ANTIHYPERTENSIVE AND ANTIOXIDANT ACTIVITY OF PEPTIDES DERIVED FROM FISH

BY

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Peptides derived from food proteins after enzymatic treatment and/or processing, are known to be bioactive in both biological and food systems; for this reason fish muscle peptides were investigated for their antihypertensive and antioxidant activity. Atlantic mackerel muscle proteins were hydrolysed with pepsin and pancreatin and the resultant hydrolysate was sequentially fractionated on 2 kDa membrane ultrafilters and further by gel filtration, ion exchange and high performance liquid chromatography and the resultant peptide fraction contained the amino acids histidine, proline, tyrosine, methionine, leucine, tryptophan and lysine. For ACE inhibitory activity, the peptide fraction (MFPH-V-JPA) had an inhibitory concentration (IC$_{50}$) of 0.15 mg/ml and showed competitive inhibition for ACE with an inhibition constant (K$_i$) of 0.32 mg/ml. In terms of antioxidant activity, the HPLC isolated peptide fraction (LC1-Z) contained the amino acids serine, histidine, tyrosine, phenylalanine, tryptophan and lysine. It inhibited the formation of both peroxides and malonaldehyde in a linoleic acid model emulsion in a dose dependent manner with lipid oxidation inhibitory concentration (IC$_{50}$) of 1.80 mg/ml. At concentration of 8 mg/ml, the inhibition of linoleic acid oxidation was more than that of 0.01 % butylated hydroxytoluene (BHT) and trolox (p<0.001). The mechanism of antioxidant activity of the peptide (LC1-Z) was by carbon centered radical scavenging (5.34 %), hydroxyly radical scavenging (IC$_{50}$ value of 1.60 mg/ml), metal chelating (5.72 %) and reducing ability. In caco-2 cells, 1 mg/ml of the peptide (LC1-Z) was not toxic to the cells seeded at 2 x 10$^4$ cells/well. Proxidant tBHP (2.5 mM) reduced cell viability significantly (79.3 %) but this increased to 94.7 % in the presence of the
peptide or trolox. The peptide (1 mg/ml) also reduced TBARS formation (33.18 µg/ml) in cells compared to cells treated with tBHP alone (38.18 µg/ml). The activity of caspases-3 and -7, was higher in caco-2 cells treated with tBHP only (157.5 ± 7.99 %) compared with those treated with the peptide (25.7 ± 3.92 %). Morphological modification of the caco-2 cells treated with tBHP was evident as the cells appeared detached from the flask surface compared to those treated with the peptide (LC1-Z) which were healthy and attached to the flask surface. In Ea.hy 926 cells, reactive oxygen species were reduced by 26 % and 39 % in the lucigenin-chemiluminescence and fluorescence methods respectively in cells treated with the peptide. In a caco-2 cell monolayer, transepithelial transport of the peptide was observed in both directions with a basolateral to apical apparent permeability of 0.95 ± 0.12 cm⁻¹ and apical to basolateral flux of 0.74 ± 0.20 cm⁻¹. HPLC chromatograms of the buffer solution taken from the apical and basolateral side showed the presence of the peptide in both sides i.e. 11.5% for apical to basolateral flux and 12.2 % for basolateral to apical. These results demonstrate that peptides with antihypertensive and antioxidant activity can be derived from Atlantic mackerel muscle proteins, with potential for nutraceutical applications.
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CHITUNDU KASASE, 2009.
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DEDICATIONS

Chisomo Chitundu Kasase and Elizabeth Maiwase Kasase

My life and inspiration;

Lillian Z Kasase – the reason for everything!
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<tr>
<td>ACE</td>
<td>Angiotensin I converting enzyme</td>
</tr>
<tr>
<td>ACEI</td>
<td>ACE inhibitory</td>
</tr>
<tr>
<td>Ang-I</td>
<td>Angiotensin I</td>
</tr>
<tr>
<td>Ang-II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>BAPs</td>
<td>Bioactive peptides</td>
</tr>
<tr>
<td>BHA</td>
<td>Butylated hydroxyanisole</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Caco-2</td>
<td>Human colon carcinoma cell line</td>
</tr>
<tr>
<td>Caspases</td>
<td>Cysteine aspartate proteases</td>
</tr>
<tr>
<td>COML</td>
<td>Census of Marine Life</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic Blood Pressure</td>
</tr>
<tr>
<td>DHE</td>
<td>2-Hydroxyethidium</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Media</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DPPH</td>
<td>1,1-diphenyl-2-picrylhydrazyl</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Cell Cultures</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin gallate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and agriculture organisation</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FRS</td>
<td>Free radical scavengers</td>
</tr>
<tr>
<td>FTC</td>
<td>Ferric thiocyanate</td>
</tr>
<tr>
<td>Glu-DMEM</td>
<td>Glutamax Dulbecco’s Modified Eagle Media</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>HA</td>
<td>Hippuric acid</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks buffer saline solution</td>
</tr>
<tr>
<td>HHL</td>
<td>Hippuryl- histidyl-leucine</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HRSA</td>
<td>Hydroxyl radical scavenging activity assay</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Inhibitory concentration</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>KNOS</td>
<td>Kinin nitric oxide system</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid Chromatography-Mass spectroscopy</td>
</tr>
<tr>
<td>LOX</td>
<td>Lipoxygenase enzyme</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MTT</td>
<td>2-Amino-7-dimethylamino-3-methyl phenazine hydrochloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non essential amino acids</td>
</tr>
<tr>
<td>NEPS</td>
<td>Neutral endopeptidase system</td>
</tr>
<tr>
<td>Papp</td>
<td>Apparent permeability coefficient</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PITC</td>
<td>Phenyl isothiocyanate</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>PV</td>
<td>Peroxide value</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin angiotensin system</td>
</tr>
<tr>
<td>RCS</td>
<td>Renin chymase system</td>
</tr>
<tr>
<td>ROOH</td>
<td>Lipid peroxide</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reversed-phase high-performance liquid chromatography</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic Blood Pressure</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneous hypertensive rat</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive species</td>
</tr>
<tr>
<td>t-BHP</td>
<td>tert-butyl hydroperoxide</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TEER</td>
<td>Transepithelial electrical resistance</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
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UV  Ultra violet
Vmax  Maximum velocity
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CHAPTER ONE
1. GENERAL INTRODUCTION

Fish are aquatic vertebrates that use gills to obtain oxygen from water and have fins with variable number of fin rays also called skeletal elements (Thurman and Webber, 1984). The Census of Marine Life (COML) research group indicates that there are over 20,000 different species of fish in the world's seas and oceans, about a quarter of which are still to be discovered and classified (Mason, 2003).

The consumption of fish has been linked to a number of health benefits as a result of its unique content of proteins, oils, vitamins and minerals. Globally, fish provides more than 2.6 billion people with approximately 20% of the average animal protein requirements (Sidhu, 2003, FAO, 2004). However, the projected increase in world population is likely to lead to shortages in fish consuming areas. In addition, a large part of the fish harvested is wasted. The FAO estimates that post-harvest losses, as discards at sea and losses due to deterioration, are as high as 27 million tonnes of the total catch (110 million tones) (Kelleher, 2004). Fish processing waste has been well documented as a rich source of under-utilized protein, with well over 75 percent (by weight) of round fillet being discarded during a filleting process. The protein content in cod (Gadus morhua) fillet and offals was found to be 14.3% and 17.8%, of wet weight, respectively (Shahidi, 1994). Therefore, reducing these substantial losses by utilizing and upgrading the discards from processing into valuable food products remains a challenge and a primary objective of fish processors.
Traditionally, food ingredients including herbs have been used to treat diseases in addition to conventional medicines. Recently, however, there has been a drive to acquire a detailed knowledge of the composition and mechanisms of the active ingredients like polyphenols. Thus food and nutritional sciences can make a contribution to the reduction of this disease burden and a part of this science is the role played by proteins and peptides.

The nutritional and functional properties of food proteins have been investigated for many years. The nutritional quality of a protein depends on its amino acid content and on the physiological utilization of specific amino acids after digestion and absorption (Friedman, 1996). In terms of their functional properties, proteins contribute to the physicochemical and sensory properties of various protein-rich foods. Scientific evidence has shown that proteins and in particular peptides exert specific biological activities besides the established nutritional function (Korhonen and Pihlanto, 2003). These peptides are specific protein fragments that have a positive effect on body function and state and therefore impact on the health of individuals. These peptides are classified as bioactive peptides (BAPs) and they are generally considered to be inactive in their sequence in the protein. Upon hydrolytic proteolysis, by gastrointestinal enzymes or during processing through enzyme-mediated proteolysis of the parent protein, bioactive peptides are released which when absorbed exert a biological function in the human body and impact on the health of the individual (Korhonen and Pihlanto, 2003; Yoshikawa et al, 2000). BAPs are relatively resistant to hydrolysis and can either be transported across the intestinal mucosa due to their small size to exert biological
activity or they can exert local effects in the gastrointestinal tract (Vermeirssen et al., 2002).

*In vivo*, these peptides have been documented to exhibit a number of biological activities and this is heavily dependent on the nature and the sequence of amino acids in peptides. Activities exhibited include Angiotensin I converting enzyme (ACE) inhibition, antioxidant activity, antimicrobial activity, mineral binding, opioid and immunomodulatory activities. Bioactive peptides of food origin therefore have the potential to be utilized and delivered to consumers as active ingredients in functional foods, conventional foods and nutraceuticals (Meisel, 1997; Korhonen and Pihlanto, 2003; Mine and Shahidi, 2006). This review seeks to highlight the biological activity *in vivo* and *in vitro*, of fish derived peptides in inhibiting the Angiotensin converting enzyme (ACE) and antioxidant activity.

1.2. Proteins and peptides

1.2.1. Protein structure

Each protein consists of one or more unique polypeptide chains. Most proteins do not remain as linear sequences of amino acids; rather, the polypeptide chain undergoes a folding process. The process of protein folding is driven by thermodynamic considerations. This means that each protein folds into a configuration that is the most stable for its particular chemical structure and its particular environment. The final shape will vary but the majority of proteins assume a globular or fibrous configuration. In
order to maintain their function, proteins maintain this conformation (Belitz et al, 2009). Four levels of protein organization are described: primary, secondary, tertiary and quaternary (Figure 1.1).

1.2.1.1. Primary structure

The primary structure refers to the linear sequence of amino acids that make up the polypeptide chain. That bond between two amino acids is a peptide bond. This bond is formed by the removal of a H₂O molecule from two different amino acids, forming a dipeptide. The sequence of amino acids determines the positioning of the different side chain R groups relative to each other. This positioning therefore determines the way that the protein folds and the final structure of the molecule (Belitz et al, 2009).

1.2.1.2. Secondary structure

The secondary structure of protein molecules refers to the formation of a regular pattern of twists or kinks of the polypeptide chain. The regularity is due to hydrogen bonds forming between the atoms of the amino acid backbone of the polypeptide chain. The two most common types of secondary structure are known as the α-helix and β-pleated sheet (Belitz et al, 2009).
1.2.1.3. Tertiary structure

Tertiary structure refers to the three dimensional globular structure formed by folding of the linear polypeptide chain. The folding of the polypeptide chain is stabilized by multiple weak, non-covalent interactions including hydrogen bonds, electrostatic interactions and hydrophobic interactions. During folding of the polypeptide chain, amino acids with a polar (water soluble) side chain are often found on the surface of the molecule while amino acids with non-polar (water insoluble) side chains are buried in the interior. This means that the folded protein is soluble in water or aqueous solutions. Covalent bonds including disulphide bonds may also contribute to tertiary structure. The amino acid, cysteine, has a SH group as part of its R group and, therefore, the disulphide bond (S-S) can form with an adjacent cysteine (Belitz et al, 2004).

1.2.1.4. Quaternary structure

Quaternary structure refers to the association of several polypeptide chains giving an additional level of structural organization: Each polypeptide chain in the protein is called a subunit which may comprise the same or different polypeptide chains (Belitz et al, 2004).
1.2.2. Fish proteins

The proteins of fish tissue can be divided into three groups namely structural, sarcoplasmic and connective tissue proteins.

1.2.2.1. Structural proteins

Structural proteins, that include actin, myosin, tropomyosin and actomyosin, constitute 70-80 % of the total protein content and make up the contractile apparatus responsible
for muscle movement. Myosin and actin are the predominant constituents in structural proteins and account for more than 70% of total protein (Pearson and Young, 1989). Myosin is a very large protein (about 500 kDa) consisting of two identical heavy chains (about 200 kDa each) and two pairs of light chains (about 20 kDa each). Each heavy chain consists of a globular head region and a long α-helical tail. The α-helical tails of two heavy chains twist around each other in a coiled-coil structure to form a dimer, and two light chains associate with the neck of each head region to form the complete myosin molecule. The amino acid composition of structural proteins is similar to mammalian muscle proteins; however the physical properties differ slightly (Spinelli et al, 1972). Myosin has 17% basic amino acids, 18% acidic amino acids and about 42 thiol residues that contribute to its function. Actin represents about 15% of meat proteins. It has 3 isoforms α, β and γ and in its monomeric form it’s referred to as G-actin. Four hundred (400) G-actin molecules are joined to form the non covalent filament called F–actin and these form the backbone of the thin filaments in muscles (Offer et al, 1989).

1.2.2.2. Sarcoplasmic proteins

Sarcoplasmic proteins include albumins, globulins and enzymes; and constitute 25-30% of the protein, the majority being enzymes participating in cell metabolism. They are soluble in water and dilute aqueous solutions (Belitz et al, 2009).
1.2.2.3. Connective tissue proteins

Fish connective tissue proteins consist predominantly of fibrillar collagen, constituting approximately 3 - 10 % total proteins compared with 17 % in most mammals. Collagen contains large quantities of hydroxyproline, with the primary structure having a repeating sequence of glycine-proline-hydroxyproline-glycine amino acids. In vertebrates, at least twelve different forms have been identified, each with a characteristic sequence of amino acids which allows the formation of a left-handed helical secondary structure. Three helices combine to form tropocollagen, a right handed super helix, which assemble and aggregate to form fibrils and consequently fibers (Lawrie, 1991).

The chemical and physical properties of collagen proteins differ depending on the tissues such as skin, swim bladder and the myocommata in muscle. Fish collagen fibrils form a delicate network structure with varying complexity in the different connective tissues. Compared to mammals, the hydroxyproline content is lower varying from 4.7 - 10 % of total amino acids (Sato et al, 1989). However, different fish species contain varying amounts of collagen in the body tissues. This difference is believed to reflect the swimming behaviour and influences the textural characteristics of fish muscle (Yoshinaka et al, 1988; Montero and Borderias, 1989).

As with all other proteins, fish proteins have nutritional, functional and biological properties. In terms of nutrition, fish proteins are a source of energy and contain all the essential amino acids (with a high biological value) that are essential for growth and
maintenance. Fish proteins are also known to contribute to the physicochemical and sensory properties of various protein-rich and enriched foods (Belitz et al, 2009). Besides the nutritional and functional properties, fish proteins are reported to exhibit specific biological activities which make them potentially useful nutraceuticals and functional food components. These properties are attributed to physiologically active peptides encrypted in fish protein molecules which when liberated affect numerous physiological functions of the organism.

1.3. Protein and peptide separation

Proteins and peptides are typically characterized by their size (molecular weight) and shape, amino acid composition and sequence, isoelectric point (pI), hydrophobicity, and biological affinity. The chemical composition of the unique R groups is responsible for the important characteristics of amino acids, chemical reactivity, ionic charge and relative hydrophobicity. Therefore protein properties relate back to number and type of amino acids that make up the protein. The amino acid composition is the percentage of the constituent amino acids in a particular protein while the sequence is the order in which the amino acids are arranged. Differences in these properties can be used as the basis for separation methods in a purification strategy (Belitz et al, 2009).

1.3.1. Size

Protein size is usually measured as molecular weight (mass), although occasionally, the length or diameter of a protein is given in Angstroms. The molecular weight of a protein
is the mass of one mole of protein, usually measured in units called daltons; one dalton is the atomic mass of one proton or neutron. The molecular weight can be estimated by a number of different methods including electrophoresis, gel filtration, and more recently by mass spectrometry. Separation methods that are based on size and shape include gel filtration chromatography (size exclusion chromatography) and polyacrylamide gel electrophoresis (Belitz et al, 2009).

1.3.2. Charge

Each protein has an amino group at one end and a carboxyl group at the other end as well as numerous amino acid side chains, some of which are charged. Therefore each protein and peptide carries a net charge. The net protein/peptide charge is strongly influenced by the pH of the solution. Different proteins have different number of each of the amino acid side chains and therefore have different isoelectric points. So, in a buffer solution at a particular pH, some proteins will be positively charged, some proteins will be negatively charged and some will have no charge. Separation techniques that are based on charge include ion exchange chromatography, isoelectric focusing and chromatofocusing (Belitz et al, 2009).
1.3.3. Hydrophobicity

In aqueous solutions, proteins and peptides tend to fold so that areas of the protein with hydrophobic regions are located in internal surfaces next to each other and away from the polar water molecules of the solution. Polar groups on the amino acid are called hydrophilic (water loving) because they will form hydrogen bonds with water molecules. The number, type and distribution of nonpolar amino acid residues within the protein determine its hydrophobic character. A separation method that is based on the hydrophobic character of proteins and peptides is hydrophobic interaction in most solid-liquid chromatography (Belitz et al, 2009).

1.3.4. Solubility

The 3-D structure of a protein affects its solubility properties. Cytoplasmic proteins and peptides have mostly hydrophilic (polar) amino acids on their surface and are therefore water soluble, with more hydrophobic groups located on the interior of the protein, sheltered from the aqueous environment. On the other hand, proteins and peptides that reside in the lipid environment of the cell membrane have mostly hydrophobic amino acids (non-polar) on their exterior surface and are not readily soluble in aqueous solutions.

Each protein has a distinct and characteristic solubility in a defined environment and any changes to those conditions (buffer or solvent type, pH, ionic strength, temperature, etc.)
can cause proteins and peptides to lose solubility and precipitate out of solution. The environment can be manipulated to bring about a separation of proteins e.g. the ionic strength of the solution can be increased or decreased, which will change the solubility of some proteins (Belitz et al, 2009).

1.3.5. Biological affinity

Proteins and peptides often interact with other molecules in vivo in a specific way. They have a biological affinity for particular molecules and these molecular counterparts are called ligands. These specific interactions are often exploited in protein purification procedures. Affinity chromatography is a common method for purifying recombinant proteins (e.g. proteins produced by genetic engineering). Several histidine residues can be engineered at the end of a polypeptide chain. Since repeated histidines have an affinity for metals, a column (250μm i.d) of the metal can be used as a bait to “catch” the recombinant protein (Belitz et al, 2004).

1.4. Angiotensin I converting enzyme (ACE) and blood pressure

Blood pressure (BP) within the body is controlled by several physiological processes, which either increase or decrease it. The major physiological pathways involved in the regulation of BP are the renin–angiotensin system (RAS), the kinin–nitric oxide system (KNOS), the renin–chymase system (RCS), and neutral endopeptidase system (NEPS). The RAS is perhaps the most important of various humoral vasoconstrictor and vasodilator mechanisms implicated in blood pressure regulation. RAS regulates BP,
electrolyte balance; and renal, neuronal and endocrine functions associated with cardiovascular control in the body. RAS plays a vital role in regulating blood volume and systemic vascular resistance; these two functions together control cardiac output and arterial pressure. The RAS has three important components namely, renin, angiotensin and aldosterone (Meisel et al, 2006).

In the RAS, through the action of kallikrein, renin is released from the precursor compound prorenin (Beldent et al, 1993; Ondetti and Cushman, 1982). Renin cleaves angiotensinogen to release angiotensin I (Ang-I) a decapeptide, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu. The Angiotensin Converting Enzyme (ACE) hydrolyzes Ang-I by removing the C-terminal dipeptide His-Leu to give an octapeptide angiotensin II (Ang-II) Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, a potent vasoconstrictor (Figure 1.2).

Ang II is known to interact with at least two distinct Ang II receptor subtypes, designated AT₁ and AT₂. Angiotensin II acts directly on a G-coupled receptor known as the AT₁ causing a negative effect. When the Ang II binds to the complementary receptor, it stimulates vascular smooth muscle contraction and release of noradrenaline thus constricting arteries and increasing the heart rate. It also stimulates the adrenal cortex, releasing the hormone aldosterone. Once aldosterone is released, the electrolyte balance of sodium and water is lost, resulting in retention of sodium and water (Laurence et al, 1997).
The three-dimensional structure reveals that ACE is composed of α-helices for the most part, and incorporates a zinc ion and two chloride ions (Figure 1.3). The active site consists of a deep, narrow channel that divides the molecule into two subdomains. On top of the molecule is an amino-terminal 'lid', which seems to allow only small peptide
substrates (2530 amino acids) access to the active site cleft. This accounts for the inability of ACE to hydrolyse large, folded substrates (Natesh et al, 2003).

Three different forms of ACE are identified namely somatic ACE, testicular ACE and ACE homologue (ACEH). The somatic and testicular forms of ACE consist of two homologous domains, N- and C-domain (Inagami, 1992). Both domains contain active sites which are responsible for the catalytic hydrolysis of angiotensin I (Wei et al, 1992). The C-domain appears to be responsible for controlling blood pressure, signifying that the C-domain is the principal angiotensin-converting site (Natesh et al, 2003).

ACE is one of a number of biologically important ectoproteins existing in both membrane-bound and soluble forms. Localized on the surface of various cells, ACE is inserted at the cell membrane via its carboxyl terminus. Human plasma ACE originates from endothelial cells while other body fluids may contain ACE that originates from
epithelial, endothelial or germinal cells. The two isoforms of ACE, the two-domain somatic form and the single domain germinal form, convert angiotensin I to angiotensin II, and metabolize kinins and many other biologically active peptides, including substance P, chemotactic peptide and opioid peptides. The broad spectrum of substrates for ACE and its wide distribution throughout the body indicates that this enzyme, in addition to an important role in cardiovascular homeostasis, may be involved in additional physiological processes such as neovascularization, fertilization, atherosclerosis, kidney and lung fibrosis, myocardial hypertrophy, inflammation and wound healing (Igic and Behnia, 2003)

Thus, ACE is a key enzyme in the regulation of BP and has been targeted in the control of hypertension. ACE inhibition prevents the formation of the vasoconstrictory (hypertensive) agent angiotensin II and enhances the vasodilatory (hypotensive) properties of bradykinin, leading to a concerted lowering of the blood pressure. As a result of Ang II production, several physiological processes are elicited that include systematic vasoconstriction, cardiac and vascular hypertrophy, increased blood volume and renal sodium and fluid retention (Figure 1.4). This process consequently leads to the lowering of blood pressure and forms the basis for the use of the inhibitors of ACE in the treatment of hypertension, heart failure, myocardial infarction and diabetic nephropathy (Mine and Shahidi, 2006).
1.4.1. ACE inhibitors

The first naturally occurring Angiotensin-I-Converting Enzyme Inhibitors (ACEI) of peptide nature were isolated from snake venom (Ferreira et al, 1970, Ondetti et al, 1971). Ferreira et al (1970) isolated nine biologically active peptides from Bothrops jararaca containing 5 to 13 amino acid residues. Ondetti et al (1971) also isolated from venom of Bothrops jararaca and reported other strong ACE inhibitors having an antihypertensive effect in vivo from the venom. However, since it was not orally active, a synthetic inhibitor had to be designed. In the 1970s, Ondetti et al (1977) developed
inhibitors of ACE by rational drug design, where they constructed the active site of ACE, using computer assisted studies.

Varying terminal amino acid sequences of peptides may serve as inhibitors of ACE. The shortest peptides that serve as inhibitors of ACE are tripeptides. It is suggested that the C-terminal tripeptide residue of peptide inhibitors, as well as the nonpeptide inhibitors such as captopril, interact with an "obligatory binding site" on ACE, which is necessary for effective binding. Peptide inhibitors, on the other hand, bind not only to the obligatory binding site of the enzyme but also interact with regions of the enzyme adjacent to the obligatory binding site (Antonaccio, 1982) (Figure 1.5).

Figure 1.5. Hypothetical model of ACE and suggested binding of peptides to the active site of the enzyme.
Many prototypes of ACE inhibitors were developed, which eventually led to the development of the first synthetic ACE inhibitor, Captopril (Laurence et al, 1997). However, due to many reported zinc deficient adverse reactions (Golik et al, 1998), subsequent ACE inhibitors were designed that includes Enalapril, Lisinopril and Ramipril (Figure 1.6).

![Figure 1.6. Synthetic ACE inhibitors.](image)

Despite these positive developments in synthetic ACEI, their use is questioned due to side effects such as skin rash, headache, dry cough and loss of taste perception. As a
result, another dimension to current research is now directed to naturally occurring inhibitors from natural sources. Until now, several ACE inhibitory (ACEI) peptides have been isolated from various food proteins such as milk, animal (non-milk), plant, insect and different protein sources and these are reviewed in the literature (Meisel et al., 2006; Mine and Shahidi, 2006). ACE inhibitory peptides have been isolated and characterized from a number of fish sources, from the muscle proteins themselves as well as from waste and discards from fish processing plants. ACE inhibitory peptides have been isolated and characterized from several fish species that include salmon, sardine, bonito, tuna, Alaska pollack, sea bream, pelagic thresher, fish sauce, blue whiting and yellowfin sole (Table 1.1).
Table 1.1. ACE inhibitory peptides derived from fish proteins: source, amino acid sequence, parent protein, enzyme used for hydrolysis, and IC_{50}-value.

<table>
<thead>
<tr>
<th>Source</th>
<th>Parent Protein</th>
<th>Enzyme treatment</th>
<th>Amino Acid Sequence</th>
<th>IC_{50} (µM)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Sardine</td>
<td>Meat</td>
<td>A.oryzae protease</td>
<td>-</td>
<td>3.97</td>
<td>Suetsuna and Osajima 1986</td>
</tr>
<tr>
<td>Tuna</td>
<td>Muscle</td>
<td>Acid</td>
<td>PTHIKWG D</td>
<td>N/A</td>
<td>Kohama and Osajima 1988</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>Acid</td>
<td>IF</td>
<td>70.0</td>
<td>Kohama et al., 1988</td>
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<td>Kohama et al., 1988</td>
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<td>Kohama et al., 1988</td>
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<td>IFG</td>
<td>&gt;100</td>
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<td>Bonito</td>
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<td>IY</td>
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<td></td>
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<td>VY</td>
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21
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<th>Protein Source</th>
<th>Type</th>
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<th>Activity (U/mg)</th>
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<td>Okamoto et al, 1995</td>
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<td>Astawan et al, 1995</td>
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<td>(gelatin protease)</td>
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<td>Chymotrypsin</td>
<td>MIFPGAG GPEL</td>
<td>28.7 µg/ml</td>
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<td>Muscle Papain</td>
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<td>Hydrolysate</td>
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<td>3.7 mg/ml</td>
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<td>Coho salmon</td>
<td>muscle Thermolysin</td>
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<td>0.138 mg/ml</td>
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</table>

NSIR*, Not specified in reference

The spontaneous hypertensive rat (SHR), a strain of *Rattus norvegicus* with elevated blood pressure, is extensively used as a model for studying hypertension in experimental animal models. SHR have been used to investigate the efficacy of ACEI peptides to regulate blood pressure. When orally or intravenously administrated to SHR, various animal and plant peptides have demonstrated antihypertensive effects through the reduction in blood pressure (Meisel et al, 2006). Peptides derived from fish proteins with ACE inhibitory activity are reported in literature and some show hypotensive activity in SHR (Table 1.2).
Table 1.2. Hypotensive effects of fish derived peptides in Spontaneously Hypertensive Rats (SHR).

<table>
<thead>
<tr>
<th>Origin of peptide</th>
<th>Main peptides</th>
<th>Dose</th>
<th>Study design</th>
<th>Results</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Sardine muscle</td>
<td>VY</td>
<td>20 mg/kg</td>
<td>SHR, 12 wk old, Japan, i.v.</td>
<td>SBP - 7.2 mmHg &amp; DBP - 28.8 mmHg</td>
<td>Matsufuji et al, 1995</td>
</tr>
<tr>
<td>(alkaline protease)</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;: 7.1 μM</td>
<td>50 mg/kg</td>
<td>SHR, 12 wk old, Japan, i.v.</td>
<td>SBP - 18 mmHg and BP - 35 mmHg</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BW</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dried bonito (Thermolysin)</td>
<td>LKPNM, IC&lt;sub&gt;50&lt;/sub&gt;: 2.4 μM</td>
<td>8 mg/kg</td>
<td>SHR, 16–25 wk old, Japan, SOD</td>
<td>SBP - 23 mmHg at 4 h</td>
<td>Fugita and Yoshikawa 1999</td>
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<tr>
<td></td>
<td>LKP, IC&lt;sub&gt;50&lt;/sub&gt;: 0.32 μM</td>
<td>2.25 mg/kg BW</td>
<td>SHR, 16–25 wk old, Japan, SOD</td>
<td>SBP - 18 mmHg at 2 h</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LKPNM</td>
<td>100 mg/kg BW</td>
<td>SHR, 16–25 wk old, Japan, i.v.</td>
<td>SBP - 30 mmHg</td>
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<td>LKP</td>
<td>30 mg/kg BW</td>
<td>SHR, 16–25 wk old, Japan, i.v.</td>
<td>SBP - 50 mmHg</td>
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<td>Bonito muscle (Thermolysin)</td>
<td>IY, IC&lt;sub&gt;50&lt;/sub&gt;: 2.1 μM</td>
<td>10 mg/kg BW by i.v and 60 mg/kg BW for SOD</td>
<td>SHR, Japan, i.v. and SOD</td>
<td>SBP - 45.0 mmHg (i.v.), SBP - 19.0 mmHg (SOD+ 2 h)</td>
<td>Fugita et al, 2000</td>
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<tr>
<td></td>
<td>IW</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;: 5.1 μM</td>
<td></td>
<td>SBP - 55.0 mmHg (i.v.), SBP - 22.0 mmHg (SOD+ 2 h)</td>
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<tr>
<td></td>
<td>IKP, IC&lt;sub&gt;50&lt;/sub&gt;: 1.6 μM</td>
<td></td>
<td></td>
<td>SBP - 70.0 mmHg (i.v.), SBP - 20.0 mmHg (SOD+ 6 h)</td>
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<tr>
<td></td>
<td>IWH, IC&lt;sub&gt;50&lt;/sub&gt;: 3.5 μM</td>
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<td></td>
<td>SBP - 70.0 mmHg (i.v.), SBP - 30.0 mmHg (SOD+ 4 h)</td>
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<td></td>
<td>IVGRPR, IC&lt;sub&gt;50&lt;/sub&gt;: 300 μM</td>
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<td>SBP - 25.0 mmHg (i.v.), SBP - 17.0 mmHg (SOD+ 6 h)</td>
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<tr>
<td></td>
<td>LKPNM</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;: 2.4 μM</td>
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<td>SBP - 80.0 mmHg (i.v.), SBP - 23.0 mmHg (SOD+ 6 h)</td>
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<tr>
<td>Protein</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Treatment</td>
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<td>----------------------------------</td>
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<tr>
<td>Yellowsole</td>
<td>MIFPGAGGPEL</td>
<td>10mg/kg BW</td>
<td>SBP - 22mmHg +3h</td>
<td>Jung &lt;i&gt;et al&lt;/i&gt;, 2006</td>
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<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;: 28.7 µg/ml</td>
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<tr>
<td>Mackerel Hydrolysate</td>
<td>0.1mg/ml</td>
<td>10mg/kg BW</td>
<td>SBP Decreased + 2-4h</td>
<td>Itou and Akahane, 2004</td>
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<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;: 0.1mg/ml</td>
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<tr>
<td>Tuna Hydrolysate</td>
<td>0.63 mg/ml</td>
<td>-</td>
<td>SBP Decreased</td>
<td>Astawan &lt;i&gt;et al&lt;/i&gt;, 1995</td>
<td></td>
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<tr>
<td>Sea breams</td>
<td>0.57mg/ml</td>
<td>300 mg/kg BW</td>
<td>SBP - 20mmHg +3h</td>
<td>Fahmi &lt;i&gt;et al&lt;/i&gt;, 2004</td>
<td></td>
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<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;:</td>
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<td></td>
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<tr>
<td>Salmon Hydrolysate</td>
<td>27 µg/ml</td>
<td>500-2000 mg/kg BW</td>
<td>SBP -28 to 38mmHg +4h</td>
<td></td>
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<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;:</td>
<td></td>
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</table>

BW, body weight; SOD, single oral dose; SBP, systolic blood pressure; DBP, disystolic blood pressure.

1.4.2. Method for ACE inhibition activity

Angiotensin-converting enzyme (ACE) is a dipeptidyl carboxypeptidase that catalyzes the conversion of Angiotensin I to Angiotensin II. Therefore, the formation of the Angiotensin II is a measure of the enzyme activity, and the inhibition of the enzyme by certain compounds consequently indicates the potential and ability of these compounds to inhibit the activity of the ACE enzyme. Several methods for the measurement of ACE activity are reported and among them are included those based on spectrophotometry (Cushman and Cheung, 1971; Hayakari <i>et al</i>, 1978; Neels <i>et al</i>, 1983, Holmquist <i>et al</i>, 1979), fluorimetry (Friedland and Silverstein, 1976; Friedland and Silverstein, 1977; Persson and Wilson, 1977), high-performance liquid chromatography (HPLC) (Neels <i>et al</i>, 1983).
al, 1982, Doig and Smiley, 1993; Meng et al, 1995; Hyun and Shin, 2000; Wu et al, 2002), and internally quenched fluorogenic methods (Araujo et al, 1999). However, spectrophotometry methods are widely used in food and pharmaceutical industries. The Cushman and Cheung (1971) protocol is the most widely used method and is based on the hydrolysis of a synthetic substrate hippuryl-histidyl-leucine (HHL) by ACE to give hippuric acid (HA) and histidyl-leucine as products. ACE-inhibitory activity is quantified through the formed HA. Among the HPLC methods, the method of Wu et al, (2002), has recently been developed to improve on the Cushman and Cheung method. It utilises C18 reversed phase HPLC columns and a gradient acetonitrile-water-trifluoroacetic acid (TFA) mobile phase system.

1.5. Lipid oxidation inhibition and peptides

1.5.1. Lipids and ROS in biological systems

Lipids are a large and diverse group of naturally occurring organic, biological molecules that are related by their solubility in non-polar organic solvents and insolubility in aqueous solutions. Lipids are generally defined as fatty acids and their derivatives, and substances related biosynthetically or functionally to them, and include fatty acids, triacylglycerols, steroids, phospholipids, sphingolipids, plasmalogens, eicosanoids, waxes and terpenes. Lipids occur in both plants and animals serving a number of physiological functions including as structural components of biological membranes, energy reserves, precursors in vitamins and hormone synthesis and aiding lipid solubilization (Belitz et al, 2009).
Fatty acids contain an even numbers of carbon atoms in straight chains and may be saturated or unsaturated (with or without double bonds) and can have a range of substituent groups, and possess a carboxylic acid moiety at one end. Fatty acids have two major roles in the body, as constituents of more complex membrane lipids and as major components of stored fat in the form of triglycerides/triacylglycerols. These triglycerides constitute fats and oils that are found in both plants and animals, forming a major food group in human diets. Unsaturated lipids are unstable and oxidize easily leading to a loss of functionality and consequently changes in structural arrangement of biological membranes and food systems (Belitz et al, 2009).

The human body and other aerobic organisms have reactive oxygen species (ROS) that can induce in vivo lipid oxidation; they comprise singlet oxygen $^1$O$_2$, hydroperoxyl radical HO$_2^*$, superoxide radical anion O$_2^{-}$, hydroxyl radical •OH, hydrogen hydroperoxide H$_2$O$_2$, hydroperoxide ROOH, peroxyl radical ROO•, alkoxyl radical RO' and hypochlorous acid HOCl. ROS participate in a number of in vivo biological processes, are products of enzymatic reactions of oxidases such as xanthine oxidase and NAD (P) H oxidase, and may be produced by various cells. In the body, oxidative metabolism is indispensable for the survival of cells and the consequence of this dependence is the formation of ROS that can cause oxidative changes. These ROS, when formed in excess, overpower the protective enzymes like superoxide dismutase, catalase and peroxidase and cause destructive and lethal cellular effects (e.g. apoptosis) in membrane lipids, cellular proteins, DNA and enzymes, impairing cellular respiration. Due to these effects, lipid oxidation is a risk factor for diseases such as cardiovascular
diseases, cancers, diabetes, neurological diseases, immune diseases and eye diseases (Wilcox et al, 2004). Several studies have shown increased oxidative damage to all the main classes of biomolecules in the brains of Alzheimer’s patients (Halliwell, 2001). Atherosclerosis, diabetes and rheumatoid arthritis are also associated with free radical mediated injury (Halliwell, 2000; Halliwell and Whiteman, 2004). Some types of cancer are possibly a consequence of oxidative DNA-damage (Collins, 2005). Exposure to ultraviolet radiation, air pollution and cigarette smoke can also generate free radicals. Nitrogen dioxide, one of the major oxidants in smog, is also found in cigarette smoke. Two free radicals are found in cigarette smoke, one in the tar portion and the other in the gaseous phase. The NO\textsuperscript{-} is found in the tar portion and is able to reduce oxygen to the superoxide radical. Highly reactive oxygen and carbon-centered radicals are found in the gas phase (Chow, 1993).

In food systems, lipid oxidation causes deterioration in food quality producing rancid flavours, unacceptable taste, toxic compounds and shortening of shelf life. However, physiological processes and food processing techniques are employed to mitigate the negative effects of lipid oxidation through the use of antioxidants. In food processing, major natural antioxidants include tocopherols, ascorbic acid and phenolic compounds, and synthetic ones such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate and ethoxyquin. Synthetic antioxidants are cheap and potent and therefore popular in food processing. However, current research has shown that they interfere with DNA, proteins and lipids and may cause diseases. Thus there is a shift to
using naturally occurring antioxidants including peptides and amino acids from animal and plant proteins.

1.5.2. Lipid oxidation

Lipids, and in particular fatty acids that are unsaturated, can be oxidized under appropriate conditions. The mechanisms by which lipid oxidation is induced are multifaceted but are similar for many lipids. Three different mechanisms are postulated, namely autoxidation, photooxidation and enzymatic oxidation (Kolakowska, 2002).

1.5.2.1. Autoxidation

Autooxidation of lipids is a radical-chain process involving free radicals, defined as any species capable of independent existence and that contains one or more unpaired electrons. Free radicals are, therefore, highly reactive species due to the presence of these unpaired electrons as they need another electron to fill the orbital to assume stability. Oxidation is initiated by radicals present in living organisms (e.g., hydroperoxide HO₂⁺, hydroxide ·OH, peroxide ROO⁺, alkoxyl RO⁺, alkyl R⁺) or by thermal or photochemical homolytic cleavage of an R–H bond. The classical autoxidation route involves three stages: initiation, propagation, and termination (Kolakowska, 2002).
Initiation: In the presence of an initiator (In), unsaturated lipids (RH) lose hydrogen to form a lipid radical (R*):

$$\text{RH} + \text{In} \rightarrow \text{R}^* + \text{InH}$$

Propagation: The alkyl radical lipid (R*) reacts with molecular oxygen to form peroxyl radical (ROO*):

$$\text{R}^* + \text{O}_2 \rightarrow \text{ROO}^*$$

Peroxyl radicals abstract hydrogen from another molecule of unsaturated lipid (RH) to form hydroxyperoxides (ROOH) and a new lipid radical (R*):

$$\text{ROO}^* + \text{RH} \rightarrow \text{ROOH} + \text{R}$$

Termination: The peroxyl radicals react with each other to form non-radical products:

$$\text{ROO}^* + \text{ROO}^* \rightarrow \text{ROOR} + \text{O}_2$$

1.5.2.2. Photooxidation

Photooxidation involves the highly electrophilic singlet oxygen ($^1\text{O}_2$) that can react with unsaturated fatty acids, but by a different mechanism to free radical autoxidation. In the presence of photosensitizers (e.g. chlorophyll, porphyrins, myoglobin), a double bond in the unsaturated fatty acid interacts with singlet oxygen produced from $\text{O}_2$ by light.
Ultraviolet (UV) light, may be involved in initiation of the classical free radical oxidation of lipids and catalyse other stages of the process (Kolakowska, 2002).

1.5.2.3. Enzymatic oxidation

Enzymatic oxidation processes are driven by the lipoxygenase enzyme (LOX) system. LOX catalyzes reactions between oxygen and unsaturated fatty acids to form hydroperoxides which can be transformed into hydroxy products. LOX-catalyzed lipid oxidation is different from the free radical reactions as the formation of hydroperoxides is at a certain position of the chain of usually a free fatty acid (Kolakowska, 2002).

1.5.3. Secondary oxidation products

In lipid oxidation, free radicals and hydroperoxides are regarded as primary oxidation products, and the products derived from them are thus termed secondary oxidation products. These formed products vary in composition and differ both quantitatively and qualitatively as they depend on the types of lipids, the presence of pro- and antioxidants and conditions of oxidation. Hydroperoxides are transformed into ketones, aldehydes and hydroxides with a functional group situated at different positions, depending on the unsaturated FA, epoxides, dimers, and oligomers (Frankel, 1998). Volatile compounds that include aldehydes, alcohols, and hydrocarbons are formed as a result of homolytic
β-scission of fatty acids hydroperoxides. Unsaturated aldehydes and ketones undergo autoxidation and supply further volatile compounds (Kolakowska, 2002).

Oxidation of a single pure fatty acid gives rise to several tens of volatile compounds. Propanal and hepta-2,4-dienal are characteristic of the oxidative decomposition of n-3 polyunsaturated fatty acids, while hexanal and pentane are typical for oxidative decomposition of n-6 polyunsaturated fatty acids (Frankel, 1998). The rancid lipid odour profile is made up of a mixture of several volatile compounds. Among them, the trans, cis-alkadienals, and vinyl ketones have the lowest flavor threshold in oils, while the threshold of hydrocarbons (alkanes and alkenes) is the highest (Min, 1998). The sensory effects depend on the composition of the participating compounds and on the composition of the food matrix. The rancid off-odors and off-flavors of foods emanate from the interactions between lipids and other components, especially proteins (Kolakowska, 2002).

1.5.4. Mechanisms of antioxidant action

Based on their mode of action, antioxidants are classified as free radical terminators, chelators of metal ions, or oxygen scavengers. For this reason, primary antioxidants react with high-energy lipid radicals to convert them to thermodynamically more stable products. On the other hand, secondary antioxidants, also known as preventive antioxidants, function by retarding the rate of chain initiation by breaking down hydroperoxides (Kolakowska, 2002).
1.5.4.1. Free radical terminators

In free radical termination, free radical scavengers (FRS) or chain-breaking antioxidants such as phenolic compounds (AH) impede lipid oxidation by rapidly donating a hydrogen atom to lipid radicals such ROO•, RO•.

\[ \text{ROO}^\bullet + \text{AH} \rightarrow \text{ROOH} + \text{A}^\bullet \quad (1) \]
\[ \text{RO}^\bullet + \text{AH} \rightarrow \text{ROH} + \text{A}^\bullet \quad (2) \]
\[ \text{ROO}^\bullet + \text{A}^\bullet \rightarrow \text{ROOA} \quad (3) \]
\[ \text{RO}^\bullet + \text{A}^\bullet \rightarrow \text{ROA} \quad (4) \]
\[ \text{RO}^\bullet + \text{RH} \rightarrow \text{ROOH} + \text{R}^\bullet \quad (5) \]

The resulting phenoxy radical (A•) does not start a new free radical reaction or, is not subjected to rapid oxidation by a chain reaction. This property makes phenolic antioxidants excellent hydrogen or electron donors. Besides this activity, the radical intermediates of phenolic antioxidants are relatively stable due to resonance delocalization and lack of suitable sites for attack by molecular oxygen (Belitz et al, 2009).

The reactions of FRS with peroxy radicals are favoured due to a number of reasons. The propagation reaction is a slow step in lipid oxidation and accordingly, peroxy radicals are often found in the greatest concentration of all radicals in the systems. The interaction between FRS and peroxy radicals is favourable because peroxy radicals
have lower energies than radicals such as alkoxyl radicals, and also react more readily with the low energy hydrogens of FRS than with polyunsaturated fatty acids. As FRS are generally found at low concentrations, they do not compete effectively with initiating radicals. Therefore, FRS generally inhibits lipid oxidation by more effectively competing with other compounds (especially unsaturated fatty acids) for peroxyl radicals (Kolakowska, 2002; Belitz et al, 2009).

With phenolic antioxidants, the stability of the phenoxy radical is increased by bulky groups at the ortho positions as seen in butylated hydroxyanisole (BHA) (Hall, 2001). These groups increase the steric hindrance in the region of the radicals. They additionally reduce the rate of possible propagation reactions that may involve antioxidant free radicals (Gordon, 1990):

\[
\begin{align*}
A^\bullet + O_2 & \rightarrow AO^\bullet \\
AOO^\bullet + RH & \rightarrow AOOH + R^\bullet \\
A^\bullet + RH & \rightarrow AH + R^\bullet
\end{align*}
\]

The introduction of a second hydroxyl group at the ortho or para position of the hydroxyl group of a phenol also increases antioxidant activity of phenolic antioxidants. The antioxidant efficiency of a 1,2-dihydroxybenzene derivative is amplified by the stabilization of the phenoxy radical through an intramolecular hydrogen bond. This
property makes catechol and hydroquinone more effective in their peroxynitrite scavenging activity than phenol (Hall, 2001).

Multiple hydroxyl groups also confer significant antioxidant, chelating, and, in some cases, pro-oxidant activity to the molecule. These methoxyl groups introduce unfavorable steric effects but the presence of a double bond and carbonyl functionality in the C ring increases the activity by affording a more stable flavonoid radical through conjugation and electron delocalization (Heim et al, 2002).

In some instances, the antioxidant activity of phenolic antioxidants is influenced by pH e.g. hydroxyflavones. The antioxidant potential of hydroxyflavones increases upon deprotonation of the hydroxyl group. This shows that the mechanism of action of flavonoids is variable and electron (not hydrogen) atom donation is involved in the deprotonated species although abstraction of the hydrogen atom is involved for underprotonated species (Lemanska et al, 2001). Furthermore, the hydroxyl radical scavenging activity of phenolics involves multiple mechanisms, including hydroxyl bond strength, electron donating ability, enthalpy of single electron transfer and spin distribution of the phenoxy radical after hydrogen abstraction (Cheng et al, 2003).

The effect of antioxidant concentration on autoxidation rates depends on many factors, including the structure of the antioxidant, oxidation conditions, and nature of the sample oxidized. In certain cases, phenolic antioxidants lose their activity at high concentrations and behave as pro-oxidants by getting involved in initiation reactions (Gordon, 1990).
In food systems, phenolic antioxidants are more effective in extending the induction period when added to foods that have not deteriorated to any great extent. Phenolic antioxidants are generally ineffective in delaying decomposition of already deteriorated lipids. Therefore, antioxidants should be added to foodstuffs as early as possible to realize utmost protection against oxidation (Coppen, 1983).

1.5.4.2. Metal chelators

Metal chelating agents inhibit lipid oxidation by blocking the pro-oxidant metal ions, and as a consequence limit the formation of chain initiators by preventing metal-assisted homolysis of hydroperoxides.

\[
\text{ROOH} + \text{M}^{n+} \rightarrow \text{RO}^{*} + \cdot \text{OH} + \text{M}^{(n+1)+}
\]

\[
\text{ROOH} + \text{M}^{(n+1)+} \rightarrow \text{ROO}^{*} + \text{H}^{+} + \text{M}^{n+}
\]

Ascorbic, phosphoric, citric, tartaric and malic acids possess pronounced chelating activities and are effective in improving the oxidative stability of aqueous food emulsion systems (Frankel, 1998). The salts of phytic acid, phospholipids, and oxalates especially from plant materials are also common representatives of this group.
Amino acids and peptides in their antioxidant behavior have been shown to be metal chelating agents (Fujimoto et al., 1984; Pokorny, 1987). In peptides, the characteristic amino acid sequences are important for them to show lipid oxidation inhibitory effects (Chen et al., 1995). In peptides containing the amino acid histidine, their antioxidant behavior is related to their metal chelating ability and to lipid-radical trapping potential of the imidazole ring (Uchida and Kawakishi, 1992; Murase et al., 1993). Dipeptides carnosine and anserine that contain histidine involve not only singlet oxygen and free radical scavenging, but also metal chelation (Egorov et al., 1992). Phenolics, such as flavonoids also exhibit metal chelating characteristics in their antioxidant activities (Afanasev et al., 1989; Morel et al., 1993; Nardini et al., 1995; Chen and Ahn, 1998).

1.5.4.3. Oxygen scavengers

Foote and Denny (1968) first demonstrated that carotenoids, such as β-carotene, lycopene, zeaxanthin, lutein and canthaxanthin, can quench the singlet oxygen $^{1}\text{O}_2$. This discovery was an important advance in understanding how effectively carotenoid pigments prevent the damage of photobiological systems. The quenching mechanism is understood to proceed by two routes. In the first route, a carotenoid is thought to undergo no ultimate chemical change (physical quenching) whilst the second route involves a chemical reaction that results in new products (Young and Brewer, 1978).
The deactivation of \( ^1\text{O}_2 \) by carotenoids results primarily from a physical quenching process involving transfer of excited energy from \( ^1\text{O}_2 \) to the carotenoids. This results in the formation of ground state oxygen \( ^3\text{O}_2 \) and triplet excited carotenoid \( ^3\text{Car}^* \) (Stahl and Sies, 1993). The energy is dissipated through rotational and vibrational interactions between \( ^3\text{Car}^* \) and the solvent to recover the ground state of the carotenoid.

\[
^1\text{O}_2 + \text{Car} \rightarrow ^3\text{O}_2 + ^3\text{Car}^* 
\]

\[
^3\text{Car}^* \rightarrow \text{Car} + \text{thermal energy}
\]

One molecule of \( \beta \)-carotene is estimated to quench up to 1000 molecules of singlet oxygen (Foote and Denny, 1968).

In the other mechanism, where there is no hydrogen abstraction, carotenoids are postulated to scavenge peroxyl radicals through addition of the radical to the conjugated system. As such, the resulting radical, which is carbon-centered, is stabilized by resonance (Burton and Ingold, 1984). Once oxygen concentrations are low, a second peroxyl radical is added to the carbon-centred radical to produce a non-radical product (Erickson, 1998). However, at high oxygen pressures, carotenoids work as pro-oxidants (Jorgensen and Skibsted, 1993).

In photosensitised oxidation, the inhibition of oxidation by carotenoids is rather complex as they are very prone to autoxidation themselves. They are rapidly destroyed during the

1.6. Antioxidation defense systems

1.6.1. Endogenous antioxidants

Living organisms possess a network of defense systems consisting of intracellular and extracellular antioxidants that include molecules and enzyme systems with diverse roles within each area of defense. The levels and locations of these antioxidants are highly regulated for cell survival. The enzymatic system that controls the levels of oxygen reactive species is comprised of three major enzyme systems: superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). They work within the cells to suppress radical formation, removing most superoxides and peroxides before they react with metal ions to form more reactive free radicals.
1.6.1.1. Superoxide dismutases (SOD)

Superoxide dismutases are enzymes that are found universally in oxygen-metabolising organisms. SODs are generally classified according to the metal species which acts as the redox-active centre to catalyse the dismutation reaction. Four different SOD are classified namely, copper- and zinc-containing SOD (with copper as the catalytic active metal), manganese-containing SOD, iron-containing SOD, and nickel containing SOD (Miller, 2004). Plant prokaryotes contain two types of SOD, MnSOD and FeSOD whilst mammals contain three types of SOD CuZnSOD in the cytoplasm, MnSOD in the mitochondria and extracellular SOD (Kolakowska, 2003).

The substrate of superoxide dismutases (SODs) is the superoxide radical anion \( \cdot {O^-} \) which is generated by the transfer of one electron to molecular oxygen which is responsible both for direct damage of biological macromolecules (such as proteins and DNA for example) and for generating other reactive oxygen species. SOD catalyses the dismutation of two molecules of \( \cdot {O^-} \) to yield one molecule of molecular oxygen and one molecule of peroxide via the following reaction:

\[
2 \cdot {O^-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

SODs keep the concentration of superoxide radicals in low limits and therefore play an important role in the defense against oxidative stress.
1.6.1.2. Catalases

Catalase is a heme containing redox enzyme found in virtually all organisms, in cells called peroxisomes. Through a scavenging reaction, catalase catalyses the conversion of hydrogen peroxide, a powerful and potentially harmful oxidizing agent, to water and molecular oxygen. It also utilizes hydrogen peroxide to oxidise toxins that include phenols, formic acid, formaldehyde and alcohols.

\[
2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2
\]

1.6.1.3. Glutathione peroxidase (GPx)

Glutathione peroxidase is a selenium-containing tetrameric glycoprotein, that is, a molecule with four selenocysteine amino acid residues bestowing the catalytic activity. Glutathione scavenges hydrogen peroxide and organic hydroperoxides including peroxides of fatty acids to form water, alcohols and oxidized glutathione (GSSG), thus stopping the oxidation reaction.

\[
2 \text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2 \text{H}_2\text{O}.
\]

\[
2 \text{GSH} + \text{ROOH} \rightarrow \text{GSSG} + \text{H}_2\text{O} + \text{ROH}
\]
GSSG is harmful to cells as it can interact with proteins or oxidized thiol groups to form disulphides, thus forming disulphide bonds in proteins. A subsequent reaction follows that reduces the GSSG back to GSH by enzyme glutathione reductase (Wilcox et al, 2004).

\[
\text{GSSG + NAD (P) H + H}^+ \rightarrow 2 \text{GSH} + \text{NADP}^+
\]

1.7. Dietary antioxidants

1.7.1. Synthetic antioxidants

The most common synthetic antioxidants utilized in food systems possess phenolic group(s) and include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), butyl hydroquinone (BHQ) and propyl gallate (PG) (Figure 1.7). The antioxidant mechanisms they exhibit involve the formation of resonance-stabilized phenolic radicals. The formed radical neither catalyzes the oxidation of other molecules nor reacts with oxygen to form antioxidant peroxides that autooxidize. Synthetic phenolic radicals react with each other in a way similar to that of tocopherols. The phenolic radicals also react with other peroxyl radicals in termination reactions resulting in phenolic–peroxyl species. In addition, oxidized synthetic phenolics undergo numerous degradation reactions resulting in degradation products with active hydroxyl groups. These products may retain antioxidant activity because of the presence of the phenolic group. Therefore, the net antioxidant activity of synthetic phenolics in food systems represents the activity
of the original phenolic in addition to some of its degradation products. This property makes synthetic phenolics effective antioxidants that are widely used in food. However, their application in the food industry has recently declined due to consumer safety concerns and demand for all-natural products (Yanishlieva-Maslarova, 2001).

1.7.2. Natural antioxidants

Diet plays a vital role in the production of the exogenous antioxidants defense system by providing essential nutrient antioxidants supplied by plants and food additives. Vegetables, fruits, herbs, spices, teas, oilseeds, nuts, cereals, legumes are rich sources of natural antioxidants (Yanishlieva-Maslarova, 2001). These dietary antioxidants include vitamins (vitamin E, C, and \( \beta \)-carotene), plant phenols (flavanoids, other phenolic compounds) and essential minerals (selenium, zinc, manganese and copper) that form important antioxidant enzymes (Figure 1.5). Diet also provides a significant function in the oxidative process by affecting the substrates that are subject to oxidation. In the oxidation of lipids, polyunsaturated fatty acids (PUFA) having two or more double bonds are more susceptible to free radical attack. Therefore, antioxidants available at the site of radical attack break the chain of oxidation by being preferentially oxidized by the attacking radical, thereby preventing oxidation of the adjacent fatty acid (Wilcox et al., 2004).

Phenolic and polyphenolic compounds are the most active dietary antioxidants contained in plants and utilized in both body and food systems. The most important compounds,
along with tocopherols and tocotrienols (tocols), are flavonoids. Flavonoids that include flavones, flavonols, isoflavones, and chalcones, with over 8000 compounds among them, remain the most abundant group of natural antioxidants. Antioxidants inhibit lipid oxidation reactions by various mechanisms, including radical (ROO*, RO*, HO*, $^{1}$O$_2$) scavenging, protein (enzymes and their metal binding sites) complexing, and synergistic effects by reducing oxidized antioxidants and metal chelation (Frankel, 1999).

Ascorbic acid

Tocopherol

Trolox

Epigallocatechin gallate (EGCG)
1.7.3. Antioxidant peptides from food sources

Various plant- and animal-derived proteins, individual peptides and amino acids, and protein hydrolysates possess significant antioxidant activity against the peroxidation of lipids and fatty acids. Proteins, peptides and amino acids have been utilized both in vitro and in vivo systems to inhibit lipid oxidation. These include, for example, hydrolyzed milk proteins (Pihlanto, 2006), fish proteins (Je et al., 2005; Wu et al., 2003), egg proteins (Davalos et al., 2004; Sakanaka et al., 2004) and pork protein (Carlsen et al., 2003; Saiga et al., 2003). Plant sources with proteins, peptides and amino acids possessing antioxidant activity include oilseed proteins (Rhee et al., 1979), wheat gliadin (Iwami et al., 1987), maize zein (Kong and Xiong 2006), soy bean protein (Chen et al., 1995).
Peptides and amino acids from fish proteins are emerging as potential sources of antioxidants. The diversity of fish species and their valuable contribution to protein and lipid content of diets has precipitated large scale production and processing. This has led to increased fish consumption and, as a result of processing, an increase in by products and waste. Both the fish muscle and waste products from filleting and processing are potential sources of bioactive peptides that may be used as antioxidants. Peptides derived from enzymatic digestion of various fish proteins are reported to possess antioxidant activity based on the nature, size and composition of the different peptide fractions and the protease specificity (Table 1.3).
<table>
<thead>
<tr>
<th>Fish type</th>
<th>Source of Peptide</th>
<th>Hydrolytic Enzyme</th>
<th>Peptide sequence</th>
<th>Scavenging Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capelin</td>
<td>Protein</td>
<td>NSIR*</td>
<td>Hydrolysates</td>
<td>NSIR*</td>
<td>Amarowiz and Shahidi 1996</td>
</tr>
<tr>
<td>Mackerel</td>
<td>Protein</td>
<td>Protease N</td>
<td>Hydrolysates</td>
<td>N</td>
<td>Chuang et al, 2000</td>
</tr>
<tr>
<td>Alaska pollack</td>
<td>Skin gelatin</td>
<td>Alcalase, pronase E, collagenase</td>
<td>repeating motif – GPP-</td>
<td>NSIR*</td>
<td>Kim et al, 2001a</td>
</tr>
<tr>
<td>Tuna</td>
<td>Cooking juice</td>
<td>Protease XXIII</td>
<td>4 - 8 amino acid residues hydrolysate</td>
<td>Carbon centered</td>
<td>Jao and Ko 2002</td>
</tr>
<tr>
<td>Herring</td>
<td>Protein</td>
<td>NSIR*</td>
<td></td>
<td>Scavenging and Reducing ability</td>
<td>Sathivel et al, 2003</td>
</tr>
<tr>
<td>Mackerel</td>
<td>Protease N</td>
<td>NSIR*</td>
<td></td>
<td></td>
<td>Wu et al., 2003</td>
</tr>
<tr>
<td>Yellowfin sole</td>
<td>Protein</td>
<td>Pepsin and MICE*</td>
<td>RPFDLLEPPY</td>
<td></td>
<td>Jun et al, 2004</td>
</tr>
<tr>
<td>Alaska pollack</td>
<td>Protein</td>
<td>MICE*</td>
<td>LPHSGY</td>
<td>Hydroxyl radical</td>
<td>Je et al, 2005</td>
</tr>
<tr>
<td>Hooky</td>
<td>Skin gelatin</td>
<td>Commercial enzymes</td>
<td>HGPLGPL</td>
<td>Superoxide, carbon-centered</td>
<td>Je et al, 2005; Mendis et al, 2005</td>
</tr>
<tr>
<td>Giant squid</td>
<td>Muscle</td>
<td>Pepsin, trypsin, chymotrypsin</td>
<td>NADFGLNLEGLA NGLEGLK</td>
<td>Radicals carbon-centered, hydroxyl and superoxide</td>
<td>Rajapakse et al, 2005</td>
</tr>
<tr>
<td>Chum salmon</td>
<td>Cartilage and skin</td>
<td>NSIR*</td>
<td>NSIR*</td>
<td>Superoxide, hydroxyl, carbon centered</td>
<td>Nagai et al, 2006</td>
</tr>
<tr>
<td>Jumbo flying squid</td>
<td>Skin gelatin</td>
<td>Properase, pepsin, Mw &lt; 2 kDa</td>
<td>Hydrolysates</td>
<td></td>
<td>Lin and Li 2006</td>
</tr>
<tr>
<td>Shell fish</td>
<td>Muscle protein</td>
<td>Gastrointestinal enzymes</td>
<td>LVGDEQAVPAVCVP</td>
<td>Hydroxyl, super-oxide and carbon-centered</td>
<td>Jung et al, 2007</td>
</tr>
<tr>
<td>Yellow stripe trevally</td>
<td>Meat</td>
<td>Alcalase 2.4L</td>
<td>Hydrolysates</td>
<td></td>
<td>Klompong et al, 2007</td>
</tr>
<tr>
<td>Fish</td>
<td>Part</td>
<td>Enzyme</td>
<td>Peptide Sequence</td>
<td>Chelating</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------</td>
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<td>----------------------</td>
<td>----------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Walleye</td>
<td>Pollack</td>
<td>GI proteases, pronase, thermolysin</td>
<td>Pepsin, VKAGFAWTANQLS</td>
<td>Superoxide and hydroxyl radicals</td>
<td>Nagai et al, 2007</td>
</tr>
<tr>
<td>Tuna</td>
<td>Back bone protein</td>
<td>Alcalase</td>
<td>Hydrolysates</td>
<td>Radical scavenging, superoxide radicals</td>
<td>Je et al, 2007</td>
</tr>
<tr>
<td>Round scad</td>
<td>Muscle</td>
<td>Alcalase</td>
<td>Hydrolysates</td>
<td>Radical scavenging, reducing power, and Fe²⁺ chelating</td>
<td>Thiansilakul, et al, 2007</td>
</tr>
<tr>
<td>Grass carp</td>
<td>Muscle</td>
<td>Alcalase 2.4L</td>
<td>PSKYEPFV</td>
<td>Hydroxyl radical scavenging</td>
<td>Ren et al, 2008</td>
</tr>
<tr>
<td>Smooth hound</td>
<td>Muscle</td>
<td>GI proteases</td>
<td>Mw &lt; 3.5kDa</td>
<td>Radical scavenging</td>
<td>Bougatief et al, 2009</td>
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<tr>
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<td>Skin gelatin</td>
<td>Alcalase</td>
<td>Mw &lt; 6.5kDa</td>
<td>Metal chelating, Fe³⁺ reducing, radical scavenging</td>
<td>Gimenze et al, 2009</td>
</tr>
</tbody>
</table>

* MICE, Mackerel intestine crude enzyme; NSIR*, Not specified in reference

### 1.7.4. Methods for the determination of lipid oxidation

#### 1.7.4.1. Peroxide value (PV).

Determination of peroxide value (PV) is the oldest method of lipid oxidation evaluation and is based on the quantification of primary oxidation products. It is carried out using the iodometric technique. The result depends on the amount of oxygen, availability of light, and the number of double bonds in the substrate. To improve the sensitivity of the method, several iodine spectrophotometric and potentiometric techniques have been developed (Oishi et al., 1992; Lovaas, 1992). The ferric thiocyanate (FTC) technique of

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peroxide determination involves $\text{Fe}^{2+} \rightarrow \text{Fe}^{3+}$ oxidation. The red Fe3+ complexes formed is determined spectrophotometrically at 470 nm. The FTC is ten times more sensitive than the iodometric technique (Wolff, 1994).

1.7.4.2. Carbonyl Compounds

Carbonyl compounds in lipid oxidation comprise the secondary products of lipid oxidation. The determination of carbonyl compounds mostly involves the formation of colour products that result from the reaction of the carbonyl group with the amine group of a cyclic leuco-compound. The most popular old method involves determination of carbonyl compounds by reaction with 2,4-dinitrophenylhydrazine (carbonyl value). The most popular method used currently involves 2-thiobarbituric acid (TBA). In this method, 2 molecules of 2-thiobarbituric acid are condensed with Malondialdehyde (Figure 1.8). The developed chromogen, the two tautomeric structures of the red TBA-Malondialdehyde adduct, is determined at 532 nm, and also often at 450 nm, to analyse for alkenals and alkanals, respectively (Ohkawa et al, 1979).
1.8. Peptide sequencing

1.8.1. Edman degradation

Edman degradation is one of the methods of sequencing amino acids in a peptide or protein. In this method, the amino-terminal residue is labeled and cleaved from the peptide without disrupting the peptide bond between other amino acid residues. Phenylisothiocynate is commonly used to label the N-terminal amino residue. The phenylisothiocynate reacts with an uncharged terminal amino group, forming a cyclical phenylthiocarbamoyl derivative. This derivative of the terminal amino acid under acidic conditions is cleaved as a thiazolinone derivative that is selectively extracted into an organic solvent and treated with an acid to form the more stable phenylthiohydantoin (PTH) - amino acid derivative that can be identified by chromatography or electrophoresis. This is repeated again to identify the next amino acid. The Edman degradation can accurately sequence up to 30 amino acids. The advantage of the Edman degradation is that it can be used at levels of 10 – 100 picomoles of peptides (Belitz et al, 2004).
1.8.2. Liquid Chromatography-Mass spectroscopy (LC-MS).

Liquid Chromatography-Mass spectroscopy (LC-MS) is a powerful and useful analytical technique providing information about the structure and composition of a compound. LC-MS has been applied to many fields. LC combined with ESI-MS has become a standard approach for the separation and identification of many macromolecules like peptides and proteins (Careri and Mangia, 2003). Electrospray ionization has facilitated the direct analysis of peptides as they elute from a liquid chromatograph (LC) in the LC/MS experiment (D'agostino et al, 1997).

LC-MS is based on the separation of compounds by LC chromatography followed by MS. The ions produced by MS are subsequently filtered according to their mass-to-charge (m/z) ratio and then detected (Van der Greef and Niessen, 1992). To achieve this target, two ionization modes have been used in current commercial instruments which are electrospray ionization and atmospheric pressure chemical ionization. In an electrospray interface, the eluent from LC is passed through a capillary held at a high voltage, generating an aerosol of charged droplets. Nitrogen gas flow is usually used to desolvate the droplets leading to increase in the charge density at the droplet surface. The ions overcome the liquid's surface tension and attain the gas phase in a process commonly referred to as ion evaporation. Therefore, the ions which are highly charged can be directly sampled into the mass spectrometer for mass and peptide sequencing through appropriate programs.
1.9. Cytotoxicity effects of ROS in epithelial and endothelial cells

Apoptosis and necrosis are two types of cell death. Apoptosis is programmed cell death process and is common in cell development in living cells (Banerjee et al, 2005). In apoptotic cell death, the cell shrinks, forms blebs and detaches from its neighboring cells; the mitochondria undergo modifications including release of cytochrome c into the cytosol, reduction in the membrane potential and deterioration of membrane permeability with opening of specialized pores and diffusion of diverse pro-apoptotic proteins; in the nucleus, the chromatin condenses at the nuclear membrane. Finally the cell splits into apoptotic bodies and caspases are activated (Green and Kroemer, 1998). Stimulated apoptosis together with defective DNA repair mechanisms and cell cycle alteration contribute to several disorders such as cancer, AIDS, autoimmune diseases, degenerative disorders, ageing, liver and heart diseases (Ducasse et al, 2005). Apoptosis is controlled by a network of positive and negative signal pathways via the intrinsic or extrinsic pathways (Figure1.9). In the intrinsic pathway, cysteine proteases activating proteins are released into the cytosol from the mitochondria and this triggers apoptosis. This family of intracellular cysteine proteases is also known as caspases; with at least 14 members identified. Among them, caspase-3 is responsible for many of the nuclear changes associated with apoptosis, including DNA fragmentation (Leung et al, 2005). The extrinsic pathway is regulated by proteins such as Fas and the tumour-necrosis factor receptor (TNF-R) inducing apoptosis at the cell surface. Necrosis is considered as a disordered cellular death or non-programmed death. It is characterized by cell swelling, lysis and inflammatory response (Girotti, 2001).
Figure 1.9. Mechanisms of oxidant induced apoptosis through caspases.

1.10. Protein hydrolysis and digestion

Food proteins are currently one of the sources of a range of bioactive peptides, encrypted within the primary structure of their individual proteins. Proteolysis releases the bioactive peptides from their precursors during gastrointestinal transit or during food processing. Three strategies are generally utilized to identify and characterize
biologically active peptides: isolation from \textit{in vitro} enzymatic digests of precursor proteins, isolation from \textit{in vivo} gastrointestinal digests of precursor proteins; and chemical synthesis based on combinatorial library designs of peptides that have a structure identical to that of those known to be bioactive (Mine and Shahidi, 2004).

The digestion of proteins begins in the acidic environment of the stomach by pepsin. The resultant polypeptides are additionally cleaved by the pancreatic proteases namely trypsin, \(\alpha\)-chymotrypsin, elastase and carboxypeptidase A and B at alkaline pH in the small intestine. The action of these enzymatic actions results in a concoction of free amino acids and predominantly oligopeptides. Free amino acids absorption into the enterocytes takes place across the brush border membrane via distinctive amino acid transport systems. On the other hand, the oligopeptides go through additional hydrolysis by the action of an array of peptidases present at the brush border.

The aminopeptidase activity present at the intestinal brush border membrane provides functional complementation to the carboxypeptidases present in the pancreatic juice. Peptides of different amino acid sequences and chain length \textit{in vivo} studies have been documented, exhibiting resistance to digestive enzymes. Tripeptides with the C-terminal proline-proline resist degradation by proline-specific peptidases. Proline- and hydroxyproline-containing peptides are also, in general, resistant to hydrolysis by digestive enzymes (Vanhoof \textit{et al.} 1995; FitzGerald and Meisel, 2000). Several researchers have shown that dietary peptides exert biological activity \textit{in vivo} after release during gastrointestinal digestion and absorption into the blood. Bioactive
peptides have been isolated from the stomach, small intestine and blood of adult human subjects fed with milk or yoghurt. Peptides derived from αs1-, β - or κ-caseins were detected in the stomach, and smaller peptides derived from casein and lactoferrin from the small intestine. Two long peptides, casein glycomacropeptide, k-casein f(106-117) and the N-terminal peptide f(1-23) of αs1-casein, were believed to be absorbed and hence detected in plasma (Chabance et al. 1998).

1.11. Peptide intestinal absorption

Small peptides (primarily di- and tripeptides) and amino acids are both absorbed from the small intestine against a concentration gradient. The energy-dependent transport of amino acids is linked with the co-transport of Na⁺; for peptides, energy-dependent transport is linked with the co-transport of protons. Peptides are absorbed more rapidly than amino acids (Webb, 1990; Webb et al., 1992). Yang et al (1999) postulated that peptides consisting of two or three amino acids are absorbed intact across the brush border membrane by a specific peptide transport system. The peptide transporter PepT1, with a broad substrate specificity, uses a transmembrane electrochemical proton gradient as the driving force. When they enter the enterocyte, the peptides are generally hydrolysed to free amino acids in the cytoplasm by various intracellular peptidases. Besides the peptide transporter path, peptides are considered to be absorbed whole across the intestinal mucosa through other mechanisms – paracellular, passive diffusion, endocytosis and carrier mediated transport (Gardner, 1998). Literature evidence exists
supporting both paracellular and transcellular routes for passage of intact peptides, however, there are still questions as to the relative significance of these. Paracellularly, large water-soluble peptides pass via the tight junctions between cells, whilst the more lipid-soluble peptides appear to be able to diffuse via the transcellular pathway. Peptides are also thought to enter the enterocytes by means of endocytosis, which entails that there is membrane binding and vesiculisation of the material (Ziv and Bendayan, 2000).

The intestinal basolateral membrane also has a peptide transporter, which facilitates the exit of hydrolysis-resistant small peptides from the enterocyte into the portal circulation (Gardner, 1984). Peptides brought about through the diet can be absorbed whole through the intestine and bring about particular biological effects at the tissue level. Nevertheless, chain length appears to be a factor in the absorption mechanism as the potency of the peptides to be absorbed decreases as the chain-length increases (Roberts et al. 1999).

The caco-2 cell monolayer model is widely used in intestinal transport studies of drugs and food compounds (Augustijns et al. 1998; Boisset et al. 2000, Rubio & Seiquer, 2002). This confluent cell monolayer caco-2 cell line, which is derived from a human colon carcinoma, has characteristics that closely resemble intestinal epithelial cells and displays several properties typical of differentiated intestinal epithelial cells (Wilson et al. 1990). Caco-2 cell monolayers are thought to be tighter than mammalian intestinal tissues (Boisset et al. 2000). The brush border membrane-associated enzyme activities are generally thought to be lower (Bolte et al. 1998).
1.12. Summary and aims

In recent times, consumers have started to look for foods that are capable of improving their health and reducing their risk of developing chronic diseases as they become more aware of the direct link between diet and health. Drug-based approaches are currently available to cushion the development of chronic diseases but their use is increasingly being questioned because of their side effects. This has led to innovative research to derive natural compounds from natural sources such as foods.

One research thrust is the isolation of bioactive peptides (BAPs) and proteins from several sources to impart positively on diseases such as cardiovascular diseases and cancers. These BAPs have been reported to possess antihypertensive and antioxidant activities. Marine organisms represent a valuable source for BAPs and nutraceuticals. The biodiversity of the marine environment in its fish species amount to a practically unlimited resource of new active substances in the field of the development of functional foods. In particular Atlantic mackerel, a fatty fish, is readily available and highly consumed especially in countries of the Atlantic Ocean in the north. Its muscle protein remains a potential source of BAPs after hydrolysis with enzymes or processing.

Therefore, the aim of this study is to investigate the antihypertensive and antioxidant activity of peptides derived from Atlantic mackerel with gastrointestinal enzymes, and in particular:
To isolate and characterize peptides from Atlantic mackerel (Scomber scombrus) muscle protein with ACE inhibitory activity and study the mechanism of ACE inhibition.

To isolate and characterize peptides from Atlantic mackerel (Scomber scombrus) muscle protein with antioxidant activity and its mechanisms of lipid oxidation inhibition.

To study the antioxidant behavior of peptides derived from Atlantic mackerel in caco-2 cells exposed to a proxidant.

To examine the ROS scavenging activity of peptides derived from Atlantic mackerel in endothelial Ea.hy 926 cells and

To investigate the absorption of peptide in a caco-2 cell monolayer model.
CHAPTER TWO
2. ISOLATION OF ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORY PEPTIDE FROM ATLANTIC MACKEREL (SCOMBER SCOMBRUS) FISH PROTEIN

2.1. INTRODUCTION

Angiotensin I-converting enzyme (ACE, dipeptidyl carboxypeptidase, EC 3.4.15.1) plays an important role in the regulation of blood pressure. ACE hydrolyzes Ang-I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) by removing the C-terminal dipeptide His-Leu to give an octapeptide angiotensin II (Ang-II) Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, a potent vasoconstrictor (Manger and Page, 1982). ACE is a key enzyme in the regulation of BP and has been targeted in the control of hypertension. Ang II production leads to several physiological processes that include systemic vasoconstriction, cardiac and vascular hypertrophy, increased blood volume and renal sodium and fluid retention. This cascade of events leads to the elevation of blood pressure and forms the basis for hypertension, heart failure, myocardial infarction and diabetic nephropathy (Mine and Shahidi, 2006). Thus, inhibition of ACE results in a decrease in blood pressure. Hypertension is a worldwide problem of epidemic proportions mostly in adults. It is the most common serious chronic health problem because it carries with it a high risk of cardiovascular complication. About 70% of over 65 year olds show ≥ Systolic Blood Pressure (SBP) 140 and/or Diastolic Blood Pressure (DBP) 90 mmHg or are on antihypertensive medication in the UK (Spronston and Primatesa, 2003).
Several potent synthetic ACE inhibitors such as captopril, benazepril, enalapril, fosinopril, lisinopril, moexipril, perindopril, quinapril, ramipril, and trandolapril are widely used in the clinical treatment of hypertension in humans (Sica, 2003). However, these synthetic ACE inhibitors can have side effects such as skin rash, headache, dry cough and loss of taste perception (Fotherby and Panayiotou, 1999). As a result, there is an increasing interest in nutraceuticals and diet-related preventive measures for hypertension. Peptides from food proteins with ACE inhibitory activity are potential candidates for such products. As they are not known to have any side effects, peptides from food proteins may represent an alternative or an additional treatment to antihypertensive drugs and may be applied in the prevention of high blood pressure (Fitzgerald and Meisel, 2000).

The three-dimensional structure reveals that ACE is composed mainly of α-helices, and incorporates a zinc ion and two chloride ions. The active site consists of a deep, narrow channel that divides the molecule into two subdomains. On top of the molecule is an amino-terminal 'lid', which seems to allow only small peptide substrates access to the active site cleft (Natesh et al, 2003). Although the N-domain and C-domain are highly similar in protein sequence and share many enzymatic properties, they can be differentiated by substrate and inhibitor preferences and by the extent to which they are activated by Cl⁻ (Bingham et al, 2006).
ACE inhibitory peptides have been isolated from enzymatic hydrolysates or fermentation of different food proteins (Mine and Shahidi, 2004) that include fish proteins (Kasase and Howell, 2009). However, a combination of pepsin and pancreatin is rarely used to investigate if ACE inhibitory peptides can be produced in an *in vitro* digestion model system with enzymes similar to those in the gastrointestinal digestive system of humans.

Atlantic mackerel (*Scomber scombrus*) is a pelagic species that varies between 180 to 210 cm in length and 325 to 360 g in body weight. Atlantic mackerel has a large proportion of dark muscle. The main chemical composition of muscle includes water (66 – 84 %), proteins (15 – 25 %), lipids (0.1 – 22 %), carbohydrate (0.3 %), minerals (0.8 – 2 %) and vitamins (Kolacowska, 2002).

In this study, the objective was to hydrolyse Atlantic mackerel (*Scomber scombrus*) muscle protein using digestive enzymes pepsin and pancreatin; to isolate and characterize peptides with respect to ACE inhibitory activity and to investigate the mechanism of ACE inhibition.

### 2.2. MATERIALS AND METHODS

#### 2.2.1. Materials

Atlantic mackerel (*Scomber scombrus*) fillets were supplied by M & J Seafood, Farnham, UK and delivered in ice to the laboratory. Pancreatine, pepsin, Sephadex G-
25, Sephadex C-25, captopril, phenyl isothiocyanate (PITC), triethylamine (TEA), hippuric acid (HA), histidyl hippuryl leucine (HHL), sodium borate, sodium acetate, sodium chloride, acetonitrile and Trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (Dorset, UK). Vivaspin membrane ultrafiltration cartridges were obtained from Sartorius (Epsom Surrey, UK). All other reagents were of analytical grade and were obtained commercially.

2.2.2. Methods

2.2.2.1. Preparation of Atlantic mackerel Fish Protein Hydrolysates (FPH)

Fish fillets were skinned and the muscle part only was washed in distilled water, and a portion was mixed with distilled water in the ratio (1:2.5 w/w) and homogenised in a blender (Omni mixer homogeniser, USA) for about 2 min. The mixture was adjusted to pH 2.0 with 1.0 M HCl. Pepsin (1:25 w/w enzyme to substrate) was added and the mixture was incubated for 1 h at 37 °C with shaking. After 1 h, the pH of the mixture was adjusted to 5.3 with 0.9 M NaHCO₃ and then to pH 7.5 with 1.0 M NaOH. Pancreatine (1:35 w/w enzyme to substrate) was added and incubated for 2 h at 37 °C with shaking. After 2 h, the mixture in the beaker was immersed in boiling water for 10 min and centrifuged at 12000 x g for 15 min. The resultant hydrolysate was freeze dried.
2.2.2. Membrane ultrafiltration

The freeze-dried protein hydrolysate from the enzymatic hydrolysis was dissolved in a minimum amount of milli-Q water and ultrafiltered using a 2, 5 and 10 kDa molecular weight cut off (MWCO) Vivaspin membrane ultrafilter (Sartorious, UK). Fractionation was effected by centrifuging at 3,500 x g on a centrifuge (Beckman, UK) for 30 minutes. The collected fractions were quantified and freeze-dried.

2.2.2.3. Gel filtration chromatography

The 2 kDa fraction was dissolved in 5 ml distilled water and loaded onto a gel filtration column (2.5 x 90 cm) filled with Sephadex G-25 previously equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The column was eluted with 50 mM sodium phosphate buffer (pH 7.0) at 1 ml/min for 10 h. Fractions were collected in 5 ml portions and the absorbance of the collected fractions was monitored at 250 nm. The fractions showing ACE activity were collected, pooled and freeze-dried.

2.2.2.3. Ion exchange chromatography

The freeze-dried fraction with the highest ACE activity from gel filtration was dissolved in 5 ml of 20 mM sodium phosphate buffer (pH 4.0) and loaded onto an ion-exchange column (3.0 x 40 cm) packed with SP-Sephadex C-25 previously equilibrated with 20
mM sodium phosphate buffer (pH 4.0). The components were eluted with 20 mM sodium phosphate buffer (pH 4.0) and a linear gradient of 0 to 2.0 M NaCl at 1 ml/min. Fractions were collected in 5 ml portions and the absorbance of the collected fractions monitored at 250 nm. The fractions showing ACE activity were collected, pooled and freeze dried.

2.2.2.4. High performance chromatography

The fraction exhibiting the highest ACE inhibitory activity was further purified using a reversed-phase high-performance liquid chromatography (RP-HPLC) on a Partisil 10 ODS-1 (9.5 mm x 500 mm) column (Whatman plc, UK) with a linear gradient of acetonitrile (0-70 % in 50 min) containing 0.1 % trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. The elution peaks were detected at 215 and 250 nm and fractions were collected in 2 ml portions. The fraction from this HPLC separation with highest ACE inhibitory activity was re-chromatographed on a Phenomenex C18 (10 mm x 250 mm) column and fractions were collected. RP-HPLC analysis was performed on the HPLC system comprising a pump (Thermoseparation Products), autosampler (Spectra Physics AS1000), detector (Thermoseparation Products UV6000LP) and integration software (Chromquest, 2004). The fractions showing ACE activity were collected, pooled and freeze dried.
2.2.2.5. ACE inhibitory activity

The ACE inhibitory activity assay was performed according to the method of Wu et al (2002) with some modifications. In this method, the total reaction volume was 70 μL, made up of 50 μL of 2.17 mM hippuryl histidyl leucine (HHL), 10 μL of 2 mU of ACE and 10 μL of different peptide fractions obtained after 2, 5, 10 kDa, gel filtration, ion exchange and HPLC (all prepared with 100 mM borate buffer, containing 300 mM NaCl, pH 8.3). The HHL and peptide fractions were combined and maintained at 37 °C for 10 min in 2 ml polyethylene microcentrifuge tubes. ACE was also maintained at 37 °C for 10 min before the two solutions were combined and incubated at 37 °C with agitation. The reaction was terminated after 30 min by adding 85 μL of 1.0 M HCl and the mixture kept for HPLC analysis. A positive control of HHL and enzyme; and blank of HHL and buffer were also prepared in the same manner.

Samples (10 μL) were analyzed on a C18 column (3.0×150 mm, 5 μm, Phenomenex); and HA and HHL were detected at 228 nm. The column was eluted (1 ml/min) with a two solvent system: (A) 0.05 % TFA in water and (B) 0.05 % TFA in acetonitrile, with a 5–60 % acetonitrile gradient for the first 10 min, maintained for 2 min at 60 % acetonitrile, then returned to 5 % acetonitrile for 1 min. This was followed by isocratic elution for 4 min at the constant flow-rate of 1 ml/min. RP-HPLC analysis was performed on the HPLC system comprising a pump (Thermoseparation Products), autosampler (Spectra Physics AS1000, detector (Thermoseparation Products UV6000LP) and intergration software (Chromquest, 2004).
The ACE inhibitory activity was calculated as

(i) ACE activity (%) 

$$= \frac{\text{PeakArea}_{\text{Sample}} - \text{PeakArea}_{\text{blank}}}{\text{PeakArea}_{\text{control}} - \text{PeakArea}_{\text{blank}}} \times 100$$

(ii) ACE inhibitory activity (%) = 100 – ACE activity (%).

The IC$_{50}$ of fractions from each purification stage exhibiting highest ACE inhibitory activity was determined. The IC$_{50}$ value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity under the assayed conditions and determined by regression analysis of ACE inhibition (%) versus inhibitor concentration (mg/ml).

### 2.2.2.6. ACE inhibition mechanism

Two different concentrations of ACE inhibitory peptide were added to each reaction mixture (0.1 and 1.4 mg/mL). The enzyme activity was measured with different concentrations of the substrate (0 – 0.05 μM HHL). The resultant mixtures were analysed for hippuric acid peaks by HPLC as described above in section 2.2.2.5. The kinetics of ACE in the presence of the inhibitor was determined by the Lineweaver-Burk plots. Standard comprising captopril and negative controls were also included in the analysis. A standard curve of hippuric acid was determined with a concentration range of 0.02 to 0.1μM.
2.2.3. Amino acid composition determination

2.2.3.1. Preparation of samples and standards in determination of amino acids
Ten milligrams of each sample were placed in screw-cap tubes (16 mm x 125 mm) and hydrochloric acid (6 M, 10 ml) was added. The tubes were then closed under nitrogen, and placed in an oven at 110 °C for 24 h. After hydrolysis, tube contents were vacuum-filtered to remove solids.

2.2.3.2. Derivatization with PITC
The derivatization procedure was a modification of the method used by (Gonzalez-Castro et al, 1997). Amino acid standard solution and hydrolysed sample (20 μl) were placed in a vial and dried under vacuum for 20 min at room temperature. After this, 20 μl drying agent (200 μl methanol + 200 μl 1 M sodium acetate + 100 μl triethylamine) were added to the residue and the resulting solution was vacuum-dried for a further 10 min. Next, 20 μl of the derivatizing reagent (140 μl methanol + 20 μl water + 20 μl + 20 μl PITC) were added, and the vials were vortex-mixed for 30 s and left to stand at room temperature for 20 min. Finally, the resulting solution was vacuum-dried for 20 min and reconstituted in eluent A (0.22 M sodium acetate buffer containing 0.05 % (v/v) TEA (pH adjusted to 6.2 with glacial acetic acid).
2.2.3.3. HPLC equipment and conditions

RP-HPLC analysis was performed on the HPLC system comprising a pump (Thermoseparation Products), autosampler (Spectra Physics AS1000, detector (Thermoseparation Products UV6000LP) and integration software (Chromquest, 2004).

Compounds were separated on a 3.9 x 150 mm, 5 μm particle size, C18 reversed-phase column (Nova-Pak, Waters separations, Elstree, UK). The mobile phase was a gradient prepared from two solutions, A and B. Solution A was 0.22 M sodium acetate buffer containing 0.05 % (v/v) TEA (pH adjusted to 6.2 with glacial acetic acid). Solution B was 60:40 acetonitrile:water. The gradient profile was as shown in Table 2.1. The flow rate was 1 ml min/ml and the detection wavelength 254 nm.

**Table 2.1. Gradient profile for chromatographic run**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (ml/min)</th>
<th>% Solution A</th>
<th>% Solution B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10.0</td>
<td>1.0</td>
<td>54.0</td>
<td>46.0</td>
</tr>
<tr>
<td>10.5</td>
<td>1.0</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>11.5</td>
<td>1.0</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>12.0</td>
<td>1.5</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>12.5</td>
<td>1.5</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>20.0</td>
<td>1.5</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>20.5</td>
<td>1.0</td>
<td>100.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
2.2.4. Statistical analysis

Statistical analyses were performed with Minitab statistical software (13.1). Comparisons were made by one-way analysis of variance. P< 0.05 was considered statistically significant.

2.3. RESULTS AND DISCUSSION

2.3.1. Purification and amino acid sequencing of ACE inhibitory peptide

Atlantic mackerel fish muscle protein was successfully hydrolysed with pepsin and pancreatin (trypsin and chymotrypsin) to yield a protein hydrolysate (MFPH) that was fractionated using 2, 5 and 10 kDa molecular weight cutoff (MWCO) ultrafiltration membrane cartridges.

The ACE inhibitory activity of the fractionated ultramembrane fractions < 2 kDa (MFPH-V), 3-5 kDa (MFPH-III), 5-10 kDa (MFPH-II) and crude enzymatic hydrolysate (MFPH-I) is shown in figure 2.1. MFPH-V having a molecular mass (MW) below 2 kDa showed the highest ACE inhibitory activity of 95.0 %. Consequently, MFPH-V fraction was chosen for further chromatographic purification and ACE inhibitory activities.
Figure 2.1. ACE inhibitory activity (%) of mackerel fish protein crude enzyme hydrolysate and 2, 5 and 10 kDa fractions obtained by membrane ultrafiltration.

The MFPH-V fraction was loaded onto the column packed with Sephadex G25 and was eluted with 50 mM sodium phosphate buffer (pH 7.0). Five milliliter (5 ml) portions were collected in test tubes and the absorbance of the resultant peptide fractions was monitored at 215 nm.

The 2kDa fraction (MFPH-V fraction), after separation on a Sephadex G-25 gel filtration column (2.5 x 90 cm) previously equilibrated with 50 mM sodium phosphate buffer (pH 7.0), showed 19 peptide fractions (A – T) as shown by the chromatogram in figure 2.2A. These gel filtration peptide fractions were freeze-dried and analysed for their ACE inhibitory activity by the HPLC method according to Wu et al (2002). Fraction J (MFPH-V-J) showed the highest ACE inhibitory activity of 36.09 % (figure 2.2B). This fraction (MFPH-V-J) was subjected to ion exchange chromatography on a
SP-Sephadex C-25 column and elution with a 0 - 2.0 M sodium chloride gradient. Sixteen fractions denoted by letters were identified as shown in the chromatogram in figure 2.3A and their corresponding ACE inhibitory activities were measured. The fraction labelled P showed the highest ACE inhibitory activity of 86.1 % (figure 2.3B); this fraction (MFPH-V-JP) was freeze-dried and stored at -20 °C for liquid chromatography purification. When fractionated on a C18 (9.8 x 50 cm) reversed phase column, MFPH-V-JP gave 5 fractions that showed ACE inhibitory activity (figure 2.4A). The fraction with highest ACE inhibition activity of 34.9 % was tube A (MFPH-V-JPA) (figure 2.4B).
Figure 2.2. (A) Gel filtration chromatography of the 2kDa mackerel muscle protein hydrolysate (MFPH-V) separated on Sephadex G-25 column and detected at 215 nm. (B) ACE inhibitory activity of the 2 kDa mackerel fish peptides from gel filtration. Separation was performed at a flow rate of 1 ml/min and 5 ml fractions were collected. Aliquots, drawn from each fraction, were used to measure ACE inhibitory activity. The fractions showing peptides are designated with letters.
Figure 2.3. (A) Ion Exchange chromatography of MFPH-V-J fraction on the SP-Sephadex C-25 column and detected at 215nm. (B) ACE inhibitory activity of the fractions from ion exchange chromatography. Separation was performed at a flow rate of 1 ml/min and 5 ml fraction collected. Aliquots, drawn from each fraction, were used to measure ACE inhibitory activity. The fractions showing peptides are designated with letters.
Figure 2.4. (A) High performance liquid chromatography of MFPH-V-JP on Partisil 10 ODS-1 (9.5 mm x 500 mm) column and detected at 215 nm. (B) ACE inhibitory activity of the fractions on Partisil 10 ODS-1 (9.5 mm x 500 mm) column. Separation was performed at a flow rate of 1 mL/min and 2 mL fraction collected. Aliquots, drawn from each fraction, were used to measure ACE inhibitory activity. The fractions showing peptides are designated with letters.
Fraction MFPH-V-JPA was rechromatographed on the C18 9.8 x 250 mm reversed phase column as shown in chromatogram in figure 2.5. One identifiable peak was evident in the chromatogram (figure 2.5), showing the presence of peptide(s). This fraction was collected and freeze dried. The amino acid composition of the peptide fraction was determined and contained the amino acids – Histidine, proline, tyrosine, methionine, leucine, tryptophan and lysine (Figure 2.6 and Table 2.2).

Figure 2.5. Chromatogram of MFPH-V-JPA rechromatographed on Partisil 10 ODS-1 (9.5 mm x 250 mm) column and detected at 215 nm.
Figure 2.6. (A) Chromatogram of amino acid standards and (B) amino acids from MFPH-V-JPA2.
Table 2.2. Amino acid composition of standard and peptide fraction (MFPH-V-JPA2)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Standards</th>
<th></th>
<th>Peptide Fraction</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retention time (min)</td>
<td>Amount (µg)</td>
<td>Retention time (min)</td>
<td>Amount (µg/mg)</td>
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<tr>
<td>Asp</td>
<td>3.5</td>
<td>1.33</td>
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<td>-</td>
</tr>
<tr>
<td>Glu</td>
<td>3.8</td>
<td>1.47</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ser</td>
<td>5.7</td>
<td>1.05</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gly</td>
<td>6.0</td>
<td>0.75</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>His</td>
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<td>1.55</td>
<td>6.2</td>
<td>0.16</td>
</tr>
<tr>
<td>Thr</td>
<td>6.7</td>
<td>1.19</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ala</td>
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<td>0.89</td>
<td>-</td>
<td>-</td>
</tr>
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</tr>
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<td>Try</td>
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<td>1.81</td>
<td>8.0</td>
<td>0.59</td>
</tr>
<tr>
<td>Val</td>
<td>9.0</td>
<td>1.17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Met</td>
<td>9.3</td>
<td>1.49</td>
<td>9.2</td>
<td>0.15</td>
</tr>
<tr>
<td>Cys</td>
<td>9.8</td>
<td>1.21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ile</td>
<td>10.5</td>
<td>1.31</td>
<td>-</td>
<td>-</td>
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<td>Leu</td>
<td>10.7</td>
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<td>Phe</td>
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<td>-</td>
<td>-</td>
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<tr>
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<td>11.9</td>
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</tr>
<tr>
<td>Lys</td>
<td>12.2</td>
<td>0.73</td>
<td>12.3</td>
<td>0.12</td>
</tr>
</tbody>
</table>
2.3.2. ACE inhibitory activity

The purification and ACE inhibitory activity of peptides derived from Atlantic mackerel muscle hydrolysed with gastrointestinal enzymes is the first to be reported in this study. The inhibitory potential was quantified through the inhibitory concentration (IC\textsubscript{50}) that is the concentration required to inhibit 50% of the enzyme. The IC\textsubscript{50} of 2 kDa, gel filtration, liquid chromatography1 fractions and sequenced peptide from Atlantic mackerel fish were determined with the validated HPLC method (Table 2.3). The (IC\textsubscript{50}) values of MFPH-V, MFPH-V-J, MFPH-V-JPA and MFPH-V-JPA2 were significantly different (p<0.0001) and were found to be 2.76, 0.43, 0.36 and 0.15 mg/ml respectively.

Table 2.3. Inhibitory concentration (IC\textsubscript{50}) mg/ml of the different fractions (2 kDa, gel filtration fraction, liquid chromatography1 and liquid chromatography 2) of Atlantic mackerel fish protein peptides.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Inhibitory concentration (IC\textsubscript{50}) mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFPH-V (2kDa Fraction)</td>
<td>2.76</td>
</tr>
<tr>
<td>MFPH-V-J (Gel Filtration Fraction)</td>
<td>0.43</td>
</tr>
<tr>
<td>MFPH-V-JPA (HPLC Fraction)</td>
<td>0.36</td>
</tr>
<tr>
<td>MFPH-V-JPA2 (HPLC 2 Fraction)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Research on bioactive peptides derived from Atlantic mackerel with gastrointestinal enzymes has not been reported previously. In the present study, an ACE inhibitory peptide fraction was purified with an IC\textsubscript{50} value of 0.15 mg/ml. This IC\textsubscript{50} activity value was higher or similar to those of peptides derived from protein sources hydrolysed with
one or two of the gastrointestinal enzymes. In peptides (5-6 amino acid sequence) derived from tuna fish with trypsin, chymotrypsin, pronase and pepsin hydrolysis, IC\textsubscript{50} values obtained ranged from 0.019 to 0.106 mg/ml (22.2 to 156.3 µM). Among these, 2 peptides CWLPVY and SKVPP with a characteristic proline on the C-terminal tripeptide had IC\textsubscript{50} values 0.019 and 0.106 mg/ml (22.2 and 74.22 µM) respectively (Astawan \textit{et al}, 1995); these values are comparable to the IC\textsubscript{50} value 0.15 mg/ml of the isolated peptide in this study. Itou and Akahane (2004) obtained a hydrolysate obtained by fermentation from mackerel with an IC\textsubscript{50} value of 0.1 mg/ml; this is comparable to the HPLC fraction isolated in this study of 0.15 mg/ml.

The release of bioactive peptides from their immediate precursor sequence is a requirement for any functional role they will play in biological systems. Bioactive peptides can be released by enzymatic proteolysis of food proteins, so gastrointestinal enzymes, pepsin and pancreatine (chymotrypsin and trypsin) in combination or alone have been used to derive bioactive peptides from proteins. Among these gastrointestinal digestive enzymes, pepsin is known to cleave peptide bonds with phenylalanine and tyrosine whilst trypsin is specific for lysine and arginine. In contrast, \(\alpha\)-chymotrypsin is specific for peptide bonds involving amino acids with bulky side chains and non-polar amino acids (Jung \textit{et al}, 2004). Therefore, natural peptides derived by gastrointestinal hydrolysis can have varied C-terminus amino acids. The peptide isolated in this study shows that the peptide bonds present in the peptide can survive the intestinal peptic and pancreatine enzyme hydrolysis and be available for absorption in the lumen and exert the ACE inhibitory activity. As most food derived ACE peptides are derived from non-
gastrointestinal enzymes, their potential to be used as nutraceuticals is limited as they may be prone to peptic and pancreatic hydrolysis with consequential loss of bioactivity. The use of gastrointestinal enzymes in this study eliminates this hurdle and strengthens the nutraceutical potential of the isolated peptide in this study.

The somatic form of the ACE comprises two homologous domains, generally known as the N-domain and C-domain, arranged in tandem and joined by a short connecting peptide sequence. ACE inhibitors may preferentially act on either ACE domain. However, the C-domain seems to be necessary for controlling blood pressure (Ondetti and Cushman, 1982) suggesting that this domain is the dominant angiotensin-converting site. It is expected that the peptide conformation, that is, the structure adopted in a specific environment of the binding site, should contribute to ACE inhibitor potency (Natesh et al, 2005). Therefore the peptide isolated in this study, comprising 7 kinds of amino acid can access the active site of ACE and may enhance to lower blood pressure. This is in agreement with the results which have been shown from crystallography studies that the active site of ACE cannot accommodate large peptide molecules comprising more than 2530 amino acids (Natesh et al, 2005).

It is thought that ACE inhibition is strongly influenced by the C-terminal tripeptide sequence of the peptide in question. It is proposed that peptides with hydrophobic amino acids at these positions are potent inhibitors. Furthermore, the presence of a positive charge as in the guanidine group of the C-terminal also contributes to the ACE inhibitory potency of several peptides (Wu et al, 2006a, Wu et al, 2006b). In this study,
the observed IC$_{50}$ value of the purified peptide can be attributed to its C-terminal tripeptide. The presence of a hydrophobic amino acid in the C-terminal tripeptide may explain the observed ACE inhibitory bioactivity. Several peptides derived from fish with gastrointestinal enzymes with hydrophobic amino acids such as proline, histidine, tyrosine and phenylalanine in the tripeptide position in the C-terminal show a range of IC$_{50}$ values; for example CWLPVY 0.019 mg/ml, VAWKL 0.021 mg/ml, SKVPP 0.044 mg/ml, YSKVL 0.106 mg/ml (Astawan et al., 1995); MIFPGAGGP 0.029 mg/ml (Jung et al., 2006). In particular, pancreatic digests of salmon and and Pollack had IC$_{50}$ ranging between 3.69 to 5.0 mg/ml (Nakajima et al., 2009). It appears that the position of a hydrophobic amino acid may be a factor for the ACE inhibitory activity of the peptide. The ultimate goal for ACE inhibition is the reduction of blood pressure and gastrointestinal enzymatic hydrolysates of mackerel (IC$_{50}$ 0.1 mg/ml), yellowfin sole (IC$_{50}$ 0.028 mg/ml) and Tuna (IC$_{50}$ 0.63 mg/ml) have been shown to reduce systolic blood pressure (SBP) in spontaneously hypertensive rats (SHR) (Itou and akahane, 2004; Jung et al., 2006; Astawan et al., 1995).

2.3.3. **Competitive or non-competitive binding of the peptides to ACE**

Derivation of peptides with ACE inhibitory activity and the determination of the amino acid sequence and composition can help to further understand the mechanisms by which the specific sequenced peptides effect the bioactivity. Peptides inhibit the ACE either by binding to the active site (competitive inhibition) or to another site on the enzyme (non-competitive inhibition). In this study, the mechanism of ACE inhibition by the isolated
peptide was characterised through Lineweaver-Burk plots. The Lineweaver-Burker plots of ACE with or without the peptide (at 2 different concentrations of 0.1 and 1.42 mg/ml) are shown in figure 2.7. The calculated $V_{\text{max}}$ for control (without inhibitor/peptide), 0.1 and 1.42 mg/ml was 0.18, 0.21 and 0.23 respectively (Table 4.1). The values are not significantly different ($p < 0.01$) and therefore have the same kind of ACE inhibition mechanism. These results indicate that the peptide acted as a competitive inhibitor with respect to HHL as substrate, implying that the peptide competed with the substrate for the active site of ACE. The competitive mechanism exhibited is similar to that exhibited by captopril ($IC_{50}$, 0.022 µM), the most potent synthetic ACE inhibitor as the $V_{\text{max}}$ values for the peptide, control and captopril are not significantly different ($p<0.01$).

The calculated $K_m$ value of ACE was 0.50 mM for HHL in the absence of the peptide. In the presence of the peptide at concentrations of 0.1 and 1.42 mg/ml, the calculated $K_m$ value of ACE was 0.79 and 1.97 mM respectively (Table 2.4). Therefore, in the presence of the peptide (inhibitor), the $K_i$ value was 0.32 mg/ml. This inhibition constant represents the dissociation for the enzyme – inhibitor complex; therefore the lower the value, the greater the affinity of the inhibitor for the enzyme. The $K_i$ obtained in this study shows considerable affinity of the peptide for ACE and will consequently infer considerable inhibition as observed in the $IC_{50}$ value obtained.
This type of inhibition shows that the peptide competitively binds to the active site of ACE and consequently impairs the production of Angiotensin II. Therefore, in the RAS system, the peptide will compete with angiotensin I for the active site of ACE. As a result limited or no angiotensin II will be produced. In this way, the vasoconstriction reaction cascade is impaired and blood pressure consequently lowered in a hypertensive condition.
Table 2.4. Vmax and Kmax values for the isolated peptide and captopril

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$V_{max}$ (mM/min)</th>
<th>$K_m$ (mM)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.18</td>
<td>0.50</td>
<td>0.997</td>
</tr>
<tr>
<td>Peptide 1.42 mg/ml</td>
<td>0.23</td>
<td>1.97</td>
<td>0.884</td>
</tr>
<tr>
<td>Peptide 0.1 mg/ml</td>
<td>0.21</td>
<td>0.79</td>
<td>0.992</td>
</tr>
<tr>
<td>Captopril</td>
<td>0.19</td>
<td>0.41</td>
<td>0.975</td>
</tr>
</tbody>
</table>

Peptides are known to exhibit both competitive and non-competitive inhibition of ACE. ACE appears to prefer substrates or competitive inhibitors containing hydrophobic (aromatic or branched side chains) amino acid residues at each of the three C-terminal positions (Cheung et al, 1980) and many naturally occurring peptidic inhibitors contain proline at the C-terminus (Ondetti et al, 1977). Peptides with Trp as the C-terminal residue, Ala–Trp, Val–Trp, Met–Trp, Ile–Trp, Leu–Trp showed non-competitive inhibition. But when the sequence is reversed with Trp at the N-terminal they exhibit competitive inhibition, except Trp–Leu. (Ono et al, 2006). Peptides, Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala, isolated from Alaska Pollack (Je et al, 2004) and Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu, from yellowfin sole (Jung et al, 2006) were found to be non-competitive. These results show that the sequence of amino acids in peptides can affect both inhibitory potency and the type of inhibition mechanisms.
The isolated peptide in this study has hydrophobic amino acids and when positioned in the C-terminal tripeptide may possibly explain the observed inhibitory mechanism. For example, peptides with proline in the C-terminal tripeptide are known to be competitive inhibitors. ACE inhibitors with a competitive inhibition are preferred as the peptidic fractions resulting from their hydrolysis by ACE are docile or inert and do not have any negative physiological effects. In some instances, the resulting peptidic fragments may also exhibit some ACE inhibitory effects (Yokoyama et al, 1992, Fugita and Yoshikawa, 1999).

2.4. CONCLUSIONS

The results from this study show that in vitro digestion of Atlantic mackerel (Scomber scombrus) fish protein with pepsin and pancreatin produced peptides with ACE inhibitory activity. The inhibitory mackerel fish peptides with the highest inhibitory activity, produced after 3 h of in vitro sequential digestion with pepsin and pancreatin, were all smaller than 2 kDa and after further purification by gel filtration, ion exchange and liquid chromatography, resulted in a peptide with amino acids histidine, proline, tyrosine, methionine, leucine, tryptophan and lysine; and a significant ACE inhibitory activity. The binding of the peptide to ACE was found to be competitive. This finding suggests the potential production of ACE inhibitory mackerel fish peptides upon consumption and digestion of Atlantic mackerel, since the pepsin and pancreatin enzymes used in this study are similar to enzymes in a gastrointestinal digestive system.
CHAPTER THREE
3 ANTIOXIDANT PROPERTIES OF PEPTIDES DERIVED FROM ATLANTIC MACKEREL (*SCOMBER SCOMBRUS*) PROTEIN

3.1 INTRODUCTION

Oxidation of proteins and lipids in biological systems is harmful and is reported to be linked to several diseases such as atherosclerosis, inflammation, and cancer (Frlich and Riederer, 1995). In food, lipid oxidation produces rancid flavours and toxic undesirable compounds, leading to quality deterioration and shortened shelf life. Oxidation of lipids and proteins can be retarded by antioxidants both *in vivo* and *in vitro*. An antioxidant is defined as any substance that significantly delays or inhibits oxidation of a substance when present at low concentrations compared to that of an oxidisable substrate (Belitz *et al*, 2009).

Many synthetic antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, are used as food additives to prevent food deterioration. Despite these synthetic antioxidants showing stronger antioxidant activity than that of natural antioxidants such as α-tocopherol and ascorbic acid, there is concern about their safety with regard to human health (Ito *et al*, 1986; Becker, 1993). Therefore, current research focuses on identifying antioxidants from natural sources that includes plant polyphenols as well as novel peptides from dietary proteins such as zein, fish, egg and milk (Kong and Xiong, 2006; Mendis *et al*, 2001; Yamamoto, *et al*, 1996). Marine products and by-
products have been shown to be good sources of antioxidant peptides, such as jumbo squid skin (Mendis et al., 2005), hoki frame protein (Kim et al., 2007), yellowfin sole frame protein (Jun et al., 2004), yellow stripe trevally (Klompong et al., 2007), round scad (Thiansilakul et al., 2007), Pacific hake (Samaranayaka and Li, 2008), Alaska pollack skin (Kim et al., 2001) among many.

Antioxidant peptides have been characterized from enzymatic hydrolysates of different food proteins, produced by fermentation or enzymatic hydrolysis of food proteins (Kasase and Howell, 2009). Peptides obtained from a combination of gastrointestinal proteases (pepsin and pancreatin) have rarely been used to investigate if antioxidant peptides can be produced in an in vitro digestion model system. The digestion of Atlantic mackerel muscle with gastrointestinal enzymes is a potential strategy that can be employed to produce antioxidant peptides. This offers an advantage in that the formed peptides will resist physiological digestion after oral intake when targeted as nutraceuticals.

Atlantic mackerel (Scomber scombrus) is a pelagic or fatty species found on both sides of the northern hemisphere of the Atlantic Ocean. It varies in size between 180 to 210 cm in length and 325 to 360 g in body weight. Its proximate composition include 15-25% proteins, 0.1-22% lipids, 0.8-2% minerals, 0.3% carbohydrates and 66% and 84% water (Kolokowska, 2003).
Therefore, the aim of this study is to investigate the antioxidant activity of enzymatically prepared peptides from Atlantic mackerel fish protein. The antioxidative activity of the peptide against lipid peroxidation was tested in a linoleic acid model system. It is hoped that understanding of the antioxidative properties of Atlantic mackerel fish peptides may lead to their potential use as potent natural antioxidants.

3.2. MATERIALS AND METHODS

3.2.1 Materials

Atlantic mackerel (*Scomber scombrus*) fillets were supplied by M & J Seafood, Farnham, UK and delivered in ice to the laboratory. Sodium phosphate, hydrochloric acid, sodium hydrogen carbonate, sodium hydroxide and ethanol, were obtained from Fisher Scientific, UK. Vivaspin Membrane ultrafilters (Sartorius (Epsom Surrey, UK). Pancreatine, pepsin, α-tocopherol, trolox, ammonium thiocyanate, ferrous chloride, Sephadex G-25 and Sephadex C-25 were obtained from Sigma-Aldrich, Dorset, UK.

3.2.2. Methods

3.2.2.1. Enzymatic hydrolysis of Atlantic mackerel fish protein

See section 2.2.2.1
3.2.2.2. Membrane ultrafiltration

See section 2.2.2.2

3.2.2.3. Gel filtration

See section 2.2.2.3

3.2.2.4. Measurement of lipid peroxidation in linoleic acid model system.

Linoleic acid was oxidized in a linoleic acid model system to measure the antioxidative activity following the method of Osawa et al (2001) with some modifications.

3.2.2.4.1. Preparation of reaction mixture

Each freeze-dried hydrolysate or peptide sample (60 mg) was mixed with 4.87 mL distilled water, 0.13 mL linoleic acid, 10 mL ethanol, and 10 mL 50 mM phosphate buffer (pH 7.0) in glass flasks. The flasks were sealed tightly with silicone rubber caps and kept at 40 °C in the dark. The analyses were performed in triplicate with a positive control (α-tocopherol and trolox) and a negative control (without the peptide).
3.2.2.4.2. Ferric thiocyanate method for peroxide formation

At 24 h intervals, 100 μL aliquots of the emulsion were withdrawn with a microsyringe into test tubes to which 4.7 ml of 75 % ethanol (v/v), 100 μL 30 % ammonium thiocyanate (w/v) and 100 μL 20 mM ferrous chloride solution was added. Precisely after 3 minutes after the addition of ferrous chloride solution, the absorbance was measured at 500 nm on a spectrophotometer (UV Mini 1240, Shimadzu Europa, Milton Keynes, UK).

3.2.2.4.3. Thiobarbituric acid reactive species (TBARS) method

The TBARS were measured using a modified version of the method of Ohkawa et al (1979). At 24 h intervals, 50 μL aliquots of the emulsion were withdrawn with a microsyringe into test tubes to which 0.8 ml distilled water, 0.2 ml 8.1 % sodium dodecyl sulfate (w/v), 1.5 ml of 20 % acetic acid (v/v), and 1.5 ml of 0.8 % TBA (w/v) solution were added. The mixture was then heated at 100 °C for 1 h in the dark. After cooling, the tubes were centrifuged and the supernatant was measured for TBARS on a spectrophotometer by reading the absorbance at 532 nm.

3.2.2.4.3. Lipid oxidation inhibitory concentration

Different concentration of peptide, 0.4, 0.8, 4, 6 and 8 mg/ml were prepared in a linoleic acid emulsion system. After 3 days, the peroxide values through the FTC method were
determined. A standard of 0.01 % BHT and 0.01 % trolox were also prepared and treated in the same way.

3.2.3. Amino acid composition of isolated peptide determination

Amino acids composition was determined according to section 2.2.3.

3.2.4. Statistical analysis

Statistical analyses were performed with Minitab statistical software (13.1). Comparisons were made by one-way analysis of variance. P< 0.05 was considered statistically significant.

3.3. RESULTS AND DISCUSSION

3.3.1. Isolation and purification of antioxidative peptides

The functional properties of bioactive peptides are highly influenced by the molecular structure, molecular mass, as well as conditions of processing. Enzymatic hydrolysis has become the most important tool for modifying the functionality of dietary proteins to identify different bioactivities (Korhonen et al, 1998). Therefore, Atlantic mackerel
protein was hydrolyzed with pepsin and then with pancreatin, the enzymes that are involved in the gastrointestinal digestive system in humans.

To purify the antioxidant peptides from the mackerel protein hydrolysate, membrane ultrafiltration (2 kDa MWCO), gel filtration chromatographic and HPLC techniques were utilized. The lipid oxidation inhibition (%) by the ferric thiocyanate method was used as the selection criteria for choosing the peptide fractions for further purification. Figure 3.1, 3.2 and 3.3 show the chromatographic profiles obtained during the purification steps of a < 2 kDa Atlantic mackerel fish hydrolysate peptide as well as their lipid oxidation inhibition (%).

In the lipid peroxidation inhibition activity assay, peroxyl (ROO•) and alkoxyl (RO•) radicals, derived from the pre-existing lipid peroxide, were employed directly to initiate lipid peroxidation in the emulsified linoleic acid system (Cheng et al, 2003). Previous studies with soya protein isolates (SPI) hydrolysed with pepsin and pancreatin and fractionated into >2 kDa, 2-5 kDa, 5-10 kDa, and the crude hydrolysate, shows >2 kDa fraction exhibits the highest lipid oxidation inhibition (Kasase and Howell, 2008). Therefore, in this study, the Atlantic mackerel fraction was first purified on a 2 kDa MWCO ultrafiltration membrane and then purified by gel filtration on Sephadex G 25, collecting 5 ml fractions. When the freeze dried fractions where determined for their lipid oxidation inhibition, fraction G (in figure 3.1) gave the highest lipid oxidation inhibition of 47.0 %. Consequently, this fraction was pooled, freeze dried and purified on a C18 (9.8 x 500 mm) column by HPLC (Figure 3.2). Of the four fractions collected,
the fraction denoted by the letter Z gave the highest lipid oxidation inhibition of 19.05 % (Figure 3.3). Fraction Z was then rechromatographed by HPLC on a C18 (9.8 x 250 mm) column, collected, pooled and freeze dried. The amino acid composition of the peptide fraction was determined and found to contain 7 amino acids - serine, histidine, tyrosine, phenylalanine, tryptophan and lysine (figure 3.4 and Table 3.1).
Figure 3.1(A). Gel filtration chromatography of the 2kDa Atlantic mackerel fish peptides on the Sephadex G-25 column and detected at 215 nm and (B) the corresponding lipid oxidation inhibition. Separation was performed at a flow rate of 1 ml/min and collected at a fraction volume of 5 ml.
Figure 3.2. (A) HPLC of fraction G from gel filtration detected at 215nm and (B). The corresponding % lipid oxidation inhibition. Separation was performed on a C18 (9.8 x 500 mm) column at a flow rate of 1 ml/min and 2 ml fraction collected.
Figure 3.3. HPLC of fraction Z from HPLC rechromatographed C18 (9.8 x 250 mm) column and detected at 215 nm.
Figure 3.4. (A) Chromatogram of amino acid standards and (B) amino acids from LC1-Z.
Table 3.1. Amino acid composition of standard and peptide fraction (LC1-Z).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Standards Retention time (min)</th>
<th>Amount (μg)</th>
<th>Peptide Fraction (LC1-Z) Retention time (min)</th>
<th>Amount (μg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>3.5</td>
<td>1.33</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glu</td>
<td>3.8</td>
<td>1.47</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ser</td>
<td>5.7</td>
<td>1.05</td>
<td>5.5</td>
<td>0.31</td>
</tr>
<tr>
<td>Gly</td>
<td>6.0</td>
<td>0.75</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>His</td>
<td>6.2</td>
<td>1.55</td>
<td>6.2</td>
<td>0.31</td>
</tr>
<tr>
<td>Thr</td>
<td>6.7</td>
<td>1.19</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ala</td>
<td>6.8</td>
<td>0.89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pro</td>
<td>7.0</td>
<td>1.15</td>
<td>-</td>
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</tr>
<tr>
<td>Try</td>
<td>8.1</td>
<td>1.81</td>
<td>8.0</td>
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</tr>
<tr>
<td>Val</td>
<td>9.0</td>
<td>1.17</td>
<td>-</td>
<td>-</td>
</tr>
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<td>Met</td>
<td>9.3</td>
<td>1.49</td>
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<td>Cys</td>
<td>9.8</td>
<td>1.21</td>
<td>-</td>
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</tr>
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<td>Ile</td>
<td>10.5</td>
<td>1.31</td>
<td>-</td>
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<td>Leu</td>
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<td>-</td>
</tr>
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<td>Phe</td>
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<td>11.2</td>
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</tr>
<tr>
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<td>11.4</td>
<td>0.16</td>
</tr>
<tr>
<td>Lys</td>
<td>12.2</td>
<td>0.73</td>
<td>12.0</td>
<td>0.08</td>
</tr>
</tbody>
</table>
3.3.2. Inhibitory effects of isolated peptide on lipid peroxidation

To assess the inhibitory effects of the isolated peptide (LC1-Z) on lipid peroxidation, linoleic acid was oxidized in an emulsified model system. The mixture was incubated in the dark at 40 °C and primary (peroxides) and secondary oxidation products (TBARS as Malondialdehyde) of linoleic acid determined for 7 days. In lipid oxidation process, there is a sequential formation of peroxides and hydroperoxides as primary products which are then transformed into carbonyl secondary products also referred to as thiobarbituric acid reactive substances (TBARS) and include Malondialdehyde (MDA). Peroxides/hydroperoxides and TBARS are considered as biomarkers of lipid peroxidation (Liu et al, 1997). In this study, the inhibition of linoleic acid oxidation by the isolated peptide was followed by monitoring peroxides through the ferric thiocyanate method and MDA through the thiobarbituric acid method. Figure 3.5 and 3.6, shows that the oxidation of linoleic acid results in both peroxides and MDA (control) being formed and that the formation of both primary and secondary products is markedly and effectively inhibited in the presence of the isolated peptide (LC1-Z). The trend in the inhibition of linoleic acid oxidation is similar to that of the standards, butylated hydroxytoluene (BHT) and trolox. The trend in the formation of MDA in the presence of the peptide (LC1-Z) and standards (BHT and trolox) correlated well as it appears to follow the same pattern (Figure 3.6).
Figure 3.5 Antioxidant activity of LC1-Z. LC1-Z was incubated in a linoleic acid oxidation system for 6 days and the degree of linoleic acid oxidation was assessed by measuring peroxides equivalents (mg/ml FeCl₂) at every 24-h interval. Butylated hydroxytoluene (BHT) and trolox were used as positive controls.

Figure 3.6 Antioxidant activity of LC1-Z. LC1-Z was incubated in a linoleic acid oxidation system for 6 days and the degree of linoleic acid oxidation was assessed by measuring malondialdehyde (μg/ml) at every 24-h interval. Butylated hydroxytoluene (BHT) and trolox were used as positive controls.
A dose dependent inhibition of linoleic acid oxidation by the peptide was observed through the formed peroxides by the ferric thiocyanate method (figure 3.7). An increase in the peptide concentration had a corresponding increase in linoleic acid oxidation inhibition. This dose dependent phenomenon in figure 3.7 gives an inhibitory concentration (IC<sub>50</sub>) of the peptide of 1.80 mg/ml assay volume (y = 8.254x + 35.239, R<sup>2</sup> = 0.85). However, at peptide concentration of 8 mg/ml, the inhibition of linoleic acid oxidation inhibition is markedly more than (p<0.001) BHT and trolox (0.01 % in each) at concentrations normally used in food systems (figure 3.8).

**Figure 3.7.** Dose dependent lipid oxidation inhibition of LC1-Z. LC1-Z was incubated in a linoleic acid oxidation system and lipid oxidation inhibition assessed by measuring peroxides equivalents (mg/ml FeCl<sub>2</sub>) after 72 h.
Figure 3.8. Lipid oxidation inhibition of LC1-Z. LC1-Z was incubated in a linoleic acid oxidation system and lipid oxidation inhibition was assessed by measuring peroxides equivalents (mg/ml FeCl$_2$) after 72 h. Butylated hydroxytoluene (BHT) (0.01%) and trolox (0.01%) were used as positive controls.

Peptides exhibiting antioxidant activity have been isolated from a number of fish species. The antioxidant activity of these peptides is generally attributed to the size of the peptide, sequence of amino acids and most importantly the composition of the amino acids. Amino acids, as individual compounds, do not show marked antioxidant activity (Elias et al, 2008). The observed activity is due to their increased free radical scavenging activity, metal chelation and aldehyde adduction activity (Chan et al, 1994; Zhou and Decker, 1999a and b). In free radical scavenging, peptides impart their protective actions in lipid peroxidation by scavenging the lipid-derived (R*, RO* or ROO*) radicals through
the donation of hydrogen thereby inhibiting the propagation cycle of lipid peroxidation. Some peptides are reported to exhibit higher antioxidative activities against lipid peroxidation in the presence of specific hydrophobic amino acids such as Gly, Leu, Phe, and Pro (Saiga et al, 2003).

In peptides containing histidine, the antioxidant activity is attributed to hydrogen donating ability, lipid peroxyl radical trapping, and/or the metal ion-chelating ability of the imidazole group (Chan and Decker, 1994). The anti-oxidative activities of histidine-containing peptides exceed that of histidine itself. This trend is partly due to the increased hydrophobicity of the peptides, that increases the interaction between the peptides and fatty acids (Jun et al, 2004; Yee et al, 1980). The observed antioxidative activity of the peptide in this study can therefore be attributed to the presence of histidine hydrophobicity, which may lead to a higher interaction between the peptide and linoleic acid, and hydrogen donating ability and peroxyl radical trapping.

The environment surrounding the imidazole group of histidine in a peptide affects the antioxidant activity of the individual peptides. N-(longchain-acyl) histidine-containing compounds restrain the oxidation of phosphatidylcholine liposomes and methyl linoleate as the hydrophobicity of the compounds is important for the accessibility to the hydrophobic targets (Murase et al, 1993). In the lipid oxidation inhibition with Asp-Arg-Val-Tyr-Ile-His-Pro-Phe mediated by copper (II)/ascorbate, the N-terminal Asp-Arg-Val-Tyr sequence adds extensively to the reactivity of the histidine residue, which is converted to the 2-imidazolone derivative upon oxidation. The histidine residue in Ile-
His-Pro-Phe shows no activity against lipid oxidation inhibition without the N-terminal segment (Uchida and Kawakishi, 1992). Also, a potent antioxidative peptide Ala-His-Lys from the egg white albumen hydrolysate, in which neither His-Lys nor a constituent amino acid mixture has activity, but Ala-His is as potent as the parent peptide (Tsuge et al, 1991). Therefore, the amino acids in the environment of the hydrophobic amino acid in the peptide are contributing to the overall observed antioxidant activity.

In addition to hydrophobicity, the specific positioning of amino acid residues in the peptide sequence plays an important role in the antioxidative activity observed. In residue-activity relationship studies of synthetic antioxidative peptide mimics, in linoleic acid peroxidation system, a loss of the antioxidative activities was observed after deletion of the terminal histidine residue (Chen et al, 1996; Chen et al, 1998). A peptide from yellow fin sole fish with 10 amino acid residues, RPDFDLEPPY contained a tyrosine residue, which is a potent hydrogen donor and considered to be responsible for the antioxidant activity (Jun et al, 2004). Another peptide, LPHSGY, from Alaska Pollack fish has histidine and tyrosine (Je et al, 2005). Histidine was reported to be responsible for the chelating and lipid radical-trapping ability due to the imidazole ring while the tyrosine residue was thought to be responsible for the hydrogen donating ability (Je et al, 2005). Besides peroxo radical scavenging, some histidine-containing peptides can act as a metal-ion chelator, singlet oxygen quencher and/or hydroxy radical scavenger (Chen et al, 1998). Similar antioxidative properties have been reported for carnosine, a unique histidine-containing dipeptide in which the antioxidant activity is attributed to the donation ability of the histidine imidazole group (Chen et al, 1998;
Decker et al, 1992). The presence of histidine contributes to the overall antioxidant activity. Therefore, we can speculate that the particular size, amino acid composition and sequence, and hydrophobicity in the terminal positions of the peptide contributes to the overall observed antioxidant activity of LC1-Z peptide.

3.4. CONCLUSIONS

Enzymatic digestion of Atlantic mackerel protein with gastrointestinal enzymes (pepsin and pancreatin) and subsequent purification of hydrolysate with membrane ultrafiltration, gel filtration and HPLC yielded a peptide with lipid oxidation inhibition activity. The antioxidant activity was dose-dependent and peptide concentrations of 4 - 8 mg/ml were comparable to 0.01 % BHT and 0.01 % trolox. These results suggest that antioxidant peptide fractions from Atlantic mackerel protein may have useful applications in the food and pharmaceutical industries when specifically targeting the inhibition of lipid oxidation in emulsion systems.
CHAPTER FOUR
4 ANTIOXIDANT MECHANISMS OF PEPTIDE DERIVED FROM ATLANTIC MACKEREL (SCOMBER SCOMBRUS) FISH PROTEIN.

4.1 INTRODUCTION

Oxidation of biological molecules especially lipids has been recognized as a process that is mediated through free radicals. This process is known to impact adversely on biological systems and food. In biological systems, damaging free radicals that are formed during the metabolism of oxygen are associated with the incidence of several disease conditions such as atherosclerosis, inflammation, Alzheimer’s disease and cancer (Frlich and Riederer, 1995). In food systems, lipid oxidation is of great concern because it leads to the development of undesirable off-flavours, odours and potentially toxic reaction products (Alghazeer and Howell, 2008).

The formation of free radicals and reactive oxygen species (ROS) in aerobic organisms during respiration is a process that cannot be avoided. These free radicals are very unstable and react rapidly with other biomolecules in the organism, leading to cell or tissue injury. However, in normal conditions, ROS are effectively eliminated by the antioxidant defense system, such as antioxidant enzymes, antioxidant compounds and non-enzymatic factors. On the other hand, in pathological conditions, the balance between the generation and the elimination of ROS is impaired and as a result, biomacromolecules, including DNA, membrane lipids and proteins, are damaged by
ROS-mediated oxidative stress. Scientific evidence shows that this imbalanced oxidation is the trigger for the occurrence of numerous chronic diseases (Halliwell, 2001).

Lipid peroxidation in foods affects nutritive value and may cause disease conditions following consumption of potentially toxic reaction products (Alghazeer and Howell, 2008). To overcome the effects of lipid peroxidation, synthetic antioxidants are widely used in the food industry. While these synthetic antioxidants show stronger antioxidant activity than that of natural antioxidants such as α-tocopherol and ascorbic acid, there is concern about their safety with regard to health (Yanishlieva-Maslarova, 2001). As a result, in the recent past, the research fields of human nutrition and biochemistry have focused on antioxidants sourced from food ingredients that could retard lipid peroxidation. Also the antioxidant properties of proteins and peptides derived from plant, animal and aquatic sources are documented in the literature (Mine and Shahidi, 2006).

Since the antioxidant activity of the peptide derived from Atlantic mackerel (Scomber scombrus) has been established (LC1-Z), understanding of its antioxidant mechanisms may lead to the utilisation of the fish muscle as a source of potent natural antioxidants. Therefore, in this study we investigated the mechanisms of antioxidant activity of peptides prepared from Atlantic mackerel fish (Scomber scombrus) using gastrointestinal enzymes.
4.2. MATERIALS AND METHODS

4.2.1. Materials

Atlantic mackerel (*Scomber scombrus*) fillets were supplied by M & J Seafood, Farnham, UK and delivered in ice to the laboratory. 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, ferrous sulphate, α-deoxyribose, trichloroacetic acid, potassium ferricyanide, ferric chloride, ferrozine and trolox were obtained from Sigma-Aldrich, Dorset, UK. All other chemicals and solvents used were of Analytical grade and commercially available.

4.2.2. Methods

4.2.2.1. Isolation and purification

The peptide LC1-Z was isolated and purified as described in 3.2.2.1 to 3.2.2.3.

4.2.2.2. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity of the peptide was determined as described by Bersuder *et al* (1998). A volume of 500 μL of peptide LC1-Z (1mg/ml) and standards BHT and ascorbic acid (0.01 %) were mixed with 500 μL of 99.5 % ethanol and 125 μL of 0.02 % DPPH in 99.5 % ethanol. The mixture was
then kept at room temperature in the dark for 60 min, and the reduction of DPPH radical was measured at 517 nm using a UV–Visible spectrophotometer (UV Mini 1240, Shimadzu Europa, Milton Keynes, UK).

The DPPH radical-scavenging activity was calculated as follows:

\[
\frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100\%.
\]

The control was conducted in the same manner, except that distilled water was used instead of peptide. A lower absorbance of the reaction mixture indicated a higher DPPH radical-scavenging activity. The test was carried out in triplicate.

4.2.2.3. Hydroxyl radical scavenging activity assay (HRSA)

The HRSA assay was performed as previously described by Chung et al (1997) with some modifications. The reaction mixture consisted of 0.1 ml of 10 mM FeSO₄, 0.1 ml of 10 mM EDTA, 0.5 ml of 10 mM α-deoxyribose, 0.9 ml of sodium phosphate buffer (pH 7.4) and 0.2 ml of different concentrations of peptide (0.01, 0.05, 0.1, 0.15 and 0.2 mg/ml) that were thoroughly mixed in a tube. Hydrogen peroxide (0.2 ml, 10 mM) was then added and the reaction mixture was incubated at 37 °C for 1 h. One milliliter of 2.8 % trichloroacetic acid (TCA) and 1.0 ml of 1.0 % thiobarbituric acid (TBA) were added to the test tubes and boiled for 15 min. After cooling the mixture, the absorbance was measured at 532 nm using the spectrophotometer (UV Mini 1240, Shimadzu Europa,
Milton Keynes, UK). Sodium phosphate buffer (pH 7.4) instead of sample was used as blank. The HRSA was evaluated as the inhibition rate of α-deoxyribose oxidation by hydroxyl radical,

\[
\text{HRSA \%} = \left( \frac{A_0 - A_i}{A_0} \right) \times 100
\]

Where \( A_0 \) was the absorbance of the blank and \( A_i \) was the absorbance in the presence of the test compound (LC1-Z). These values of HRSA were plotted against the concentration of peptide and the concentration to scavenge 50% of radical activity was defined as the IC\textsubscript{50} value.

4.2.2.4. Reducing power assay

The ability of the hydrolysate to reduce iron (III) was determined according to the method of Yildirim et al. (2001). An aliquot of 1 ml sample (LC1-Z) of each hydrolysate at different concentrations was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1 % potassium ferricyanide (w/v). The mixture was incubated at 50 °C for 30 min, followed by the addition of 2.5 ml of 10 % trichloroacetic acid (w/v). The mixture was then centrifuged at 1650 x g for 10 min. Finally, 2.5 ml of the supernatant solution were mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride (w/v). After 10 min reaction time, the absorbance of the resulting solution was measured at 700 nm using a spectrophotometer (UV Mini 1240, Shimadzu Europa, Milton Keynes,
UK). Increased absorbance of the reaction mixture indicates increased reducing power.
The values are presented as the means of triplicate analyses.

4.2.2.5. Fe$^{2+}$ chelating activity assay

The chelating activity of the peptide for ferrous ions Fe$^{2+}$ was measured according to the
method of Boyer and McCleary (1987) with slight modifications. To 0.5 ml of LC1-Z
peptide (1 mg/ml), 1.6 ml of deionized water and 0.05 ml of FeCl$_2$ (2 mM) was added.
After 30 s, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron
to form stable magenta complex species that were very soluble in water. After 10 min at
room temperature, the absorbance of the Fe$^{2+}$-ferrozine complex was measured at 562
nm on a spectrophotometer (UV Mini 1240, Shimadzu Europa, Milton Keynes, UK).
The chelating activity of the peptide for Fe$^{2+}$ was calculated as:

\[
\text{Chelating ability (\%) = } \left( \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \right) \times 100
\]

Where $A_{\text{control}}$ was the absorbance of the control (without peptide) and $A_{\text{sample}}$ was the
absorbance in the presence of the LC1-Z peptide. The values are presented as the means
of triplicate analyses.
3.2.3. Statistical analysis

Statistical analyses were performed with Minitab statistical software (13.1). Comparisons were made by one-way analysis of variance or Student's t test as appropriate. P< 0.05 was considered statistically significant.

4.3. RESULTS AND DISCUSSION

4.3.1. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule and shows maximum absorbance at 517 nm. When DPPH radicals encounter a proton-donating substrate such as an antioxidant, the radicals would be scavenged and the absorbance reduced. The reduction in absorbance is used as a measure of the radical-scavenging activity of the antioxidant under study. DPPH is often used as a substrate to evaluate antioxidant activity of potential antioxidants (Bersuder et al, 1998). Therefore, this DPPH based method was used in this study to investigate the radical scavenging activity of fish peptide with antioxidant activity, together with BHT and ascorbic acid as standards.
The results showed that the peptide (LC1- Z) has radical scavenging activity just like BHT and ascorbic acid (figure 4.1). The radical scavenging activity of the peptide (1mg/ml) of 5.34 % is comparable to that of the 0.01 % BHT (10.03 %) and 7 times more than ascorbic acid (35.2 %).

![Radical scavenging activity of Atlantic mackerel peptide (LC1- Z) and standards BHT and ascorbic acid. Each value represents means ± % CV (n=3).](image)

**Figure 4. 1** Radical scavenging activity of Atlantic mackerel peptide (LC1- Z) and standards BHT and ascorbic acid. Each value represents means ± % CV (n=3).

During the oxidation of lipids, carbon-centered free radicals such as ROO$^*$, RO$^*$ and R$^*$ are generated and peptides like antioxidants, are reported to interact with these compounds. Generally, the scavenging of free radicals by antioxidants is reported to be effected via the donation of hydrogen atoms. Amino acids histidine, leucine, methionine and tyrosine are known to enhance the radical-scavenging activities of antioxidant peptides (Park et al, 2001). Several peptides isolated from fish proteins containing these amino acids have been shown to exhibit radical scavenging activities (Je et al, 2005;
Mendis et al, 2005; Rajapakse et al, 2005; Jung et al, 2007; Je et al, 2007). Histidine-containing peptides such as anserine, balenine and carnosine are reported to demonstrate high radical-scavenging activity. This activity is attributed to the proton-donation ability of the imidazole group of histidine (Park et al, 2001). A peptide His-Gly-Pro-Leu-Gly-Pro-Leu derived from gelatin prepared from hoki fish skin also displayed radical scavenging activity due to the presence of the N-terminal histidine as a strong proton donating residue in the sequence. In another study, the radical scavenging activity of peptides isolated from tuna fish with amino acids tyrosine and leucine exhibited radical scavenging activity (Je et al, 2007). The peptide isolated in this study contains the amino acids histidine and tyrosine and the observed radical scavenging activity may therefore be attributed to these amino acids.

Although the exact mechanism that underlies the scavenging of radicals by the isolated peptide is not well understood, the observed radical scavenging activity of the isolated peptide in this study can be attributed to the donation of hydrogen by amino acids such as histidine, leucine, methionine and tyrosine in the sequence of the peptide. As peptides can interact with free radicals and ROS, Figure 4.1 suggests that peptides may protect lipids from oxidation if they are oxidized preferentially to unsaturated fatty acids. Preferential oxidation of proteins and peptides can occur if available amino acids are more oxidatively labile than unsaturated fatty acids (Saeed et al, 2006), or if the physical location of a protein or peptide places it near the site of free radical or ROS generation where the protein or peptide would be able to rapidly scavenge the free radical prior to the migration of the radical to lipids (Elias et al, 2008). The isolated peptide under
investigation is less than 2 kDa in size and is therefore stereochemically less bulky and will have its amino acids such as histidine, leucine, methionine and tyrosine exposed to exert antioxidant activities. These results agree with those of other studies in which peptides and hydrolysates from fish proteins (tuna, shell fish, smooth hound, Giant squid) hydrolysed with gastrointestinal enzymes exhibited radical scavenging activity (Rajapakse et al, 2005; Je et al, 2007; Bougatef et al, 2009; Jung et al, 2007).

4.3.2. The hydroxyl radical-scavenging activity

The hydroxyl radical is very reactive and can be generated in biological systems via the Fenton reaction. Hydroxyl radical is the most active free radical and attacks all biological molecules by setting off a free radical chain reaction, resulting in cell damage and hence human diseases (Halliwell, 2000). It interacts easily with biomolecules such as amino acids, proteins and DNA (Cacciuttolo et al, 1993). For this reason, its removal can perhaps be one of the most effective defenses of a living body against various diseases.

In the HRSA assay, the Fenton reactions proceed as Iron(II) (ferrous) is oxidized by hydrogen peroxide to iron(III) (ferric), a hydroxyl radical and a hydroxyl anion. Iron (III) is then reduced back to iron (II), a peroxide radical and a proton by the same hydrogen peroxide

\[
(1) \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-
\]

\[
(2) \text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{OOH}^- + \text{H}^+
\]

116
The resultant radical interacts with the antioxidant through a radical quenching mechanism and this is monitored spectrophotometrically. In this study, the peptide showed hydroxyl radical scavenging activity. As the concentration of a scavenging molecule is proportional to the radical scavenging activity, different concentrations of the peptide (LC1-Z) showed an increased scavenging activity (figure 4.2). The observed scavenging activity was linear with respect to peptide concentration and expressed through a linear relationship, \( y = 31.605x - 0.5408 \) (\( R^2 = 0.9279 \)) and accordingly an IC\(_{50}\) value of 1.60 mg/ml was obtained. The obtained results suggest that the peptide contains amino acids which are hydroxyl radical scavengers and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

![Graph](image)

**Figure 4.2.** Hydroxyl radical scavenging activity (HRSA) of Atlantic mackerel peptide (LC1-Z). Each value represents means ± % CV (n=3).
The potential scavenging abilities of phenolic substances is reported to be due to the active hydrogen donating ability through hydroxyl substitution. Also, high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical scavenging by specific functional groups (Korycka-Dahl and Richardson, 1978). Similarly, it has been reported that amino acids cysteine, histidine and methionine are central to the hydroxyl radical scavenging activity of peptides. This activity is due to their characteristic special structure. In histidine, the imidazole group has the proton-donation ability (Tsuge et al, 1991). In methionine, the methionine sulfoxide is susceptible to oxidation and cysteine can donate the sulfur hydrogen (Hernandez-Ledesma et al, 2005). Tyrosine and phenyalanine have been shown to act positively as direct radical scavengers (Rajapakse et al, 2005). The antioxidant activity of tyrosine is explained by the unique ability of its phenolic group to serve as a hydrogen donor, which is one mechanism of inhibiting radical-mediated oxidation chain reactions (Jung et al, 1995).

It therefore follows that the observed hydroxyl radical scavenging activity of the peptide is attributed to the characteristic amino acids such as histidine, tyrosine and phenyalanine present in the peptide. This observation concurs with similar studies that show that peptides containing such amino acids exhibit hydroxyl radical scavenging activity as seen in yellowfin sole (Jun et al, 2004), Alaska pollack (Je et al, 2005, giant squid (Rajapakse et al, 2005), chum salmon (Nagai et al, 2006), jumbo flying squid (Lin and Li 2006), shell fish (Jung et al, 2007), yellow stripe trevally (Klompong et al, 2007),
walleye pollack (Nagai et al, 2007), tuna (Je et al, 2007), round scad (Thiansilakull et al, 2007) and grass carp (Ren et al, 2008).

4.3.3. The reducing power activity

Antioxidants can be explained as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species (oxidant) is reduced at the expense of the oxidation of another antioxidant. Therefore, the reducing power assay is often used to evaluate the ability of antioxidant to donate an electron. This assay measures the antioxidant effect of any substance in the reaction medium as reducing ability. Different studies have reported that there is a direct correlation between antioxidative activities and reducing power of certain bioactive compounds (Yildirim et al, 2000).

In this study, the ability of peptide (LC1-Z) to reduce Fe3+/ferrieyanide complex to the ferrous form was determined. Figure 4.3 shows that the reducing power of peptide increased with increasing concentrations (0 – 25 mg/ml) and the peptide exhibited reducing ability and was expressed linearly as $y = 0.0301x + 0.0322$ ($R^2 = 0.9943$).

These results suggest the presence of amino acids in the peptide which functioned as electron donors and which may react with free radicals to form more stable products. These results concur with several studies in which fish derived peptides and protein hydrolysates isolated from Mackerel (Wu et al, 2003), Yellow stripe trevally (Klompong...
et al, 2007) and Round scad (Thiansilakul et al, 2007) exhibited reducing power activity in a linear relationship.

![Graph showing the reducing power of peptide isolated from Atlantic mackerel peptide.](image)

**Figure 4.3.** Reducing power of peptide ((LC1-Z)) isolated from Atlantic mackerel peptide. Each value represents means ± % CV (n=3).

### 4.3.4. Fe²⁺ chelating activity assay

Transition metal ions, such as Fe²⁺/³⁺, Cu²⁺, Co²⁺, in biological systems affect both the rate of autoxidation and breakdown of hydroperoxide to volatile compounds. Transition metal ions react very quickly with peroxides by acting as one-electron donors to form alkoxy radicals (Gordon, 2001) as shown in the scheme below.
Metal\(^{n+}\) + Lipid-OOH → Metal\(^{(n+1)+}\) + OH\(^{-}\) + Lipid – O\(_2\).

Therefore, chelation of transition metal ions by antioxidant peptides would retard this oxidation reaction.

In this method used to determine the chelating activity of the peptide, the ferrozine makes complexes with ferrous ions. In the presence of chelating agents (peptide), the complex (red coloured) formation is interrupted and, as a result, the red colour of the complex is decreased. As a consequence, the chelating effect of the coexisting chelator can be determined by measuring the rate of colour reduction.

The isolated peptide demonstrated appreciable iron chelating activity of 5.72 % and was comparable to the potent antioxidants trolox (11.11 %) and ascorbic acid (12.67 %) as shown in figure 4.4. Similarly, peptides and hydrolysates with chelating activity have been reported from fish proteins from yellow stripe trevally (Klompong et al, 2007), round scad (Thiansilakull et al, 2007) and sole (Gimenze et al, 2009).
Figure 4.4. Ferrous ion chelating activity of Atlantic mackerel peptide 1 mg/ml (LC1-Z) and 0.01 % of standards trolox and ascorbic acid. Each value represents means ± % CV (n=3).

It has been reported that peptide chelators can inhibit lipid oxidation by changing the physical location of transition metals (e.g. partitioning metals away from oxidatively labile lipids or hydroperoxides), forming insoluble metal complexes, reducing the chemical reactivity of transition metals, and/or sterically hindering the interaction of metals and dispersed lipids (Diaz et al, 2003; Diaz and Decker, 2005).

Amino acids and peptides are typical metal chelating agents (Fujimoto et al, 1984). Chen et al (1995) has reported that the characteristic amino acid sequence of peptides is important for metal chelating activity of peptides. The antioxidant activity observed in
histidine-containing peptides is thought to be related to their metal chelating ability, besides the lipid-radical trapping potential of the imidazole ring (Uchida and Kawakishi, 1992; Murase et al, 1993). In carnosine and anserine (histidine-containing dipeptides), it has been shown that histidine is involved in metal chelation in addition to free radical scavenging and singlet oxygen activities it exhibits (Egorov et al, 1992). Therefore, the observed chelating activity of the peptide (LC1-Z) may be a result of the presence of the amino acid histidine in the peptide. Chelation of prooxidants such as iron and copper by the histidine containing peptide in biological systems will consequently lead to decreased lipid oxidation in biological and food systems.

Peptides, in general, can reduce lipid peroxidation through biologically designed mechanisms (e.g. iron-binding peptides) or by nonspecific mechanisms. Both of these types of peptides contribute to the endogenous antioxidant capacity of foods and could also be used as potential antioxidant additives. Overall, the antioxidant activity of peptides is due to complex interactions between their ability to inactivate reactive oxygen species, scavenge free radicals, chelate pro-oxidative transition metals, reduce hydroperoxides and alter the physical properties of food systems in a way that separates reactive species. Peptides therefore are somewhat distinctive in this way, compared to other food antioxidants as they can potentially act as multifunctional antioxidants that can inhibit several different lipid oxidation pathways (Elias et al, 2008).
4.4. CONCLUSIONS

The results of the present study show that the peptide (LC1-Z) containing amino acids serine, histidine, tyrosine, phenylalanine and lysine, isolated from Atlantic mackerel exhibits the antioxidant activity through the scavenging of free radicals such as carbon centered radicals and hydroxyl radicals, reducing power activity and chelating activity; and the presence of amino acids such as histidine, tyrosine and phenylalanine is highly possible. Overall, the peptide is a potential natural antioxidant that can be important in food preservation, disease prevention and health preservation.
CHAPTER FIVE
5. ANTIOXIDANT ACTIVITY OF PEPTIDES ISOLATED FROM ATLANTIC MACKEREL \textit{(Scomber scombrus)} FISH PROTEIN IN CACO-2 AND Eh.Hy 926 CELLS, AND ABSORPTION OF PEPTIDE IN A CACO-2 MONOLAYER MODEL

5.1. INTRODUCTION

In normal physiological conditions, cellular systems are constantly challenged by stress arising from both internal and external sources. In aerobic organisms, the potential sources of cell stress (oxidative stress) are the reduced derivatives of oxygen, also referred to as reactive oxygen species (ROS). ROS are produced in living cells as a result of normal cell metabolism, xenobiotic detoxication, UV- and X-ray irradiation. The effects of oxidative stress in mammalian cells range from rapid cell mortality to disturbance of many cell signaling processes (Klotz \textit{et al}, 2003). As a result, ROS are implicated in the pathogenesis of many diseases that include cardiovascular diseases, certain cancers, rheumatoid arthritis, diabetes, Alzheimer’s disease, neurological disorders and the ageing process. On the other hand, aerobic organisms possess a protective system that limits their exposure to ROS. These protective components, also referred to as antioxidant defense system, include an array of enzymes and essential nutrients whose role is to prevent the generation of ROS and/or intercept the generation of ROS. Despite the presence of the antioxidant defence system, the prooxidant-antioxidant balance favours the ROS and consequently creates oxidative stress with potential cell damage and onset of chronic diseases (Morrissey and O’Brien, 1998).
The human colon carcinoma cell line (Caco-2) spontaneously exhibits structural and functional characteristics of mature small bowel enterocytes under standard culture conditions. Normal intestinal cell turnover can be disrupted by lipid hydroperoxides and this underscores the tumorigenic potential of oxidized lipids. Peroxidized lipids, as dietary oxidants, can initiate intestinal degenerative processes via generation of oxygen radicals (Parks et al, 1983). Subtoxic low levels of lipid hydroperoxide (1–5 µM) induce phase transition of intestinal cells from a quiescent to a proliferative state that is mediated by peroxide-induced disruption of cellular redox balance (Wang et al, 2002). Also, oxidized lipids have been shown to have a cytotoxic effect on caco-2 cells (Alghazeer et al, 2008). At lipid hydroperoxide concentrations that were a 100-fold higher than those that elicited proliferative responses (0.1 – 0.2 mM), caco-2 cells were significantly injured (Wang et al, 2002). The organic hydroperoxide, tert-butyl hydroperoxide (t-BHP), is a useful model compound to study mechanisms of oxidative cell injury. Exposure of mammalian cells to the membrane-permeable organic peroxide t-BHP induces an array of toxic events, such as depletion of reduced glutathione (GSH) and protein thiols, peroxidation of membrane lipids, DNA strand breakage, significant cellular ATP depletion, an alteration in intracellular calcium homeostasis and Ca^{2+}-signalling and a loss of mitochondrial membrane potential. As a result of these events, cell necrosis is necessitated (Lapshina et al, 2005).

In addition, the caco-2 cell monolayer model has been used to study intestinal transport system. The model is widely used in intestinal transport studies of drugs and food
compounds (Augustijns et al, 1998; Boisset et al, 2000; Rubio and Seiquer, 2002). This confluent caco-2 cell monolayer, has characteristics that closely resemble intestinal epithelial cells and displays several properties typical of differentiated intestinal epithelial cells (Wilson et al, 1990). Caco-2 cell monolayers are thought to be tighter than mammalian intestinal tissues (Boisset et al, 2000) and the brush border membrane-associated enzyme activities are generally thought to be lower (Bolte et al, 1998). Small peptides (primarily di- and tripeptides) and amino acids are both absorbed through various systems that include the energy-dependent transport linked with the co-transport of Na⁺ (Webb, 1990), specific peptide transport system PepT1 (Yang et al, 1999) paracellular, passive diffusion, endocytosis and carrier mediated transport (Gardner, 1998).

The human endothelial-like immortalised cell line Ea.hy926, derived from the fusion of HUVEC (human umbilical vein endothelia cells) with the lung carcinoma cell line A549, is a human endothelial cell line that shows characteristics of differentiated endothelial cells (Edgell et al, 1983). Endothelial cells (EC) express constitutively the NADPH oxidase enzyme system, which by generating reactive oxygen species (ROS) such as O₂⁻ and H₂O₂, are involved in the regulation of cellular redox-signalling. NADPH oxidase activation and increased ROS production are implicated in Angiotensin II-dependent hypertension and the associated endothelial dysfunction (Wang et al, 2001).
This study aims to investigate the antioxidant behavior of peptides in caco-2 cells exposed to prooxidant t-BHP, scavenging of ROS in Ea.hy 926 exposed to angiotensin II, and absorption of peptides in a caco-2 cell monolayer.

5.2. MATERIALS AND METHODS

5.2.1. Materials

Human colorectal carcinoma (caco-2) cells lines were obtained from the European Collection of Cell Cultures (ECACC). The human endothelial-like immortalised cell line Ea.hy926 was a donation from the nutrition group, FHMS, University of Surrey, UK. Phosphate buffered saline (PBS) was obtained from Oxoid (Hampshire, UK); foetal bovine serum (FBS), trypsin EDTA solution, non-essential amino acids, Dulbecco’s Modified Eagle Media (DMEM), Hanks buffer saline solution (HBSS), glutamine, penicillin/streptomycin and non essential amino acids (NEAA) were obtained from Invitrogen (Paisley, UK). Tert-butyl hydroperoxide (tBHP), 2-Amino-7-dimethylamino-3-methylphenazine hydrochloride (MTT) dye, dimethyl sulphoxide (DMSO) were obtained from Sigma-Aldrich (Poole, UK). Caspase 3/7 reagent was obtained from Promega, Southampton, UK.
5.2.2. Methods

5.2.2.1 Isolation and purification of peptide

The peptide (LC1-Z) was isolated as described in section 3.2

5.2.2.2. Antioxidant activity of peptide in caco-2 cells

5.2.2.2.1. Cell viability of caco-2 cells treated with peptide.

Caco-2 cells were cultured in Dulbecco’s Modified Eagle Media (DMEM) supplemented with 20 % heat-inactivated foetal bovine serum (FBS), 5 % glutamine, 5 % penicillin/streptomycin and 5 % NEAA. The cells were grown in a 25-cm² tissue-culture flask (Corning Co., Cambridge, MA) at concentrations 1 x 10⁶ cells/mL and sub cultured every 48 hours, at 37 °C in 5 % CO₂/95 % air in a humidified incubator. Cells were harvested from the tissue-culture flask by trypsinization (1ml trypsin/EDTA) after a 5 ml PBS wash, counted and seeded in a 96-well plate at 2 x 10⁴ cells/well. After 24 hours, 1.0 mg/ml peptide in DMEM without FBS was added to the wells. Plates were incubated for 24 hours at 37 °C in 5 % CO₂/95 % air. Following removal of media from wells, cells were washed with PBS. MTT (5 μL of 10 mg/mL PBS) was added to each well. Following an additional 4 h of incubation at 37 °C, dimethyl sulfoxide (DMSO) reagent (150 μL) was added to dissolve the formazan crystals. The plates were shaken at...
37 °C for 20 min and the absorbance was read at 492 nm using a plate reader (Behring Co., Marburg, Germany). Standards of trolox, BHT and EGCG were also treated in the same way.

5.2.2.2.2. Treatment of caco-2 cells with peptides and tert-butyl hydroperoxide (tBHP).

From the cell suspension, caco-2 cells were counted, and seeded in a 96-well plate at 2 x 10⁴ cells/well. After 24 hours, 1.0 mg/ml peptide in DMEM without FBS was added to the wells. Plates were incubated for 24 hours at 37 °C in 5 % CO₂/95 % air. Following removal of media from wells, cells were washed with PBS and then subjected to oxidant stress by incubation with 200 μL of 2.5 mM tert-butyl hydroperoxide (t-BHP) for 150 min.

To determine the effect of antioxidative peptides on t-BHP-induced injury, MTT assay of cell viability was carried out as described by Sladowski et al. (1993). After 150 min of incubation with t-BHP, cells in 96-well plates were rinsed with PBS. MTT (5 μL of 10 mg/mL PBS) was added to each well. Following an additional 4 h of incubation at 37 °C, dimethyl sulfoxide (DMSO) reagent (150 μL) was added to dissolve the formazan crystals. The plates were shaken at 37 °C for 20 min and the absorbance was read at 492 nm using a plate reader (Boehringer Co., Marburg, Germany). A control (without tBHP) and a standard with trolox were also treated in the same way.
5.2.2.3. TBARS formation in caco-2 cells

The formation of TBARS (level of lipid peroxidation) in control and treated cell samples was measured according to the method of Ohkawa et al. (1979) with slight modifications. A caco-2 cell suspension was prepared as in 5.2.2.1.1 and seeded at 1 x 10^6 cells in 25 cm^2 cell culture flasks. After reaching confluence (80%), the mono-layer of caco-2 cells were treated with peptide (1 mg/ml) for 24 h. After incubation, DMEM was removed and cells were washed twice with PBS and then treated with 2.5 mM tBHT for 2.5 hours. After washing with PBS, the cells were scraped and lysed with 20 % trichloroacetic acid (TCA). Then, 2 ml of 0.7 % TBA were added and sample heated at 100 °C for 1 hour. After cooling, the solutions were centrifuged at 1500 x g for 10 min. The absorbance of the pink supernatant was measured at 532 nm using a spectrophotometer (UV Mini 1240, Shimadzu Europa, Milton Keynes, UK). Malondialdehyde (MDA) concentration was calculated using 1,1,3,3-tetramethoxypropane as standard and was expressed as µmol of MDA.

5.2.2.4. The Caspase-Glo 3/7 Assay

Caco-2 cells were prepared according to section 5.2.2.2.1. After seeding in a 96 well plate with a white background, the cells were treated with 1 mg/ml peptide for 24 hours followed by 2 hours of tBHP. To the 100 µl tBHP treated cells in each well, 100 µl
caspase 3/7 reagent was added and incubated for 30 minutes. Luminescence was measured using a luminometer (GloMax® 96 Luminometer, Promega, USA).

5.2.2.5. Morphological changes in caco-2 treated with peptide

A caco-2 cell suspension was prepared as in 5.2.2.1.1 and seeded at 1 x 10^6 cells in 25 cm cell culture flasks. After reaching confluence (80%), the mono-layer of caco-2 cells were treated with peptide (1 mg/ml) for 24 h. After incubation, DMEM was removed and cells were washed twice with PBS and then treated with 2.5 mM tBHP for 2.5 hours. The cells were investigated for changes in morphology with a phase contrast microscope (Zeiss Telaval inverted microscopy) fitted with a camera (Nikon, Japan).

5.2.2.6. Reactive oxygen species (ROS) scavenging in Ea.hy926 cells treated with peptides

5.2.2.6.1. Cell culture preparation and cell viability of Ea.hy 926 cells treated with peptides

Ea.hy 926 cells were cultured in glutamax Dulbecco's Modified Eagle Media (Glu-DMEM) supplemented with 10 % heat-inactivated foetal bovine serum (FBS), 5 % glutamine, 5 % penicillin/streptomycin and 5 % NEAA. The cells were grown in a 25-cm^2 tissue-culture flask (Corning Co, Cambridge, MA) at concentrations 1 x 10^6 cells/mL and subcultured every 48 hours, at 37 °C in 5 % CO_2/95 % air in a humidified
incubator. Cells were harvested from tissue-culture flask by trypsinization (1 ml trypsin/EDTA) after a 5 ml HBSS wash, counted, and seeded in 96-well flat bottomed plates at different cell concentrations (1 x 10^4 to 5 x 10^4 cells/well). After 24 hours, 1.0 mg/ml peptide in Glu-DMEM without FBS was added to the wells. Plates were incubated for 17 h at 37 °C in 5% CO₂/ 95 % air in a humidified incubator. Following removal of media from the wells, cells were washed with HBSS. The MTT assay of cell viability was carried out as described by Sladowski et al (1993). After 17 hours, 5 µL MTT (10 mg/mL in PBS) was added to each well for 4 hours. The cells were washed with HBSS and 150 µL dimethyl sulfoxide (DMSO) reagent was added to dissolve the formazan crystals. The plates were shaken at and the absorbance was read at 492 nm using a plate reader (Behring Co, Marburg, Germany).

5.2.2.6.2. Lucigenin-enhanced chemiluminescent measurement of ROS

Specific superoxide (O₂⁻) production was assessed by lucigenin chemiluminescence assay as described previously by Li et al (1998) with some modifications. Ea.hy 926 cells were cultured in 25 cm² tissue flask until confluent. The cells were treated with peptide (1 mg/ml) in FBS free media with and without 100 nM angiotensin II for 1 h, washed twice with HBSS and detached using trypsin. The cells were resuspended in FBS free medium followed by sonication. The cells were redistributed at 5 x 10^4 in 96 well plates with a white background, and immediately 200 µM lucigenin added. Light
emission was read using the luminometer (GloMax® 96 Luminometer, Promega) and expressed as chemiluminescence arbitrary light units.

5.2.2.6.3. ROS measurement by fluorescence method with dihydroethidium (DHE)

Ea.hy 926 cells were cultured in 25 cm² tissue flask until confluent. FBS free DMEM was added to the flask a day before the treatments. Cells were then treated with peptide (1 mg/ml) in FBS free DMEM, and with and without 100nM angiotensin II for 1 h. The cells were washed with HBSS, and 25 μM 2-hydroxyethidium was added and the cells were incubated for 20 min. The cells were then washed with HBSS and again incubated with HBSS for 1 h. The cells were then scraped with 500 μl cold methanol, homogenized and filtered on a 0.22 μm membrane filter. Ethidium and 2-hydroxyethidium (DHE) were detected with a fluorescence detector using an emission wavelength of 580 nm and an excitation of 480 nm using a fluorimeter (Varian, USA).

5.2.2.7. Peptide transport in a caco-2 monolayer model

5.2.2.7.1. Caco-2 monolayer preparation

Caco-2 cells were cultured in Dulbecco’s Modified Eagle Media (DMEM) supplemented with 20 % heat-inactivated foetal bovine serum (FBS), 5 % glutamine, 5 % penicillin/streptomycin and 5 % NEAA. The cells were grown in a 25-cm² tissue-culture flask (Corning Co., Cambridge, MA) at a concentration 1 x 10⁶ cells/mL and
subcultured every 48 h, at 37 °C in 5 % CO₂/95 % air in a humidified incubator. Cells were harvested from the tissue-culture flask by trypsinization (1 ml trypsin/EDTA) after a 5 ml PBS wash. The suspended cells were seeded onto 0.3 μm pore, 1.2 cm diameter polycarbonate cell culture inserts with filters (Transwell, Costar) at 1.0×10⁶ cells/cm² in 12-well plates. The cells on inserts were maintained in a humidified atmosphere of 5% CO₂-95% air at 37 °C and DMEM was changed every two days after seeding. Cell confluence was monitored by transepithelial electrical resistance (TEER) measurements using a TEER EVOM meter (World Precision Instrument, USA). The TEER value is an indication of the integrity of the alveolar epithelial cell monolayers. The cells became completely confluent on day 5 of cultivation and were used for transport experiments on day 7 with TEER values greater than 200 Ω.

5.2.2.7.2. Peptide transport in caco-2 monolayer model

Transport experiments were carried out using PBS as the transport medium. The cell monolayers were washed twice with PBS and the apical (A) and basolateral (B) compartments of the filter cups were filled with 0.75 ml of transport medium. The cells were equilibrated in a 5 % CO₂/95 % air atmosphere at 37 °C for 1 h. Stock solutions of the peptide (1 mg/ml) were placed in either apical or basolateral reservoir and allowed to stand in a humidified atmosphere of 5 % CO₂/95 % air at 37 °C for 180 min. The TEER across each monolayer was measured at the beginning and after 180 min.
The apparent permeability coefficient (Papp, cm/s) for peptide was calculated using the following equation.

\[ P_{\text{app}} = V_r \times \frac{dC}{dt} \times \frac{1}{A_{\text{Co}}} \]

Where \( V_r \) is the volume of the receiver chamber, \( \frac{dC}{dt} \) is the solute transfer rate, \( A \) is the surface area of the membrane (1.2 cm\(^2\); the normal surface area of the Transwell filter), and \( C_0 \) is the initial concentration of solute (peptide).

5.2.2.7.3. HPLC of absorbed peptide in caco-2 monolayer model

From both the apical and basal side of insert from each well, 300 \( \mu \)L was collected at 180 min and put in vials. Each sample was then run on a reversed-phase high-performance liquid chromatograph (RP-HPLC) on a Phenomenex C18 (3.0 mm x 250 mm) column with a linear gradient of acetonitrile (0-70 % in 50 min) containing 0.1 % trifluoroacetic acid (TFA) and milliQ water containing 0.1 % TFA at a flow rate of 1 mL/min. RP-HPLC analysis was performed on the HPLC system comprising a pump (Thermoseparation Products), autosampler (Spectra Physics AS1000), detector (Thermoseparation Products UV6000LP) and integration software (Chromquest, 2004).
5.2.3. Statistical analysis

Statistical analyses were performed with Minitab statistical software (13.1). Comparisons were made by one-way analysis of variance or Student’s t test where appropriate. P < 0.05 was considered statistically significant.

5.3. RESULTS AND DISCUSSION

5.3.1. Antioxidant activity of peptide in caco-2 cells

5.3.1.1 Cell viability of caco-2 cells treated with peptide

The MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolioum bromide] cell proliferation and viability assay is a safe, sensitive, \textit{in vitro} assay for the measurement of cell proliferation or, when metabolic events lead to apoptosis or necrosis, a reduction in cell viability. Since proliferating cells are metabolically more active than non-proliferating (resting) cells, the assay is suitable not only for the determination of cell viability and factor mediated cytotoxicity but also for the determination of cell activation and proliferation (Sladowski \textit{et al}, 1993).

The principle of this assay is that the tetrazolium salt MTT is cleaved to formazan by the succinate-tetrazolium reductase which belongs to the mitochondrial respiratory chain
and is active only in viable cells. MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals which are solubilized and read spectrophometrically. The rate of tetrazolium reduction is proportional to the rate of cell proliferation. In this study, the proliferative status of caco-2 cells was assessed by the metabolic activity of viable cells using the MTT assay.

Figure 5.1 shows the viability of caco-2 cells treated with the peptide and standards. Caco-2 cells were prepared and treated with 1.0 mg/ml and 0.01 % of trolox, BHT and epigallocatechin gallate (EGCG). Cell viability is expressed as the percentage of the proliferating cells in respective treatments based on the control and values expressed as the mean ± % CV. At these concentrations, there was no difference observed between the control (100 %), peptide (96.6 %) and trolox (95.7 %) in the cell viabilities of the caco-2 cells (p<0.10). However, a significant difference (p<0.0004) was observed with BHT (81.9 %) and EGCG (80.4 %) where a marked reduction in the cell viability was observed in those treated with BHT and EGCG compared to those treated with trolox. This demonstrates that the peptide in this concentration range and under the study conditions are not toxic to caco-2 cells, and neither do they have a proxidant activity on caco-2 cells. Some phenolic based antioxidants are reported to have proxidant activity in higher concentrations and in these experiments BHT and EGCG may have had proxidant activity judging by the reduced cell viability (Alghazeer et al, 2008). Due to the observed effects of 1.0 mg/ml of peptide and 0.01 % trolox, these concentrations were chosen for antioxidant activity studies of the peptide in caco-2 cells.
Figure 5.1. Cell viability of caco-2 cells treated with peptide (1.0 mg/ml) and 0.01% of trolox, BHT and EGCG. Each value represents means ± % CV (n=3).

5.3.1.2. Antioxidant activity in caco-2 cells treated with tert-butyl hydroperoxide (tBHP)

Peroxides including tBHP are known to have cytotoxic effects on several types of mammalian cells after penetration (Makino *et al*, 1995). With tBHP, the proposed mechanisms of induced toxicity include decomposition of organic peroxide by heme-containing proteins, membrane alterations, lipid-peroxidation, GSH depletion, and damages to cell proteins (protein-bound carbonyl formation and protein thiol oxidation), as well as arachidonic acid cascade mobilization and DNA damage (Lapshina *et al*, 2005). In this study, tBHP induced cytotoxic effects in caco-2 cells are seen from the
reduced cell viability (Figure 5.2). When caco-2 cells were treated with different concentrations of 0.5, 1.0, 5.0 and 10 mM tBHP, there was a marked reduced viability of 88.64, 78.18, 60.34 and 52.50% respectively (Figure 5.2). There was a significant difference (p<0.0001) in the caco-2 cells viability when treated with the different tBHP concentrations in a dose dependent manner (y = -28.39 + 79.32, R² = 0.9973). Therefore, 2.0 μM tBHP concentration was required to effect 30% reduction in cell viability and this concentration was used in preceding experiments on antioxidant activity of the peptides.

Figure 5.2. Proliferation of caco-2 cells incubated with t-BHP for 150 min at different concentrations. Each value represents means ± % CV (n=6).
In experiments to see the effect of peptides on caco-2 cells in the presence of an oxidant (tBHP), caco-2 cells were prepared and incubated with 1.0 mg/ml of peptide for 24 h and then with 2.5 mM tBHP for 2.5 h. In cells treated with tBHP alone, the cell viability reduced significantly (79.3 %) but in the presence of the peptide and/or trolox no significant reduction was observed (94.7 % and 90.2 % respectively) (Figure 5.3). It is clear that the peptide provided some protective effects against the prooxidant effects of tBHP and consequently the viability of the caco-2 cells. This shows that when cells are exposed to oxidants such as tBHP, cell death is induced, but in the presence of the peptide the cytotoxic effect of tBHP is reduced, due to the antioxidant behavior of the peptides. The cytotoxic effects of tBHP occur through the generated hydroperoxide radical. In the presence of the peptide, the radical is believed to be scavenged and hence its ability to oxidize cell components and membrane lipids is avoided. In this way the integrity of the cell is maintained and cells proliferate normally. Since peptides are absorbed across caco-2 cell membranes (Lapshina et al, 2005; Shimizu et al. 1997; Makino et al, 1995), and from our own studies (section 5.3.3), the antioxidant protection can be presumed to occur both inside and outside the cell, thus offering protection to cell constituents such as DNA and at the surface of the cell membrane. These results concur with those observed with peptides isolated from Alaska pollack (Kim et al, 2001), hoki fish (Kim et al, 2007), and giant squid (Rajapakse et al, 2005).
5.3.1.3. TBARS formation in caco-2 cells

In vivo studies of tBHP show that it is a membrane permeable oxidant and can be easily metabolized into free radical intermediates forming ROO$^*$ and RO$^*$ that initiates oxidation of membrane lipids and consequently forms TBARS (Barr and Mason, 1995). From figure 5.4, it can be seen that more TBARS ($p<0.05$) were formed in cells treated with tBHP (38.18 $\mu$g/ml) than in those treated with the peptide (33.18 $\mu$g/ml). As tBHP forms covalent bonds with cellular molecules and consequently oxidise membrane lipids, the cell integrity is compromised resulting in t-BHP-induced toxicity and cell death. Therefore the protective effect of the peptide from prooxidant tBHP is presumed to be due to its ability to scavenge lipid-derived radicals that would otherwise oxidize PUFAs in cell membranes. This effect is considered to be similar to the action of a common lipid soluble antioxidant vitamin E to inhibit radical-mediated membrane lipid oxidation in human umbilical vein endothelial cells (HUVEC) (Shimpuku et al, 2000).

On the other hand, Rajapakse et al (2005) attributed the antioxidant activity of a peptide derived from giant squid in human embryonic lung fibroblast to its size. The research showed that a lower molecular weight improves contact ability with membrane lipids and/or permeability. The peptide isolate in this study was fractionated on a less than 2 kDa MWCO membrane and therefore fits with the theory that the observed antioxidant activity in caco-2 cells is an element of its size.
Figure 5.3. Cell viability of caco-2 cells treated with 1 mg/ml peptide (LC1-Z) and 0.01% trolox, with/without tBHP (2.5mM). Each value represents means ± % CV (n=3).

Figure 5.4. TBARS as Malondialdehyde formed in caco-2 cells incubated with 1mg/ml peptide (LC1-Z) and with/without tBHP.
5.3.1.4. Caspase activity in caco-2 cells treated with tBHP

Necrosis and apoptosis are two types of cell death distinguished in mammalian cells. Necrosis is characterized by cell swelling and disruption of cell membrane leading to the release of cellular components and consequently into an inflammatory response and reduction of intracellular glutathione content (Girotti, 2001; Saiato et al, 2005). Apoptosis is the process of programmed cell death that may occur in multicellular organisms and involves a series of biochemical events that lead to a variety of morphological changes, including blebbing, changes to the cell, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Apoptosis is triggered by a variety of stimuli, including cell surface receptors, mitochondrial response to stress, and cytotoxic T cells. Caspases are a class of cysteine proteases that includes several representatives involved in apoptosis. The caspases convey the apoptotic signal in a proteolytic cascade, with caspases cleaving and activating other caspases that then degrade other cellular targets that lead to cell death. The caspases at the upper end of the cascade include caspase-8 and caspase-9. Caspase-3, -6 and -7 are downstream caspases that are activated by the upstream proteases and act themselves to cleave cellular targets (Muzio, 1998). This caspase family plays a key effector role in apoptosis in mammalian cells (Garcio-Calvo et al 1999; Mooney, et al, 2002).

In this study, the Caspase-Glo 3/7 Assay, a homogeneous, luminescent assay that measures caspase 3 and 7 activities was used to measure apoptosis. The assay provides a
luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity, and cell lysis. Adding a single Caspase-Glo 3/7 Reagent in an add-mix-measure format results in cell generation of a glow-type luminescent signal, produced by luciferase. The measured luminescence is proportional to the amount of caspase activity present.

Therefore, to verify apoptosis associated with caspase 3 and 7, we determined the activity in caco-2 cells. Table 5.1 shows that caspases 3 and 7 activity was 6 times more in the cells treated with tBHP only (157.5 ± 7.99 %) compared to those treated with the peptide (25.7 ± 3.92 %). Wang et al (2000) have reported that redox perturbation, induced by subtoxic levels of lipid hydroperoxide, leads to activation of caspase 3, oxidative DNA damage, and enhanced apoptosis. Therefore, in this study, the observed caspase activity is as a result of peroxide tBHP induced apoptosis and is linked to DNA damage. As tBHP is membrane permeable, it is prone to attack by cellular components that include DNA. DNA damage, and in particular apoptosis, was impaired by the peptide as can be seen by the caspases 3 and 7 activity observed in the cells treated with the peptide. Similar results are reported with polyphenol extracts and it is believed that phenolic substances with antioxidant activity have a protective effects from oxidants against DNA damage induced by \( \text{H}_2\text{O}_2 \) (Giovannelli et al, 2000). However, the mechanism of DNA protection is not clear (Wang et al, 2000).
Table 5.1 Caspase activity in caco-2 cells treated with peptide (LC1-Z) in the presence of tBHP. Each value represents means ± % CV (n=3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Caspase 3/7 activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td>38.5 ± 6.83</td>
</tr>
<tr>
<td>Peptide + tBHP</td>
<td>25.7 ± 3.92</td>
</tr>
<tr>
<td>tBHP</td>
<td>157.5 ± 7.99</td>
</tr>
</tbody>
</table>

5.3.1.5. Morphological changes in caco-2 treated with peptide

The protective effects of the peptide from the oxidant activity of tBHP were investigated by microscopy. Figure 5.5 shows the morphological modification in the caco-2 cells treated with tBHP (D) where the cells are detached from the flask surface, indicating cell death due to the cytotoxic effect of tBHP. On the other hand, cells that were treated with the tBHP in the presence of peptide (Figure 5.5 C) appear healthy and were attached to the flask surface and compared well to the control (A) and to those cells treated with peptide only (B). This observation further confirms the protective effects of the peptides on cells subjected to oxidants like peroxides.
Figure 5.5. Caco-2 cells control (A), and those treated with peptide (B), peptide and tBHP (C) and tBHP only (2.5mM).
5.3.2. Reactive oxygen species (ROS) scavenging in Ea.hy926 cells treated with peptide

Figure 5.6 shows the viability of Ea.hy 926 cells treated with the peptide and the control (no peptide). Ea.hy 926 cells were seeded at different concentrations in a 96 well plate and treated with 1.0 mg/ml. The cell viability is expressed as the absorbance of the proliferating cells at respective cell seeding concentrations and values are expressed as the mean ± SD. At these seeding concentrations, there were no differences observed in the cell viability of the peptide and control (p< 0.01). These results show that the peptide at this concentration was not cytotoxic to the Ea.hy 926 cells and therefore this peptide concentration was used further in ROS scavenging studies in Ea.hy 926 cells.
Figure 5.6. The cell viability of Ea.hy 926 treated with peptide (LC1-Z) and control. Data points are means ± SD, n=3

One mechanistic pathway in which the intracellular reactive oxygen species (ROS) are produced is through the signal transduction of Angiotensin II-dependent cellular responses via activation of redox sensitive signalling cascades. In endothelial cells, this activation is through a phagocyte-type NADPH oxidase enzyme system that is relatively active but is stimulated acutely by agonists such as Angiotensin II and cytokines (Li and Shah, 2003). The ROS produced in this cascade are the electron-reduction product superoxide \( \left( \text{O}_2^+ \right) \) and the electron-reduction product hydrogen peroxide \( \left( \text{H}_2\text{O}_2 \right) \). These serve as progenitors for other ROS that consequently leads to oxidative stress. Numerous methods are utilised for ROS detection, each with potential pitfalls and advantages. All of these techniques can yield errors, and it is advisable to use at least 2 methods to avoid...
an erroneous conclusion (Dikalov et al, 2007). In this study, the lucigenin-chemiluminescence and fluorescence methods were utilized to measure ROS in endothelial cells, Ea.hy 926. In the lucigenin-chemiluminescence method, on exposure to superoxide ($O_2^{*-}$), a chemiluminescent probe (lucigenin) releases a photon that is detected by a luminometer. Since most of these compounds are cell permeable, the $O_2^{*-}$ measured reflects the extracellular, as well as intracellular, $O_2^{*-}$ production. In the fluorescence method, the reaction of dihydroethidium with $O_2^{*-}$ yields a very specific product; 2-hydroxyethidium can then be used to estimate the intracellular production of $O_2^{*-}$.

In this study, both methods showed that the treatment of Ea.hy 926 cells with angiotensin II resulted in increased ROS production (figure 5.7 and 5.8). When the Ea.hy 926 cells were stimulated with angiotensin II and later treated with the peptide, $O_2^{*-}$ were reduced by 26 % and 39 % in the lucigenin-chemiluminescence and fluorescence methods respectively. This study postulates that the reduction in $O_2^{*-}$ is due to a scavenging mechanism as several in vitro studies show that some peptides with particular amino acid sequences have superoxide radical scavenging activities (Rajapakse et al, 2005; Je et al, 2007; Bougatef et al, 2009; Jung et al, 2007). This shows the potential of the isolated peptide to impact positively on oxidative stress in endothelial cells through superoxide radical scavenging. However, the scope of this study has not shown the possible mechanism(s) in endothelial cells to explain the reduced $O_2^{*-}$ and this is worth exploring in further studies.
Figure 5.7. ROS production in Ea.hy 926 cells in the presence of peptide (LC1-Z) and angiotensin II (Ang II) by the lucigenin-chemiluminescence method. Each value represents means ± SD (n=3).

Figure 5.8. ROS production in Ea.hy 926 cells treated with peptide (Pep) and Angiotensin II (Ang II) by the DHE fluorescence method. Each value represents means ± SD (n=3).
5.3.3. Peptide transport in a caco-2 monolayer

Culturing of caco-2 cells on a semipermeable filter inserts for between 5 – 7 days resulted in the successful formation of a monolayer with transepithelial electrical resistance (TEER) values above 200 ohm (Ω) x cm². Therefore peptide transport studies were done in inserts that had a TEER value higher than 200 Ω. The peptide (1mg/ml) was added to either side, apical and/or basolateral (donor side); transepithelial transport was expressed as apparent permeability and the ratio of the peak areas of the peptide from recipient and donor side (apical to basolateral and basolateral to apical) was determined. The basolateral to apical flux of 0.95± 0.12 cm s⁻¹ of the peptide was slightly higher (p< 0.01) than that of the apical to basolateral flux of 0.74 ± 0.20 cm s⁻¹ (Table 5.2).

Table 5.2. Apparent permeability of peptide in a caco-2 monolayer.

<table>
<thead>
<tr>
<th>Movement of peptide</th>
<th>Apparent permeability* (cm s⁻¹)</th>
<th>% Ratio of Peak areas*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical/basolateral</td>
<td>0.74 ± 0.20</td>
<td>11.5</td>
</tr>
<tr>
<td>Basolateral/apical</td>
<td>0.95± 0.12</td>
<td>12.2</td>
</tr>
</tbody>
</table>

* the ratio of the peak area of the peptide in the recipient side to that of the donor side (where peptide was added). Data are mean ± SD, n=3.

Figure 5.9 shows the chromatographic trace of the buffer taken from the apical and basolateral side respectively when the peptide was added to the apical side. However,
the apical to basolateral flux and basolateral to apical flux of the peptide is not significantly different as the ratio of the peak areas (recipient/donor) is the same, i.e. 11.5% for apical to basolateral and 12.2 % for basolateral to apical (Table 5.1). These results show that the transport of the peptide in the caco-2 monolayer model is not unidirectional.

Figure 5.9. Chromatogram of peptide added to apical side of insert. Black line represent the apical side, blue line represents the basolateral side.
Three mechanisms for intestinal transport of oligopeptides are reported namely a) through the transporter (PepT1) - mediated transport for di and tripeptides b) transcytosis and c) paracellular transport through the intercellular junctions. These transport systems, together with their respective enzyme systems, are all present in caco-2 cells, making the caco-2 cell model an ideal one for transport studies (Hidalgo et al, 1989). It has been demonstrated that Caco-2 cells express at least 8 aminopeptidases (Howell et al, 1992). Satake et al (2002) has reported that the paracellular route is the favoured for intestinal absorption for intact Val-Pro-Pro and that the transport is strongly dependent on their structure and properties.

The susceptibility to cellular peptidases is an important factor in determining the bioavailability of the peptide and hence a peptide which is highly susceptible to surface peptidases will have negligible transepithelial transport. Therefore, the digestion by cellular peptidases is the primary factor affecting intact peptide flux across the epithelial cell layer. In this study, we observed the presence of the intact peptide in the basolateral side when the peptide was added to the apical side as evidenced by the same retention times in the HPLC chromatograms (Figure 5.10). We therefore postulate that the peptide isolated in this study is probably transported intact across the caco-2 monolayer and to some degree survives the caco-2 endopeptidases. This study therefore infers that this peptide produced using gastrointestinal enzymes has the potential to impart its antioxidant bioactivity in vivo.
5.4. CONCLUSIONS

This study showed that peptides from pepsin and pancreatin digestion of fish muscle in caco-2 cells possess antioxidant activity and in the presence of peroxides, offer protection to caco-2 cells against apoptosis and necrosis. This protection was observed by a significant proliferation of the caco-2 cells treated with peptides in the presence of oxidants tBHP. In Ea.hy 926 endothelial cells, the isolated peptide scavenged ROS after stimulation with angiotensin II. This study also showed that the isolated peptide can survive the endopeptidases of the caco-2 monolayer and be absorbed across the caco-2 monolayer.
CHAPTER SIX
6. GENERAL DISCUSSION AND CONCLUSIONS

6.1. Atlantic mackerel fish protein fractionation and peptide purification

The consumption of fish has been linked to a number of health benefits as a result of its unique content of proteins, omega-3 oils, vitamins and minerals. Atlantic mackerel is a particularly good source of lipids and proteins, and forms an important part of people's diets especially in the northern hemisphere countries near the Atlantic Ocean. The proximate composition of Atlantic mackerel muscle was 19.9% protein, 3.7% oil, and 73.7% water.

The aim of this study was to investigate the potential positive effects of Atlantic mackerel by investigating particularly the bioactivity of peptides derived from its muscle protein. Bioactive peptides are released from the protein by hydrolysis, and when absorbed, exert a biological function in the human body and impact on the health of the individual (Korhonen and Pihlanto, 2003, Yoshikawa et al., 2000). Many different enzyme combinations have been used to produce bioactive peptides from fish proteins (Howell and Kasase, 2009). In the present study, the muscle only (flesh) of Atlantic mackerel was hydrolysed with gastrointestinal enzymes, pepsin and pancreatine (mixture of trypsin and chymotrypsin). A combination of these digestive enzymes has seldom been used to obtain peptides to investigate ACE inhibition and antioxidant activity. The resultant hydrolysate was sequentially fractionated on 10, 5 and 2 kDa molecular weight cut off (MWCO) membrane ultrafilters and the ACE inhibition and antioxidant activities were determined separately, as discussed below. For both
activities, the fractions collected from the less than 2 kDa membrane filter exhibited the highest activities. This fraction was then purified by gel filtration, ion exchange and high performance liquid chromatography to identify the peptide exhibiting highest ACE inhibition and antioxidant activity. The amino acid composition of the isolated peptide was then determined by HPLC.

6.2. Angiotensin converting enzyme inhibitory activity of peptide isolated from Atlantic mackerel

The ACE inhibitory activity of fractionated ultrafiltration membrane fractions < 2 kDa (MFPH-V), 3-5 kDa (MFPH-III), 5-10 kDa (MFPH-II) and crude enzymatic hydrolysate (MFPH-I) were determined and found to be 95.0, 52.7, 33.3 and 26.6 % respectively. The < 2 kDa (MFPH-V) fraction showed the highest ACE inhibitory activity and was consequently further purified by gel filtration chromatography with Sephadex G-25 (Figure 2.1). The MFPH-V fraction showed 19 peptide fractions and when analysed for their ACE inhibitory activity, (MFPH-V-J) showed the highest ACE inhibitory activity of 36.09 % (Figure 2.2). This fraction (MFPH-V-J) was subjected to ion exchange chromatography and 16 fractions were identified and analysed for ACE inhibitory activity (Figure 2.3). The fraction P MFPH-V-JP) showed the highest ACE inhibitory activity of 86.1 %. This fraction was purified by liquid chromatography and 5 fractions that showed ACE inhibitory activity were identified. The fraction with highest ACE inhibition with activity of 34.9 % was fraction denoted MFPH-V-JPA. This fraction was
subjected to HPLC analysis for amino acid composition and contained the amino acids – histidine, proline, tyrosine, methionine, leucine, tryptophan and lysine.

The inhibitory potential was quantified through the inhibitory concentration (IC$_{50}$), i.e. the concentration required to inhibit 50% of the enzyme. The (IC$_{50}$) values of MFPH-V, MFPH-V-J, MFPH-V-JPA and MFPH-V-JPA2 were significantly different (p>0.0001) and were found to be 2.76, 0.43, 0.36 and 0.15 mg/ml respectively. This is the first time that a peptide with this IC$_{50}$ has been obtained from Atlantic mackerel with gastrointestinal enzymes hydrolysis and is comparable to 2 peptides CWLPVY (0.019 mg/ml, 22.2μM) and SKVPP (0.106 mg/ml, 74.22 μM) from tuna fish with trypsin, chymotrypsin, pronase and pepsin enzymatic hydrolysis (Astawan et al, 1995); 0.10 mg/ml for an hydrolysate from mackerel (Itou and Akahane, 2004), 0.53 mg/ml for a seabream hydrolysate and 0.027 mg/ml for a salmon hydrolysate (Fahmi et al, 2004).

The inhibition of ACE by peptides is strongly influenced by the amino acid sequence of the C-terminal tripeptide, hydrophobicity in the C-terminal tripeptide and size of the peptide. Cheung et al. (1980) suggested that the most favorable amino-terminal residues are branched amino acids such as Val and Ile and that the most preferred C-terminal residues are among Trp, Tyr, Pro, or Phe. Peptides with hydrophobic amino acids in the C-terminal tripeptide are reported to be potent inhibitors (Wu et al, 2006a, Wu et al, 2006b). The presence of a positive charge as in the guanidine group of the C-terminal tripeptide also contributes to the ACE inhibitory potency of several peptides. Therefore, the observed ACE inhibitory activity of the isolated peptide (MFPH-V-JPA2) can be
attributed partly to the presence of proline and tyrosine, hydrophobic amino acids in the C-terminal tripeptide. A number of peptides from fish sources with proline in the C-terminal tripeptide have also been reported and show a range of IC$_{50}$ values; FRVFTPN (0.01 mg/ml/9.59 μM), SKVPP (0.044 mg/ml/74.22 μM), YRPY (0.21 mg/ml/320 μM), FRVFTPN (0.01 mg/ml/9.59 μM) and MIFPGAGGPEL (0.029 mg/ml) Astawan et al, 1995; Fugita et al, 2000; Matsumura et al, 1993; Jung, et al, 2006). It appears indeed that the position of the proline residue is just one factor that determines the ACE inhibitory activity of these peptides.

Obtaining peptides with ACE inhibitory bioactivity and determination of the amino acids sequence can help to further understand the mechanisms through which the sequenced peptides exert the bioactivity. Peptides inhibit the ACE either by binding to the active site (competitive inhibition) or to another site on the enzyme (non-competitive inhibition). In this study, the mechanism of ACE inhibition by the isolated peptide was characterised through Lineweaver-Burk plots and the isolated peptide showed competitive inhibition. In the RAS, the inhibition of ACE by peptides leads to the reduction in blood pressure. Hydrolysates from tuna and mackerel with IC$_{50}$ values of 0.029 mg/ml (Itou and Akahane, 2004) and 0.63 mg/ml (Astawan et al, 1995) respectively have been shown to reduce systolic blood pressure in SHR. The peptide isolated in this study with an IC$_{50}$ value of 0.15 mg/ml may potentially reduce SBP in SHR.
6.3. Antioxidant activity and mechanism of isolated peptide from Atlantic mackerel

When lipids are oxidized, peroxides and hydroperoxides are formed as primary products which are then transformed into carbonyl secondary products also referred to as thiobarbituric acid reactive substances (TBARS) that include Malondialdehyde (MDA). Therefore, peroxides/hydroperoxides and TBARS are considered as biomarkers of lipid peroxidation (Liu et al, 1997; Orhan, et al, 2004). In this study, the inhibitory effects of the isolated peptide (LC1-Z) containing amino acids serine, histidine, tyrosine, phenylalanine, tryptophan and lysine, on oxidizing linoleic acid emulsion model system was investigated by monitoring peroxides using the ferric thiocyanate method and MDA by the TBARS method over 7 days. The isolated peptide showed a marked reduction in the formation of both primary and secondary oxidation products from linoleic acid. The formation of both peroxides and MDA was markedly and effectively inhibited in the presence of the isolated peptide (LC1-Z). The trend in the inhibition of linoleic acid oxidation was similar to that of the antioxidant standards, 0.01 % butylated hydroxytoluene (BHT) and 0.01 % trolox. A dose dependent inhibition of linoleic acid oxidation by the peptide was observed. From this dose dependent trend, an inhibitory concentration (IC \textsubscript{50}) of the peptide of 1.80 mg/ml assay volume \( (y = 8.254x + 35.239, R^2 = 0.85) \) was observed. However, at a peptide concentration of 8 mg/ml, the inhibition of linoleic acid oxidation inhibition is markedly more than \( (p<0.001) \) BHT and trolox (0.01 %) at concentrations normally used in food systems, indicating the potential of using bioactive fish peptides as natural antioxidants.
The lipid oxidation inhibitory ability and mechanism of inhibition of the peptide is attributed to the type of amino acids present in the peptide, sequence of amino acids and size of peptide. The presence of hydrophobic amino acids such as Gly, Leu, Phe, and Pro has been shown to exhibit higher antioxidative activities against lipid peroxidation (Saiga et al., 2003). The antioxidant activity of peptides containing histidine is attributed to hydrogen donating ability, lipid peroxyradical trapping, and/or the metal ion-chelating ability of the imidazole group (Chan and Decker, 1994). The observed higher antioxidative potency of the peptide in this study can therefore be attributed to the presence of hydrophobicity, which may lead to a higher interaction between the peptide and linoleic acid. In addition to hydrophobicity, the specific positioning of amino acid residues in the peptide sequence plays an important role in the antioxidative activity observed. In residue-activity relationships for synthetic antioxidative peptide mimics in linoleic acid peroxidation systems, a loss of the antioxidative activity was observed after deletion of terminal histidine residue (Chen et al., 1996; Chen et al., 1998).

The mechanism of antioxidant activity of the peptide in this study was investigated and observed to be through carbon centered radical scavenging, hydroxyl radical scavenging, metal chelating and reducing ability. In the carbon centered radical scavenging technique that was used, 1,1-diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. When DPPH radicals encounter a proton-donating substrate such as an antioxidant, the radicals are scavenged and the absorbance is reduced. The reduction in absorbance is used as a measure of the radical-scavenging activity of the antioxidant under study.
(Shimada et al, 1992). The peptide (LC1-Z) (1mg/ml) exhibited radical scavenging activity of 5.34 % and was comparable to that of the 0.01 % BHT (10.03 %) and 7 times more than ascorbic acid (35.2 %). The scavenging of free radicals by antioxidants is reported to be effected via the donation of hydrogen atom. Amino acids histidine, leucine, methionine and tyrosine are known to enhance the radical-scavenging activities of antioxidant peptides (Park et al, 2001, Je et al, 2007). In this context, several peptides isolated from fish proteins, containing these amino acids, have exhibited radical scavenging activities (Je et al, 2005; Mendis et al, 2005; Rajapakse et al, 2005; Jung et al, 2007; Je et al, 2007). Therefore, the observations from this study suggest that the peptides interact with free radicals and protect lipids from oxidation by being preferentially oxidized rather than the lipids. This happens if the available amino acids of the peptide are more oxidatively labile than lipids, or if the physical location of the peptide places itself near the site of free radical generation where the peptide is able to rapidly scavenge the free radical prior to the migration of the radical to lipids (Elias et al, 2008, Saeed et al, 2006).

The hydroxyl radical is very reactive and can be generated in biological systems via the Fenton reaction; it is one of the most active free radicals that attacks all biological molecules by setting off a free radical chain reaction, resulting in cell damage and hence human diseases (Halliwell, 2000). Hydroxyl radicals interact easily with biomolecules such as amino acids, proteins and DNA (Cacciuttolo et al, 1993). In this study, we have shown that the isolated peptide has hydroxyl radical scavenging ability. As the concentration of a scavenging molecule is proportional to the radical scavenging
activity, this scavenging activity of the peptide was linearly expressed ($y = 31.605x - 0.5408, R^2 = 0.9279$) and accordingly an IC$_{50}$ value of 1.60 mg/ml was obtained. Amino acids cysteine, histidine, methionine, tyrosine and phenyalanine are key to the hydroxyl radical scavenging activity of peptides due to their characteristic structure. The imidazole group in histidine has the proton-donation ability (Tsuge et al., 1991), the sulfoxide group in methionine is susceptible to oxidation, and cysteine can donate the sulfur hydrogen (Hernandez-Ledesma et al., 2005). Tyrosine and phenyalanine act positively as direct radical scavengers by the unique ability of their phenolic groups that serve as hydrogen donors (Jung et al., 1995; Rajapakse et al., 2005, Saeed et al., 2006).

Antioxidants can be explained as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species (oxidant) is reduced at the expense of the oxidation of another antioxidant. Therefore, the reducing power assay is often used to evaluate the ability of antioxidants to donate electrons. In this study, the ability of the isolated fish peptide to reduce Fe$^{3+}$/ferricyanide complex to the ferrous form was determined and showed that the reducing power of the peptide increased with increasing concentrations (0 – 25 mg/ml) and that the peptide exhibited reducing ability in a dose dependent manner ($y = 0.0301x + 0.0322, R^2 = 0.9943$). The results propose that the peptide has amino acids that work as electron donors and react with free radicals to form more stable products.

Transition metal ions, such as Fe$^{2+/3+}$, Cu$^{3+}$, Co$^{3+}$, in biological systems affect both the rate of autoxidation and breakdown of hydroperoxide to volatile compounds. Therefore,
chelation of transition metal ions by antioxidant peptides would retard this oxidation reaction. The isolated peptide has demonstrated appreciable iron chelating activity of 5.72 % and is comparable to potent antioxidants 0.01 % trolox (11.11 %) and 0.01 % ascorbic acid (12.67 %). It has been reported that peptide chelators can inhibit lipid oxidation by changing the physical location of transition metals (e.g. partitioning metals away from oxidatively labile lipids or hydroperoxides), forming insoluble metal complexes, reducing the chemical reactivity of transition metals, and/or sterically hindering the interaction of metals and dispersed lipids (Diaz et al, 2003; Diaz and Decker, 2005). Amino acids and peptides are typical metal chelating agents (Fujimoto et al, 1984) and the characteristic amino acid sequence of peptides is important for antioxidant activity (Chen et al, 1995). The antioxidant activity of histidine-containing peptides is related to their metal chelating ability, besides the lipid-radical trapping potential of the imidazole ring (Uchida and Kawakishi, 1992; Murase et al, 1993).

Overall, the observed antioxidant activity and consequent mechanism of antioxidant ability of peptides is generally explained by the peptide comprising amino acids such as histidine, tyrosine, and phenyalanine. The observed antioxidant activity of the peptide isolated from Atlantic mackerel has been reported from other fish sources such as Mackerel (Wu et al, 2003), yellow stripe trevally (Klompong et al, 2007) and round scad (Thiansilakul et al, 2007), yellowfin sole (Jun et al, 2004), Alaska pollack (Je et al, 2005, giant squid (Rajapakse et al, 2005), chum salmon (Nagai et al, 2006), jumbo flying squid (Lin and Li 2006), shell fish (Jung et al, 2007), yellow stripe trevally
(Klompong et al, 2007), walleye pollack (Nagai et al, 2007), tuna (Je et al, 2007), round scad (Thiansilakul et al, 2007) and grass carp (Ren et al, 2008).

6.4. Antioxidant activity of peptides isolated from Atlantic mackerel in epithelial and endothelial cells

Cell proliferation and viability was used to determine the safety and antioxidant activity in epithelial (caco-2 cell line) and endothelial cells (Ea.hy 926 cell line). When Caco-2 cells were treated with either the peptide (LC1-Z) 1.0 mg/ml, 0.01 % trolox, 0.01 % BHT or 0.01 % epigallocatechin gallate (EGCG) there were no differences observed in cell viability between the control (100 %), peptide (96.6 %) and trolox (95.7 %) (p<0.10). However, there was a marked reduction with, a significant difference (p<0.0004), in the cells treated with BHT (81.9 %) and EGCG (80.4 %). This observation indicates that the peptide at this concentration and under the study conditions is not toxic to caco-2 cells and do not have a proxidant effect in caco-2 cells. Some phenolic based antioxidants have been shown to exhibit proxidant activity at higher concentrations (Alghazeer et al, 2008) and in this study BHT and EGCG concentrations used exhibited lower cell viability and could imply that they acted as proxidants.

Peroxides including tBHP have cytotoxic effects on several types of mammalian cells after penetration (Makino et al, 1995). With respect to tBHP, its toxicity is due to its decomposition by heme-containing proteins, membrane alterations, lipid-peroxidation,
GSH depletion, damage to cell proteins and DNA damage (Lapshina et al, 2005). In this study, tBHP induced cytotoxic effects in caco-2 cells when treated with different concentrations of tBHP (0.5, 1.0, 5.0 and 10 mM) resulting in reduced viability of 88.64, 78.18, 60.34 and 52.50% respectively. To observe the effects of peptides on caco-2 cells in the presence of an oxidant (tBHP), caco-2 cells were treated with 1.0 mg/ml of peptide and 2.5 mM tBHP. Caco-2 cells treated with tBHP had cell viability reduced significantly (79.3 %) but in the presence of the peptide and trolox no significant reduction was observed (94.7 % and 90.2 % respectively). Our peptides were absorbed across caco-2 cell membranes and this was supported by other studies (Lapshina et al, 2005; Shimizu et al. 1997; Makino et al, 1995). Therefore, the antioxidant activity is deduced to occur both inside and outside the cell, thus offering protection to cell constituents such as DNA as well as at the surface of the cell membrane. These results agree with those observed with peptides isolated from Alaska Pollack (Kim et al, 2001), hoki fish (Kim et al, 2007), and giant squid (Rajapakse et al, 2005).

*In vivo* studies of tBHP have shown that it is a membrane permeable oxidant and can be easily metabolized into free radical intermediates forming ROO* and RO* that initiates oxidation of membrane lipids and consequently forms TBARS (Barr and Mason, 1995). The formation of TBARS was observed to be greater in cells that were treated with tBHP (38.18 μg/ml) than in those treated with the peptide (33.18 μg/ml).

Necrosis and apoptosis are two types of cell death distinguished in mammalian cells. Part of the apoptotic cell death process is due to a class of cysteine proteases, also called
caspases that include caspase-3 and -7. The redox perturbation induced by subtoxic levels of lipid hydroperoxide leads to the activation of caspase 3, oxidative DNA damage, and enhances apoptosis (Wang et al. 2000). Therefore, the activities of caspase-3 and -7 were determined in caco-2 cells as biomarkers of apoptosis; the activity was observed to be greater in cells treated with tBHP only (157.5 ± 7.99 %) compared with that in cells treated with the peptide (25.7 ± 3.92 %). Thus apoptosis and consequently DNA damage were reduced by the peptide. This result agrees with studies in which phenolic substances with antioxidant activity have exhibited protective effects from oxidants against DNA damage induced by H₂O₂ (Giovannelli et al., 2000). In microscopy studies, the morphological modification in the caco-2 cells treated with tBHP was evident as the cells were detached from the flask surface as a result of the cytotoxic effect of tBHP. In the presence of the peptide, the cells appeared healthy and were attached to the flask surface. This observation further confirms the protective effects of the peptide from peroxides such as tBHP.

In endothelial cells, intracellular reactive oxygen species (ROS) are produced through the signal transduction of angiotensin II-dependent cellular responses via activation of redox sensitive signalling cascades (Li and Shah, 2003). The ROS produced in this cascade are the electron-reduction product superoxide (O₂⁻⁻) and the electron-reduction product hydrogen peroxide (H₂O₂); these serve as progenitors for other reactive oxygen species (ROS) that consequently lead to oxidative stress. In this study, production of ROS in the endothelial cell line, Ea.hy 926, was investigated after stimulation with angiotensin II in the presence of the peptide (LC1-Z). When the Ea.hy 926 cells were
stimulated with angiotensin II and later treated with the peptide, ROS as $O_2^{-*}$ were reduced by 26% and 39% in the lucigenin-chemiluminescence and fluorescence method respectively. This study postulates that the reduction in $O_2^{-*}$ is due to scavenging mechanism as several in vitro studies show that some peptides with particular amino acid sequences have superoxide radical scavenging activities (Rajapakse et al, 2005; Je et al, 2007; Bougatef et al, 2009; Jung et al, 2007). The present study shows the potential of the isolated peptide to impact positively on the oxidative stress in endothelial cells through superoxide radical scavenging.

The benefit of nutraceuticals depends on their ability to survive the gastrointestinal enzymes, lumen epithelial cells endopeptidases, and be absorbed into the lumen. Therefore, in this study we investigated the absorption and survival of the antioxidant peptide in caco-2 cell monolayer model. A monolayer of caco-2 cells was developed in a 12 well cell culture plate with permeable filter inserts and 1 mg/ml of peptide was added to both the apical and basolateral side of the insert. Apparent permeability (transepithelial transport) was observed in both directions (apical to basolateral and basolateral to apical) with a basolateral to apical flux of 0.95 ± 0.12 cm s$^{-1}$ and 0.74 ± 0.20 cm s$^{-1}$. Chromatographic traces of the buffer solution taken from the apical and basolateral side showed the presence of the peptide on both sides i.e., 11.5% for apical to basolateral and 12.2% for basolateral to apical. This indicates that the peptide that can be transported across a caco-2 monolayer unidirectionally can survive the caco-2 endopeptidases. This peptide produced from fish muscle using gastrointestinal enzymes has the potential to impart its antioxidant and antihypertensive bioactivity in vivo.
6.5. General conclusions

- The *in vitro* digestion of Atlantic mackerel (*Scomber scombrus*) fish protein with pepsin and pancreatin produced several useful peptide fractions with the most potent peptide containing amino acids histidine, proline, tyrosine, methionine, leucine, tryptophan and lysine and an ACE inhibitory activity IC$_{50}$ value of 0.15 mg/ml.

- The peptide with the amino acid composition histidine, proline, tyrosine, methionine, leucine, tryptophan and lysine is a competitive inhibitor for ACE and has inhibition constant (Ki) of 0.32 mg/ml.

- The *in vitro* digestion of Atlantic mackerel (*Scomber scombrus*) fish protein with pepsin and pancreatin produced several useful peptide fractions and in particular the peptide (LC1-Z) containing the amino acids serine, histidine, tyrosine, phenylalanine and lysine had the highest antioxidant activity, comparable to trolox and BHT and higher than vitamin C.

- The peptide (LC1-Z) exerted its antioxidant activity through carbon centered radical scavenging, hydroxyl radical scavenging, metal chelating and reducing ability.
- Peptide (LC1-Z), in the presence of peroxides, offered protection to caco-2 cells against apoptosis and necrosis.

- In Ba.hy 926 endothelial cells, the peptide (LC1-Z) scavenged ROS after stimulation with angiotensin II.

- The peptide (LC1-Z) survived the endopeptidases of the caco-2 cells and can be absorbed across the caco-2 monolayer and therefore exert its effect in the vascular system.

- Peptides obtained from the muscle of Atlantic mackerel have the potential to lower hypertension and enhance antioxidant activity which may have major potential benefits for cardiovascular and other diseases as well as for food safety.
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7. REFERENCES


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APPENDICES
Appendix 1. Standard curve of BSA

![Graph showing the standard curve of BSA. The equation of the line is \( y = 0.0206x + 0.1244 \) with a coefficient of determination \( R^2 = 0.9746 \).]
Appendix 2. Standard curve of DHE

\[ y = 9.0713x - 0.9703 \]

\[ R^2 = 0.9857 \]
Appendix 3. Standard curves for hippuryl, histidyl leucine (HHL) and hippuric acid (HA)

HHL, \( y = 15.05x + 7.12, R^2 = 0.9877 \) and HA, \( y = 15.44x + 7.12, R^2 = 0.9712 \)
Appendix 4. Recovery of HA in reaction mixture.

Recovery of hippuric acid in assay conditions spiked with a concentration of hippuric acid (0 – 1.0mM).
Appendix 5. HPLC chromatogram of HHL and HA

Eluted with acetonitrile (v/v, containing 0.1% TFA) and milliQ water (v/v, containing 0.1% TFA) on Phenomenex C18 (150 x 3.2 mm) column.
Appendix 6. Standard curve of FeCl₂.
Appendix 7. Standard curve of Malondialdehyde (MDA)

\[ y = 0.0084x + 0.0113 \]

\[ R^2 = 0.9999 \]
### Appendix 8. Table of Amino acids

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<tr>
<th>AA Codes</th>
<th>MW.</th>
<th>Structure</th>
<th>AA Codes</th>
<th>MW.</th>
<th>Structure</th>
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Appendix 9. Conferences, presentations and publications

1. Posters


2. Presentations

i. Kasase C. Isolation of Angiotensin Converting Enzyme (ACE) inhibitory peptides from Atlantic Mackerel (Scomber scombrus) fish. 4th June 2009. Nutrition research group seminar, division of nutrition and food science, FHMS, university of surrey, UK.


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3. Publications
