepsin might cleave Asp-Pro-His-Ile (DPHI) and release combination of Asp-Pro-His (DPH), Asp-Pro (DP), and His-Ile (HI). It is, however, unclear why pepsin could not digest Trp-Asp-Asp-Met-Glu (WDDME), although this peptide contained tryptophane (Trp, W) and aspartic acid (Asp, D) in their primary structures. A possible reason for this phenomenon is that pepsin was actually able to cleave this peptide and release active peptide Asp-Asp-Met-Glu (DDME) with similar activity. Further digestion of the peptic digest with trypsin/chymotrypsin results in increased IC_{50} values for both peptide fractions indicating further cleaving of peptides previously produced through pepsin hydrolysis. Trypsin prefers amino acid residues lysine (Lys, K) and arginine (Arg, R), while chymotrypsin prefers amino acid residues phenylalanine (Phe, F), tyrosine (Tyr, Y), and tryptophane (Trp, W) at C-terminal residue after cleaving (Adler-

Nissen, 1986). Peptide Asp-Pro-His-Ile (DPHI) lack all these amino acid residues in the sequence, while Trp-Asp-Asp-Met-Glu (WDDME) has only tryptophan (W) in the sequence, hence it is possible that these two enzymes could break other peptide bonds in addition to their preferred peptide linkage.

Stability of ACE inhibitory peptides have been studied and described by other researchers. Megías et al., (2009) reported that the IC_{50} values of hydrolysate from 5 min Alcalase hydrolysis of sunflower defatted protein remained unchanged after 1 h hydrolysis with pepsin. The IC_{50} values, however, increased in time-dependant manner after additional hydrolysis with pancreatin for 3 h. Pancreatin is a mixture of proteinases/peptidases that is active at around neutral pH (pH 7.5). Similar results were reported by Wu and Ding (2002) when studying Alcalase hydrolysate of soy protein. Other researchers, however, reported mixed results of increased, unchanged, or decreased IC_{50} values after hydrolysis with simulated gastrointestinal enzymes. Thus they are indicating the presence of specific target of digestive enzymes in the sequences of the peptides under investigation (Gómez et al., 2004). The peptide fractions used in this experiment contain very small peptides and lack certain amino acid residues such as phenylalanine (Phe, F), histidine (His, H), lysine (Lys, K), and leucine (Leu, L), that are more sussceptible to pepsin, trypsin, and chymotrypsin hydrolysis. Further study, however, is needed to evaluate the stability of proline-containing peptides against prolinase (an iminopeptidase) hydrolysis. Prolinase degradation of ACE inhibitory peptides may result in either weaker or stronger active peptides depending on the amino acid residues at the N- and C-terminal positions of the new peptides.

5.8. Conclusion

Research on the ACE inhibitory peptides of hydrolysates and peptide fractions derived from hydrolysis of leatherjacket and trevally soluble and insoluble proteins resulted in the finding of active peptide fractions of 5 kDa or less and eight active peptide fractions. A total of 120 hydrolysates of both fish protein fractions were subjected for screening of ACE inhibitory peptides. The IC_{50} values of leatherjacket soluble protein hydrolysates ranged from 1.35 mg/ml to 1.89 mg/ml, while the IC_{50} values of the leatherjacket insoluble protein fractions ranged from 0.77 mg/ml to 6.78 mg/ml. The IC_{50} values of trevally soluble protein hydrolysates ranged from 1.35 mg/ml to 1.89 mg/ml, while the IC_{50} values of the trevally insoluble protein fractions ranged from 0.77 mg/ml to 6.78 mg/ml. Eight leatherjacket hydrolysates and 13 trevally hydrolysates were selected for further fractionation using molecular weight cut off (MWCO) membranes. The selection was based on the IC_{50} values of the hydrolysates where only hydrolysates that showed strong inhibition (lower IC_{50} values) were selected. After further ACE inhibition screening, three leatherjacket hydrolysates and three trevally hydrolysates were fractionated and 12 active peptide fractions with significant ACE inhibitory activities were isolated. The 12 fraction were labelled as LPI5, LPI6, LBI2, LBI5, and LFI5 from leatherjacket hydrolysates, and TPI3, TPI4, TBS1, TBS2, TBS6, TBI2, and TBI4 from trevally hydrolysates.

Additional ACE inhibition and stability assays revealed that leatherjacket hydrolysate fractions LPI5 and LPI6, LBI5, and LFI5 showed inhibitor characteristics, while trevally hydrolysate fractions TBS1 and TBI2 showed pro-drug characteristic whereas TBS2, TBS6, and TBI4 showed inhibitor type characteristics, indicating their ability to resist further cleaving by ACE or if ACE were able to cleave them, the resulting new peptides have stronger activity against ACE. Peptide

fractions LPI5, LPI6, LBI5, LFI5, TBS1, TBS2, and TBI2 were subjected for Edman amino acid sequence and complimentary ESI/MS analyses. The primary structures of the four leatherjacket peptide fractions are Glu-Pro-Leu-Tyr-Val (EPLYV), Asp-Pro-His-Ile (DPHI), Ala-Glu-Arg (AER), and Trp-Asp-Asp-Met-Glu (WDDME), having molecular weights of 619.71, 480.52, 374.40, and 694.71 Da, respectively. The primary structures of the three trevally peptide fractions are Ala-Arg (AR), Ala-Val (AV), and Ala-Pro-Glu-Arg (APER), having molecular weight of 245.28, 188.23, and 471.51 Da, respectively. These peptides have not been identified and isolated from any other published research. The presence of these peptides have confirmed by ESI/MS and originated from various fragments of actin, actin related alkali chain, myosin heavy chain.

Evaluation of the structure-activity relationship was conducted using the primary structure of the peptides based on the assertion that ACE prefer hydrophobic (aromatic or branched side chain) residues at the C-terminal tripeptide positions. The first three leatherjacket hydrolysates Glu-Pro-Leu-Tyr-Val (EPLYV), Asp-Pro-His-Ile (DPHI), and Ale-Glu-Arg (AER) have known amino acid residues at their C-terminal tripeptide positions, such as proline (Pro, P), valine (Val, V), and arginine (Arg, R) that are usually have strong affinity towards the active site of ACE, while Trp-Asp-Asp-Met-Glu (WDDME) has glutamic acid (Glu, E) residues at the C-terminal position that, although is not in accordance with the structure-activity relationship principle, may have a chelating effect on the zinc ion bound at the active site of ACE. An additional ACE inhibitory peptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) was also isolated as an anti-microbial peptide, but has also showed ACE inhibition activity with an IC_{50} value of 0.24 mg/ml. As for Trp-Asp-Asp-Met-Glu (WDDME), this peptide is not in accordance with the structure-activity principle

outlined above, and its activity was probably due to the glutamine (Gln, Q) C-terminal residue that behaves in a manner similar to the chelating effect of glutamic acid (Glu, E) or due to the glutamic acid residue at the N-terminal position.

The active peptides from trevally hydrolysates are small and have either arginine or valine at the C-terminal residues that usually have strong affinity towards the active site of ACE. Results from simulated gastrointestinal enzyme degradations revealed that all of peptide fractions and their 5 kDa crude fractions were stable against pepsin, trypsin, and chymotrypsin. These results indicated that the peptides could resist further breakdown by the mentioned gastrointestinal enzymes, which show their potential efficacy *in vivo*. However, as prolinase was not used in this investigation, it is worthwhile to study its hydrolysis impact on proline containing ACE inhibitory peptides. The active peptides found in this study are quite small and were stable against both ACE and gastrointestinal enzyme degradation hence may have potential use in the formulation of functional foods or supplements for mild hypertensive subjects.

Chapter 6

Structure-Activity Relationship of Peptide EQIDNLQ

6.1. Abstract

Heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) was found to be able to inhibit the growth of Gram-negative *Bacillus cereus* and *Staphylococcus aureus* (Chapter 4) as well as inhibit the hydrolytic action of angiotensin-I converting enzyme (ACE) (Chapter 5). This peptide was then subjected to structure-activity relationship studies that involve kinetic study to observe its mode of action against ACE and nuclear magnetic resonance (NMR) study to determine its secondary structure. Results from kinetic study indicated that this peptide is a competitive peptide that will only bind to the active site of ACE. Results from NMR studies in aqueous solution showed that this peptide lack the classical secondary structures such as α-helix, β-strand, or loop. This was supported by chemical shift index (CSI) based structure analysis, as the CSI (both in aqueous and sodium dodecyl sulfate (SDS) solution) fell short of 70% dense grouping of either '1' or '-1' uninterupted marks (Wishart et al., 1992). Further NOE based structure analysis showed unbroken connectivities between $H_N(i)$ and $H\alpha(i-1)$, hence confirm the sequential assignments of amino acids of the heptapetide. Weak NOE connectivities were observed between H_N of aspartic acid (Asp-4, D) and H_β and H_δ of isoleucine (Ile-3, I) which was not strong enough to support either β-strand or loop conformation but indicates the tendency of the peptide to have a bent structure in this location. The connectivity between H_N of aspartic acid (Asp-4, D) and H_δ of isoleucine (Ile-3, I), however, disappeared when the peptide was dissolved in SDS solution indicating the loss of bent structure of the peptide. Therefore, the heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-

Gln (EQIDNLQ) possess an extended structure both in aqueous solution and also in SDS micelles that mimic bacterial membrane.

6.2. Introduction

Proteins are linear polymers of amino acids, with each amino acid unit (also known as residue) chosen from 19 natural amino acids and one imino acid (proline), in such an arrangement and combination to produce macromolecules with unique structures and functions. The polymer formed by these 19 amino acids and one imino acid are, however, similar in their backbone structure and only differ in their side chains. These differences make proteins with different secondary and tertiary structures, which in turn determine their functions and to some extent their biological activities of significant importance. Numerous studies have reported the structure-activity relationship of active peptides/proteins by studying the stereochemistry and conformation of the peptides/proteins (Chakraborty et al., 2004; Galanis et al., 2004; Tomita et al., 2008; Torres et al., 2002; Venkatraman et al., 2002; Wu et al., 2009) This is particularly true for anti-microbial proteins and peptides such as fowlicidin-1 (Xiao et al., 2006) and polymyxin-B (Meredith et al., 2009).

Most of the structure-activity relationship studies of anti-microbial proteins/peptides were carried out using nuclear magnetic resonance (NMR) spectroscopy, while the structure-activity relationship of ACE inhibitory peptides were studied based on their primary structure with replacement technique often applied to examine the differences of inhibition strength of a peptide after certain amino acid residue had been replaced with another amino acid. A kinetic study to examine the binding preference of an ACE inhibitory peptide to ACE is more often carried out and the information obtained is used to determine the mode of inhibition. NMR technique

has also been used to study enzyme-substrate interaction of ACE and decapeptide angiotensin I (Galanis et al., 2004).

In protein ^{1}H NMR analysis identification of proton spin systems of each amino acid and their assignment is an important first step. Each amino acid residue spin system starts with the proton of the backbone nitrogen (H_N) and moves to the proton on the α-carbon (H_α), then out to the side chain (H_β, H_γ, H_δ, H_ε, …etc.). These protons show chemical shifts at typical regions of 7-11 ppm (H_N, peptide linkages), 6-7.5 (side chain amide H_N such as in glutamine and aspargine), 6.5-8 ppm (aromatic protons), 3.5-5.5 ppm (H_α), -0.5-3.3 ppm (side chain protons not close to oxygen), and -0.3-1.3 ppm (methyl group not connected to sulphur) (Jacobsen 2007). The chemical shifts of protons in common amino acid residues of random coil peptides/proteins are presented in Table 1.5 (Wishart et al., 1992; Wuthrich, 1986).

The amino acid sequence and the secondary structure of peptides are two important characteristics of bioactive peptides that are thought to be responsible for the mechanism and mode of action of an active peptide. Various studies to characterise ACE inhibitory peptides have been undertaken to determine the way the inhibitory peptides bind to ACE (Lee et al., 2009; Muguruma et al., 2009; Qian et al., 2007; Tsai et al., 2008a; Tsai et al., 2008b; Wang et al., 2008; Yu et al., 2006). These studies involved comparisons of the kinetic behaviour of the peptides at different substrate and inhibitor concentrations. The resulting data are plotted into Lineweaver-Burk plot. This plot yields information on the mode of inhibition. There are three major modes of inhibition that give rise to distinctly different kinetic behaviours. They are competitive inhibition, uncompetitive inhibition, and non-competitive or mixed inhibition. The competitive inhibition occurs when the inhibitor binds only to the active site of an enzyme and inhibits the attachment of the

substrate. The uncompetitive inhibition occurs when the inhibitor binds to a site of an enzyme remote from the active site, but only if the substrate is already present. In the non-competitive inhibition, the inhibitor binds to a site other than the active site, and its presence reduces the ability of the substrate to bind to the active site (Atkins and Paula, 2006).

The assessment of the active mode of binding as mentioned above gives an insight into the place where the active peptides bind to the enzyme. However, it gives little information why these peptides bind to a certain site of the enzyme and whether this mode of binding related to the structure, the secondary structure in particular, of the active peptides. Nuclear magnetic resonance (NMR) is a technique of choice to study the structure characteristics of active peptides such as anti-microbial peptides, but to the best of our knowledge there is no report of the use of this technique in determining the mode of inhibition of ACE inhibitory peptides. In recent decades, the NMR technique has been used to study structure and dynamics of proteins (including peptides) and nucleic acids (Wüthrich, 1986). The advancements in the NMR technique have potentially increased its use in study of structure and activity relationship of bioactive peptides, and numerous positive reports are available in various scientific publications. These studies have been carried out both in aqueous solution and in membrane-mimic solution such as sodium dodecyl sulfate (SDS) micelles (Rozek et al., 2000; Torres et al., 2002), or in trifluoroethanol (TFE) micelles (Meredith et al., 2009; Xiao et al., 2006).

NMR spectroscopy involves the study of resonance frequency that arise from the interaction of magnetic field with nuclei such as ^{1}H, ^{2}H, ^{13}C, ^{14}C, ^{15}N, ^{17}O, ^{19}F, and ^{31}P, that possess spin. The most commonly used NMR technique to determine the structure of small peptides is ^{1}H NMR that has proven to be a uniquely powerful tool

for this purpose. The method employed in this chapter include a kinetic behaviour study of synthetic ACE inhibitory heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) and an NMR based structure-activity relationship study to characterise this active ACE inhibitory and anti-microbial heptapeptide.

6.3. Materials and methods

6.3.1. Materials

Unless otherwise stated, all chemicals used in these experiments were reagent grade purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). These included hippuryl-histidyl-leucine (HHL), angiotensin-I converting enzyme (ACE), deuterated sodiumdodecylsulfate (SDS), D_2O, and HPLC grade methanol and tetrahydrofuran. The synthetic heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) was purchased from GenScript (Piscataway, NJ, USA).

6.3.2. Analysis of mode of inhibition

6.3.2.1. Kinetic analysis

The mode of inhibition of ACE by Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) was carried out by reacting 0.1 U ACE against different concentrations (5, 2.5, 1.25, and 0.625 mM) of HHL substrate in the presence of two different concentrations (1 mg/ml and 0.5 mg/ml) of peptide inhibitors. The reaction was monitored at every 10 min interval by quantification of the HA released using multi-injection HPLC technique at 5, 15, 25, 35, and 45 minutues of incubation time. The HPLC solvent consists of 1% tetrahydrofuran, 19% methanol, and 80% Milli-Q water containing 50 mM sodium acetate in the final volume. The pH of the solvent was adjusted to 5.9 by dropwise addition of 30% acetic acid. The activity was transferred into velocity of

ACE (μmol/min of HA released) catalysis. The data collected were plotted into Lineweaver-Burk plot. The mode of inhibition of ACE by peptide inhibitors were estimated by comparing their data with the data without the presence of peptides. The intercept on the horizontal axis is the value of the inhibition constant (K_i) (Atkins and Paula, 2006, 2009; Tsai et al., 2006; Wu and Ding, 2002).

6.3.2.2. Statistical analysis

All values for the mode of inhibition analysis are reported as mean of at least four observations ± standard deviation. A linear regression analysis was performed using Minitab 15.1 (Minitab Inc., USA) to evaluate the mode of inhibition and to determine the dissociation constant (K_i).

6.3.3. NMR studies

Heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) solution was prepared by disolving the peptide powder in 90% H_2O and 10% D_2O to a final concentration of 2.5 mM (pH 3.98). This sample was used for the NMR studies in water solution. Standard homonuclear proton one dimension (1D) and two dimension (2D) NMR experiments have been conducted for assignment and structural studies. All NMR experiments have been performed on a Bruker Avance 500 MHz NMR spectrometer (Bruker BioSpin, Alexandria, NSW, Australia) as detailed in section 2.4.7. All spectra were recorded at 25^{o}C.

6.3.3.1. 1D-NMR experiments

One-dimentional ^{1}H spectra were recorded with water suppresion by presaturation and also by watergate sequence. The FID was zero-filled to 4K points before Fourier transformation.

6.3.3.2. 2D total correlation spectroscopy (TOCSY) experiments

All TOCSY spectra were recorded with mixing times, t_m, of 90 ms to ensure the magnetisation transfer throughout the coupled spin networks. The spectra were collected with 256 x 1024 data points. The numbers of transients collected for each FID were 16, and the FIDs were zero-filled to 1024 points in F1 dimension and to 4096 data points in F2 dimension before Fourier transformation.

6.3.3.3. 2D rotating-frame Overhauser effect spectroscopy (ROESY) experiments

ROESY spectra were recorded with mixing times, t_m, of 300, 500, and 700 ms. The spectra were collected with 256 x 1024 data points. The numbers of transients collected for each FID were 32, and the FIDs were zero-filled to 1024 points in F1 dimension and to 4096 data points in F2 dimension before Fourier transformation.

6.3.3.4. NMR experiments in sodiumdedocylsulfate (SDS) micellar environment

For the NMR experiments in membrane mimic environment, the heptapeptide EQIDNLQ was dissolved in 90% H_2O and 10% D_2O containing 220 mM deuterated SDS (Torres et al., 2002) to a final peptide concentration of 2.2 mM. The pH was adjusted to 4.02 by adding small amount of 1 N HCl. 1D-proton, 2D-TOCSY, and

2D-ROESY spectra were collected with the same experimental parameters as detailed above.

6.4. Results

6.4.1. The mode of inhibition of EQIDNLQ

In addition to primiary structure analysis as discussed in Chapter 4 and Chapter 5, synthetic heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) has been shown to be able to inhibit the activity of ACE and had pro-drug type characteristics. This peptide was subjected to kinetic study to evaluate its mode of inhibition or mode of action toward ACE. This study was carried out by reacting two level concentrations of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) with ACE at different concentration of HHL. The hippuric acid release was then quantified using HPLC (Figure 6.1.) and reciprocally plotted against the reciprocal of HHL concentration (Lineweaver-Burk plot). Blank reaction between different concentrations of HHL and ACE was used as comparison for the mode of action, and the peptide was further classified based on its mode of binding.

Results from the study of the mode of inhibition of synthetic Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) are shown in Plate 6.1. The complete data can be seen in Appendix 6. The plot for Blank, hippuryl-histidyl-leucine (HHL) of different concentrations, and peptide inhibitor 1 mg/ml and 0.5 mg/ml intercept the Y axis ($1/V_{max}$) at a same point, while $-1/K_m$ and the slope (K_m/V_{max}) were of different values thus indicating the peptide to be competitive. The calculated $1/V_{max}$ were 1.7 $\mu g.ml^{-1}.min^{-1}$; $-1/K_m$ were -0.22, -0.18, and -0.14 $mg.ml^{-1}$ for blank, 0.5 mM HHL, and 1.0 mM HHL; and the slopes were 7.17, 10.29, and 12.75, respectively. Therefore, the combination between substrate (HHL) and the enzyme (ACE) did not

affect the inhibition of ACE by EQIDNLQ, while increasing the concentration of the active peptide will increase both slope and the V_{max}. This observation is consistence with the finding of other competitive ACE inhibitory peptides (Muguruma et al. 2009, Yu et al. 2006).

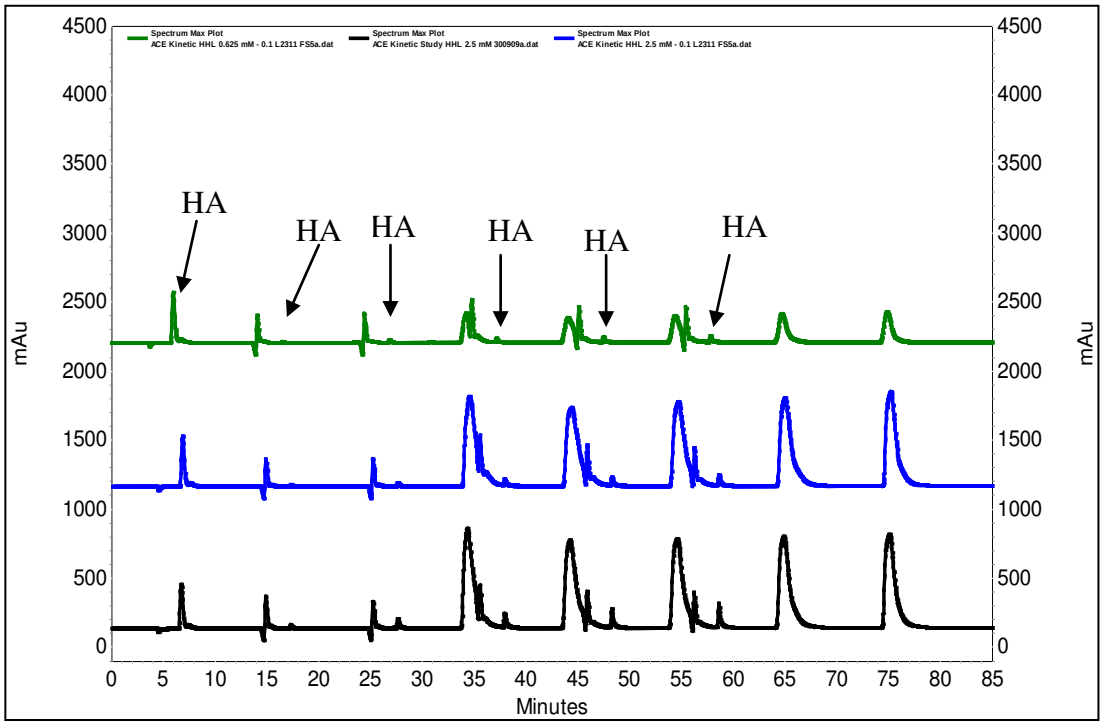


Figure. 6.1. Chromatograms of hippuric acid (HA) released during kinetic study of the mode of action of ACE inhibitory peptide. Pure 0.44 mg/ml HA was injected first followed by injection of sample every 10 min interval.
Top : 0.625 mM HHL with 0.5 mg/ml peptide sample
Middle : 1.25 mM HHL with 0.5 mg/ml peptide sample
Bottom : 2.5 mM HHL with 0.5 mg/ml peptide sample

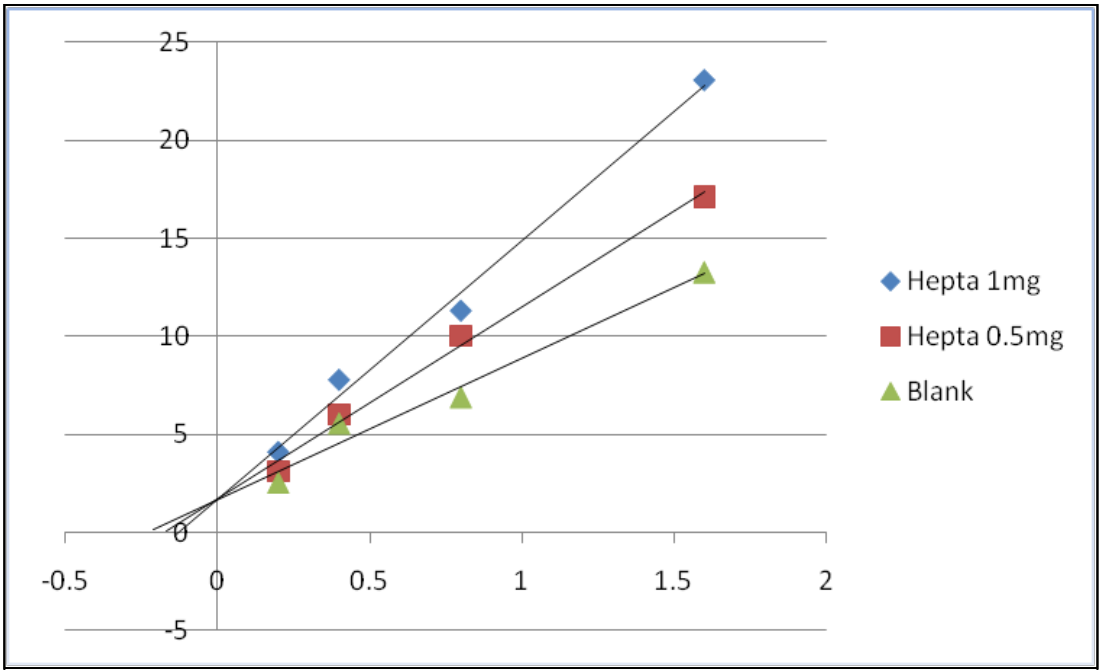


Plate 6.1. Lineweaver-Burk plot of Flavourzyme hydrolysate of synthetic heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ). The Blank plot is the products from hippuryl-histidyl-leucine (HHL) hydrolysis by ACE without the presence of the inhibitory peptide.

6.4.2. NMR based structural analysis of EQIDNLQ in water

Data collected from the NMR experiments were analysed to obtain the structure of the heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in water solution. Two approaches were used to obtain the structures: the simple chemical shift index (CSI) technique and the conventional nuclear-Overhouser-effect (NOE) based technique. The former was achieved by using H_α chemical shift of the amino acid residues alone in comparison to the H_α random coil chemical chift of the same residues (Wishart et al. 1992). The later involved proton resonance assignments using 1D-proton and 2D-TOCSY spectra followed by an extensive analysis of ROESY cross-peaks.

6.4.2.1. Proton assignment

The assignments of all protons in heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) were performed mainly by examining the TOCSY cross-peaks. TOCSY provided total correlation of proton spin system in each amino acid residue starting from H_N. Figure 6.2 shows the TOCSY spectra (H_N-H_α and side chain proton region) and gives the assignments of protons of all amino acid residues in Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in water. The H_N protons of different amino acid residues appeared in the spectrum in the following order (upfield in *F2* scale): glutamine (Gln-2, Q; 8.83 ppm), aspartic acid (Asp-4, D; 8.49 ppm), aspargine (Asn-5, N; 8.38 ppm), isoleucine (Ile-3, I; 8.37 ppm, which was very close to Asn-5), leucine (Leu-6, L; 8.16 ppm), and glutamine (Gln-7, Q; 8.01 ppm). Table 6.1 shows the chemical shifts of all protons in heptapetide EQIDNLQ. From the known assignments of H_N protons, it is straight forward to assign H_α and other side chain protons of individual amino acid residues (Figure 6.2).

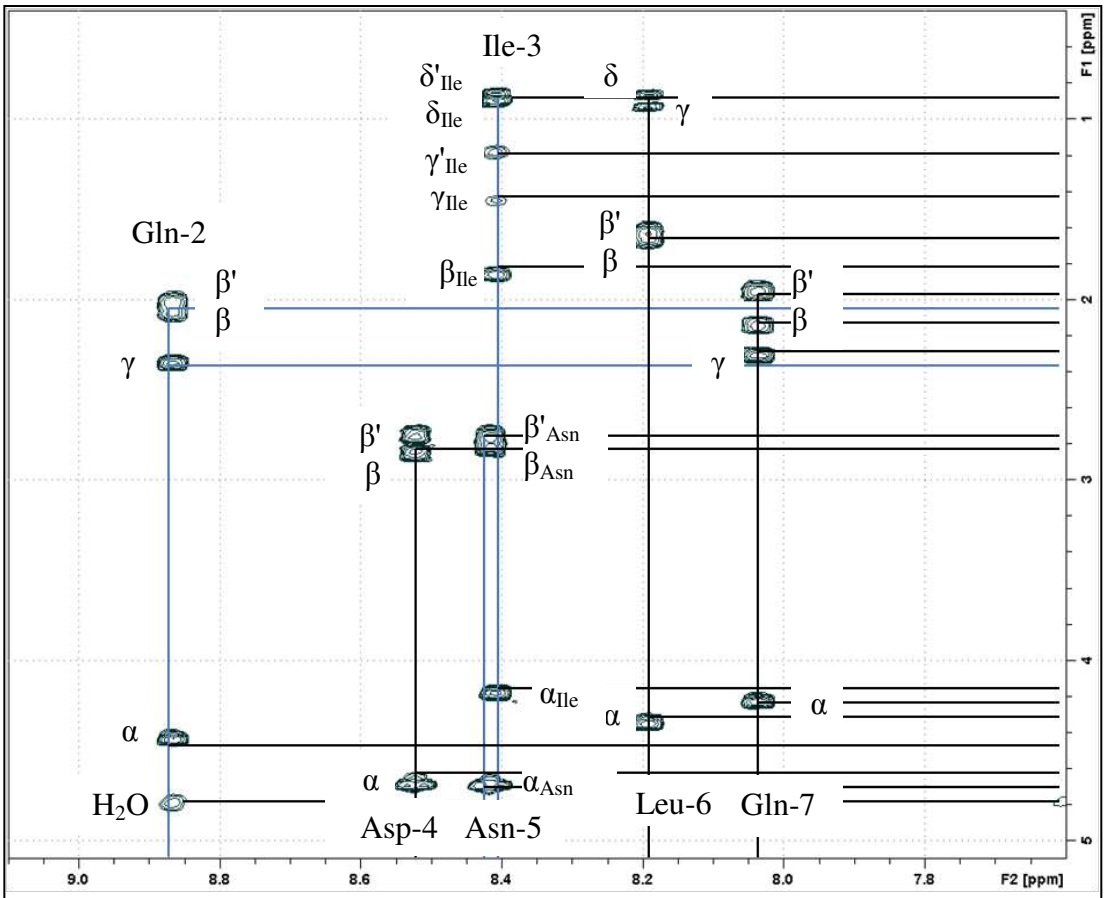


Figure 6.2. Total correlation spectroscopy (TOCSY) spectrum (H_N-H_α region) of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in water at 25°C and 90 ms mixing times.

Table 6.1. The chemical shifts (δ in ppm) of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in water (δ in ppm).

Protons*	Experimental chemical shifts (δ) of amino acid residues						
	E[a]	Q	I	D	N	L	Q
H_N		8.83	8.37	8.49	8.38	8.16	8.01
H_α	4.05	4.42	4.15	4.66	4.66	4.32	4.20
H_β	2.10	2.04	1.82	2.79	2.71	1.61	2.11
H_β		1.98		2.75	2.73	1.61	1.92
H_γ	2.41	2.32	1.42			0.90	2.28
H_γ			1.15				
H_δ			0.87			0.83	
H_δ			0.82				

* Obtained from TOCSY experiments in conjunction with 1D-NMR spectra.
[a] Not detected due to very fast exchange of H_N proton with water protoins.

6.4.2.2. Sequential assignments

The sequential assignment of heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) was carried out by examining the strong rotating frame Overhauser effect (ROE) cross-peaks between H_N (*i*) to H_α (*i-1*). An expansion of ROESY spectrum in water showing ROE cross-peaks originated from H_N protons is presented in Figure 6.3 and were used to assign the sequential ROE connectivity ($H_N(i)$-$H_\alpha(i-1)$ of the protons of the heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ). Strong ROE connectivities between all pairs of $H_N(i)$ to $H_N(i-1)$ in the heptapeptide have linked all N_H protons without interruption. These sequential connectivities (Table 6.2) confirm the sequence of the amino acid residues in the peptide under investigation.

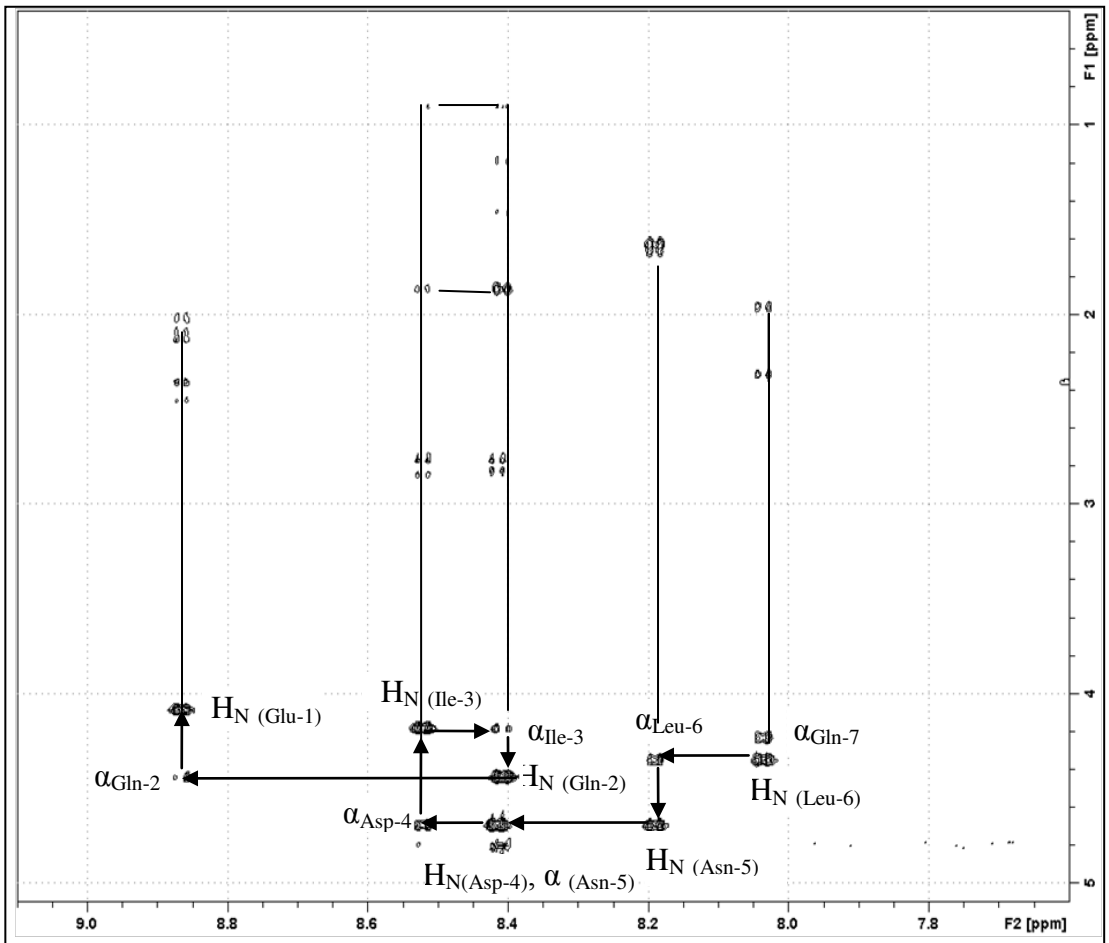


Figure 6.3. Rotating frame nuclear Overhouser effect spectroscopy (ROESY) spectrum (H_N-H_α region) of heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in water at 25°C and 500 ms mixing times.

Table 6.2. Sequential NOE connectivity of $H_N(i)$ to $H_\alpha(i\text{-}1)$ of the amino acid residues of the heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in water.

No.	Sequential NOE Connectivities
1.	H_N(Gln-2) ↔ H_α(Glu-1)
2.	H_N(Ile-3) ↔ H_α(Gln-2)
3.	H_N(Asp-4) ↔ H_α(Ile-3)
4.	H_N(Asn-5) ↔ H_α(Asp-4)
5.	H_N(Leu-6) ↔ H_α(Asn-5)
6.	H_N(Gln-7) ↔ H_α(Leu-6)

6.4.2.3. NOE based secondary structure analysis

NOE cross-peak analysis also showed connectivity between $H_\beta(i)$ and $H_\delta(i)$ of isoleucine (Ile-3, I) with $H_N(i+1)$ of aspartic acid (Asp-4, D) as indicated by the appearance of the cross-peaks between these two isoleucine (Ile-3, I) protons with the spin system of aspartic acid (Asp-4, D) that were not identified in the TOCSY spectra. These NOE connectivities suggest the tendency of the peptide to take a turn between isoleucine (Ile-3, I) and aspartic acid (Asp-4, D).

6.4.2.4. Structure analysis

6.4.2.4.1. Chemical shift index (CSI) based structure

The structure study based on the CSI method (Wishart et al., 1992) was carried out by comparing the H_α chemical shifts of each amino acid residue within the heptapeptide with the random coil chemical shifts reference. Results of such a comparison are presented in Table 6.3. In doing this comparison, a CSI mark of '1' is given if the H_α is greater (by 0.1 ppm) than the chemical shift reference; '-1' mark is given if the H_α is less than the reference; and '0' is given if the chemical shifts are the same. Using these marks, any 'dense' grouping of four or more '-1' not interrupted by a '1' is a α-helix. Any 'dense' grouping of three or more '1' not interrupted by a '-1 ' is a β-strand. All other regions are designated as coil. In addition, a local 'density' of nonzero chemical shift indices that exceeds 70% is required when designing regions of helical or extended structure. All other regions that are not identified as either helix or β-strand or regions where the local density of either '-1' or '1' fall below 70% are defined as 'coils' (Wishart et al. 1992). The chemical shift indices of H_α protons of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) are plotted in Figure 6.4.

Table 6.3. Chemical shift index (CSI) of heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in water used for the assignment of its structure.*

Amino Acid Residues[a]	H_α Coil[b]	H_α Experiment	H_α Differences[c]	CSI Values[d]
Glutamic acid (Glu, E)	4.29	4.05	-0.24	-1
Glutamine (Gln, Q)	4.37	4.44	0.07	1
Isoleicine (Ile, I)	3.95	4.15	0.2	1
Aspartic acid (Asp, D)	4.76	4.66	-0.1	-1
Aspargine (Asn, N)	4.75	4.66	-0.09	-1
Leucine (Leu, L)	4.17	4.32	0.15	1
Glutamine (Gln, Q)	4.37	4.20	-0.17	-1

* The experimental chemical shifts of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) were obtained from 2D TOCSY assignment 90 ms mixing time.

[a] Listed as in the sequence of the peptide.

[b] The H_α chemical shifts of isoleucine (Ile, I) and leucine (Leu, L) have been altered to improve the accuracy and general applicability of this CSI technique (Wishart et al. 1992).

[c] Obtained by subtracting H_α Experiment with H_α random coil.

[d] Obtained by comparing the H_α Coil and H_α Experiment where mark '1' is given if the H_α Experiment is greater than the H_α of random coil by 0.1 ppm; mark '-1' is given if the H_α Experiment is less than the H_α Coil; and '0' if the values are the same.

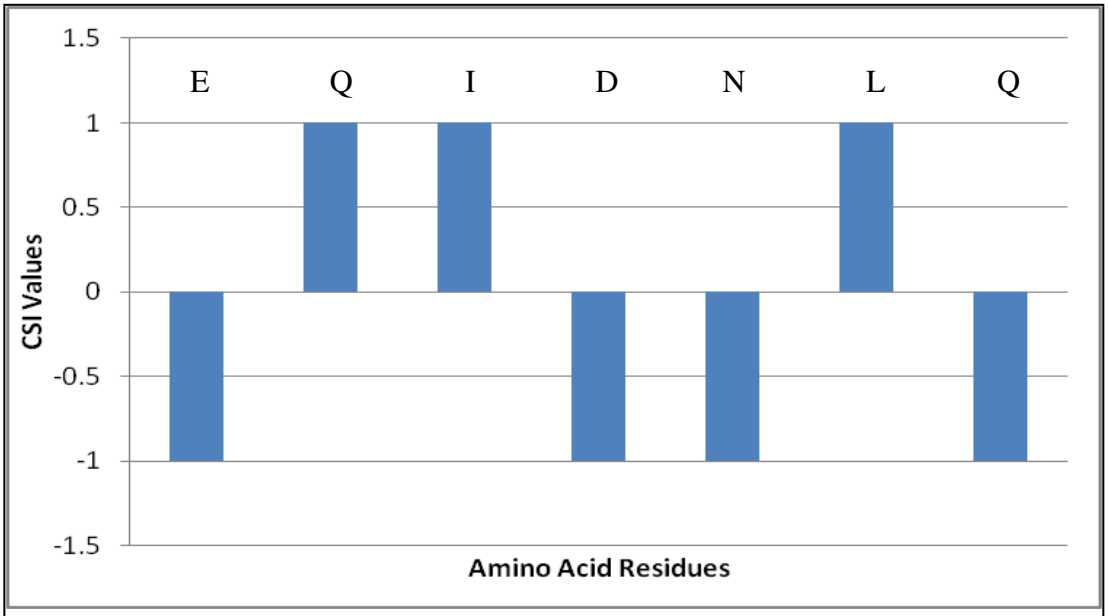


Figure 6.4. Chemical shift indices (CSI) of H_α of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in water . The chemical shift values were obtained from 2D TOCSY experiment at 25°C and 90 ms mixing time.

Results from NMR experiment in water did confirm that there are distinct chemical shift changes of α-protons of each amino acid from that of the random coil. The CSI values of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) showed an extended structure. This is supported by the fact that the above results showed a total of 'nonzero' dense grouping exceeding 70%. The CSI also showed an interrupted '1' mark dense grouping by a '-1' mark dense grouping. This in effect causes less than 70% dense groupings of either '1' marks to express a β-strand structure or '-1' marks to express helical structure.

6.4.2.4.2. NOE based structural determination

The structure of heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDLNQ) in water was then determined using spatial correlations via the long range connectivity information discussed in section 6.4.2.3. Figure 6.5 shows the primary structure of the peptide and ROE connectivities of protons. The walk form $H_N(i)$ to $H_\alpha(i\text{-}1)$

indicated an unbroken spatial connectivity between the protons. These sequential connectivities (Table 6.2) confirm the sequence of the amino acid residues in the peptide under investigation. The presence of observable NOE connectivities between $H_\beta(i)$ and $H_\delta(i)$ of isoleucine (Ile-3, I) with $H_N(i+1)$ of aspartic acid (Asp-4, D) indicate that heptapeptide takes a turn between isoleucine (Ile-3, I) and aspartic acid (Asp-4, D), but this turn is not sufficiently stabilised to for a β-strand or loop conformations. These connectivities were observed by the appearances of the cross-peaks between these two isoleucine (Ile-3, I) protons with the spin system of aspartic acid (Asp-4, D) that were not previously identified in the TOCSY spectra. These results, therefore, point to an extended structure the heptapeptide has in water solution with a tendency to bend between amino acid residues 3 and 4.

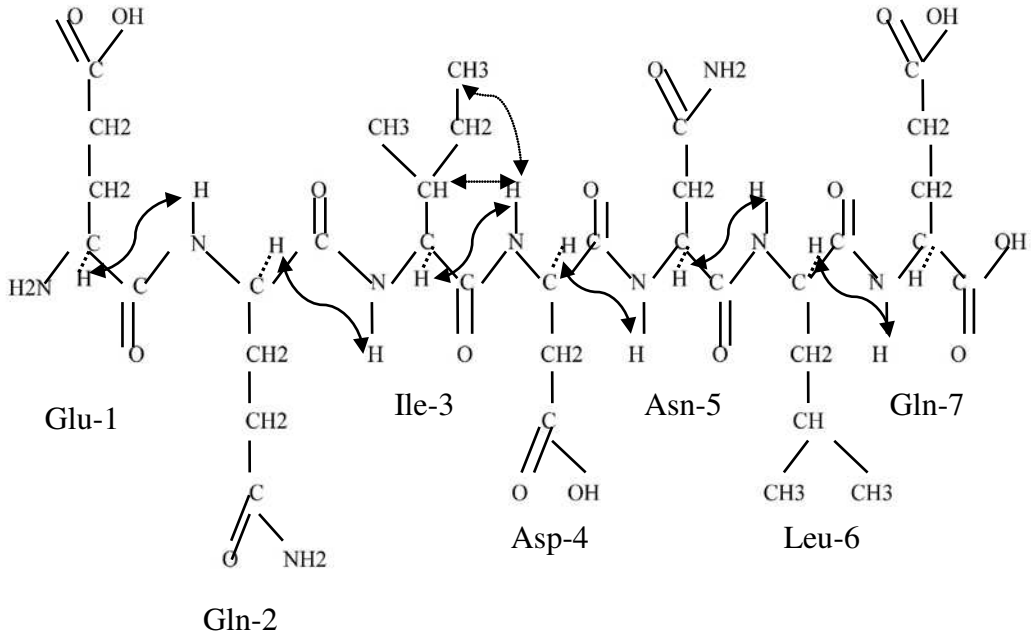


Figure 6.5. Structure of heptapetide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in water showing spatial connectivity between $H_N(i)$ to $H_\alpha(i+1)$. Arrow with broken line indicates weak connectivity between H_N of aspartic acid (Asp-4) and H_β of isoleucine (Ile-3) and between H_N of aspartic acid (Asp-4) and H_δ of isoleucine (Ile-3).

6.4.3. NMR based structural analysis of EQIDNLQ in SDS micellar solution

NMR experiments were also carried out for the heptapetide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in SDS based micellar solution, a membrane mimic, to observe how the heptapeptide behaves in an artificial membrane mimicking environment. All NMR parameters are the same as in the NMR experiments of the heptapeptide in water as mentioned earlier. 1D-proton, 2D-TOCSY, and 2D-ROESY spectra were obtained and used for the proton assignments, sequential assignments, and structural analysis of the heptapeptide in SDS solution.

6.4.3.1. Proton assignments

The proton assignments for the heptapetide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in SDS micelle were carried out mainly from the analysis of 2D-TOCSY spectra in conjunction with 1D-proton spectra. Figure 6.6 shows the TOCSY spectra (H_N-H_α and side chain proton region) and gives the assignments of all protons of the amino acid residues in Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ). The amino acid residues appeared in the spectrum in the following order (upfield *F2* scale): glutamine (Gln-2, Q-2; 8.67 ppm), aspargine (Asn-5, N-5; 8.32 ppm), isoleucine (Ile-3, I-3; 8.25 ppm), aspartic acid (Asp-4, D-4; 8.23 ppm), glutamine (Gln-7, Q-7; 7.99 ppm), and leucine (Leu-6, L-6; 7.95 ppm). Table 6.4 shows the chemical shifts of all protons of heptapetide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in SDS micellar solution. Noticeable chemical shift changes were observed for all H_N protons (upfield shift) and the order of their appearances were also altered as compared to their chemical shifts in water. The chemical shifts of H_α

protons also changed as compared with the chemical shifts of random coil as well as the chemical shifts from experiments in water. Comparing with the H_α chemical shifts of random coil, the H_α protons from heptapeptide showed upfield shifts for glutamic acid (Glu-1, E), aspartic acid (Asp-4, D), aspargine (Asn-5,N), and glutamine (Gln-7, Q); the H_α protons of glutamine (Gln-2, Q), isoleucine (Ile-3, I), and leucine (leu-6, L) changed downfield. The pattern of these changes is similar to the pattern of chemical shift changes from experiments in water. Comparing the chemical shift changes between H_α protons from experiments in SDS solution with those from the experiments in water, the chemical shifts of glutamic acid (Glu-1, E) remained unchanged, the chemical shifts of isoleucine (Ile-3, I) and aspargine (Asn-5, N) changed upfield; the chemical shifts of glutamine (Gln-2 and Gln-7, Q), aspartic acid (Asp-4, D), and leucine (Leu-6, L) changed downfield.

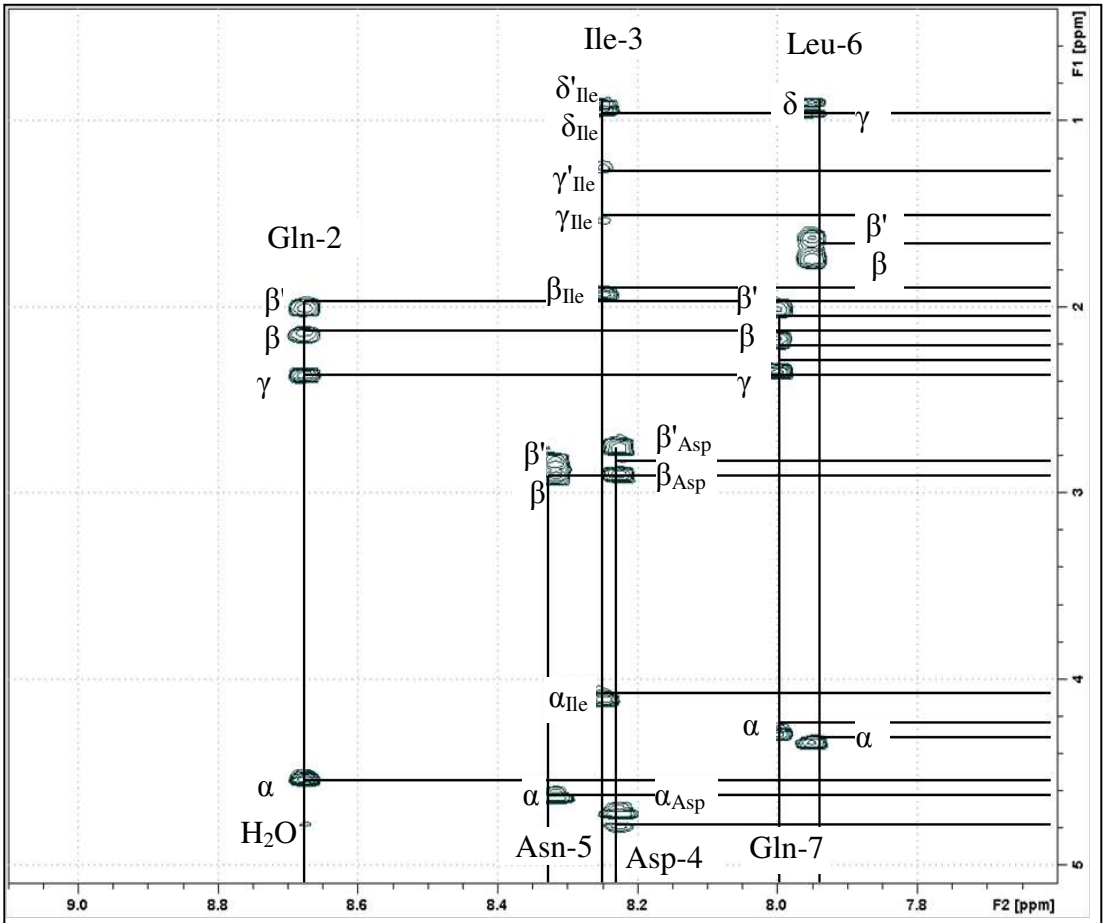


Figure 6.6. Total correlation spectroscopy (TOCSY) spectrum (H_N-H_α region) of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in SDS solution at 25°C and 90 ms mixing times.

Table 6.4. The chemical shifts (δ in ppm) of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in SDS solution (δ in ppm).

Protons*	Experimental chemical shifts (δ) of amino acid residues						
	E	Q	I	D	N	L	Q
H_N		8.67	8.25	8.23	8.32	7.95	7.99
H_α	4.04	4.54	4.10	4.70	4.63	4.34	4.29
H_β	2.09	2.14	1.93	2.90	2.91	1.74	2.18
H_β		2.01		2.74	2.83	1.63	2.00
H_γ	2.40	2.37	1.54			0.96	2.35
H_γ			1.25			0.90	
H_δ			0.95				
H_δ			0.91				

* Obtained from TOCSY experiments in conjunction with 1D-NMR spectra.

6.4.3.2. Sequential assignments

The sequential assignment of heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) was carried out by examining the strong rotating frame Overhauser effect (ROE) cross-peaks between H_N (*i*) to H_α (*i-1*). An expansion of ROESY spectrum in SDS showing NOE cross-peaks originated from H_N protons is presented in Figure 6.7 and were used to assign the sequential ROE connectivity ($H_N(i)$-$H_\alpha(i-1)$) of the protons of the heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ). Strong ROE connectivities between $H_N(i)$ to $H_N(i-1)$ that linked all N_H protons without interruption. These sequential connectivities (Table 6.5) confirm the sequence of the amino acid residues in the peptide under investigation.

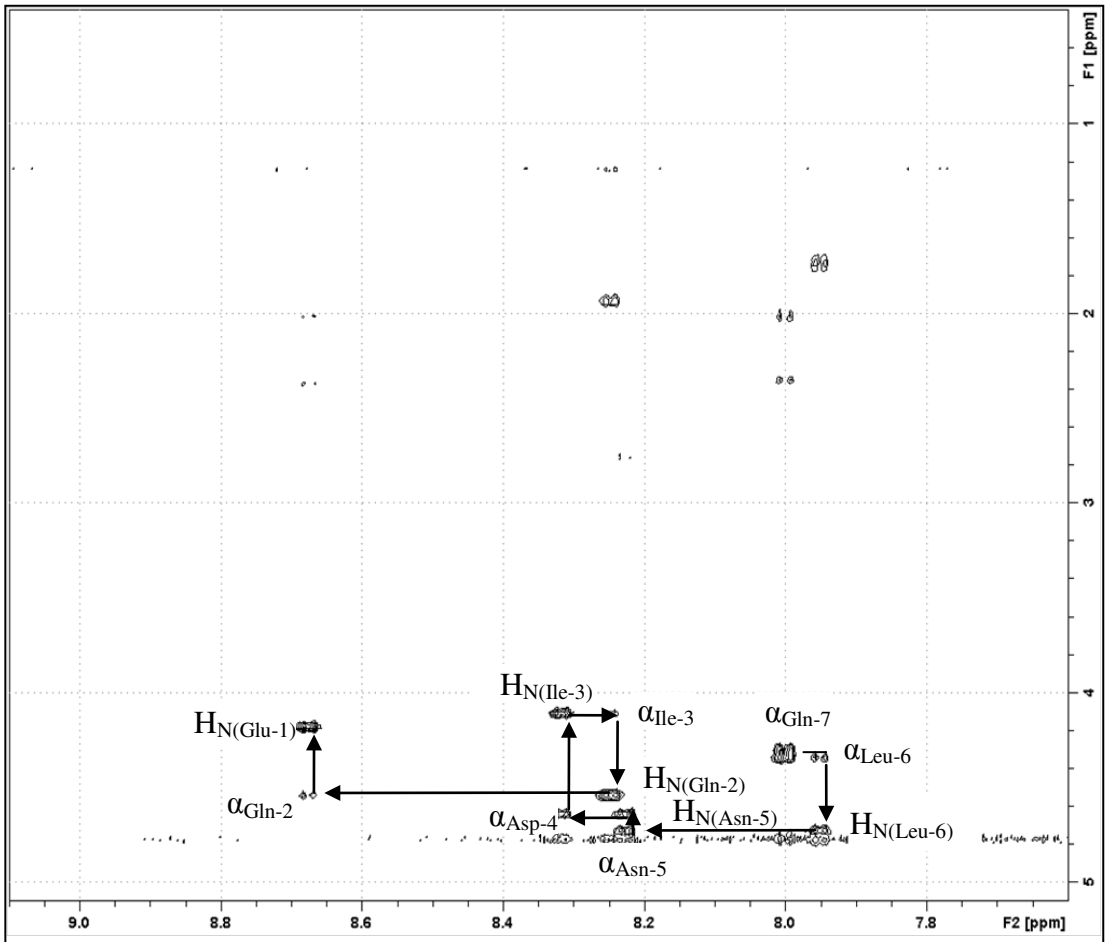


Figure 6.7. Rotating frame nuclear overhouser effect spectroscopy (ROESY) spectrum (H_N-H_α region) of heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in SDS solution at 25^{o}C and 500 ms mixing times.

Table 6.5. Sequential NOE connectivity of $H_N(i)$ to $H_\alpha(i\text{-}1)$ of the amino acid residues of the heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in SDS solution.

No.	Sequential NOE Connectivities
1.	H_N(Gln-2) ↔ H_α(Glu-1)
2.	H_N(Ile-3) ↔ H_α(Gln-2)
3.	H_N(Asp-4) ↔ H_α(Ile-3)
4.	H_N(Asn-5) ↔ H_α(Asp-4)
5.	H_N(Leu-6) ↔ H_α(Asn-5)
6.	H_N(Gln-7) ↔ H_α(Leu-6)

6.4.3.3. NOE based secondary structure analysis

Results from NOE cross-peak analysis seemed to show the presence of NOE connectivity between $H_{\beta}(i)$ of isoleucine (Ile-3, I) and $H_N(i+1)$ of aspartic acid (Asp-4, D) as indicated by the appearances of the cross-peaks between isoleucine (Ile-3, I) protons with the spin system of aspartic acid (Asp-4, D) that were not identified in the TOCSY spectra. The presence of NOE connectivity between $H_{\delta}(i)$ of isoleucine (Ile-3, I) and $H_N(i+1)$ of aspartic acid (Asp-4, D) that was identified in aqueous solution has disappeared in SDS. This indicates that the peptide structure extended further when in contact with SDS micelles that mimics the microbial membrane.

6.4.3.3. Structure analysis

6.4.3.3.1. Chemical shift index (CSI) based structure

As in the experiments in water, the structure of heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in SDS solution was carried out to investigate if the peptide has undergone any conformational change in a solution that mimicked an active bacterial membrane. Results from the calculation of chemical shift index (CSI) and structural analysis based on the CSI technique are presented in Table 6.6 and Figure 6.8. The results do indicate that the peptide structure remains largely the same in both aqueous solution and in SDS solutions.

Table 6.6. Chemical shift index (CSI) of heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in SDS solution used for the assignment of its structure.*

Amino Acid Residues[a]	H_α Coil[b]	H_α Experiment	H_α Differences[c]	CSI Values[d]
Glutamic acid (Glu, E)	4.29	4.04	-0.25	-1
Glutamine (Gln, Q)	4.37	4.54	0.17	1
Isoleicine (Ile, I)	3.95	4.10	0.15	1
Aspartic acid (Asp, D)	4.76	4.70	-0.06	-1
Aspargine (Asn, N)	4.75	4.63	-0.12	-1
Leucine (Leu, L)	4.17	4.34	0.17	1
Glutamine (Gln, Q)	4.37	4.29	-0.08	-1

* The experimental chemical shifts of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) were obtained from 2D TOCSY assignment 90 ms mixing time.

[a] Listed as in the sequence of the peptide.

[b] The H_α chemical shifts of isoleucine (Ile, I) and leucine (Leu, L) have been altered to improve the accuracy and general applicability of this CSI technique (Wishart et al. 1992).

[c] Obtained by subtracting H_α Experiment with H_α random coil.

[d] Obtained by comparing the H_α Coil and H_α Experiment where mark '1' is given if the H_α Experiment is greater than the H_α of random coil by 0.1 ppm; mark '-1' is given if the H_α Experiment is less than the H_α Coil; and '0' if the values are the same.

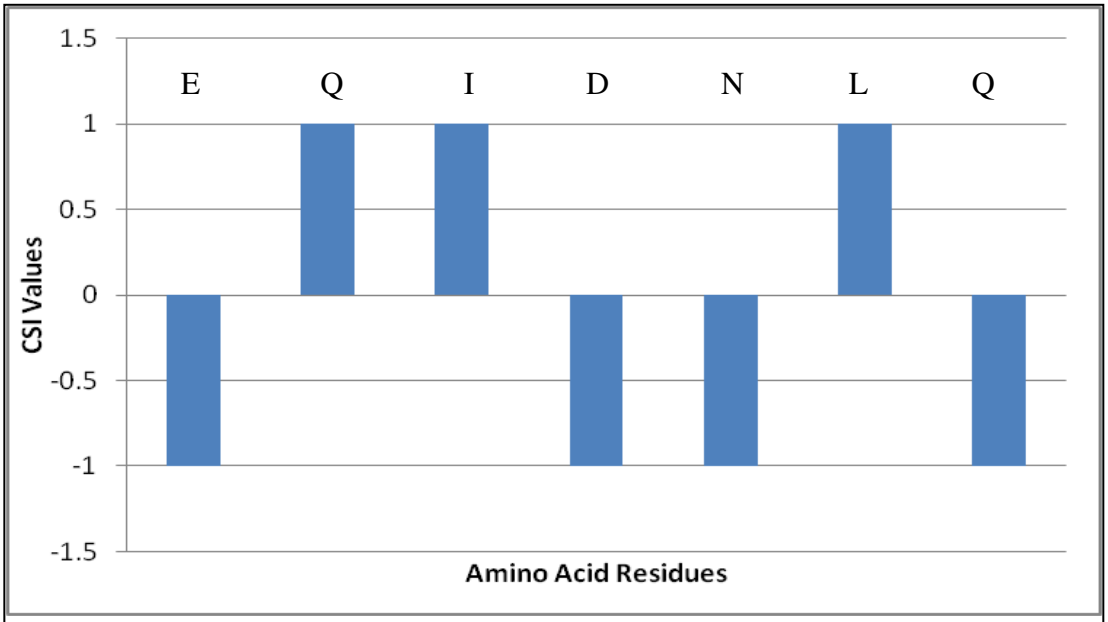


Figure 6.8. Chemical shift index (CSI) of H_α of Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in SDS solution. The chemical shift values were obtained from 2D TOCSY experiment at 25°C and 90 ms mixing

6.4.3.3.2. NOE based structural determination in SDS solution

The structure of heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDLNQ) in SDS solution was carried out through the spatial connectivity of $H_N(i)$ to $H_\alpha(i\text{-}1)$ protons based on proton assignments and ROE information previously obtained. Figure 6.9 shows the primary structure and ROE connectivity of protons. The walk form $H_N(i)$ to $H_\alpha(i\text{-}1)$ indicated an unbroken spatial connectivity between the protons. The presence of NOE connectivity between $H_\beta(i)$ of isoleucine (Ile-3, I) and $H_N(i+1)$ of aspartic acid (Asp-4, D) remained observable by the appearance of the cross-peak between isoleucine (Ile-3, I) protons with the spin system of aspartic acid (Asp-4, D) that were not identified in 2D-TOCSY spectra in SDS solution. The NOE connectivity between $H_\delta(i)$ of isoleucine (Ile-3, I) and $H_N(i+1)$ of aspartic acid (Asp-4, D) identified in aqueous solution was not observable in experiments with SDS solution. These results indicate the heptapeptide has a slight conformational change as it shows the tendency to extend further when in contact with SDS micelles.

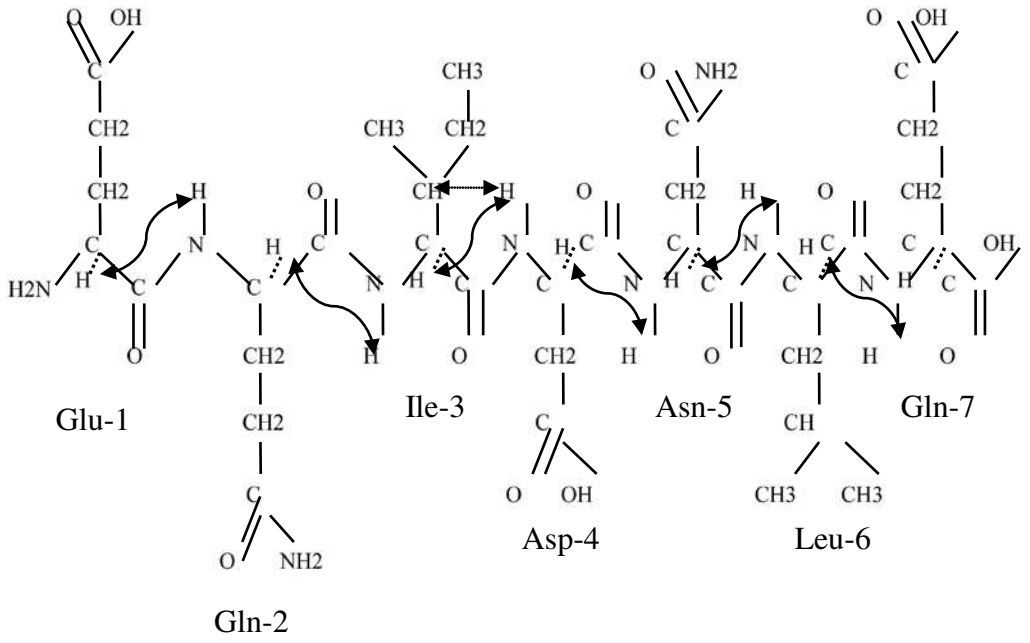


Figure 6.9. Structure of heptapetide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) in SDS solution showing spatial connectivity between $H_N(i)$ to $H_\alpha(i+1)$. Arrow with broken line indicates weak connectivity between H_N of aspartic acid (Asp-4, D) and H_β of isoleucine (Ile-3, I).

6.5. Discussion

Various studies have reported the mode of action of ACE inhibitory peptides. Most of these ACE inhibitory peptides are competitive peptides that bind to the active site of ACE. There are also non-competitive (or mixed-competitive) peptides that bind to a site other than the active site of ACE and its presence reduces the ability of the substrate to bind to the active site. These non-competitive peptides can also bind to the active site of ACE (Lee et al., 2009; Qian et al., 2007; Wang et al., 2008). The ACE inhibitory heptapeptide under investigation in this study has been found to be a competitive binding peptide based on the kinetic results. This finding adds to the growing number of ACE inhibitory peptides of competitive characteristics. Competitive inhibitor will only bind to the active site of ACE, thus competing with a

substrate. The glutamic acid (Glu, E) that is present in the heptapeptide, Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ), was postulated to have a chelating effect on the zinc ion that is bound to the active site of ACE (Wei et al., 1992) when it is in the C-terminal position. In this peptide, however, glutamic acid is at the N-terminal end while the C-terminal end is glutamine (Gln. Q). The chelating effect of glutamic acid (Glu, D) is probablly due to it side chain partially charged COO^- group that can be as effective even it is in the N-terminal position. The side chain group of glutamine (Gln, Q) can also form partially charged domain that can interact with the positively charged zinc ion that is bound to the active site of ACE.

Results from NMR experiments in water indicated that the heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDLNQ) has an extended structure. This is obtained by observing the CSI of H_α of the peptide in aqueous solution, where the dense grouping of 'nonzero' marks was predominant (more than 70%). The presence of weak NOE connectivities between H_β and H_δ of isoleucine (Ile-3, I) and H_N of aspartic acid (Asp-4, D) indicated that the peptide has displayed a tendency of taking a turn between these two amino acid residues. With such a structure, the bulkier negatively charged side chain groups of glutamines (Gln-2 and Gln-7) and glutamic acid (Glu-1, E) would arrange themselves in the space at both terminal ends of the peptide to minimise steric hindrance with other side chains. This arrangement provides partial negative charges that give a chelating effect to the zinc ion on the active site of ACE.

Results presented in section 6.4.2.3.1 indicate that there are changes in the chemical shifts of protons in each amino acid α-protons. These results in water showed that Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) takes an extended structure as it contained less that 70% of '1' dense grouping marks, or less than four '1' marks uninterrupted by any '-1' mark and vice versa (Wishart, et al. 1992); however the

total ‘nonzero’ marks exceeded 70%, hence support the formation of extended structure. Similar results were obtained from 2D-TOCSY experiments in SDS solution, in which the chemical shift index (CSI) calculation showed analogous results to that in aqueous solution. These meant the ‘nonzero’ marks dense grouping exceeded 70%, hence this peptide has an extended structure both in aqueous and SDS solutions.

Results from ROE connectivity analysis indicated that there were an unbroken strong connectivity of $H_N(i)$ to $H_N(i\text{-}1)$ and connectivity of $H_\alpha(i)$ to H_N $(i\text{-}1)$ in ROESY spectra from both experiments in water and in SDS solutions. The presence of weak connectivity between H_β and H_δ of isoleucine (Ile-3, I, *i*) and H_N of aspartic acid (Asp-4, *i-1*) was observed in water. The connectivity between H_δ of isoleucine (Ile-3, I, *i*) and H_N of aspartic acid (Asp-4, D; *i-1*) disappeared when the peptide was disolved in SDS solution, while the connectivity between H_β of isoleucine (Ile-3, I, *i*) and H_N of aspartic acid (Asp-4, *i-1*) remained unaffected. This observation would most likely point that isoleucine (Ile-3, I) and aspartic acid (Asp-4, D) as a bending site, but this bend was weakened when the peptide was dissolved in SDS solution. As these connectivities were weak, an extended structure would be an appropriate form for this peptide. The disappearance of NOE connectivity between H_δ of isoleucine (Ile-3, I, *i*) and H_N of aspartic acid (Asp-4, D; *i-1*) when the peptide is dissolved in SDS solution supports the formation of extended structure. Anti-microbial peptide such as indolicidin was reported to have extended structure (Rozek et al., 2000). The extended class of peptides lack the classical secondary structures such as α-helix, β-strand, and loop. These are generally due to their high proline and/or glycine contents in the peptides (Powers and Hancock 2003). In this investigation, however, the lack of classical structure may be due to the length of the peptide as this peptide does not contain any proline (Pro, P) or glycine (Gly, G).

The tendency of this heptapeptide to take a turn that occurs between isoleucine (Ile-3) and aspartic acid (Asp-4), therefore, is likely to give the negatively charged side chain of glutamines (Gln-2 and Gln-7) and glutamic acid (Glu-1, E) a free space at both terminal ends of the peptide and provide negatively charged active binding domain to the Gram positive bacterial membranes. The structure-activity relationship of anionic anti-microbial peptides is not completely revealed in this investigation and is the subject of further investigation. However, for the extended cationic anti-microbial peptides, such as indolicidin, the mechanism includes interaction with the membrane lipids of bacteria through hydrogen bonds and van der Waals forces (Powers and Hancock 2003).

Since the van der Waals forces depend on the surface areas of molecules and their polarisability (Smith 2006), this peptide (as expected) exerts weak activity. Having only seven amino acid residues, the surface area that is available for the distribution of the negative charge of this peptide is limited to amino acid residues with polar side chains i.e. glutamic acid (Glu-1, E), glutamine (Gln-2, Gln-7; Q), aspartic acid (Asp-4, D), and aspargine (Asn-5, N). In addition, these side chains must align in such a way to produce a surface with partial negative charge. The presence of weak NOE connectivities between H_N of aspargine (Asn-4, N) and H_β and H_δ of isoleucine (Ile-3, I) is likely to bend the molecule near the N-terminal domain. This, in effect, will form two rather compact domains. The first is the N-terminal domain that is less polar since it has only two amino acid residues i.e. glutamic acid (Glu-1, E) and glutamine (Gln-2, Q) that are neutralised further by the positive charge of the N-terminal ammonium ion (NH_3^+). The second domain is the C-terminal domain that is more polar, having three amino acid residues i.e. aspartic acid (Asp-4, D), aspargine (Asn-5, N), and glutamine (Gln-7, Q) that contribute to the negativity of the domain. This domain is further negated by the negative carboxyl ion COO^-. Having this

formation, therefore, will place the C-terminal domain as the main contributor to the negativity of the peptide. The compact molecule orientation will produce smaller negative surface area, hence smaller attractive force or van der Waals interaction that in turn produces weaker inhibitory activity.

6.6. Conclusion

The kinetics and NMR experiments under taken to investigate the relationship between structure and the activity of synthetic heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) revealed that this peptide is a competitive anti-hypertensive peptide that will bind to the active site of ACE. This activity is related to the partially negative charge of glutamic acid (Glu-1, E) as well as glutamine (Gln-2 and Gln-7, Q) that provides negative domain to bind to the zinc ion that is bound at the active site of ACE. NMR experiments in aqueous solution showed that the peptide has an extended structure, a characteristic lack of classic structures such as α-helix, β-strand, or loop.

The structure analysis based on CSI of H_α chemical shifts showed that the peptide is a coil with more than 70% 'nonzero' marks. Such mixed arrangements of amino acid orientation in the CSI system strongly pointed to an extended structure that the peptide formed in water. The CSI analysis of H_α chemical shifts in SDS micelle revealed similar results. Therefore, it is concluded that extended structure is the most appropriate conformation this peptide forms in both water and in SDS solution.

Results from the NOE based structure analysis showed an unbroken $H_N(i)$ to $H_\alpha(i\text{-}1)$ sequential connectivities, thus confirm the sequence of the amino acids in the heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ). The presences of weak NOE connectivities between H_N aspartic acid (Asp-4, D) and H_β and H_δ of isoleucine

(Ile-3, I) show their closeness in space thus indicating a bent structure. As this bend was initiated by weak NOE connectivity of H_N aspartic acid (Asp-4) to H_β and H_δ of isoleucine (Ile-3), there is not sufficient information to form β-strand or loop conformation, thus support the formation of an extended structure. The activity of an extended anti-microbial peptide is acquired possibly through hydrogen bonding or van der Waals interaction with bacterial membrane. As indicated by the results from CSI analysis of the peptide both in aqueous and in SDS solutions and the NOE connectivities, the peptide forms two rather compact domains at the two ends of the peptide. The C-terminal domain is the site mostly contributes to the negative charge of the peptide. However, these conformations produce, in general, a rather compact molecule with smaller van der Waals force, hence weaker activity.

Chapter 7

Conclusion and Future Direction

Hydrolysis of food proteins has been practised for many years in the preparation of food protein hydrolysates (FPHs). These hydrolysates are used in many formulations such as microbial media, food supplements, and animal feed. In recent decades, more attention has been given to physiologically functional properties of food protein hydrolysates of all sources of proteins owing to their bioactive peptide content obtained through hydrolysis. These physiological properties include body defence properties such as anti-microbial and anti-oxidative peptides, body regulating properties such as anti-hypertensive, anti-thrombotic, and cholesterol lowering peptides, modulating properties such as immunomodulatory peptides, mineral binding property such as anti-cariogenic peptides, property that related to emotional state such as opioid peptides, and prebiotic property of certain peptides.

Aiming to produce anti-microbial and anti-hypertensive peptides through papain, bromelain, and Flavourzyme™ hydrolysis of fish proteins, this research was carried out without pH adjustment at an optimal condition obtained through preliminary experiments. Results from the preliminary studies showed that hydrolysis of trevally and leatherjacket soluble and insoluble protein can be optimally carried out at 50^{o}C with papain, bromelain, and Flavourzyme™. The presence of these enzymes could play determining effect in preventing possible microbial spoilage through enzymic lyses while continue hydrolyse the proteins to 10 h and reached degree of hydrolysis (DH) of around 30% which was suitable for the purpose of this study. Various reports suggest that hydrolysis of food protein up to 30% DH will likely produce anti-hypertensive peptides. There is no report, however, to link the release of anti-

microbial peptides and degree of hydrolysis. This is understandably as there is very few finding of anti-microbial peptides through protein hydrolysis.

Hydrolysis of fish protein with papain, bromelain, Flavourzyme™ up to 10 h did change the pH significantly ($p < 0.05$) but did not cause the pH of the hydrolysates to fall beyond the optimum pH of all enzymes. This is of significant importance as it can minimise production time and cost. Data from DH analysis showed that hydrolysis time significantly ($p < 0.05$) affected the DH values of the proteins under investigation, but hydrolysis up to 10 h did not increase the DH values beyond 35%. SDS-PAGE patterns of all hydrolysates did indicate that the hydrolysis produced peptides with molecular weights of around 3 – 10 kDa. Therefore, hydrolysis of fish proteins with papain, bromelain, and Flavourzyme™ can be achieved without pH adjustment and the products were subjected to screening for their activities against angiotensin I-converting enzymes (ACE) and some pathogenic microbial strains. The results from this study indicated a possibility of production fish hydrolysate at a lower cost.

Screening for anti-microbial peptides were carried out using three pathogenic bacteria i.e. *Staphylococcus aureus*, *Bacillus cereus*, and *Escherichia coli*, and one fungi i.e. *Candida albicans*. Results from preliminary screening showed that hydrolysates of both fish proteins did not show any inhibition on the growth of both *E. coli* and *C. albicans*, while the growths of *S. aureus* and *B. cereus* were inhibited at different extents. Of all the hydrolysates, 8 h bromelain hydrolysate of leatherjacket insoluble proteins showed the strongest inhibition against *S. aureus* and *B. cereus* (42% and 23%, respectively). The hydrolysate was then subjected to fractionation, further screening, and minimum inhibition concentration (MIC) assay. A total of 13 fractions were collected and assayed, of which fraction 9 (labelled as LBI9) and fraction 12 (labelled as LBI12) showed stronger inhibition than the other

fractions. Results from MIC assay revealed that fraction LBI9 could inhibit the growth of *B. cereus*, however, a MIC value could not be reached at a concentration of 5.35 mg/ml. Fraction LBI12, on the other hand, exerted growth inhibition of *S. aureus* and *B. cereus* with a MIC value of 4.3 mg/ml. Fraction LBI12 was then purified and subjected to structure elucidation by Edman N-terminal sequence analysis and complimentary electronspray ionisation mass spectrometry (ESI/MS) after the activity of the purified fraction was confirmed.

Results from primary structure analysis revealed that the purified fraction LBI12 contained mainly heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ; MW = 858.9 Da; net charge = -2) and another minor peptide Ala-Pro-Ile-Asp-Asn-Leu-Gln (APIDNLQ; MW = 769.85 Da; net charge = -1). Further ESI/MS analysis confirm the presence of these two peptides where the heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) showed a peak at 859.8 m/z, while Ala-Pro-Ile-Asp-Asn-Leu-Gln (APIDNLQ) showed a peak at 770.8 m/z. The anti-microbial activity of synthetic Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) was confirmed having MIC values of 5.3 mg/ml and 7.96 mg/ml for against *B. cereus* and *S. aureus*, respectively. This heptapeptide is, therefore, a weak anionic anti-microbial peptide. These MIC values are higher than the MIC values of the crude fraction LBI12, indicating decrease in activity. This might be due to its sole action as compared to combined action of the two peptides in fraction LBI12. Study to evaluate synergism of this anionic anti-microbial peptide with other agents and/or metal ions or to synthetically modify its structure may be needed to further characterise it.

The ACE inhibitory activities of 120 hydrolysates obtained from hydrolysis of leatherjacket and trevally soluble and insoluble proteins were also carried out and expressed as the concentration of hydrolysates to inhibit 50% the activity of ACE

(IC_{50} values). The IC_{50} values of leatherjacket soluble and insoluble protein hydrolysates ranged from 1.35 mg/ml to 1.89 mg/ml, and from 0.77 mg/ml to 6.78 mg/ml, respectively. The IC_{50} values of trevally soluble and insoluble hydrolysates ranged from 1.99 mg/ml to 3.34 mg/ml and from 2.45 mg/ml to 4.74 mg/ml, respectively. Fractionation with molecular weight cut off (MWCO) membranes were carried out to eight leatherjacket hydrolysates and 13 trevally hydrolysates that showed strong ACE inhibitory activities. Results from ACE inhibitory screening of the MWCO fractions were then used to limit the number of hydrolysates further to three leatherjacket and three trevally hydrolysates for fractionation and purification with high performance liquid chromatography (HPLC). The fractionation and further ACE inhibitory assays resulted in the collection of 12 active fractions labelled as LPI5, LPI6, LBI2, LBI5, and LFI5 from leatherjacket hydrolysates, and TPI3, TPI4, TBS1, TBS2, TBS6, TBI2, and TBI4 from trevally hydrolysates.

Stability assays against ACE showed that fractions TBS1 and TBI2 showed pro-drug characteristics, fractions LPI5, LPI6, LBI5, LFI5, TBS2, TBS6, and TBI4 showed inhibitor characteristics, while fractions LBI2, TPI3, and TPI4 showed substrate type characteristics. Peptide fractions that showed pro-drug and inhibitor type characteristics are of great interest as they indicate the ability to produce stronger ACE inhibitory peptides or resist ACE hydrolysis. Peptide fractions LPI5, LPI6, LBI5, LFI5, TBS1, TBS2, and TBI2 were subjected to primary structure analysis. The four leatherjacket peptide fractions contained Glu-Pro-Leu-Tyr-Val (EPLYV), Asp-Pro-His-Ile (DPHI), Ala-Glu-Arg (AER), and Trp-Asp-Asp-Met-Glu (WDDME), having molecular weights of 619.71, 480.52, 374.40, and 694.71 Da, respectively. The three trevally peptide fractions contained Ala-Arg (AR), Ala-Val (AV), and Ala-Pro-Glu-Arg (APER), having molecular weight of 245.28, 188.23, and 471.51 Da, respectively. They originated from various fragments of actin, actin

related alkali chain, myosin heavy chain. These peptides have not been identified and isolated from any other published research; however they share similar amino acid residues that were reported to have strong affinity towards ACE in their C-terminal tripeptide residues.

The activity of these peptides were evaluated based on the assertion that ACE prefer hydrophobic (aromatic or branched side chain) residues at the C-terminal tripeptide positions. Peptides Glu-Pro-Leu-Tyr-Val (EPLYV), Asp-Pro-His-Ile (DPHI), Ala-Asp-Arg (AER), Ala-Arg (AR), Ala-Val (AV), and Ala-Pro-Asp-Arg (APER) have known amino acid residues at their C-terminal tripeptide positions, such as proline (Pro, P), valine (Val, V), and arginine (Arg, R) that are usually have strong affinity towards the active site of ACE. Peptide Trp-Asp-Asp-Met-Glu (WDDME) has glutamic acid (Glu, E) residue at the C-terminal position that may have a chelating effect on the zinc ion bound at the active site of ACE. Heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ), the anionic anti-microbial peptide mentioned earlier, has also showed ACE inhibition activity with an IC_{50} value of 0.24 mg/ml. This peptide's activity was probably due to the glutamine (Gln, Q) C-terminal residue that behaves in a manner similar to the chelating effect of glutamic acid (Glu, E) or due to the glutamic acid residue at the N-terminal position.

The active peptides from leatherjacket and trevally hydrolysates found in this study are small and could mostly resist degradation by gastrointestinal enzymes pepsin, trypsin, and chymotrypsin. The effect of gastrointestinal degradation of <5 kDa fractions were noted by the decrease of the IC_{50} values after incubation with pepsin and incubation with trypsin and chymotrypsin decreased the IC_{50} values even further. This decrease pointed to breakdowns of peptides these MWCO fractions contain and produced smaller, more active peptides. Mixed results, however, were observed from the gastrointestinal stability assays of purified active fractions LPI5, LPI6, LBI2,

LBI5, LFI5, TBS1, TBS2, TBS6, TBI2, and TBI4. Fractions LPI5 and TBI2 have had their IC_{50} values decreased by 80% and 22% after similar incubation trypsin/chymotrypsin. The IC_{50} values of LBI5 and LBI12 decreased by 18% and 75% after incubation with pepsin, and then increased by 18% and 33% after further incubation with trypsin/chymotrypsin. The IC_{50} values of LFI5 remained unchanged after incubation with pepsin, and then increased by 50% after further incubation with trypsin/chymotrypsin. The IC_{50} values of TBS1 and TBS2 fraction remained almost unchanged after similar incubation, but IC_{50} values of LPI6 increased by 67% and 75% after incubation with pepsin and trypsin/chymotrypsin, respectively. These mixed results may originate from different amino acid residues in the sequences of peptides contained in these active purified fractions that are more prone to hydrolysis by pepsin, trypsin, and chymotrypsin. These results in general, however, indicate that the peptide fractions may have potential use in the formulation of functional foods or supplements for mild hypertensive subjects.

The heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) was subjected to kinetic and NMR experiments to investigate its mode of action and relationship between structure and activity. Kinetic study showed that heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) is a competitive anti-hypertensive peptide that will bind to the active site of ACE. Chemical shift index (CSI) from NMR experiments in aqueous solution showed that the peptide has an extended structure. With such an open structure, this small peptide can attach into the active site of ACE though partially negative charge of glutamic acid (Glu-1, E) as well as glutamine (Gln-2 and Gln-7, Q) to bind to the zinc ion that is bound at the active site of ACE.

The structure analysis based on CSI of H_α chemical shifts showed that the peptide has a ‘nonzero’ (a combination of ‘1’ and ‘-1’ marks) dense grouping of more than 70%. Such mixed arrangements of amino acid orientation in the CSI values strongly

support an extended structure this peptide formed in water. The CSI analysis of H_α chemical shifts in SDS micelle revealed similar results. Therefore, it is concluded that extended structure is the most appropriate structure this peptide forms either in water or in SDS solution.

In addition to the CSI results, structural analysis based on the NOE connectivities revealed that an unbroken $H_N(i)$ to $H_\alpha(i\text{-}1)$ sequential connectivities, thus confirm the sequence of the amino acids in the heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ). The presences of weak NOE connectivities between H_N aspartic acid (Asp-4, D) and H_β and H_δ of isoleucine (Ile-3, I) were observed which indicates their closeness in space. This also indicates a bend in the molecule orientation. As this bend is possibly initiated by weak connectivity of H_N aspartic acid (Asp-4) to H_β and H_δ of isoleucine (Ile-3), it is not strong enough to accommodate a rigid and close enough space to form β-strand or loop conformation. Therefore, an extended structure with a slight bend between isoleucine (Ile-3, I) and aspartic acid (Asp-4, D) is the most likely structure for heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ). The NOE connectivity between H_N aspartic acid (Asp-4, D) and H_β of isoleucine (Ile-3, I) remained unaffected when the peptide was dissolved in SDS solution, while NOE connectivity between H_N aspartic acid (Asp-4, D) and H_δ of isoleucine (Ile-3, I) has disappeared. These results showed that the peptide was extended further when dissolved in SDS. As SDS acts as a membrane mimic, the peptide may gain more surface area that provide van der Waals interaction to bind to the positively charged Gram-positive bacterial membrane. However, the surface area of this peptide is not large enough to exert stronger van der Waals forces to interact with the bacterial membrane, thus produce weaker activity.

The findings of various ACE inhibitory peptides and one anti-microbial peptide from hydrolysis of leatherjacket and trevally proteins extent the numbers of ACE

inhibitory peptides of fish origin and food protein derived anti-microbial peptides. The ACE inhibitory peptides found in this study are small peptides containing seven or less amino acid residues. Results from stability assays against gastrointestinal enzymes gave insight into the possibility of utilising these peptides. Various analyses and application can be proposed further to the finding of these peptides. These include the following things:

- To evaluate possible dual or multiple functions these peptides may exert. Reports of ACE inhibitory peptides that also show other activity have been forthcoming. These include activity of ACE inhibitor peptide against pathogenic microbial and human cancer cells (Jang et al. 2008). Therefore, it is worthwhile to attempt an evaluation for other possible physiological function such anti-cancer, anti-microbial, and oxidative activities of ACE inhibitory peptides.
- To evaluate possible incorporation of active ACE inhibitory <5 kDa hydrolysate fraction into real food systems such as breakfast cereal. The use of <5 kDa fractions is more feasible as they offer advantages in terms of isolating them, they may contain more than one active peptides in the fraction, and lower production cost.
- To evaluate possible incorporation of ACE inhibitor peptides into real food system such breakfast cereal. The active ACE inhibitory peptides can be encapsulated prior to application.
- To study the efficacy of synthetic ACE inhibitory peptides. ACE inhibitory peptides such as Ala-Arg (AR), Ala-Val (AV), and Ala-Asp-Arg (AER) are very small peptides and can be synthesised and used as nutraceutical in functional food formulation.

- To study the blood pressure lowering effect of <5 kDa, isolated peptide fractions or synthetic peptides. Animal model such as spontaneously hypertensive rats (SHRs) or hypertensive human subjects can be employed for this purpose.

The anionic anti-microbial Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) found here is very small compared to other reported anti-microbial peptides. The weak activity this peptide exerted was possibly due to its relatively small size, extended structure, and its negativity. Various attempts can be made to further increase its activity and function as follows:

- To elongate the peptide and increase its net negative charge by looking into the sequence of the parent protein and identify additional prospective sequence around the N- or C-terminal of the peptide.
- To use a replacement technique and synthesise its analogue with increased negative charge. This can be achieved by replacing amino acid residues such as isoleucine (Ile-3, I) or leucine (Leu-6, L) with basic amino acid in particular aspartic acid (Asp, D).
- To study the synergism between Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) with other anti-microbial peptides or metal ions such as zinc.

It is expected that the information found in this study will be useful for improvement of knowledge based application of science and technology in bioactive peptide production and further characterisations of the active peptides found in this research will be beneficial for development of nutraceuticals for health food formulation and production.

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Appendix 1.

Table A-1: Inhibition (%) of microbial growth by trevally soluble and insoluble protein hydrolysates.

Hydrolysates	Microorganisms			
	B. cereus	*S. aureus*	*C. coli*	*C. albicans*
Soluble Papain hydrolysates				
2 h	-10	-13	-2	-4
4 h	3	5	-22	-6
6 h	-24	-14	-59	-1
8 h	-4	-27	-11	-3
10 h	-21	-3	-19	-1
Insoluble Papain hydrolysates				
2 h	-11	-4	-7	-9
4 h	-13	-42	-27	-2
6 h	-12	-21	-39	-1
8 h	-19	-15	-11	-4
10 h	-21	15	-7	-3
Soluble Bromelain hydrolysates				
2 h	-11	-26	-9	-4
4 h	14	-17	-34	-7
6 h	-15	-26	-10	-2
8 h	1	-22	-13	-8
10 h	-18	-16	-35	0
Insoluble Bromelain hydrolysates				
2 h	-22	-23	-2	-7
4 h	-4	6	-32	-4
6 h	-49	-37	-43	-8
8 h	23	35	-25	-6
10 h	-23	15	-20	-3
Soluble Flavourzyme™ hydrolysates				
2 h	-25	-20	-35	-3
4 h	-1	-3	-63	-1
6 h	-41	-20	-12	0
8 h	-7	-3	-87	-2
10 h	-22	-12	-58	-4
Insoluble Flavourzyme™ hydrolysates				
2 h	-8	-24	-19	-3
4 h	-7	-83	-42	-7
6 h	-35	-47	-92	-9
8 h	1	-9	-17	-5
10 h	-27	-33	-20	-6

Appendix 1. (Cont.)

Table A-1: Inhibition (%) of microbial growth by leatherjacket soluble and insoluble protein hydrolysates (cont.)

Hydrolysates	Microorganisms			
	B. cereus	*S. aureus*	*C. coli*	*C. albicans*
Soluble Papain hydrolysates				
2 h	3	-8	-25	-19
4 h	-4	-2	-39	-5
6 h	-14	10	-12	-3
8 h	-3	8	-23	-1
10 h	24	-17	-7	-4
Insoluble Papain hydrolysates				
2 h	2	-8	-24	1
4 h	3	-1	-33	0
6 h	-2	8	-12	-3
8 h	-1	12	-23	2
10 h	-23	7	-7	2
Soluble Bromelain hydrolysates				
2 h	3	-9	-24	-3
4 h	-6	5	-33	1
6 h	-12	42	-13	-13
8 h	-9	36	-22	1
10 h	-12	-3	-11	-1
Insoluble Bromelain hydrolysates				
2 h	3	11	-22	1
4 h	4	-4	-36	-2
6 h	-11	19	-23	-3
8 h	23	43	-39	-1
10 h	22	13	-17	0
Soluble Flavourzyme™ hydrolysates				
2 h	-11	17	-43	-3
4 h	-16	6	-67	-6
6 h	-10	6	-58	-3
8 h	-11	-9	-60	-1
10 h	-23	3	-44	-3
Insoluble Flavourzyme™ hydrolysates				
2 h	-12	-1	-50	-3
4 h	-3	-20	-75	-2
6 h	-8	-5	-86	-1
8 h	-12	-20	-57	-2
10 h	3	-4	-63	2

Appendix 2.

```
      1     *      10     *      20     *      30     *      40     *      50
1     ASPDAEMAAFGEAAPYLRKS***EKER***IEAQNKPFDAKSSVFVVHPKESFVKG
51    TIQSKEGGKVTVKTEGGETLTVKEDQVFSMNPPKYDKIEDMAMMTHLH***EP***
101   ***V*****LY**N***LKER***YAAWMIYTYSGLFCVTVNPYKWLPVYNPEVVLAYRGKKRQE
151   A***PPHI***FSISDNAYQFMLTDRENQSILITGESGAGKTVNTKRVIQYFATIA
201   ASGEKKKEEQSGKMQGTLEDQIISANPLLEAFGNAKTVRNDNSSRFGKFI
251   RIHFGATGKLASADIETYLLEKSRVTFQL***PAERSYHI***FYQIMSNKKPELI
301   DMLLITTNPYDYHYVSQGEITVPSIDDQEELMATDSAIDILGFSADEKTA
351   IYKLTG**AV**MHYGNLKFKQKQREEQAEPDGTEVADKAAYLMGLNSAELLKA
401   LCYPRVKVGNEFVTKGQTVSQVHNSVGALAK**AV**YEKMFLWMVIRINQQLD
451   TKQPRQYFIGVLDIAGFEIFDFNSFEQLCINFTNEKLQQFFNHHMFVLEQ
501   EEYKKEGIEWEFIDFGMDLAACIELIEKPMGIFSILEEECMFPKATDTSF
551   KNKLYDQHLGKSNNFQKPKPAKGKAEAHFSLVHYAGTVDYNISGWLEKNK
601   DPLNETVIGLYQKSSVKTLALLFATYGGEAEGGGGKKGGKKKGSSFQTVS
651   ALFRENLNKLMANLRSTHPHFVRCIIPNETKTPGAMEHELVLHQLRCNGV
701   LEGIRICRKGFPSRVLYADFKQRYRVLNASAIPEGQFMDSKKASEKLLGS
751   IDVDHTQYRFGHTKVFFKAGLLGLLEEMRDDKLAEIITRTQ**AR**CRGFLMR
801   VEYRR***MVER***RESIFCIQYNVRSFMNVKHWPWMKLFFKIKPLLKSAESEKE
851   MANMKEEFEKTKEELAKSEAKRKELEEKMVVLLQEKNDLQLQVQAEADSL
901   AD***AEER***CDQLIKTKIQLEAKIKEVTERAEDEEEINAELTAKKRKLEDECS
951   ELKKDIDDLELTLAKVEKEKHATENKVKNLTEEM**AV**LDETIAKLTKEKKA
1001  LQEAHQQTLDDLQVEEDKVNTLTKAKTKLEQQVDDLEGSLEQEKKLRMDL
1051  ERAKRKLEGDLKLAHDSIMDLENDKQQLDEKLKKKDFEISQIQSKIEDEQ
1101  ALGMQLQKKIKELQ**AR**IEELEEEIE**AER**TSRAKAEKHRADLSRELEEISE
1151  RLEEAGGATAAQIEMNKKREAEFQKMRRDLEEATLQHEATAAALRKKHAD
1201  STAELG**EQIDNLQ**RVKQKLEKEKSELKMEIDDLASNMESVSKAKANLEKM
1251  CRTLEDQLSEIKTKEEQNQRMINDLNTQR**AR**LQTETGEYSRQAEEKDALI
1301  SQLSRGKQGFTQQIEELKRHLEEEIKAKNALAHALQS**AR**HDCDLLREQYE
1351  EEQEAKGELQRALSKANSEVAQWRTKYETDAIQRTEELEEAKKKLAQRLQ
1401  DAEEHVE**AV**NAKCASLEKTKQRLQNEVEDLMVDVERSNAACAALDKKQKN
1451  FDKILAEWKQKYEETQTELEASQKESRSLSTELFKMKNAYEESLDHLETL
1501  KRENKNLQQEIADLTEQIAEGGK**AV**HELEKVKKHVEQEKSELQAALEEAE
1551  ASLEHEEGKILRLQLELNQIKSEIDRKIAEKDEEIDQLKRNHLRIVESMQ
1601  STLDAEIRSRNEALRLKKKMEGDLNEMEIQLSHANRMAAEAQKNLRNTQG
1651  TLKDTQIHLDDALRTQEDLKEQVA***MVER***RANLLQAEVEELRGALE***QTER***S
1701  RKVAEQELLDATERVQLLHTQNTSLINTKKKLETDIVQIQSEMEDTIQEA
1751  NAEEKAKKAITDAAMMAEELKKEQDTSA***HLER***MKKNMDQTVKDLQLRLD
1801  AEQLALKGGKKQLQKLE**AR**VRELEGEVDAEQKRSAE**AV**KGVR***KYER***RVK
1851  ELTYQCEEDRKNILRLQDLVDKLQMKVKSYKRQAEEAEELSNVNLSKFRK
1901  IQHELEE***AEER***ADIAESQVNKLRVKSREIHGKKIEEEE
```

Figure A-1. Amino acid sequence of gi|238274|gb|AAB20215.1| myosin heavy chain [chickens, skeletal muscle, Peptide, 1938 aa]. Identified sequences of active peptides are marked in **bold underlined**. Sequence homologues of active peptides are marked in ***bold italic underlined***.
Source: http://prowl.rockefeller.edu

Appendix 3.

Amino acid sequence of gi|167837|gb|AAA33228.1| myosin light chain.

```
    1       *       10      *       20      *       30      *       40      *       50
1   MSASADQIQECFSIFDKDNDGKVSVEDIGACLRSLGKSPTMADIEALKTE
51  IGAKEFDINTLKSIYKKPNIKTPQEQQKEMLDAFKALDKEGHGTIQGAEL
101 RQLLTTLGDYLSTAEVDELFKEISVDSTTG**AV**SYASLVNTIVSGYPEFRH
151 KFQSGFRVKREHYHQF
```

Amino acid sequence of gi|189015|gb|AAA59891.1| myosin light chain.

```
    1       *       10      *       20      *       30      *       40      *       50
1   MAPKKPEPKKEAAKPAPAPAPAPAPAPAP***APEA***PKEPAFDPKSVKIDFTA
51  EQIEELKEAFSLFDRTPTGEMKITYGQCGDVLRALGQNPTNAEVLRVLGK
101 PKPEEMNVKMLDFETFLPILQHISRNKEQGTYEDFVEGLRVFEKESNGTD
151 MGAELRHVLATLGEKMTEAEVEQLLAGQEDANGCINYEAFVKHIMSG
```

Figure A-2. Amino acid sequence of some myosin light chain (http://prowl.rockefeller.edu). Identified sequences of active peptides are marked in **bold underlined**. Sequence homologues of active peptides are marked in ***bold italic underlined***.

Appendix 4.

1 * 10 * 20 * 30 * 40 * 50
1 AGRLPACVVDCGTGYTKLGYAGNTEPQFIMPSCIAIKESSKVGDQAQRR
51 MRGVDDLDFFIGDEAIDKPPYATKWPIRHGIVED***WDLME***RFMEQIIFKY
101LR***AEP***EDHYFLLTEPPLNTPENREYTAEIMFESFNVPGLYI**AV**Q**AV**LALA
151ASWTSRQVGERTLTGTVIDSGDGVTHVIPV***AEG***YVIGSCIKHIPIAGRDI
201TYFTQQLLREREVGIPPEQSLETAK**AV**KERFSYVCPDLVKEFNKYDTDGS
251KWIKQYTGINAITKKEFTIDVGYERFLGPEIFFHPEFANPDFTQPISEVV
301DEVIQNCPIDVRRPLYKNIVLSGGSTMFRDFGRRLQRDLKRTVD**AR**LKMS
351EELSGGKLKPKPIDVQVITHHMQRY**AV**WFGGSMLASTPEFYQVCHTKKDY
401EEIGPGICRHNPVFGVMS

Figure A-3. Amino acid sequence of gi|7441435|pir||JC6567 actin-related protein – Japanese pufferfish (Source: http://prowl.rockefeller.edu). Identified sequences of active peptides are marked in **bold underlined**. Sequence homologues of active peptide are marked in ***bold italic underlined***.

Appendix 5.

Table A-2. Summary of inhibition (%)of peptide samples at different concentrations.

Peptide Fractions	% Inhibition [Peptide][a]	% Inhibition [Peptide][a]	% Inhibition [Peptide][a]	% Inhibition [Peptide][a]
Leatherjacket				
LPI5	79.77 [0.074]	52.77 [0.05]	35.54 [0.03]	23.69 [0.02]
LPI6	85.39 [0.074]	57.64 [0.02]	37.46 [0.01]	25.94 [0.01]
LBI2	26.70 [0.015]	18.19 [0.04]	12.73 [0.01]	7.27 [0.004]
LBI5	19.80 [0.04]	13.04 [0.03]	8.99 [0.02]	5.84 [0.01]
LBI12	73.83 [0.36]	48.73 [0.24]	32.89 [0.16]	22.33 [0.11]
LFI5	58.38 [0.02]	39.40 [0.01]	27.58 [0.01]	15.76 [0.004]
Trevally				
TBS1	34.62 [0.07]	22.90 [0.03]	15.42 [0.02]	10.28 [0.02]
TBS2	51.53 [0.07]	34.09 [0.05]	22.96 [0.03]	15.30 [0.02]
TBS6	58.86 [0.09]	39.07 [0.06]	26.16 [0.04]	17.22 [0.03]
TPI3	74.25 [0.09]	49.74 [0.06]	32.88 [0.04]	21.92 [0.03]
TPI4	65.03 [0.05]	43.04 [0.03]	29.11 [0.02]	18.99 [0.02]
TBI2	47.70 [0.09]	31.82 [0.06]	21.20 [0.04]	13.95 [0.03]
TBI4	56.10 [0.13]	37.45 [0.09]	24.95 [0.06]	16.62 [0.04]

Appendix 6.

Table A-3. Summary of ACE heptapeptide EQIDNLQ kinetic study.

Replication	1		2		3					
1/[HHL]	Vo	1/Vo 0.5mg	Vo	1/Vo 0.5mg	Vo	1/Vo 0.5mg	Recapitulation and averages			
0.2	0.32	3.13	0.35	2.87	0.30	3.31	3.13	6.09	10.15	17.34
0.4	0.16	6.09	0.17	5.79	0.17	5.98	2.89	5.79	10.23	17.63
0.8	0.10	10.15	0.10	10.23	0.10	9.69	3.31	5.98	9.69	16.43
1.6	0.06	17.34	0.06	17.63	0.06	16.43	3.11	5.95	10.02	17.13

1/[HHL]	Vo	1/Vo 1mg	Vo	1/Vo 1mg	Vo	1/Vo 1mg	Recapitulation and averages			
0.2	0.25	3.98	0.25	4.03	0.23	4.31	3.98	8.17	11.43	23.12
0.4	0.12	8.17	0.13	7.57	0.13	7.64	4.03	7.57	10.83	23.36
0.8	0.09	11.43	0.09	10.83	0.09	11.71	4.31	7.64	11.71	22.77
1.6	0.04	23.19	0.04	23.36	0.04	22.77	4.11	7.79	11.32	23.08

1/[HHL]	Vo	1/Vo	Vo	1/Vo	Vo	1/Vo	Recapitulation and averages			
0.2	0.41	2.43	0.45	2.23	0.35	2.86	2.43	5.73	6.51	12.76
0.4	0.18	5.66	0.19	5.32	0.18	5.53	2.23	5.32	7.43	13.73
0.8	0.15	6.51	0.13	7.43	0.13	7.71	2.86	5.53	6.71	13.31
1.6	0.08	12.76	0.07	13.73	0.08	13.31	2.51	5.53	6.88	13.27

1/[HHL]	1/Vo 0.5mg	1/Vo 1 mg	1/Vo Blank
0.2	3.11	4.11	2.51
0.4	5.95	7.79	5.53
0.8	10.02	11.32	6.88
1.6	17.13	23.08	13.27

Appendix 7.

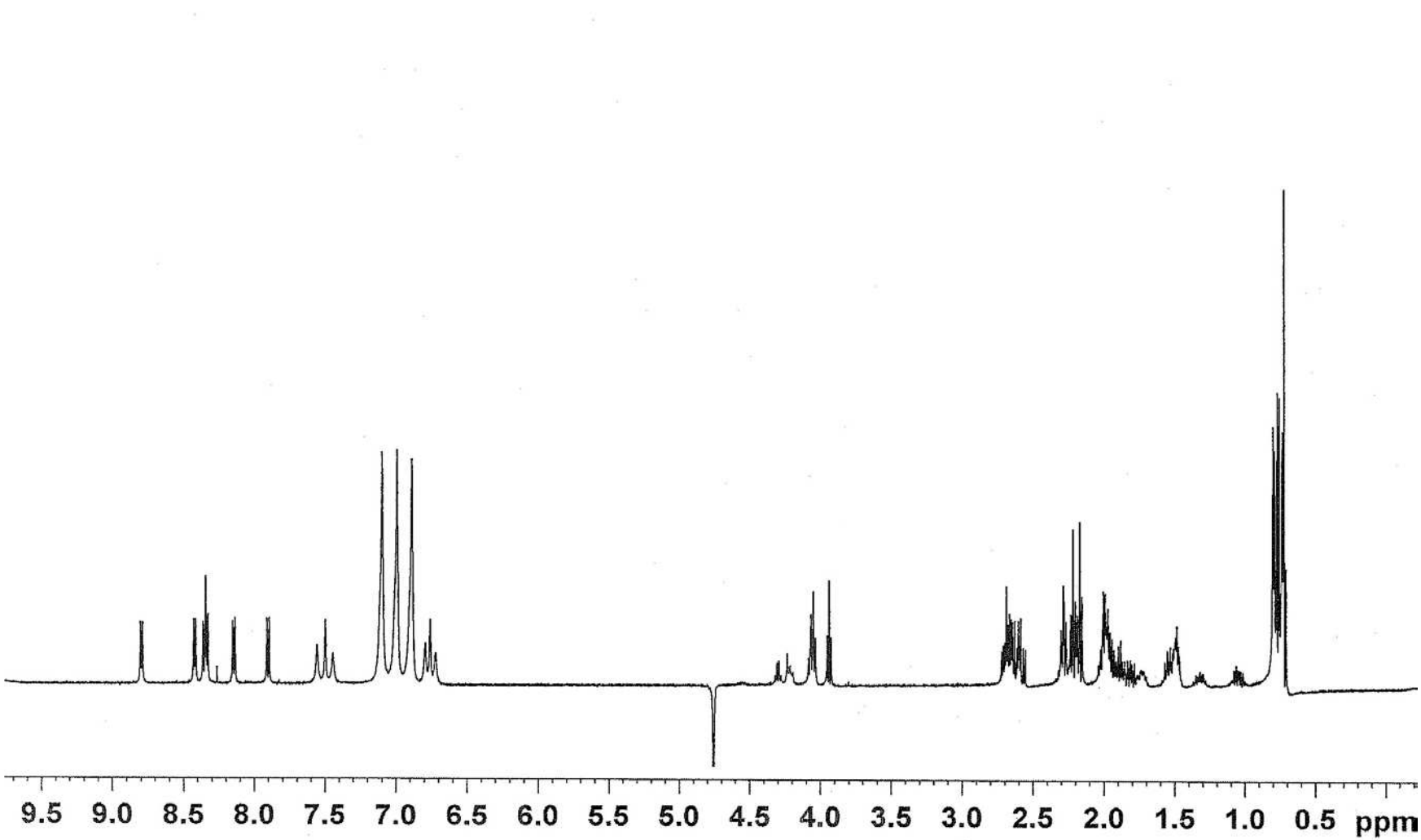

Figure A-4. 1D-NMR spectra of heptapeptide EQIDNLQ in water.

Appendix 8.

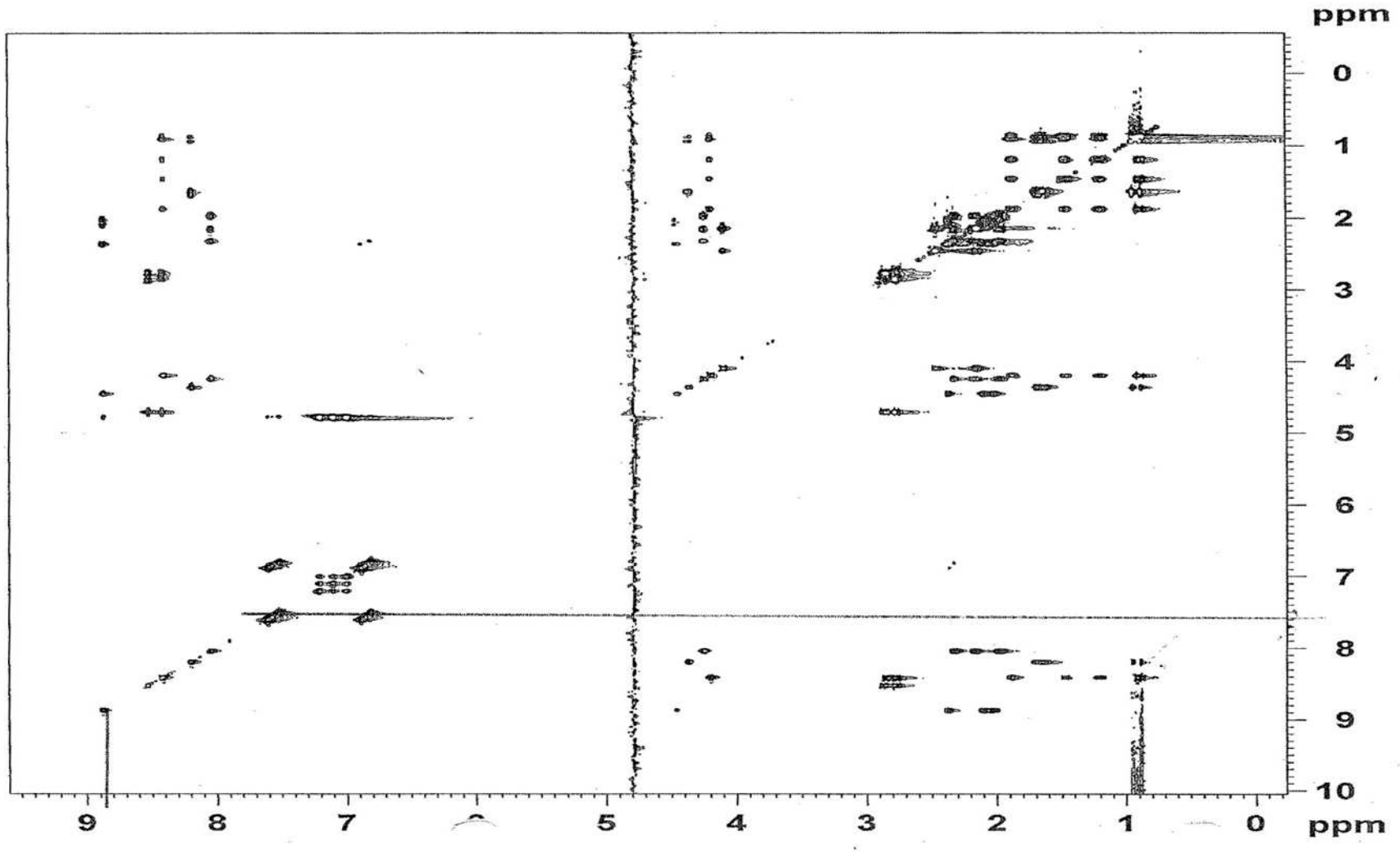

Figure A-5. 2D-TOCSY spectra of heptapeptide EQIDNLQ in water.

Enzymatic production, purification and analysis of bioactive peptides from fish proteins

A thesis submitted in the fulfilment of the requirements for the degree of Doctor of Philosophy

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DECLARATION

This is to certify that the work presented in this thesis is original, except where acknowledgment is made in the text, and has not been submitted to any other university or institution for a higher degree.

Junus Salampessy

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Finally, this Thesis would not have been possible, if not for the financial support from University of Western Sydney Australian Postgraduate Research scholarship and the Centre for Plants and the Environment.

DEDICATION

I dedicate this work with infinite gratitude to my LORD God almighty whom I know in the person of Jesus Christ my Lord and Saviour and to my beloved wife Gloria and children Sean Matthew and Shawneen Matthya, and to my family in Indonesia for their endless love, understanding, and support given to me.

Beta cinta kamorang samua.

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List of Plates

Publications and Conference Presentations

No. Title

1. Anti-hypertensive peptides derived from enzymatic hydrolysis of fish muscle proteins. Junus Salampessy, M. Phillips, and K. Kailasapathy. Abstract. International Conference of Foods and Functions, 9-11 June 2009, Zilina, Republic of Slovakia.
2. Release of anti-microbial peptides through bromelain hydrolysis of leatherjacket (*Meuchenia* sp.) insoluble proteins. Short communication. Junus Salampessy, Michael Phillips, Saman Seneweera, Kasipathy Kailasapathy. *Food Chemistry* **120** (2010) 556–560 (Appendix 10).
3. Fermented Fish Products. Junus Salampessy, Kasipathy Kailasapathy, and Namrata Thapa. In *Fermented Foods and Beverages of the World,* Chapter 10, Tamang, J. P. and Kailasapathy, K., eds. Boca Raton: CRC Press. (2010).
4. Anti-hypertensive and anti-microbial activities of bioactive peptides derived from fish muscle proteins. Junus Salampessy, Kasipathy Kailasapathy, Michael Phillips, and Narsimha Reddy. Abstract. 43rd AIFST Annual Convention, Melbourne 25-27 July 2010.
5. Fermented Dairy Ingredients. Junus Salampessy and Kasipathy Kailasapathy. In *Dairy Ingredients for Food Processing,* Chapter 13, Chandan, R. and Kilara, A., eds. New Jersey: John Wiley & Sons. In Press.
6. Recent trends in the development of bioactive peptides as physiologically functional dairy ingredients. Junus Salampessy and Kasipathy Kailasapathy. Submitted to International Journal of Dairy Technology.

List of Appendices

List of amino acids with their three and one letter codes

Amino acid names	Three letter codes	One letter codes
Alanine	Ala	A
Arginine	Arg	R
Aspargine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophane	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Abstract

Hydrolysis of food proteins with isolated enzymes has been subject to extensive research carried out with a view to produce bioactive peptides. In this study, 0.5% (w/v) of papain and bromelain, and 1.25% (v/v) of Flavourzyme™ were used to hydrolyse soluble protein fractions leatherjacket (*Meuchenia* sp.) and trevally (*Pseudocaranx* sp.) and 1% (w/v) of papain and bromelain and 2.5% (v/v) of Flavourzyme™ for insoluble protein fraction. The resulting hydrolysates were analysed for the degree of hydrolysis (DH), anti-microbial activity, and angiotensin I converting enzyme (ACE) inhibition. It was found that the DH of the hydrolysates ranged from 22- 25% for soluble protein hydrolysates, and from 30-33% for the insoluble protein hydrolysates after 10-hour hydrolysis. Results from anti-microbial assays showed that 8 h bromelain hydrolysate of leatherjacket insoluble protein fraction (labeled as LBI8H) showed strongest activity. Two active fractions, fraction 9 (labeled as LBI9) and 12 (labeled as LBI12), showed activities against *Staphylococcus aureus* and *Bacillus cereus*. Fraction LBI9 exhibited some activity against *B. cereus* without an MIC being reached at 5.35 mg/ml peptide concentration. Peptide fraction LBI12, on the other hand, showed activity against both *B. cereus* and *S. aureus* with an MIC value of 4.3 mg/ml. Edman N-terminal sequence analysis revealed active fraction LBI12 consisting mainly heptapeptide EQIDNLQ (MW = 858.9 Da, net charge = -2), an anionic peptide. Anti-microbial activity assays using synthetic EQIDNLQ revealed that the heptapeptide is active against both *B. cereus* and *S. aureus* bacteria having MIC values of 5.3 mg/ml and 7.96 mg/ml, respectively.

Results from ACE inhibition studies, expressed as IC_{50} values, showed that leatherjacket soluble protein hydrolysates have IC_{50} values ranged from 1.35 mg/ml

to 1.89 mg/ml, while the IC_{50} values of the leatherjacket insoluble protein fractions ranged from 0.77 mg/ml to 6.78 mg/ml. The IC_{50} values of trevally soluble protein fraction ranged from 1.99 mg/ml to 3.34 mg/ml, while the IC_{50} values of the trevally insoluble protein fractions ranged between 2.45 mg/ml to 4.74 mg/ml. Purification with C18 RP-HPLC column produced 5 purified fractions from leatherjacket hydrolysates and 7 purified fractions from trevally hydrolysates. The 5 leatherjacket fractions were labelled as LPI5, LPI6, LBI2, LBI5, and LFI5, while the 7 trevally fractions were labelled as TPI3, TPI4, TBS1, TBS2, TBS6, TBI2, and TBI4. The primary structures of the four leatherjacket peptide fractions LPI5, LPI6, LBI5, and LFI5 were determined as EPLYV, DPHI, AER, and WDDME, having molecular weights of 619.71, 480.52, 374.40, and 694.71 Da, respectively. The primary structure of three trevally peptide fractions TBS1, TBS2, and TBI2 are AR, AV, and APER, having molecular weight of 245.28, 188.23, and 471.51 Da, respectivelly. In addition, leatherjacket fraction LBI12 with sequence of EQIDNLQ, an anionic anti-microbial peptide also showed ACE inhibitory activity with an IC_{50} value of 0.24 mg/ml.

Kinetic study showed that heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ) is a competitive anti-hypertensive peptide that will bind to the active site of ACE. Chemical shift index (CSI) nuclear Overhauser effect (NOEs) from NMR experiments in aqueous and SDS solutions showed that the peptide has an extended structure with a tendency to take a turn between isoleucine (Ile-3, I) and aspartic acid (Asp-4, D). With such an open structure, this small peptide can attach into the active site of ACE though partially negative charge of glutamic acid (Glu-1, E) as well as glutamine (Gln-2 and Gln-7, Q) to bind to the zinc ion that is bound at the active site of ACE and to bind to microbial membrane through hydrogen bonds or van der

Waals interactions. Results from this study suggest that papain, bromelain, Flavourzyme can be used in production of ACE inhibitory peptides. In addition, bromelain is also able to release anionic anti-microbial peptide from leatherjacket proteins. Further study is needed to investigate the possibility to utilise these active ACE inhibitory peptides in preparation of anti-hypertensive functional foods. Further characterisation of the anionic anti-microbial peptide may be beneficial to understand and to improve its activity.

Introduction

Better utilisation of food proteins using hydrolysis has been practiced for many years in different parts of the world. In Japan, for instance, fermented soy bean products called 'shoyu' (soy sauce) and 'miso' (soy paste) are popular and are produced on an industrial scale. In Indonesia, a partially fermented soy bean product called 'tempeh' is a well known food, while soy sauce, a fermented soy bean product, is a popular ingredient in Chinese cooking.

Almost all food proteins can be proteolytically modified to produce different food ingredients. Yogurt, soy sauce, fish sauce and miso, to mention a few, are products made through proteolytic modification of food proteins either of plants or animal origin. Some products are produced with modern technology, while others use traditional processes. Both process, however, involve the use of proteolytic enzymes to hydrolyse the protein. These enzymes are either of plant origin, such as papain and bromelain, or of microbial origin, such as neutrase, alcalase, and Flavourzyme™. The use of papain and bromelain in food protein hydrolysis has been practiced for many years. The end products are considered inferior (lack of flavour) when compared to products obtained through traditional processes and are used mainly to enhance the production of sauces such as fish sauce. This disadvantage should, however, be considered as an opportunity, as papain or bromelain hydrolysates may be used as food ingredients in other food systems. The lack of flavour can easily be masked by other flavour active compounds in different food system to obtain products of varying flavour profiles.

In recent decade, many studies have investigated the bioactivity of peptides derived from hydrolysing food proteins (Aimutis, 2004; Arihara, 2006; Meisel et al., 2006).

Two possible uses of bioactive peptides are inhibition of angiotensin I-converting enzyme (ACE), which can lower the blood pressure of hypertensive people (Foltz et al., 2007) and inhibition of microbial growth hence as a food preservative, and as medicine (peptide antibiotic) (Chan and Li-Chan, 2006; Hancock and Chapple, 1999). There are, however, many other possible uses of bioactive peptides including anticariogenic, anti-anaemia, opioid-like, antithrombotic and cholesterol-lowering agents (Aimutis, 2004; Deng et al., 2004; Gerdes et al., 2001; Turpeinen et al., 2009). Hydrolysis of food proteins with different enzymes produces different peptides with different inhibitory activities towards ACE (Fujita et al., 2000). The peptides derived from hydrolysing fish protein, such as from sardine (*Sardinella sp.*), have been reported to show strong ACE inhibitory activity. Similar results were reported for products from a few other fish proteins. However, most of the knowledge of ACE inhibitory peptides was derived from research with milk and milk products that accounted for about 34% and only about 7.7% came from research with various species of fish and seafood (Meisel et al., 2006).

Most of the antibiotic peptides are the non-ribosomally synthesised peptides usually produced by micro-organisms and the ribosomally synthesised peptides that are found in various animals (including human), plants, and bacteria. The ribosomally synthesised group of peptide antibiotics are mostly from skin such as frog skin, gill of fish, mucus and other secretion of animals (including human) and are part of innate immune defence system. There are, however, a few antibiotic peptides that are derived from hydrolysis of food proteins such as isracidin, an antimicrobial peptide derived from chymosin hydrolysis of casein (Hill et al., 1974) and a pentadecapeptide derived from clostripain hydrolysis of chicken lysozyme

(Pellegrini et al., 1997). These findings indicate the possibility of producing antimicrobial peptides from hydrolysis of food proteins.

Although there are reports on the possible use of fish hydrolysates as dietary products with a potential health benefit, there is no report of the application of these products. This is probably due to its off-flavour characteristics such as bitterness and other off-flavours arising from the raw materials used. The use of washed fish-mince is thought to decrease these off-flavour characteristics. Some research findings have indicated the production of bioactive peptides from fish myofibril proteins that is obtained after washing out the sarcoplasmic protein in fish-mince. With this background information, this research was attempted to produce fish protein hydrolysate from less commercial fish protein with the aid of papain, bromelain and Flavourzyme™, to investigate the possible presence of bioactive peptides with special emphasis on ACE inhibitors and antimicrobial activity with a view to utilise them for functional, nutraceutical and clinical purposes.

Specifically this study was designed to:

- Optimise condition for hydrolysis of fish muscle protein with papain, bromelain and Flavourzyme™
- Evaluate the degree of hydrolysis
- Purify the bioactive peptides produced from the hydrolysis
- Analyse the bioactivity of derived peptides through *in vitro* assay for ACE inhibitory and antimicrobial activities
- Elucidate the structures of the active peptides

It is expected that this study will provide information on the use of the three proteolytic enzymes in the production of bioactive peptides from fish muscle proteins

that may be extended to be used for producing bioactive peptides from other proteins. It will also provide information on the characteristics of the active peptides and the possible use of the bioactive peptides in the production of food or other type of products with potential health benefits.

This thesis consists of seven chapters. The first chapter is an introduction and review of scientific literature with emphasis given to muscle food proteins and bioactive peptides derived from hydrolysis of muscle food proteins. The second chapter provide details of materials and methods used in this study. The third chapter gives information on the preliminary studies and optimisation of hydrolysis condition. The fourth chapter focuses on antimicrobial analysis, purification, and structure analysis of the active peptides. The fifth chapter focuses on the analysis ACE inhibitory and structure elucidation of ACE inhibitory bioactive peptides. The sixth chapter covers structural characteristics and activity studies of the selected active heptapeptide Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ). The seventh chapter includes discussion, summary of the findings and future directions of research in this area.