BIOACTIVE PROPERTIES OF PEPTIDES DERIVED FROM EGG YOLK PROTEINS

MARWA NASR YOUSR

A thesis submitted for the degree of Doctor of Philosophy
Faculty of Health and Medical Sciences
School of Bioscience and medicine
University of Surrey

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Dedication

I dedicate this thesis to the memory of my much loved and greatly missed father; the Late Nasr Abdel-Hameed Yousr who always had the faith, and confidence in me to succeed, I wish he was still alive to see me achieve my dream.

Gone now but never forgotten
I will miss him always and love him forever
Thank you Dad for all you did
I miss you so much
God bless his soul
Abstract

Bioactive peptides can be released, during gastrointestinal digestion or food processing, from many plant and animal proteins and represent an important source of potential health-enhancing components. Therefore, the aim of this study is to investigate antioxidant, anticancer and antihypertensive activities of proteins and peptides extracted from egg yolk. Defatted egg yolk (EY) was hydrolysed using pepsin and pancreatin and sequentially fractionated by ultrafiltration, followed by gel filtration (EYGF), resulting in three different fractions of which two (EYGF-23 and EYGF-33) effectively inhibited the oxidation of linoleic acid in a dose-dependent manner. Both fractions (80 mg/ml) exhibited antioxidant activity comparable to trolox and butylated hydroxytoluene (0.2 mg/ml). The antioxidant mechanism involved superoxide anion and hydroxyl radicals scavenging, ferrous chelating and reducing ability. The protective effect of one of the fractions (EYGF-23) was also tested in Caco-2 colon cancer epithelial cells, exposed to 3 mM of tert-butyl hydroperoxide (t-BHP) to induce oxidative stress. Induced cell toxicity by t-BHP was significantly inhibited when Caco-2 cells were treated with 1.0 mg/ml of the fraction for 24 hours. This fraction protected the cells by inhibiting intracellular ROS as well as lipid peroxidation products by 60.0% (p <0.001) and 24.15%, (p <0.05). In addition, total glutathione level and superoxide dismutase activity were significantly elevated to 0.29 μM (p <0.001) and 55.10% (p <0.01), respectively, in comparison to cells treated with t-BHP alone. Amino acids lysine, proline, tyrosine and tryptophan in this fraction may be responsible for antioxidant activity. The main highlight of this study was that cytotoxic effects of the second fraction (EYGF-33) was observed in vitro in cancer colon cells but not in normal colon cells. Apoptosis was mediated by mitochondrial ROS, evidenced by PARP-1 cleavage. Reduced cell viability could be explained by cell cycle arrest in the S-phase which
stopped DNA synthesis. Arginine, lysine, leucine, alanine, valine and tryptophan residues, which are mainly presented in the second fraction, may be responsible for the anti-proliferative activity. A third fraction (EYLCF-16) was successfully purified from egg yolk protein by both gel filtration and HPLC. This fraction exhibited high angiotensin converting enzyme (ACE) inhibitory activity (91.8%) at 10 mg/ml and IC\textsubscript{50} value (1.53). ACE inhibition exhibited by the fraction was probably due to the positively charged amino acid residues, lysine and arginine, and hydrophobic amino acid residues, leucine and tryptophan. These project findings indicate a substantial potential for producing peptides with antioxidant, anticancer and ACE inhibitory activity from egg yolk.
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Finally, I have left till last the mention of my greatest gratitude to my family: My kids Mohaned, Nooran, Rana, and Yusuf; you have been my inspiration, motivation and the reason for my happiness. My husband Mohamed Shahin- words fail me to express my gratitude to you, I am grateful to you for giving me an opportunity to achieve this degree, persistent confidence in me, unfailing encouragement and support throughout my study. What I owe you is priceless!
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<td>Ang-II</td>
<td>Angiotensin-II</td>
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<td>AOAC</td>
<td>Association of Official Agricultural Chemists</td>
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<td>BHA</td>
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<td>Egg yolk</td>
</tr>
<tr>
<td>EYGF</td>
<td>Egg yolk gel filtration fraction</td>
</tr>
<tr>
<td>EYGF-23</td>
<td>Egg yolk gel filtration fraction number 23</td>
</tr>
<tr>
<td>EYGF-33</td>
<td>Egg yolk gel filtration fraction number 33</td>
</tr>
<tr>
<td>EYGF-56</td>
<td>Egg yolk gel filtration fraction number 56</td>
</tr>
<tr>
<td>EYP</td>
<td>Egg yolk protein</td>
</tr>
<tr>
<td>EYPH</td>
<td>Egg yolk protein hydrolysate</td>
</tr>
<tr>
<td>EYUF-10</td>
<td>Egg yolk ultrafiltration fraction number 10</td>
</tr>
<tr>
<td>EYUF-5</td>
<td>Egg yolk ultrafiltration fraction number 5</td>
</tr>
<tr>
<td>EYUF-2</td>
<td>Egg yolk ultrafiltration fraction number 2</td>
</tr>
<tr>
<td><strong>F</strong></td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------</td>
</tr>
<tr>
<td><strong>FBS</strong></td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td><strong>FTC</strong></td>
<td>Ferric thiocyanate</td>
</tr>
<tr>
<td><strong>G</strong></td>
<td>Growth 1 phase</td>
</tr>
<tr>
<td><strong>G1 phase</strong></td>
<td>Growth 1 phase</td>
</tr>
<tr>
<td><strong>G2 phase</strong></td>
<td>Growth 2 phase</td>
</tr>
<tr>
<td><strong>GPx</strong></td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td><strong>GSH</strong></td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td><strong>GSH/GSSG</strong></td>
<td>Total reduced and oxidized glutathione</td>
</tr>
<tr>
<td><strong>GSSG</strong></td>
<td>Oxidised glutathione</td>
</tr>
<tr>
<td><strong>H</strong></td>
<td>Hippuric acid</td>
</tr>
<tr>
<td><strong>HA</strong></td>
<td>Hanks buffer saline solution</td>
</tr>
<tr>
<td><strong>HBSS</strong></td>
<td>Human colon epithelial normal cells</td>
</tr>
<tr>
<td><strong>HCEC</strong></td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td><strong>HCl</strong></td>
<td>Hippuryl-histidyl-leucine</td>
</tr>
<tr>
<td><strong>HHL</strong></td>
<td>Histidyl-leucine</td>
</tr>
<tr>
<td><strong>HL</strong></td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td><strong>HPLC</strong></td>
<td>Horseradish peroxide</td>
</tr>
<tr>
<td><strong>HRP</strong></td>
<td>Hydroxyl radical scavenging activity assay</td>
</tr>
<tr>
<td><strong>HRSA</strong></td>
<td>Hydroxyl radical scavenging activity assay</td>
</tr>
<tr>
<td><strong>IC</strong></td>
<td>Inhibitory concentration</td>
</tr>
<tr>
<td><strong>Ic</strong>(50)</td>
<td>Inhibitory concentration</td>
</tr>
<tr>
<td><strong>K</strong></td>
<td>KiloDalton</td>
</tr>
<tr>
<td><strong>KD</strong></td>
<td>KiloDalton</td>
</tr>
<tr>
<td><strong>Km</strong></td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td><strong>KNOS</strong></td>
<td>Kinin-nitric oxide system</td>
</tr>
<tr>
<td><strong>L</strong></td>
<td>Liquid Chromatography Mass Spectroscopy</td>
</tr>
<tr>
<td><strong>LC-MS</strong></td>
<td>Liquid Chromatography Mass Spectroscopy</td>
</tr>
<tr>
<td><strong>M</strong></td>
<td>Mitosis phase</td>
</tr>
<tr>
<td><strong>m/z</strong></td>
<td>Mass/charge</td>
</tr>
<tr>
<td><strong>MDA</strong></td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td><strong>MPA</strong></td>
<td>Metaphosphoric acid</td>
</tr>
<tr>
<td><strong>MTT</strong></td>
<td>3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl-tetrazolium bromide</td>
</tr>
</tbody>
</table>
mtROS  Mitochondrial Reactive Oxygen Species
MWCO  Molecular weight cut off
N  Nicotinamide adenine dinucleotide phosphate reduced
NADPH  Non-essential amino acids
NEAA  Neutral endopeptidase system
NEPS  Poly ADP-ribose polymerase
PBS  Phosphate buffered saline tablet
PI  Propidium iodide
PITC  Phenyl isothiocyanate
PS  Phosphatidylserine
PV  Peroxide value
PVDF  Polyvinylidene difluoride
R  Renin-angiotensin system
RAS  Renin-chymase system
RCS  Reactive oxygen species
ROS  Reversedphase highperformance liquid chromatography
RP-HPLC  DNA synthesis phase
SDS  Sodium dodecyl sulphate
SHR  Spontaneous hypertensive rat
SOD  Superoxide dismutase
S  2-thiobarbituric acid
TBARS  Thiobarbituric acid reactive species
T  Tert-butyl hydroperoxide
TBA  Trichloroacetic acid
TEA  Triethylamine
TEP  Tetraethoxy propane
TFA  Trifluoroacetic acid
U  Ultrafiltration
UF  Ultrafiltration
Chapter 1
1 Introduction

1.1 Proteins

Proteins are polymers that have a highly complex structure. Together with lipids and carbohydrates, proteins are important macro components in living systems, and are equally important in food, due to their high nutritional value and their functional properties. Nutritionally, proteins are considered as a source of energy and amino acids that are essential for well-being and growth. Additionally, the nutritional quality of proteins depends entirely on their amino acid composition and how they are used in the body following digestion and absorption. On the functional side, proteins have a strong impact on physiochemical properties such as foaming, emulsification and gelation. Scientific evidence has shown that many dietary proteins include specific biological properties that may be used as ingredients of nutraceutical or health promoting foods (Mine and Shahidi, 2005). Hence, numerous food proteins have been isolated and purified (Udenigwe and Aluko, 2012).

1.1.1 Protein biochemistry

All proteins are composed of one or more polypeptide; they are linear polymers of peptides built from a certain sequence of monomeric units called amino acids joined up by a peptide (covalent) bond (Chakrabarti and Pal, 2001).

1.1.1.1 Structure of amino acids

The total number of common amino acids is 20 and their active form is L-α. These amino acids are similar in the main structure but differ in the side-group. The central
carbon atom, which is known as α-carbon, holds carboxyl group (C-terminus), amino group (N-terminus) and variable side chain (R) (figure 1.1).

![Figure 1.1. Structure of an amino acid.](image)

Amino acids are amphoteric compounds because they carry both a negatively charged carboxylate group and positively charged protonated amino group in the same molecule. This character enables them to react either as an acid (proton donor) or as a base (proton acceptor), depending on the pH of the medium. Although the number of amino acids is limited, the properties and functions of a particular protein depend mainly on the type and the precise sequence of its amino acid which is unique to that protein. Moreover, the variability of the structure and the size of R- group play a fundamental role in the physical properties of the protein (Fennema et al., 2008).

### 1.1.1.2 Classification of amino acids

Various approaches have been employed in the classification of amino acids, but the most suitable one is to apply the classification in terms of the properties of their side-chain (R) rather than their chemical structure as indicated in figure 1.2 (Fennema et al., 2008).
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(A) Non-polar R groups

(B) Polar R groups

(C) Negatively charged R groups  
(D) Positively charged R groups

Figure 1.2. Classification of amino acids. Source: Fennema et al., 2008.

1.1.1.3 Protein synthesis

The component amino acids are linked together by substituted covalent bonds named peptide or amide bond, formed by the loss of a water molecule as a by-product. This condensation reaction occurs between amino group of one amino acid and carboxyl group of adjacent amino acid residues as depicted in figure 1.3.
Figure 1.3. Binding of amino acids via peptide bond. Source: Fennema et al., 2008.

Although the number of amino acids is limited, the nutritional and physicochemical properties of proteins are diverse. This is due to the fact that the unique properties of any protein depend entirely on the nature of its amino acid content and their sequence in the polypeptide chain (Friedman, 1996). Scientific evidence has shown that the actual reaction involved in protein synthesis in living systems is much more complicated than that illustrated in the above figure; this is because not only must the peptide bond be forged, but the amino acids must be combined in the correct sequence.

1.1.1.4 Protein structure

It has become clear that there are four distinct levels of protein structure, and each protein has a unique three-dimensional structure as illustrated in figure 1. 4 (Fennema et al., 2008).
1.1.1.4.1 Primary structure

The sequence of amino acids in the polypeptide chain is illustrated as a linear arrangement with C-terminus at one side and N-terminus on the other side. In this structure, the peptide bond places some restrictions on the shape of the molecule, as free rotation does not occur around the covalent bond.

1.1.1.4.2 Secondary structure

This structure is represented by the coiling of the long chain, which is held in a more rigid position by weak bonds (chiefly hydrogen bonds). The side chains (R groups) are extended out of the backbone structure. It has become clear that the secondary structure includes two configurations. The most common one is α-helix that occurs when intra-
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Chain hydrogen bonds connect different parts of the same chain, leading to a very stable rod-like cylinder. The second type of conformation, which is known as \( \beta \)-sheet or pleated sheet, occurs when the hydrogen bonds are inter-chain. This means that the bond in this conformation is formed between atoms of two separate chains, leading to strands lining up in a parallel or anti-parallel fashion, and results in a zigzag shape. This zigzag shape helps strengthen the polypeptide chain to a great extent as compared to \( \alpha \)-helix form (Fennema et al., 2008).

1.1.1.4.3 Tertiary structure

The tertiary structure represents the overall shape of a single protein molecule, which results from folding the secondary structure of the polypeptide chain. This structure is stabilized by non-covalent hydrogen bonds (that link various amino acid residues), hydrophobic forces (that link non-polar side groups), electrostatic interactions and covalent disulfide bridges, which link cysteine amino acids at various places in the polypeptide chain.

As the chain folds, non-polar hydrocarbon side groups tend to turn towards the interior of the molecule where they can form hydrophobic bonds with one another. In contrast, the polar side chains tend to move towards the exterior of the molecule, and give water-dispersible properties to proteins either by forming hydrogen bonds with water molecules or with other parts of the chain. Therefore, the tertiary structure determines the basic function of protein (Fennema et al., 2008).
1.1.4.4 Quaternary structure

The quaternary structure represents the non-covalent associations that occur when two or more proteins in the form of tertiary structure are merged to form a multi-subunit protein. This structure exists in proteins with complex functions. The most common example is the haemoglobin molecule which comprises four polypeptide chains (Fennema et al., 2008).

1.2 Egg yolk protein

It has been proven scientifically that the whole egg protein is nutritionally better than milk and meat protein (Yamaguchi and Matsuno, 1974; Shibata et al., 1987), and is used as a benchmark for measuring the quality of other food proteins. Although several studies have focused on albumen protein (Navarrete et al., 1977; Komatsu et al., 1983), few have examined the nutritional value of egg yolk proteins, mainly due to their lipid content that negatively affects human health.

The main components of egg yolk are phospholipids, triacylglycerols and proteins (table 1.1). The first two components are used as lecithin in food or cosmetics. It is noteworthy that egg yolk lecithin has oxidative stability that is higher than that of soybean lecithin. Scientific evidence has shown that egg yolk lecithin can be totally metabolized by humans and is non-toxic when ingested. Accordingly, lecithin is used in pharmaceutical application as a surfactant, lubricant and emulsifying agent; as a wetting, stabilizing agent; for emulsification and encapsulation, and as a good dispersing agent. Lecithin is also used as a form of choline supplement. The third component of egg yolk is protein that represents approximately 30% of dried egg yolk.
Many studies have shown that egg yolk proteins have antioxidant activities (Sakanaka et al., 2004).

<table>
<thead>
<tr>
<th></th>
<th>Fresh yolk (%)</th>
<th>Dry yolk (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>51.1</td>
<td>-</td>
</tr>
<tr>
<td>Lipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides 62%</td>
<td>3.6</td>
<td>62.5</td>
</tr>
<tr>
<td>Phospholipids 33%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol &lt;5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carotenoids &lt;1%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteins</td>
<td>16.0</td>
<td>33.0</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>0.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Minerals</td>
<td>1.7</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Table 1.1. Composition of chicken egg yolk. Source: Anton, 2007.

1.3 Bioactive peptides

Bioactive peptides are food-derived peptides that usually contain 2-20 amino acid residues per molecule resulting from specific hydrolysis of plant or animal proteins. They are released upon enzymatic hydrolysis, during food processing or during gastrointestinal digestion and, consequently, become bioactive despite the fact that they are inactive within the sequence of their parent protein (Meisel, 1997; Korhonen, 2003).

Once bioactive peptides have been liberated, they exhibit physicochemical and functional activities beyond their nutritional properties (Korhonen and Pihlanto, 2006). For nutritional aspects, small peptides such as di- or tri-peptides are considered as a suitable nitrogen source because of their absorption which is higher than that of their free amino acids (Epstein et al., 1998). From a physiological point of view, bioactive
peptides resist hydrolysis and, therefore, they are able to exert local effects in the gastrointestinal tract or directly on entering the blood circulation, after their absorption through the intestine, to exert systemic effects (Vermeirssen et al., 2002a; Vermeirssen et al., 2002b).

It is now well established that these peptides exhibit multiple beneficial health effects on the biological system, and the magnitude of these benefits is entirely dependent on the size and the sequence of amino acids inherent in the specific peptides (Samaranayaka and Li-Chan, 2011). Most recent studies have examined the impact of bioactive peptides on numerous physiologically-related activities including antithrombotic (Erdmann et al., 2008), antimicrobial (Haque and Chand, 2008), antioxidant action against unsaturated fatty acids (Je et al., 2005) and antihypertensive activities (Matsui et al., 2002). Additionally, these bioactive peptides play a fundamental role in functional and nutraceutical applications in order to supply the standard nutrients and reduce the risk of human chronic diseases (Mine and Shahidi, 2005).

Many types of plant and animal protein have been used to isolate and identify bioactive peptides. For example, sunflower (Megias et al., 2004), casein (Suetsuna et al., 2000), soybean (Gibbs et al., 2004), egg (Miguel and Aleixandre, 2006b), Pacific hake (Cinq-Mars et al., 2007), mackerel (Sampath Kumar et al., 2012), skin of sole and squid (Giménez et al., 2009) and chicken breast muscle (Saiga et al., 2006). However, there is a wide consensus among the scientists that there are other applications of bioactive peptides yet to be identified and more studies are needed in order to examine the efficacy of bioactive peptides on human health prior to their application as ingredients of the nutraceutical foods.
1.3.1 Production of bioactive peptides

Peptides are released from their parent proteins by hydrolysis using chemicals (acid or alkali) and enzymes. During hydrolysis, the peptide bond between two adjacent amino acids is cleaved to produce peptides. *In vivo*, protein digestion starts in the stomach at acidic pH by the action of a specific enzyme called pepsin, to produce polypeptides. The resultant polypeptides are cleaved further in the small intestine by the action of pancreatic proteases such as trypsin, α-chymotrypsin, elastase and carboxypeptidase at alkaline pH. As a result of the action of these enzymes, a wide variety of free amino acids and oligopeptides are produced (Korhonen and Pihlanto, 2006). While free amino acids are directly absorbed through specific amino acid transport systems, the oligopeptides undergo further hydrolysis by peptidases located in the brush border membrane. Studies have been conducted to isolate peptides from different protein sources by using different enzymes with different selectivity and specificity such as microbial enzymes, synthetic enzymes and gastrointestinal enzymes (Vioque *et al.*, 2000; Korhonen and Pihlanto, 2006). The resultant hydrolysates contain mixtures of low and high molecular weight peptides.

1.3.2 Protein and peptide separation

The characterization of proteins and peptides is determined by their molecular weight (size), electric charge, hydrophobicity, amino acid composition and their sequence. The properties of the side chain (R-group) in the amino acid structure are responsible for its chemical reactivity, polarity and its ionic charge. Therefore differences in these properties can be utilized in separation methods and purification strategy.
1.3.2.1 Molecular weight

The molecular weight (size) of a protein is the mass of one mole and is usually measured in units called daltons. The molecular weight can be measured by mass spectrometry. Separation methods, which depend on the size of molecules, include centrifugation using ultrafiltration membranes with different molecular weight cut-off, polyacrylamide gel electrophoresis and gel filtration chromatography (size exclusion chromatography) (Greaser and Warren, 2012).

1.3.2.2 Electric charge

According to the structure of proteins and peptides which consists of different amino acids with different functional groups in their side chains, each protein and peptide carries a different net charge. The net charge on the molecule is highly influenced by the pH of the surrounding medium as it can become more positively or negatively charged due to the gain or loss of protons. A separation method which depends on the charge of the molecules includes ion exchange chromatography (Hennrich et al., 2011).

1.3.2.3 Hydrophobicity

The degree of polarity of each protein and peptide is determined according to the number, type and distribution of polar and non-polar amino acids incorporated in their structure. A separation method which depends on the hydrophobic character of proteins and peptides is hydrophobic interaction in solid-liquid chromatography (Armarego and Chai, 2012).
1.3.3 Peptide sequencing

1.3.3.1 Edman degradation

Edman degradation can be used to sequence up to 30 amino acids by labelling and cleaving the N-terminal residue from the peptide without affecting peptide bonds existing between other amino acid residues. The N-terminal amino residue is labelled with phenylisothiocyanate which reacts with uncharged terminal amino groups, under alkaline conditions, to form a cyclical phenylthiocarbamoyl derivative. Acidic conditions cleave this derivative to a thiazolinone derivative that is subsequently extracted into an organic solvent and treated with acid to form the phenylthiohydantoin (PTH) - amino acid derivative, a stable compound that can be identified by using electrophoresis or chromatography technique (HPLC). By repeating this process all the amino acids can be identified. The advantage of this method is its ability to identify low levels of peptides up to 10-100 picomoles (Kinter and Sherman, 2005).

1.3.3.2 Liquid chromatography and mass spectroscopy (LC-MS)

Liquid chromatography-mass spectroscopy (LC-MS) is a powerful technique that can be used to separate and identify complex compounds and macromolecules such as peptides and proteins. The principle of LC-MS is based on the separation of compounds using liquid chromatography (LC) followed by mass analysis using mass spectroscopy (MS). Electrospray ionization (ESI) is used in mass spectroscopy in order to facilitate the direct analysis of peptides when eluted from HPLC in the LC/MS experiment by producing ions (Peng et al., 2003). The ions produced are subsequently filtered according to their mass-to-charge ratio (m/z) and then detected by MS (Allen, 2011).
1.4 Role of food-derived bioactive peptides

Peptides that are derived from food proteins may help to reduce the worldwide epidemic of chronic diseases as they have the potential to exert a high and sustainable effect on the major systems in the human body, including the cardiovascular system, nervous system, immune system and gastrointestinal system. As far as the cardiovascular system is concerned, bioactive peptides play a crucial role in reducing the risk of heart diseases through a variety of mechanisms namely antioxidant, antithrombotic, hypocholesterolemic and antihypertensive (Mine and Shahidi, 2005). Hence, the aim of this study is to investigate the antioxidant, anticancer and angiotensin-I-converting enzyme inhibitory activities of enzymatically prepared peptides derived from animal protein after ultrafiltration and purification using a combination of chromatographic techniques. The following section will elaborate further on three different mechanisms: antihypertensive effect via ACE inhibition, antioxidant and anticancer properties.

1.4.1 Angiotensin-I-converting enzyme (ACE) inhibition activity

The regulation of arterial blood pressure in human body is mainly achieved through diverse physiological systems including the kinin-nitric oxide system (KNOS), the neutral endopeptidase system (NEPS), the renin-chymase system (RCS) and the rennin-angiotensin system (RAS). Any disturbance in the blood pressure by increasing (hypertension) or decreasing (hypotension) will trigger a number of chronic diseases, such as, cardiovascular diseases, peripheral vascular disease and renal dysfunction (Mine and Shahidi, 2005).
1.4.1.1 The role of rennin-angiotensin system in cardiovascular disease

It has been recognized that the rennin-angiotensin system (RAS) regulates blood volume and systemic vascular resistance, which control cardiac output and arterial pressure. RAS exists in various human body organs, such as the brain, kidney and lung, as well as in the circulatory blood system. In the RAS system, the angiotensinogen is cleaved from the liver by the action of the renal rennin enzyme to angiotensin-I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu). One of the key elements constituting the rennin-angiotensin system is the angiotensin converting enzyme (ACE; EC3.4.15.1, dipeptidyl carboxypeptidase). ACE hydrolyzes the inactive decapeptide (angiotensin-I), through the removal of the dipeptide His-Leu from the C-terminus, to produce a potent vasoconstrictor octapeptide (Angiotensin-II) (Johnston, 1990; Crowley et al., 2006). As a result of Angiotensin-II production, many physiological processes such as systematic vasoconstriction, water and sodium retention and cardiac hypertrophy, are initiated (figure 1.5).
Angiotensin-II acts through two types of receptors termed AT-1 and AT-2. When angiotensin-II acts through the AT-1 receptor, the aldosterone hormone will be released leading to hypertensive events such as vasoconstriction. On the other hand, if angiotensin-II acts through the AT-2 receptor, it initiates antihypertensive events (de Gasparo et al., 2000) (figure1.6).
Angiotensin converting enzyme (ACE) exists in two isoforms termed somatic and germinal forms. The somatic form exists in the endothelium of multiple tissues such as lungs, kidneys and the vascular endothelium while the germinal exist in sperm (Turner and Hooper, 2002). ACE is a metalloprotease that mediates its action by the presence of zinc (Natesh et al., 2004). In addition to the ability of ACE to convert angiotensin-I to vasoconstrictor angiotensin-II, it also enhances the reabsorption of renal tubular sodium by increasing the release of adrenal aldosterone. Moreover ACE plays an important role in the depressor hormonal (Kinin-Kallikrein) system. In this system ACE hydrolyzes vasodilatory bradykinin to inactive metabolites (Tschope et al., 2002; Zhao and Xu, 2008), as depicted previously in figure 1.5. Therefore, the utilization of ACE
inhibitors will block the generation of vasoconstrictor angiotensin II and will enhance the action of vasodilator bradykinin, thus reducing blood pressure.

1.4.1.2 The ACE inhibitors

It has been recognised that the inhibition of ACE, by ACE inhibitory drugs like captopril and natural ACE inhibitory peptides, leads to an antihypertensive effect in hypertensive human subjects and animals. The IC$_{50}$ value is used to specify the effectiveness of different ACE inhibitory peptides. This value represents the concentration of inhibitory compound that inhibits 50% of ACE activity. Although the potent synthetic ACE inhibitors Captopril, Fosinopril, Lisinopril and Perindopril are used extensively in the clinical treatment of hypertension, they have significant side effects such as dry cough, dizziness, skin rashes and headache (Morimoto et al., 2004). On the other hand, the most recent studies on natural ACE inhibitors such as ACE inhibitor peptides, which are derived from food proteins, have so far indicated that these inhibitors do not have known side effects on the human body (Hong et al., 2008; Kim and Wijesekara, 2010; Norris and FitzGerald, 2013). Accordingly, more studies are now directed towards understanding how to produce food derived peptides that can act as ACE inhibitors for the clinical treatment of hypertension.

1.4.1.3 Methods used to determine ACE inhibitory activity

There are a number of methods that have been developed for the measurement of ACE inhibitory activity. These methods include spectrophotometry (Cushman and Chung., 1971), bioassay (Anderson., 1967), fluorimetry (Friedland and Siverstein., 1976), high performance liquid chromatography (Neels et al., 1983), the use of radioisotopes (Chung and Ryan, 1978), capillary electrophoresis (Vandyck et al., 2003) and internally quenched fluorogenic methods (Araujo et al., 1999).
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The most common method used is the spectrophotometric method described by Cushman and Cheung (1971). This method is based on the hydrolysis of the synthetic tripeptide substrate, hippuryl-histidyl-leucine (HHL) by ACE. ACE will release histidyl-leucine (HL) and hippuric acid (HA) which are detected by UV at 228 nm. The production of HA is directly related to ACE activity.

However, a major drawback of the Cushman and Cheung (1971) method is that the measurement of hippuric acid concentration can only be achieved after several steps including HA extraction with ethyl acetate, evaporation, redissolution in water, followed by measuring the absorbance of HA in a spectrophotometer at 228 nm (Wu et al, 2002). Furthermore, ethyl acetate is also able to extract unhydrolyzed HHL, thus resulting in the overestimation of ACE activity.

Since bioactive peptides may have the potential to affect major systems in the human body as described above, the next section will discuss further their effect as an antioxidant to protect biological and food systems against harmful reactive oxygen species.

1.4.2 Antioxidant activity

1.4.2.1 Free radicals and lipid oxidation
Free radicals are described as atoms, molecules or ions with unpaired electrons, in an open shell configuration which arises as a consequence of oxidative metabolism. The unpaired electron causes free radicals to be unstable and highly reactive, until the unpaired electron is paired up to finally reach a stable configuration. Free radicals are
classified into two major classes: reactive nitrogen species (RNS) and reactive oxygen species (ROS). Examples for the former class include the nitroxyl anion (NO\(^{-}\)) and peroxynitrite (ONOO\(^{-}\)). Examples of the latter class include the hydroxyl radical (HO\(^{+}\)) and the peroxyl radical (ROO\(^{\cdot}\)) (Michal and Schomburg, 2013).

Lipids represent a diverse group that possesses similar properties including glyceride lipids as fats, oils and phospholipids, and non-glyceride lipids such as waxes and steroids. Lipids are hydrophobic molecules associated with all living systems playing a vital role in physiological functions. They act as structural elements of cell membranes, energy reservoir, and as an important precursor for the synthesis of vitamins and hormone-like substances (Michal and Schomburg, 2013).

The main building blocks of lipids are fatty acids that are classified into saturated, with no double bonds, and unsaturated with one double bond (monounsaturated) or more than one double bond (polyunsaturated). Polyunsaturated fatty acids are more susceptible and readily oxidised by oxygen or any free radicals, than saturated fatty acids, due to the presence of multiple double bonds along the chain. The double bonds next to methylene-CH\(_2\)- groups possess a reactive centre that can be attacked easily by reactive species and cause lipid oxidation.

Free radicals can abstract electrons from lipids and produce lipid peroxide products via the free radical chain reaction mechanism (Dalrymple et al., 2010). This mechanism of lipid oxidation occurs in the following three stages, as illustrated in figure 1.7.
The initiation stage: Reactive oxygen species (ROS) initiate this step by abstracting hydrogen from the active methylene site, available in unsaturated fatty acids (RH), to produce lipid free radical (R').

The propagation stage: The conjugated diene (R') is highly unstable. Hence, it reacts with an oxygen molecule to achieve the stability and form a peroxyl radical (ROO'). Peroxyl radicals abstract hydrogen from another unsaturated lipid molecule to form another lipid radical (R') and lipid peroxides (ROOH).

The termination stage: This stage occurs when two radicals react and produce a non-radical species. This chain, as demonstrated in figure 1.7 above, will be terminated only when the concentration of radical species is high enough so there will be a high probability that two radicals will collide and form stable molecules.

Lipids in all foods are esters of long chain fatty acids with different degrees of saturation. The unsaturated fatty acids are more easily oxidised by free radicals. There
are many factors affecting lipid oxidation in food products, namely, moisture content, temperature of storage and light exposure. These factors enhance the production of free radicals and hence the process of lipid oxidation. Free radical attack on lipids in food not only causes alteration in the organoleptic properties, giving unacceptable taste, odd flavour and rancidity but also produces toxic compounds that reduce the quality and the shelf life of the products (Christen, 2000; Valko et al., 2004; Alghazeer et al., 2008). In food systems, lipid oxidation occurs when the free radicals attack the unsaturated lipids through the following different pathways:

**Autoxidation:** The process of autoxidation is triggered when free radicals present in the living cells interact with polyunsaturated fatty acids and monounsaturated fatty acids, and results in the production of intermediate substances such as alkyl and peroxyl radicals (Sikorski and Kolakowska, 2002).

**Photoxidation:** In this process, light, including ultraviolet light, works as a source of energy which raises the oxygen from the ground state (two electrons with same spin in two orbitals) to the excited energy state (two electrons with different spin in one orbital) that is referred to as singlet oxygen ($^1\text{O}_2$). The latter reacts with the unsaturated fatty acids to form free radicals. Photosensitizers like chlorophyll, riboflavin and myoglobin mediate the production of singlet oxygen, as explained above (Sikorski and Kolakowska, 2002).

**Enzymatic oxidation:** The enzymatic oxidation reaction is determined by the presence of a lipoxygenase enzyme system that catalyses the reaction between the oxygen and unsaturated fatty acids in order to produce hydroperoxides (Sikorski and Kolakowska, 2002).
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In biological systems, the existence of oxidative metabolism is crucial for the survival of cells. Consequently, a number of highly reactive free radicals arise as a by-product of enzyme and other reactions involving oxygen. Living cells have special enzymes that include superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). These enzymes help to maintain the redox state through a constant input of metabolic energy (Halliwell and Gutteridge, 1990).

Any disturbance to this normal redox state, between ROS production in the physiological system and its detoxifying ability, is referred to as oxidative stress. These imbalances lead to toxicity due to the excessive production of free radicals that destroy all cell components and their functions, including DNA, protein and lipids, causing severe human diseases (Cimen, 2008). Therefore, it is important to inhibit the occurrence of excess lipid oxidation in living organisms and food by using antioxidants.

1.4.2.2 Antioxidants

Lipid peroxidation plays a key role in the pathogenesis of many diseases such as cancer (Valko et al., 2004), cardiovascular disease (Cai and Harrison, 2000) and Alzheimer’s disease (Christen, 2000). Additionally, it plays a vital role in the loss of food quality by producing undesirable flavours and toxic compounds, which subsequently shorten the product shelf-life (Lin and Liang, 2002; Alghazeer et al., 2008). Therefore, it is important to inhibit the occurrence of lipid oxidation in food and the living body by using antioxidants.

Antioxidants are defined as substances that prevent or slow down the oxidation process (Huang et al., 2005). Antioxidants are classified as antioxidant defence systems which occur naturally in biological system, synthetic antioxidants such as butylated
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hydroxyanisol and butylated hydroxytoluene, and natural antioxidants such as vitamin C and plant phenols.

1.4.2.2.1 Antioxidants in biological systems

Aerobic cells utilize a wide array of enzymatic and non-enzymatic mechanisms that tightly control unwanted ROS accumulation. Cellular antioxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) as well as non-enzymatic antioxidants such as glutathione (GSH) can protect cell membranes and cellular content by scavenging ROS. Some of the defence system enzymes are described below:

**Superoxide dismutase** (SOD) is responsible for the dismutation of the superoxide anion to hydrogen peroxide and a molecule of oxygen according to the following reaction (Scandalios, 1993; Inoue *et al.*, 2003).

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

Superoxide anion is responsible for generating further highly reactive oxygen species which damage biological macromolecules such as DNA, lipids and protein. There are four different SOD forms according to the type of metal which acts as a cofactor to catalyse the dismutation reaction. These forms are copper/zinc-SOD (Cu/Zn-SOD), iron-SOD (Fe-SOD), manganese-SOD (Mn-SOD) and nickel-SOD (Ni-SOD) (Halliwell and Gutteridge, 1990).

**Catalase** is another enzyme in the biological defence system. This enzyme protects cells by catalysing the conversion of hydrogen peroxide, which is considered a highly
reactive free radical, to water and molecules of oxygen (Halliwell and Gutteridge, 1990) as depicted in the following reaction:

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

**Glutathione peroxidase** (GPx) is a selenium-containing tetrameric glycoprotein. This enzyme plays a major role in the elimination of hydroperoxides by catalysing their conversion to water, alcohols and oxidized glutathione (GSSG) (Halliwell and Gutteridge, 1990) according to the following reaction.

\[ \text{GPx} \]

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

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

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

In a healthy system, the high level of total glutathione plays a substantial role in protecting cells from unexpected attack by hydroxyl radicals. Under oxidative stress, the increase in the concentration of peroxides leads to a shift in thiol redox status that is represented by a severe decrease in the reduced form of glutathione (GSH) and an increase in the level of the oxidised form (GSSG) (Baud et al., 2004).

### 1.4.2.2 Synthetic antioxidants

The most common synthetic antioxidants are butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). They possess phenolic groups that form resonance stabilized phenolic radicals that have high antioxidant activities. These phenolic radicals
undergo a number of degradation reactions that result in products with active hydroxyl groups. These products also have high antioxidant activities. Although the activity shown by synthetic antioxidant in inhibiting the deterioration of lipids during storage is excellent, their usage in food is a concern to consumers due to their potential toxicity and carcinogenicity at high levels of intake (Saito et al., 2003; Vandghanoooni et al., 2013). Therefore, the demand for antioxidants from natural resources has increased in recent years.

1.4.2.2.3 Natural antioxidants

Plants and food additives are rich sources of natural antioxidants as they include vitamins (vitamin E, C and β-carotene), plant phenols (flavonoids, and other phenolic compounds), and peptides derived from proteins. Many types of plant and animal protein have been isolated and found to have antioxidant peptides. For example, sunflower protein (Megias et al., 2004), casein (Suetsuna et al., 2000), soybean protein (Gibbs et al., 2004), egg-white albumen (Miguel et al., 2004), Pacific hake protein (Cinq-Mars et al., 2007), mackerel protein (Sampath Kumar et al., 2012), skin of sole and squid protein (Giménez et al., 2009) and hoki frame protein (Kim et al., 2007) have all been identified as potential sources of antioxidant peptides. The antioxidant activity shown by food-derived compounds makes them a good natural replacement for the synthetic ones (McCarthy et al., 2001; Shahidi, 2009).

As discussed previously, food derived bioactive peptides exhibit ROS scavenging activity. ROS has been implicated in the etiology of chronic diseases such as cancer. Therefore, the ability of bioactive compounds to scavenge ROS and act as antiproliferative agents on cancer cells will be discussed further.
1.4.3 Anticancer activity

Cancer is the unrestrained growth of cells forming malignant tumours. Cells undergoing unregulated growth can easily invade surrounding tissue and can metastasize to nearby sites in the body (Surh, 2003). Due to its uncontrolled and rapid spread, cancer is considered a major cause of mortality all over the world. In 2011, about 331,487 cases were diagnosed in the UK with cancer and mortality reached 159,178 (CRUK, 2011). The four types of cancer where incidence and mortality prevail are lung, breast, prostate and bowel. Lung cancer has the highest incidence (18%) and highest mortality (22%) of any cancer. The percentage of breast, prostate and bowel cancer incidence is 15%, 13% and 13%, respectively. In cancer mortality, bowel represents 10%, while both breast and prostate cancer represent 7%. According to the latest CRUK statistics (2011), colorectal cancers are the second most common cause of cancer mortality (10 %) and the third most common cancer in men and women (14 %) (CRUK, 2011).

1.4.3.1 Cell division cycle and cancer disease

Cancer is caused as a result of changes in cells. In eukaryotic healthy cells, a series of events occur leading to cell division and produce two healthy daughter cells. The cell division cycle is represented by four important phases (figure 1.8)

Growth 1 or Gap 1 phase (G1-phase) is where the cells grow and are checked by the G1 check points control mechanism to be sure that the cell is ready for the next phase. The next phase is (S-phase) where the DNA is synthesised and duplicated. This step is followed by the growth 2/Gap 2 phase (G2-phase) where the cell continues growing and is checked by G2 check points to ensure that the cell is ready for mitosis and division. The last phase is the mitosis phase (M-phase), in which the cell stops growing
and divides into two distinct daughter cells. Metaphase check points check the cell in the middle of mitosis to ensure that the cell is ready for division. However, there is a specialised form of G1 named **G0-phase** which is considered as a resting phase where the cell leaves the cycle and stops dividing (Secko, 2003).

![Cell division cycle process](image)

**Figure 1.8. Illustration of cell division cycle process. Adapted from Secko (2003).**

The cell cycle process is controlled by a group of specific proteins such as cyclins and cyclin-dependent kinases (CDKs) that work as a check point control mechanism (Murray, 2004). Any disturbance in these check points can interrupt the cell division cycle leading to cell death or abnormal growth. Cyclins (A, B, D and E) are important groups that affect cell cycle regulation and progression through complex formation with CDKs. Cyclin D and E are responsible for controlling the G1 phase. Cyclin D/CDK4,6 is responsible for the regulation of gene transcription which is associated with DNA replication. Cyclin E activates CDK2 to ensure the transition from the G1 phase to the S
phase. In the S phase, cyclin A activates CDK2 and controls cell progression by protein phosphorylation which leads to DNA synthesis and replication. The G2 phase is regulated by cyclin A/CDK1 complex and cyclin B/CDK1, which occur at the end of this phase. In the M phase, the upregulation of cyclin A/CDK1 complex ensures the completion of this phase before cell division (Murray, 2004). Any interruption in the cell cycle process causes abnormality growth or failure in cell progression and hence cell death.

1.4.3.2 Cell death pathways

There are different cell death pathways as illustrated in figure 1.9. Apoptosis is a pathway that is also called programmed cell death (PCD). Apoptosis occurs in multicellular organisms and is characterized by biochemical events that lead to characteristic changes in cell morphology such as blebbing, cell shrinkage, chromatin condensation as well as nuclear and chromosomal DNA fragmentation (Rowan and Fisher, 1997). At the end of these events, cells split to apoptotic bodies. Some anticancer agents affect cancer cells through apoptosis induction which is controlled by intrinsic or extrinsic pathways. The intrinsic pathway is achieved by activation of the caspase family (intracellular cysteine family) which are released from mitochondria into the cytosol (Persson et al., 2009). The extrinsic pathway is induced apoptosis at the cell surface and organised by specific trans-membrane death receptors such as tumor necrosis factor receptor (TNF-R) (Seeram et al., 2005).

Necrosis is a non-programmed cell death and considered as a form of cell injury. Necrosis is activated by external factors such as toxin and infection leading to unregulated digestion of cell content. Necrotic cells are characterized by cell membrane swelling until rupture, leading to the release of cellular content causing inflammation
(Edinger and Thompson, 2004). When necrotic cells induce inflammation, an inflammatory response is initiated preventing the adjacent phagocytes from presenting and removing the dead cells by phagocytosis. Another mechanism of cell death pathways is **autophagy**; this catabolic mechanism involves the decomposition of dysfunctional cellular components *via* lysosome action. In this mechanism, dysfunctional cytoplasmic components are insulated from the rest of the cell inside the autophagosome; these components are subsequently united with lysosomes and decomposed or recycled (Edinger and Thompson, 2004).

![Cell Death Pathways](image)

**Figure 1.9. Cell death pathways. Adapted from Edinger and Thompson (2004).**

To this end, finding a suitable anticancer agent which targets the above cell death pathways is one of the aims of this project.

### 1.4.3.3 Anticancer agents

Although the causes of cancer are diverse and their mechanisms are still not fully defined, a wealth of evidence points to lifestyle and diet as risk factors contributing to the cause and progression of some types of cancer (Anand *et al.*, 2008). Lifestyle
interventions and the development of preventive approaches to control the development of this disease have gained interest in recent years. One approach is to use natural bioactive compounds such as chemopreventative occurring in dietary substances agents (Milner, 2004; Stan et al., 2008).

Bioactive compounds have a wide range of functional groups that allow them to exhibit many biological functions. In order to exhibit their biological activity, it is important that they are released or extracted from the parent food source and are resistant to degradation by gut enzymes (Kris-Etherton et al., 2002). Many studies have revealed that bioactive compounds exhibit anti-carcinogenic activity via different mechanisms. They may quench ROS and thereby protect cellular components from oxidative stress, inhibit cell growth, induce apoptosis, alter cell cycle kinetics and interfere with intracellular signal transduction events in cancer cells (Yun, 2003; Hou, 2003; Heber, 2004).

Bioactive peptides released from plant and animal protein have been the focus of increasing interest as anticancer agents due to their properties of multi-functionality and high stability (Lee et al., 2005; Leng et al., 2005). Several protein-rich sources including soy bean, milk, and cereal grains that can bear potential anticancer peptides have been reported (Kim et al., 2000; Xiao et al., 2004; Ward et al., 2005; Kannan et al., 2008).

Anticancer properties have also been documented in several egg proteins (Kovacs-Nolan et al., 2005). Ovomucin, an egg white protein, has the potential to inhibit tumour growth by limiting angiogenesis in cells (Mine and Kovacs-Nolan, 2006). The role of the egg white protein avidin as an anticancer agent may involve changes in the
host–tumour relationship or the host–mediated antitumor response (Gasparri et al., 1999). Egg cystatin was also reported as a potential novel anticancer agent through its suppression effect on cysteine proteases. These proteases, tested in cancer cells, are implicated in multiple steps of cancer progression, for example, in the early stages of immortalization and transformation, in the intermediate steps of tumour invasion and angiogenesis, and in the late steps of metastasis and drug resistance (Keppler, 2006).

1.5 Aim and objectives

Nowadays, people are increasingly aware of the benefits of healthy foods and, therefore, scientists have been working on the identification of food-derived bioactive compounds that maintain health and/or reduce the risk of chronic disease. From this point of view, the current research is built on the investigation of bioactive properties exhibited by peptides isolated from egg yolk protein. To achieve this aim the following objectives were considered:

1) To extract proteins from egg yolk.

2) To prepare protein hydrolysates from defatted egg yolk proteins through enzymatic digestion using pepsin and pancreatin.

3) To isolate and characterize peptides from egg yolk protein with antioxidant activity and to study the mechanisms of oxidation inhibition.

4) To study the antioxidant behaviour of isolated peptide in Caco-2 cells exposed to a proxidant.

5) To study the isolated peptide as an anticancer agent in Caco-2 cells and to identify the mechanism of action by the flow cytometry.
6) To isolate and characterize peptides from egg yolk protein with angiotensin converting enzyme (ACE) inhibitory activity and to study the mechanism of ACE inhibition.

7) To analyse the amino acid composition and molecular weight of isolated peptides.
Chapter 2
2 Purification and isolation of antioxidant peptides from egg yolk protein

2.1 Introduction

Lipid peroxidation plays a key role in the pathogenesis of many diseases such as cancer (Valko et al., 2004), cardiovascular disease (Cai and Harrison, 2000) and Alzheimer's disease (Christen, 2000). Additionally, lipid oxidation plays a vital role in the loss of food quality by producing undesirable flavours and toxic compounds, which subsequently shorten the product shelf-life (Lin and Liang, 2002; Alghazeer et al., 2008). Therefore, it is important to inhibit the occurrence of excess lipid oxidation in living organisms and food by using antioxidants.

Antioxidants are defined as substances that prevent or slow down the oxidation process (Huang et al., 2005). Although synthetic antioxidants show strong activity in inhibiting the deterioration of lipids during storage, there is a concern about their toxicity and carcinogenicity at high levels of intake (Saito et al., 2003; Vandghanoomini et al., 2013). Therefore, the demand for antioxidants from natural resources has increased in recent years.

In this regard, many studies have been conducted and have revealed that plant phenols and protein hydrolysates may have the ability to inhibit the oxidation process (McCarthy et al., 2001; Shahidi, 2009). The antioxidant activity shown by food-derived compounds makes them a good natural replacement of the synthetic ones. Protein hydrolysates can be obtained by hydrolysis of animal or plant protein using the process of fermentation or by using proteolytic enzymes. As a result of protein hydrolysis,
bioactive peptides are released, which impact positively on various physiological functions (Korhonen and Pihlanto, 2006).

Bioactive peptides are small protein fragments, they can exert diverse physiological effects such as antihypertensive (Matsui et al., 2002), antimicrobial (Haque and Chand, 2008), antithrombotic (Erdmann et al., 2008), hypocholesterolaemic (Zhong et al., 2007) and antioxidant actions (Je et al., 2005). The activity shown by these peptides is not only related to the structure of amino acids making up the peptide and their sequence but also to their molecular weight (Elias et al., 2008; Samaranayaka and Li-Chan, 2011).

Many types of plant and animal protein have been used to isolate and identify antioxidant peptides. For example, sunflower protein (Megías et al., 2004), casein (Suetsuna et al., 2000), soybean protein (Gibbs et al., 2004), egg-white albumen (Miguel et al., 2004), Pacific hake protein (Cinq-Mars et al., 2007), mackerel protein (Sampath Kumar et al., 2012), skin of sole and squid protein (Giménez et al., 2009) and hoki frame protein (Kim et al., 2007) have all been identified as potential sources of antioxidant peptides.

It has been suggested that the whole egg protein is nutritionally better than milk and meat protein (Miki et al., 1984), and is used as a benchmark for measuring the quality of other food proteins. Although several studies have focused on the protein albumen, (Miguel et al., 2004; Miguel and Aleixandre, 2006b), few have examined the nutritional value of egg yolk proteins, mainly due to the lipid content of yolk, which negatively affects human health.
Chapter 2: Purification and isolation of antioxidant peptides from egg yolk protein

The main components of egg yolk are phospholipids, triacylglycerols, and proteins. The first two components are used as lecithin in food or cosmetics. Accordingly, lecithin is used in pharmaceutical applications as a surfactant, a lubricant and as an emulsifying agent (Van Nieuwenhuyzen, 1976). The third component of egg yolk is protein, which represents approximately 30% of dried egg yolk. During the production of egg yolk lecithin, egg yolk protein is produced. Many studies have shown that egg yolk protein and its hydrolysates have antioxidant activities (Lee et al., 2002; Sakanaka et al., 2004; Sakanaka and Tachibana, 2006).

Park et al. (2001) conducted a study on isolating antioxidant peptides from lecithin-free egg yolk using industrial proteases. Their study showed that the isolated peptides were composed of 10 to 15 amino acid residues. Subsequently, other research groups (Sakanaka et al., 2004; Sakanaka and Tachibana, 2006) measured the antioxidant activity of egg yolk protein hydrolysates, which were obtained using a food-grade proteinase from Bacillus sp. Their research showed that egg yolk hydrolysates were powerful free radical scavengers that could be applied as natural antioxidants to prevent the oxidation of fatty acids.

While the majority of the studies related to purifying bioactive peptides have employed microbial proteases, such as those involved in milk processing, few studies have used enzymes involved in human physiological digestion. Enzymes, including pepsin and pancreatin, could be used to demonstrate the impact of physiological digestion on proteins that yield bioactive peptides. Therefore, the aim of this study has to produce an enriched antioxidant peptide hydrolysate of egg yolk protein (EYP) using enzymatic digestion by physiological proteases and to investigate its antioxidant activity.
To achieve this aim, many isolation and purification methods were employed. Briefly, the hydrolysate obtained was fractionated using ultrafiltration membranes. The antioxidant activity of peptides obtained by ultrafiltration was measured in an oxidising linoleic acid system using the ferric thiocyanate method (FTC) and the thiobarbituric acid reactive species (TBARS) reaction to select the most effective antioxidant fraction; this was subjected to gel filtration chromatography. The antioxidant activity of the resultant peptides was compared with that of commercial antioxidants such as trolox and butylated hydroxytoluene (BHT). The composition of amino acids and the molecular weight distribution of the isolated peptide were also investigated.

2.2 Materials and methods

2.2.1 Materials

<table>
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Chapter 2: Purification and isolation of antioxidant peptides from egg yolk protein

2.2.2 Methods

2.2.2.1 Protein extraction and quantification

2.2.2.1.1 Protein extraction

The method used was in accordance with Sakanaka et al. (2004) with some modifications that are detailed below. Briefly, eggs were broken and the yolk separated from the albumen using filter paper and forceps. In order to remove lipids, egg yolk was defatted eight times using hexane and 69% ethanol alternatively with slow agitation using a magnetic stirrer (SM5, Stuart Scientific, UK). The ratio of yolk to solvent was 1:3 (v/v) and 1:5 (v/v) for 69% ethanol and hexane, respectively. The isolated protein was filtered and then stored at -80 °C for further study.

2.2.2.1.2 Protein quantification

The Kjeldhal method (AOAC, 2007) was used to quantify the protein content of egg yolk before and after extraction. The amount of protein was calculated from the nitrogen content. In the first stage (digestion), 1 g of sample was digested in 20 ml concentrated sulphuric acid in the presence of a Kjeldhal catalyst. This step was conducted using a Tecator Kjeltec apparatus consisting of an autodistillation unit (FOSS Kjeltec™ 2200, Sweden) for intense heating. The mixture was left to boil at 400 °C until it became clear. Then the tubes were allowed to cool for 30 minutes. Under these conditions, organic material was oxidized and any organic nitrogen was converted to ammonium sulphate ([NH4]2SO4). In the second stage (distillation), 20 ml of 0.5 M NaOH was used to release the ammonia via steam distillation in a Tecator distillation unit. The distillate was collected in flasks containing 25 ml of 4% boric acid. In the third stage (titration), the quantity of ammonia was estimated by titration using 0.1 M
HCl in the presence of methylene red as an indicator. The percentage of nitrogen and protein was calculated using the following equations:

\[
N(\%) = \frac{100 \times [(ml \ of \ titrant \ of \ sample - ml \ of \ titrant \ of \ blank) \times 0.0014]}{Weight \ of \ sample \ in \ grams}
\]

Where, 1 ml of M HCl = 0.0014 g N

Protein content was calculated by multiplying the nitrogen percentage by a constant factor. This factor varies slightly between different foodstuffs depending on the precise amino acid composition.

\[
Protein(\%) = N \times K
\]

Where, \( K \) is the conversion factor for egg products = 6.25 (Mariotti et al., 2008).

2.2.2.2 Preparation of protein hydrolysates by enzymatic digestion

The method of Sakanaka et al. (2000) was used. Isolated yolk protein powder (prepared using methods detailed in section 2.2.2.1.1) was homogenized with milli-Q water (1:2.5 w/v) for approximately 3-5 minutes using a magnetic stirrer. The pH of the mixture was adjusted to pH 2.0 with 1 M HCl. Pepsin was added to the substrate in the ratio 1:35 (w/w) and the suspension was incubated at 37 °C with continuous stirring. After one hour, the pH was adjusted to pH 7.5 using 1 M NaOH before adding the pancreatin to the mixture in the ratio 1:25 (w/w). The mixture was incubated again at 37 °C for 2 hours with continuous stirring. After the completion of the digestion, the mixture was submerged in boiling water for 20 minutes to inactivate the enzyme and terminate the digestion process. The hydrolyzed protein was then transferred to centrifuge tubes and
centrifuged at 12000 x g (Beckman, California, UK) for 15 minutes. The supernatant egg yolk protein hydrolysate (EYPH) was then lyophilized and stored at -80 °C for further study.

2.2.2.3 Fractionation of egg yolk hydrolysate by ultrafiltration

The freeze-dried EYPH samples produced by the enzymatic digestion step were dissolved in a minimum amount of milli-Q water (1:10) and homogenized for 10 minutes. The solution was sequentially filtered using ultrafiltration membrane cartridges with molecular weight cut-offs (MWCO) of 10 kDa, 5 kDa and 2 kDa, moving from the highest to the lowest. After passage through each membrane, resultant aliquots were centrifuged at 3500 x g for 30 minutes. The fractions collected from 2 kDa, 5 kDa and 10 kDa filtrations were lyophilized and stored at -80 °C for further studies.

2.2.2.4 Gel filtration chromatography

Samples were fractionated by gel filtration chromatography using an Ultimate 3000 HPLC (Thermo Fisher Scientific Inc., Loughborough, UK). The system consists of a pump (Ultimate 3000), column (Sephadex G-25), detector (Ultimate 3000 RS variable wavelength detector), collector (Foxy R1 Fraction Collector) and software (Chromeleon). A gel filtration column (2.5 x 90 cm) was filled with Sephadex G-25 (Sigma-Aldrich, Dorset, UK) and then equilibrated with a 50 mM sodium phosphate buffer (pH 7.0). The column was loaded with 240 mg of 2 kDa hydrolysate after dissolving in 6 ml of a 50 mM sodium phosphate buffer (pH 7.0) and filtering through a 0.22 µm Millipore filter. The injected column was eluted with the same buffer for 10 hours at a constant flow rate of 1 ml/min. Resultant fractions were collected, pooled from 15 different chromatography runs, and lyophilized to be ready for measurement of inhibition of lipid oxidation activity.
2.2.2.5 Measurement of lipid oxidation in linoleic acid model system

The inhibition of lipid peroxidation by the EYPH peptides was measured in an oxidising linoleic acid model system (Osawa and Namiki, 1985). The FTC method was used to monitor the formation of peroxides as primary products from lipid oxidation while the TBARS method was used to measure the production of carbonyl compounds as secondary lipid oxidation products.

2.2.2.5.1 Preparation of reaction mixtures for the lipid oxidation assays

Antioxidant activity was measured in hydrolysate, ultrafiltration, and gel filtration fractions. In a glass tube, 50 mg of each sample was dissolved individually with 10 ml absolute ethanol, 0.13 ml linoleic acid, 4.87 ml distilled water and 10 ml 50 mM phosphate buffer (pH 7). Samples were homogenised by a sonicator (Labsonic® M, B. Braun Biotech International, Germany). The tubes were then sealed tightly with silicone rubber caps and kept in the dark at 40 °C in an oven (Raven oven, LTE Scientific Ltd, Great Britain). Aliquots for FTC and TBARS were taken from these samples daily to measure the activity over 7 days. A negative control was prepared in the same manner using distilled water in place of egg yolk hydrolysates. The commercial antioxidants, 0.2 mg/ml of BHT and trolox (highest permitted limit commercially) were also used as positive controls.

2.2.2.5.2 Ferric thiocyanate (FTC) for peroxide formation

The liberated peroxides oxidise ferrous chloride to the ferric form, resulting in the formation of a red thiocyanate complex which can be measured by spectrophotometry (Mitsuda et al., 1996). At 24 hour intervals, 100 µl aliquots were drawn from each of the reaction mixtures with a micro syringe and added to a test tube containing 4.7 ml
75% ethanol (v/v) and 100 μl 30% ammonium thiocyanate (w/v). Then, 100 μl 20 mM ferrous chloride solution was added. After exactly 3 minutes, the absorbance of the resulting ferric thiocyanate solution was read at 500 nm using a spectrophotometer (UVIKON 860, Kontron instrument, UK). The percentage (%) of lipid oxidation inhibition was calculated according to the following formula:

\[
\text{Lipid oxidation inhibition(\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100
\]

A standard curve was prepared by using serial dilution of ferrous chloride from a stock solution of 0.20 mg/ml (curve concentration range: 0.04, 0.08, 0.12, 0.16, 0.20 mg/ml) (appendix 1). The absorbance of these dilutions was used to calculate the amount of peroxide value in the samples.

2.2.2.5.3 Thiobarbituric acid reactive species (TBARS)

Malondialdehyde (MDA), which is produced as a secondary product of the lipid oxidation process, was measured according to Ohkawa et al. (1979). In acidic medium and at high temperature, thiobarbituric acid (TBA) can bind MDA to form a red-complex pigment. At 24 hour intervals, a 50 μl aliquot was drawn from each of the previous reaction mixtures with a microsyringe and added to test tubes containing 0.8 ml of distilled water, 0.2 ml of 8.1% SDS (w/v), (1.5 ml) 20% (w/v) acetic acid (pH 3.5) and 1.5 ml 0.8% 2- TBA solution in water (w/v). The mixture was heated at 100 °C for 60 minutes. After cooling, the mixture was centrifuged at 4300 x g for 10 minutes (Beckman, UK). The absorbance of the upper layer was measured at 532 nm using a spectrophotometer (UVIKON 860, Kontron instrument, UK). The % of antioxidant activity was calculated according to the following formula:
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\[ \text{Lipid oxidation inhibition(\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \]

A standard curve was prepared by using serial dilution of tetraethoxypropane (TEP) from a stock solution of 200 \( \mu \)g/ml (curve concentration range: 10, 50, 100, 150 and 200 \( \mu \)g/ml) (Appendix 2). The absorbance at these dilutions was used to calculate the amount of MDA in the samples, reported as \( \mu \)g/ml of lipid.

2.2.2.5.4 Effects of peptide concentration on lipid oxidation

In order to determine the relationship between the lipid oxidation inhibitory activity and peptide concentration, the FTC method was conducted. A wide range of isolated peptide concentrations (20, 40, 60, 80, 100 mg/ml) was prepared in a linoleic acid oxidising system and the results compared with (0.2 mg/ml) BHT and (0.2 mg/ml) trolox.

2.2.2.6 Amino acid composition of isolated egg yolk peptide

2.2.2.6.1 Sample and standard preparation

Amino acid content was determined for samples exhibiting high antioxidant activity after the gel filtration process, according to Bidlingmeyer \textit{et al.} (1987). Each sample (5 mg) was dissolved in 5 ml 6 N HCl in dark screw-cap tubes. Oxygen was expelled from the samples using a nitrogen pump before incubation in oven at 110 \( ^\circ \)C for 24 hours. The hydrolysed samples were then subjected to a derivatisation step followed by analysis using HPLC as described below.

2.2.2.6.2 Derivatisation of amino acids with phenylisothiocyanate (PITC)

The derivatization step used was according to Gonzalez-Castro \textit{et al.} (1997). Aliquots of 20 \( \mu \)l hydrolysed samples and amino acid standard solution were placed in micro-
tubes to be dried under vacuum at room temperature for 10 minutes. A 20 μl aliquot of drying reagent (see components below) was then added to dissolve the residue followed by vortexing. The solution was dried again under vacuum for another 10 minutes. The resultant residue was dissolved in 10 μl freshly prepared derivatising reagent (see components below). The sample mixture was then vortexed and left for 10 minutes at room temperature followed by further vacuum drying for another 10 minutes. The residue was dissolved in 20 μl methanol and completely dried under vacuum. Residue obtained from each sample was reconstituted in eluent A and subjected to HPLC analysis.

**Drying reagent** consists of 200 μl methanol, 200 μl 1M sodium acetate, and 100 μl triethylamine.

**Derivatising** reagent consists of 140 μl methanol, 20 μl phenylisothiocyanate (PITC), 20 μl TEA, and 20 μl water.

**2.2.2.6.3 HPLC equipment and conditions**

Samples were analysed using HPLC system (Ultimate 3000 HPLC, Thermo Fisher Scientific Inc., Loughborough, UK) that consists of a pump, autosampler, a detector and integration software (Chromleon). Amino acids were separated on a C18 reverse-phase column (3.9 × 150 mm, 5 μm particle size) using gradient mobile phase consisting of two eluents; A and B (see table 2.1). Eluent A was composed of 0.22 M sodium acetate buffer containing 0.05 % (v/v) TEA, pH6.2 (adjusted using glacial acetic acid). Eluent B was composed of 60:40 acetonitrile: water. The gradient profile used is shown in table 2.1. Amino acids were then detected at 254 nm.
Table 2.1. Gradient profile for chromatographic run of amino acids.

### 2.2.2.7 Molecular mass of egg yolk peptides

The molecular mass and amino acid sequence of the most potent antioxidant fractions identified after gel filtration were determined by quadrupole time-of-flight mass spectrometry (Q-TOF MS) (Q-STAR-XL, AB Sciex UK Limited, Cheshire, UK) equipped with a nano-electrospray ionization source (ESI). Samples of 5 mg/ml were dissolved in 500 µl of 2% acetonitrile containing 0.1% formic acid for the positive mode. Fraction solutions were filtered through 0.2 µm Millex-GV polyvinylidene difluoride (PVDF) (4 mm) filter paper (Millipore) then separately infused into the electrospray source. An aliquot of 0.75 µl from the selected fraction was then directly injected into the ESI through a harvard syringe pump using a 4 µl/min flow rate for an 8 minute injection. The system was controlled by the Analyst™ software version 1.1 and peptide sequence information was acquired over the range 250-800 mass/charge (m/z).
2.2.3 Statistical analysis

All experimental procedures were conducted with at least three independent replications on three separate occasions. Results were analysed using GraphPad Prism software version 6.0. Statistical analysis comparisons were made by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test. \( P < 0.05 \) was considered statistically significant. Data were presented as means ± SD.

2.3 Results

2.3.1 Protein extraction and quantification

Use of organic and inorganic solvents to remove lipids from egg yolk resulted in a white powder as shown in figure 2.1. The protein content of the defatted egg yolk powder was determined using the Kjeldahl method, which measures total nitrogen and provides an accurate estimate of the total protein. The percentage of protein was determined before and after the defatting process. The high percentage of protein in the defatted egg yolk sample (91.0% ± 0.23) compared with native egg yolk (30.2% ± 0.19) demonstrated that the process used to remove lipid was successful.

Figure 2.1. Protein extraction process. Egg yolk before the defatting process (left) and egg yolk isolated protein after the defatting process (right).
2.3.2 Egg yolk protein hydrolysation and ultrafiltration

In this study, the enzymes pepsin and pancreatin were chosen for the hydrolysis step. The aim of using a combination of these two enzymes was not only to mimic the digestive processes in the human body, but also to achieve a degree of hydrolysis greater than that produced when using either pepsin or pancreatin alone.

To isolate an antioxidative peptide from egg yolk, EYPH were fractionated by passing egg yolk protein sequentially through three ultrafiltration (UF) membranes with MWCOs of 10, 5, and 2 kDa. The ultrafiltered EYPH fractions were called EYUF-10 (≤ 10 kDa), EYUF-5 (≤ 5 kDa) and EYUF-2 (≤ 2 kDa). Table 2.2 shows the total yield for each fraction obtained after the ultrafiltration process. Fractions with a molecular weight equal to or less than 2 kDa represent the highest yield recovery (70%) followed by 12% for ≤ 5 kDa and 10% for ≤ 10 kDa peptides.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yield recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYUF-10</td>
<td>10 ± 1.09</td>
</tr>
<tr>
<td>EYUF-5</td>
<td>12 ± 1.2</td>
</tr>
<tr>
<td>EYUF-2</td>
<td>70 ± 2.1</td>
</tr>
</tbody>
</table>

Table 2.2. Fraction yield after ultrafiltration process. Egg yolk protein hydrolysate was fractionated using ultrafiltration membranes. EYUF-10 indicates fractions ≤ 10 kDa, EYUF-5 indicates fractions ≤ 5kDa and EYUF-2 indicates fractions ≤ 2kDa. Data correspond to the means ± SD of three independent experiments.
2.3.3 Lipid oxidation inhibition activity of ultrafiltration fractions

The linoleic acid oxidising system was used to verify the oxidation inhibition activity of fractionated EYPH using the FTC and TBARS methods to choose the most effective peptide fractions for further purification. In this study, the antioxidant effect of three fractions (table 2.2) from EYPH was investigated and compared with trolox as a natural antioxidant and BHT as a synthetic antioxidant.

When the FTC method was conducted, samples were incubated with linoleic acid in the model system at 40 °C and peroxide formation was monitored for 7 days as shown in (figure 2.2-A). Peak peroxide concentrations were detected on day 4 in all samples, thus the percentage of lipid oxidation was estimated at this time point (figure 2.2-B). All fractions significantly decreased the percentage of lipid oxidation when compared to the negative control (all at least \( p < 0.01 \)). Lipid oxidation (%) in the presence of EYUF-2, EYUF-5 and EYUF-10 was 65.86%, 77.36% and 80.74% respectively. BHT and trolox also significantly decreased the percentage of lipid oxidation to 23.32% and 32.17% respectively, when compared to the negative control (\( p < 0.001 \)).
Figure 2.2. Effect of fractionated egg yolk protein hydrolysates on lipid oxidation by ferric thiocyanate (FTC) method. Lipid oxidation was measured in a linoleic acid model system. (A) Peroxide concentration was monitored every 24 hours for 7 days at 500 nm. (B) The percentage of lipid oxidation was measured after four days incubation at 40°C. BHT and trolox (0.2 mg/ml of each) were used as positive controls, while milli-Q water was used in the control instead of sample. EYUF-10, EYUF-5 and EYUF-2 are fractions collected after passing through 10, 5 and 2 kDa ultrafiltration membranes (50 mg/ml of each fraction). Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. The result was considered statistically significant versus the water control (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
Lipid oxidation inhibition activity shown by the three fractions was compared with trolox (figure 2.3-A) and BHT (figure 2.3-B). Among the fractions, the highest inhibition activity was observed in the EYUF-2 sample, followed by EYUF-5, then EYUF-10 (34.14 %, 22.63 %, and 19.32 %, respectively) \((p < 0.001)\). Inhibitory activity was higher in fractions of lower molecular weight, although none were as effective as trolox (67.67%) or BHT (76.67%) \((p < 0.001\) for all comparisons).
Figure 2.3. Comparative antioxidant activity of fractionated egg yolk protein hydrolysates by ferric thiocyanate (FTC) method. Lipid oxidation inhibition activity of all fractions (50 mg/ml of each fraction) was compared to (A) trolox (0.2 mg/ml) as a natural antioxidant positive control and (B) BHT (0.2 mg/ml) as a synthetic antioxidant positive control. The activity was measured in a linoleic acid oxidising system using the FTC method after four days incubation in 40°C. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. The result was considered statistically significant (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
In the TBARS assay, samples were incubated with linoleic acid in the model system at 40 °C and formation of MDA was monitored for 7 days as shown (figure 2.4-A). Maximal concentrations of MDA were measured on day 4, which formed the basis of this time-point selection for investigation of percent lipid oxidation (figure 2.4-B). The percentage of lipid oxidation was significantly decreased in the presence of EYUF-2 followed by EYUF-5 then EYUF-10 when compared to the negative control (all $p < 0.001$; figure 2.4-B). Among the three fractions, the lowest lipid oxidation was observed for the 2 kDa fraction (52.54 %) followed by 5 kDa (56.28%) then 10 kDa (61.25%). The presence of BHT and trolox in a linoleic acid model system significantly inhibit lipid oxidation to 26.10% and 28.73% respectively when compared to the negative control ($p < 0.001$ for both; figure 2.4-B).
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Figure 2.4. Effect of fractionated egg yolk protein hydrolysates on lipid oxidation by thiobarbituric reactive substance (TBARS) method. (A) Malondialdehyde concentration was monitored every 24 hours for 7 days at 532 nm. (B) Percentage of lipid oxidation was measured after four days incubation at 40°C. BHT and trolox (0.2 mg/ml of each) were used as positive controls, while milli-Q water was used in the negative control instead of sample. EYUF-10, EYUF-5 and EYUF-2 are fractions collected after passing through 10, 5 and 2 kDa ultrafiltration membranes (50 mg/ml of each fraction). Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett's multiple comparisons test. The result was considered statistically significant versus the water control (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
The percentage of lipid oxidation inhibition activity shown by all fractions was significantly different to trolox (71.27%) (figure 2.5-A; \( p < 0.001 \)) as well as to BHT (73.89%) as depicted in figure (2.5-B; \( p < 0.001 \)). Lipid oxidation inhibition activity exhibited by EYUF-2, EYUF-5 and EYUF-10 was 47.46%, 43.72% and 38.75%, respectively.

**Figure 2.5. Comparative antioxidant activity of fractionated egg yolk protein hydrolysates by thiobarbituric reactive substance (TBARS) method.** EYUF-10, EYUF-5 and EYUF-2 are fractions collected after passing through 10, 5 and 2 kDa ultrafiltration membranes. Lipid oxidation inhibition activity of all fractions (50 mg/ml of each fraction) was compared to (A) trolox (0.2 mg/ml) as a natural antioxidant positive control and (B) BHT (0.2 mg/ml) as a synthetic antioxidant positive control. The activity was measured in a linoleic acid model system using the TBARS method. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Dunnett’s multiple comparisons test. The result was considered statistically significant (* = \( p < 0.05 \), ** = \( p < 0.01 \), *** = \( p < 0.001 \)).

### 2.3.4 Lipid oxidation inhibition activity of purified peptide after gel filtration

The EYUF-2 fraction with the highest antioxidant activity was collected from the ultrafiltration process and separated by gel filtration chromatography using a Sephadex G-25 column in order to obtain more purified peptides. The elution profile generated with a flow rate of 1 ml/min is shown in figure 2.6.
Figure 2.6. Elution profile of EYUF-2 separated by GPC on a sephadex G-25 column. Separation of peptides was detected at 215 nm with flow rate of 1ml/min.

Fractions obtained from gel filtration chromatography were pooled then lyophilized and their oxidation inhibition activity was assayed using the FTC method. The method was conducted as a primary overview of all fractions to test their ability to inhibit lipid oxidation when used at a concentration of 50 mg/ml. Figure 2.7 shows that oxidation inhibition activity was widely observed for all gel filtration fractions (EYGF), but the most effective fractions depicted by shaded bars were EYGF-23 (60.48% inhibition) followed by EYGF-33 (59.82% inhibition). These two fractions were, therefore, collected for further study.
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Figure 2.7. Antioxidant inhibition activity (%) of gel filtration fractions using ferric thiocyanate (FTC) method. The activity was analysed for 50 mg/ml of all fractions separated on a Sephadex G-25 column. The absorbance was measured after 4 days incubation at a wavelength of 500 nm. Data correspond to the means ± SD of three independent experiments. The results were compared with (0.2 mg/ml) trolox and (0.2 mg/ml) BHT.

FTC and TBARS assays were conducted to compare the antioxidant activity of EYUF-2 obtained from ultrafiltration, and the EYGF-23 and EYGF-33 sub-fractions obtained from gel filtration to examine if the purification process used would alter the inhibitory activity. The formation of peroxides was monitored over 7 days for selected fractions, using trolox and BHT as positive controls and a negative control containing only water (figure 2.8-A). All fractions significantly decreased lipid oxidation compared with the negative control ($p < 0.001$; figure 2.8-B). From the graph it was clearly observed that gel filtration fractions exhibited lower percentage of lipid oxidation than ultrafiltration ones. Oxidation in the presence of EYGF-23 and EYGF-33 was 39.52% and 40.18%, respectively followed by ultrafiltration fraction EYUF-2 to reach 65.86%. The percentage of lipid oxidation was markedly reduced to 23.32% and 32.17% in the
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Presence of BHT and trolox respectively when compared to the negative control ($p < 0.001$ for both; figure 2.8-B).

Figure 2.8. Effect of EYUF-2, EYGF-23, and EYGF-33 on lipid oxidation analysed by the ferric thiocyanate (FTC) method. EYUF-2 was obtained after ultrafiltration and EYGF-23 and EYGF-33 were obtained from gel filtration (50 mg/ml of each fraction). Lipid oxidation was measured in a linoleic acid model system using the FTC method. (A) Peroxide concentration was monitored every 24 hours for 7 days at a wavelength of 500 nm. (B) The percentage of lipid oxidation was measured after four days incubation at 40 °C. BHT and trolox (0.2 mg/ml of each) were used as positive controls, while milli-Q water was used in the negative control instead of sample. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. The result was considered statistically significant versus the water control (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).
Lipid oxidation inhibition activity was also calculated and compared with the activity of trolox (figure 2.9-A) and BHT (figure 2.9-B). EYGF-23 and EYGF-33 achieved 60.48% and 59.82% inhibition of lipid oxidation, respectively. The activity exhibited by the selected fractions from gel filtration was not significantly different to that of trolox (67.67%), which was used as a natural antioxidant positive control in the experiment (figure 2.9-A). On the other hand, inhibition activity exhibited by gel filtration fractions was significantly different compared with the synthetic antioxidant BHT (figure 2.9-B; \( p < 0.01 \) for both fractions). Moreover, the activity shown by both fractions was significantly more effective than EYUF-2, which inhibited lipid oxidation by only 34.13%.

![Figure 2.9](image_url)  

**Figure 2.9. Comparative antioxidant activity of EYUF-2, EYGF-23, and EYGF-33 analysed by the ferric thiocyanate (FTC) method.** EYUF-2 was after ultrafiltration and EYGF-23 and EYGF-33 were obtained from gel filtration (50 mg/ml of each fraction). Lipid oxidation inhibition activity of all fractions was compared to (A) trolox (0.2 mg/ml) as a natural antioxidant positive control and (B) BHT (0.2 mg/ml) as a synthetic antioxidant positive control. The activity was measured in a linoleic acid model system using the FTC method after four days incubation at 40 °C. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. The result was considered statistically significant (* = \( p < 0.05 \), ** = \( p < 0.01 \), *** = \( p < 0.001 \)).
The same potency of inhibitory activity was found when monitoring the formation of MDA for 7 days. Again, figure 2.10-A shows that maximum MDA concentrations were observed on day 4. The selected fractions significantly inhibited the formation of MDA, which represents one of the secondary products produced as a result of lipid oxidation (figure 2.10-B; $p < 0.001$ for all comparisons). Similar findings were observed as in FTC method that gel filtration fractions exhibited higher inhibition activity than ultrafiltration fractions. EYGF-23 and EYGF-33 inhibited MDA formation significantly to (33.59% and 44.74%, respectively) ($p < 0.001$) followed by EYUF-2 which inhibit the formation of MDA to 52.54% ($p < 0.001$).
Figure 2.10. Effect of EYUF-2, EYGF-23, and EYGF-33 on lipid oxidation analysed by thiobarbituric reactive substance (TBARS) method. EYUF-2 was obtained after ultrafiltration and EYGF-23 and EYGF-33 were obtained from gel filtration (50 mg/ml of each fraction). Lipid oxidation was measured in a linoleic acid model system using the TBARS method. (A) Malondialdehyde (MDA) concentration was monitored every 24 hours for 7 days at a wavelength of 532 nm. (B) Percentage of lipid oxidation was measured after four days incubation at 40°C. BHT and trolox (0.2 mg/ml of each) were used as positive controls, while milli-Q water was used in the negative control instead of egg yolk fraction sample. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Dunnett’s multiple comparisons test. The result was considered statistically significant versus the water control (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
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The percentage of lipid oxidation inhibition activity of selected fractions was also demonstrated in comparison with trolox and BHT. Among the fractions, EYGF-23 exhibited the highest inhibitory activity (66.4%) followed by EYGF-33 (55.26%) followed by EYUF-2 (47.45%). The activity of EYGF-23 was not significantly different when compared with trolox as shown in figure 2.11-A or BHT as depicted in figure 2.11-B.

Figure 2.11. Comparative antioxidant activity of EYUF-2, EYGF-23 and EYGF-33 analysed by thiobarbituric reactive substance (TBARS) method. EYUF-2 was obtained after ultrafiltration and EYGF-23 and EYGF-33 were obtained from gel filtration (50 mg/ml of each fraction). Lipid oxidation inhibition activity of all fractions was compared to (A) Trolox (0.2 mg/ml) as a natural antioxidant positive control and (B) BHT (0.2 mg/ml) as a synthetic antioxidant positive control. The activity was measured in a linoleic acid model system using the TBARS method. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. The result was considered statistically significant (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

2.3.5 Effect of peptide concentration on oxidation inhibition

Inhibition of linoleic acid oxidation using serial dilutions of EYGF-23 and EYGF-33 (from 20 mg/ml to 100 mg/ml) was observed by the FTC method. Inhibition of peroxide formation increased with EYGF-23 peptide concentration (figure 2.12); the activity shown by 20, 40, 60, 80 and 100 mg/ml was 53.13%, 59.82%, 67.02%, 72.58% and 80.13% respectively. At 80 mg/ml, EYGF-23 exhibited inhibition activity comparable
to trolox and BHT. Moreover, inhibition activity exhibited by 100 mg/ml peptide was significantly greater than with trolox (67.67%, $p < 0.01$) (figure 2.12-A), but not significantly different to BHT (76.67%) (figure 2.12-B).

Figure 2.12. Effect of EYGF-23 concentration on lipid oxidation inhibition. Concentration range of EYGF-23 (20, 40, 60, 80, 100 mg/ml) were incubated for 96 hours in a linoleic acid oxidising system at 40°C. The activity was measured using the ferric thiocyanate method. Trolox (A) and BHT (B) (both 0.2 mg/ml) were used as positive controls. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. The result was considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The same pattern of inhibition was observed when different doses of EYGF-33 were tested. At concentrations of 20 mg/ml, inhibition of linoleic acid oxidation was significantly less compared with trolox ($p < 0.01$), however, inhibitory activity was similar to trolox (76.42%) at higher concentrations of EYGF-33 (40 -100 mg/ml) (figure 2.13-A). Concentrations greater than 60 mg/ml were required to achieve a similar effect to BHT (figure 2.13-B).
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Figure 2.13. Effect of EYGF-33 concentration on lipid oxidation inhibition. Concentration range of EYGF-33 (20, 40, 60, 80, 100 mg/ml) were incubated for 96 hours in a linoleic acid oxidising system at 40°C. The activity was measured using the ferric thiocyanate method. Trolox (A) and BHT (B) (both 0.2 mg/ml) were used as positive controls. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Dunnett’s multiple comparisons test. The result was considered statistically significant (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

2.3.6 Amino acid analysis and determination of molecular mass

Given the substantial inhibitory effects of EYGF-23 and EYGF-33 on peroxide and MDA formation, both fractions were subjected to amino acid analysis and MS in order to elucidate the possible effect of amino acid composition and sequence on antioxidant activity. The chromatogram of amino acids and the calculated composition of the selected fractions after gel filtration are presented in figure 2.14 and table 2.3, respectively. While EYGF-23 contained large proportions of unique proline (8.01%), electrically charged lysine (11.03%), and the hydrophobic amino acids tyrosine (12.40%) and tryptophan (16.40%), EYGF-33 contained hydrophobic amino acids such as alanine (7.80%), leucine (13.82%), and tryptophan (9.44%), and positively charged lysine (8.27%) and arginine (11.80%).
Figure 2.14. Amino acid chromatograms of (A) amino acid standards, (B) EYGF-23 peptide after hydrolysis with 6 N HCl and (C) EYGF-33 peptide after hydrolysis with 6 N HCl. Amino acids were separated on a C18 reverse-phase column and detected at 254 nm. Gradient mobile phase was used consisting of two eluents; A and B. Eluent A was composed of 0.22 M sodium acetate buffer containing 0.05 % (v/v) TEA, pH 6.2 (adjusted using glacial acetic acid). Eluent B was composed of 60:40 acetonitrile: water.
## Amino acid residues composition of EYGF-23 and EYGF-33

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Amino acid residues in EYGF-23(%)</th>
<th>Amino acid residues in EYGF-33(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>3.02 ± 0.27</td>
<td>6.22 ± 0.02</td>
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<td>Glutamic acid</td>
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<td>Histidine</td>
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<td>Lysine</td>
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<td>8.27 ± 0.003</td>
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</table>

*Table 2.3. Amino acid residues composition of EYGF-23 and EYGF-33. Data correspond to the means ± SD of three independent experiments.*

EYGF-23 and EYGF-33 were subsequently subjected to MS for peptide sequence identification. Figure 2.15-A shows the ion scan mass spectrum of EYGF-23; the peptide sequence information was acquired over the range 200-750 Da m/z. EYGF-33 was also subjected to MS to identify the amino acid sequence, over the range of 250-
Chapter 2: Purification and isolation of antioxidant peptides from egg yolk protein

800 Da m/z as depicted in figure 2.15-B. However, the exact peptide sequences of the fractions were difficult to establish due to multiple possibilities and poor matching with the known peptide segments in egg yolk protein.

Figure 2.15. Molecular weight distribution profiles of EYGF-23 and EYGF-33. Molecular weight distribution profiles of (A) EYGF-23 and (B) EYGF-33 at small scale (250-750 Da m/z) performed by quadrupole time-of-flight mass spectrometry (Q-TOF MS) equipped with an electrospray ionisation (ESI) source.
2.4 Discussion

The ability of bioactive peptides to exhibit antioxidant activity and to replace synthetic antioxidants are potentially useful. Therefore, the aim of the current chapter is to investigate the antioxidant activity exhibited by peptides, which had been isolated from egg yolk protein. Egg yolk contains storage lipids as neutral lipids and conjugated lipids as phospholipids and glycolipids. Therefore, the defatting step was carried out by using a combination of hexane and ethanol. Hexane is a non-polar solvent that is considered to be a good extractor for neutral lipids, whereas ethanol is a good solvent for removing conjugated lipids. The protein content of egg yolk protein isolate was determined using the Kjeldahl method, the high percentage of protein content in defatted egg yolk sample demonstrated that the process used to remove lipid had been successful.

In this study, egg yolk protein was subjected to enzymatic hydrolysis in order to release bioactive peptides. Pepsin and pancreatin have different selectivity, therefore they were chosen in this study to offer a wide range of peptide. The high yield of the smaller molecular weight fraction, obtained from ultrafiltration process (EYUF-2), indicated that EYP was hydrolysed extensively (table 2.2). Chen et al. (1996) illustrated that the antioxidant potential of protein hydrolysates can be enhanced by hydrolysis with specific enzymes and optimal conditions used during the proteolysis step. Accordingly, many studies have been conducted using enzymatic hydrolysis to improve the functional properties and activity exhibited by isolated proteins (Vioque et al., 2000; Pedroche et al., 2004).

The ultrafiltration process was used as a first step to purify peptides depending on their molecular weight. A linoleic acid oxidising system verified the oxidation inhibition
activity of the peptides using FTC and TBARS methods; the highest activity was highlighted by the smallest molecular weight fraction EYUF-2. This finding may relate to the small molecular weight of the peptides in this fraction, as many studies have shown that peptides of smaller molecular weight exhibit greater antioxidative activity (Wu et al., 2003; Kim and Wijesekara, 2010). Therefore, only the EYUF-2 fraction with the highest antioxidative activity was chosen for further fractionation.

Further purification by gel filtration led to fractions EYGF-23 and EYGF-33 that scored the highest antioxidant activity (figure 2.7). For both FTC and TBARS methods, the inhibition activity exhibited by gel filtration fractions EYGF-23 and EYGF-33 was higher than the activity exhibited by EYUF-2 (figure 2.9 and figure 2.11). Therefore, EYGF-23 and EYGF-33 were chosen for structural characterization due to their highest antioxidant activity.

The result obtained from amino acid analysis of both EYGF-23 and EYGF-33 demonstrated that both of them have different amino acids in their structure, probably due to the use of two enzymes with different selectivity. Many studies revealed that amino acid content, their sequence in the isolated peptide and peptide size, together play an important role in determining the activity of a peptide (Elias et al., 2008; Samaranayaka and Li-Chan, 2011).

It is well known that the presence of hydrophobic amino acids in protein hydrolysates or peptides elevates their antioxidant activity by increasing peptide solubility in a lipid system (Rajapakse et al., 2005; Wang et al., 2007). In this study, the presence of hydrophobic amino acids such alanine, tryptophan and tyrosine may play a role in increasing the interaction between peptides and fatty acids (table 2.3). Chen et al.
Chapter 2: Purification and isolation of antioxidant peptides from egg yolk protein

(1995) isolated antioxidative peptides that contained the hydrophobic amino acid residues, valine and leucine at the N-terminus. Chen et al. (1996) illustrated that the presence of hydrophobic moieties in an antioxidant peptide was important to obtain access to hydrophobic targets. Tryptophan is also an important amino acid and removal of the labile hydrogen attached to the nitrogen of its indole ring produces a free radical that is easily stabilised due to electron delocalization, thereby allowing tryptophan to break the free radical chain reactions and stop the oxidation process (Tsopmo et al., 2011). Tsopmo et al. (2011) also illustrated that the presence of tryptophan in human milk hydrolysates was essential to maintain scavenging activity; activity was markedly decreased upon tryptophan deletion.

Tyrosine-containing peptides can also scavenge radicals as shown by peptides derived from pepsin-digested cow casein (Suetsuna et al., 2000), and hydrolysed beta lactoglobulin produced both tyrosine and tryptophan peptides that could quench radicals (Hernández-Ledesma et al., 2008). Saeed et al. (2006) suggested that, due to the ability of tyrosine to donate hydrogen, it was preferentially oxidised by radicals to protect lipid systems from oxidation. As a result, the antioxidant activity of EYGF-23 and EYGF-33 is thought to be related to their amino acid composition.

2.5 Conclusion

To conclude, the EYGF-23 and EYGF-33 fractions isolated from EYP using proteolytic enzymes inhibited oxidation induced in a linoleic acid oxidising system in a dose-dependent manner. The presence of hydrophobic amino acids, some of which can directly inhibit lipid oxidation due to their aromatic structure, may help to explain the mechanism of antioxidant activity of the peptides. The low molecular weight of
peptides (200 -750 Da) was also an important determinant of antioxidant activity. Free radicals and oxidative stress have been implicated as main causative factors in food deterioration. Given their antioxidant properties, these results suggest that EYGF-23 and EYGF-33 may serve as a good source of desirable antioxidant peptides.
Chapter 3
3 Antioxidant mechanisms of peptides derived from egg yolk protein

3.1 Introduction

Natural antioxidants included in the diet have drawn the attention of consumers and scientific researchers. Food-derived antioxidants play an important role in reducing the amount of free radicals and hence their adverse impact on biological systems (Yau, 1979; Stadtman, 2006; Halliwell, 2009). In food systems, when free radicals attack lipids they not only cause alteration in the organoleptic properties, giving unacceptable taste, odd flavor and rancidity but also produce toxic compounds that reduce the quality and the shelf life of the products (Lin and Liang, 2002; Alghazeer et al., 2008).

Free radicals such as the hydroxyl radical (OH\(^-\)) are generated in biological systems via the Fenton reaction. The OH\(^-\) radical can easily ignite a free radical chain reaction by attacking all biological macromolecules (Valko et al., 2007). The superoxide anion is another radical naturally generated in living cells after oxygen uptake (Stief, 2003). This radical enhances the formation of other reactive oxygen species (ROS) such as hydrogen peroxide, the hydroxyl radical and singlet oxygen. Superoxide anion can also react with nitric oxide and form reactive nitrogen species (RNS) such as peroxynitrite, which can generate toxic compounds such as nitrogen dioxide (Touyz and Briones, 2011). Therefore, antioxidants have been used to retard the harmful effect that occurs as a result of oxidation.

It is well-known that protein hydrolysates and peptides prepared from food proteins have emerged as a new source of natural antioxidants (Mine and Shahidi, 2005; Hartmann and Meisel, 2007; Shahidi and Zhong, 2008). These protein-derived
compounds have demonstrated strong antioxidant efficacy through different pathways, including radical scavenging and metal ion chelating activity (Qian et al., 2008). These pathways are discussed further below.

Radical quenching activity by antioxidant peptides is considered one of the most effective pathways in the prevention of lipid peroxidation propagation reactions. This activity is, therefore, very important as a means of protecting biological systems against various diseases and to protect food systems from deterioration. There is a structure-function relationship between the amino acid composition and the scavenging activity of the isolated peptide. Low molecular weight of the isolated peptide, the presence of specific amino acid residues such as tryptophan and tyrosine, and their sequence within the peptide are noticeably correlated with radical scavenging activity of the peptides (Peña-Ramos et al., 2004; Elias et al., 2006; Hernández-Ledesma et al., 2007).

Many studies have revealed that there is a direct correlation between the antioxidant activity of bioactive compounds and reducing power (Rice-Evans et al., 1996; Zhu et al., 2006). Under oxidising conditions, where unsaturated fatty acids and active peptides are present in the same system, peptides are more susceptible to the donation of electrons than lipids. Peptides also form more stable compounds or non-reactive polymers through radical-radical interaction (Yıldırım et al., 2000; Bougatef et al., 2009). Therefore, the reducing power assay is often conducted to evaluate the ability of an antioxidant to donate an electron to free radicals and inhibit their actions.

Chelation of transition metals, such as Fe^{2+} and Cu^{2+} is another crucial mechanism by which antioxidant peptides function (Wang and Xiong, 2005; Megias et al., 2008). In food systems, the presence of transition metal ions enhances the autoxidation rate and
formation of reactive oxygen species. Valko et al. (2006) illustrated the ability of these metal ions to react very quickly with peroxides via an electron donation process to form alkoxy radicals; these, in turn, enhance the initiation of the free radical chain reaction. Among these metals, ferrous ion (Fe^{2+}) is considered to be one of the crucial pro-oxidants that catalyses the generation of hydroxyl radicals (OH\textsuperscript{.}) through the Fenton reaction. Therefore, chelation of transition metal ions by antioxidant peptides may retard the generation of reactive oxygen species and intercept the free radical chain process.

In the current study, our potential understanding of the antioxidant mechanisms of egg yolk peptides may well lead to the utilisation of egg yolk protein as a source of natural antioxidants. Therefore, the present study investigated the antioxidant mechanism of egg yolk protein fractions prepared with pepsin and pancreatin, followed by gel filtration. Different measurements, including the scavenging effect on free radicals, Fe^{3+} chelating activity and reducing power are used to evaluate the antioxidant activities.
3.2 Materials and methods

3.2.1 Materials

<table>
<thead>
<tr>
<th>Materials</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrous sulphate</td>
<td></td>
</tr>
<tr>
<td>1, 1-diphenyl-2-picrylhydrazyl (DPPH)</td>
<td></td>
</tr>
<tr>
<td>α-deoxyribose</td>
<td></td>
</tr>
<tr>
<td>Potassium ferricyanide</td>
<td></td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>Sigma-Aldrich, Dorset, UK.</td>
</tr>
<tr>
<td>Ferrozine</td>
<td></td>
</tr>
<tr>
<td>Thiobarbituric acid (TBA)</td>
<td></td>
</tr>
<tr>
<td>Trichloroacetic acid (TCA)</td>
<td></td>
</tr>
<tr>
<td>Pyrogallol</td>
<td></td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td></td>
</tr>
</tbody>
</table>

3.2.2 Experimental methods

3.2.2.1 Isolation and purification

EYGF-23 and EYGF-33 were isolated and purified as explained in chapter 2 sections 2.2.2.2, 2.2.2.3 and 2.2.2.4

3.2.2.2 Reducing power assay

The principle of this assay is based on the reduction of ferricyanides to the ferrous form due to the presence of an antioxidant (reductant) in the reaction mixture. The resulting ferrocyanides form a complex with ferric chloride, which can be monitored by
spectrophotometry at 700 nm. Elevations in absorption readings indicate increases in electron donating ability and hence the reducing power of the antioxidant.

The ability of the EYGF-23 and EYGF-33 to reduce iron (III) was determined according to the method of Yildirim et al. (2000). An aliquot of 1 ml EYGF-23 or EYGF-33 samples at different concentrations (0.5, 1, 5, 10 and 20 mg/ml) was mixed with 2.5 ml of 0.2 M phosphate buffer previously equilibrated to pH 6.6, and 2.5 ml of 1% w/v potassium ferricyanide. The mixture was incubated at 50 °C for 30 minutes, and 2.5 ml of 10 % w/v trichloroacetic acid was added, prior to centrifugation at 1650 x g for 10 minutes. Next, 2.5 ml of the supernatant solution was added to 2.5 ml distilled water and 0.5 ml of 0.1% w/v ferric chloride. After 10 minutes of reaction time, the absorbance of the solution was measured at 700 nm using a UV-visible spectrophotometer (Kontron Instrument UNIKON 860, UK). The yellow colour of the reaction mixture changed to different degrees of greenish blue depending on the extent of reduction. The values presented are the means of triplicate experiments.

3.2.2.3 DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity assay

DPPH is a stable nitrogen-centred free radical, characterized by its violet colour. The exposure of DPPH to hydrogen- or electron-donating substrates makes its purple colour fade from violet to yellow coloured diphenylpicrylhydrazine (DPPH-H), as shown below. The degree of colour fading reflects the ability of peptides to scavenge DPPH, showing maximum absorbance at 517 nm. Substances able to scavenge DPPH can be considered as radical scavengers (Ebrahimzadeh et al., 2009).

\[
(DPPH^+) + (H-A) \rightarrow (DPPH-H) + (A^-)
\]

Purple \hspace{1cm} Yellow
Therefore, the DPPH method was conducted in this study to investigate the effectiveness of EYGF-23 and EYGF-33 as radical scavengers.

The method used was in accordance with Bersuder et al. (1998) using the synthetic free radical DPPH. Briefly, a volume of 500 µl EYGF-23 or EYGF-33 at different concentrations (0.5, 1, 5, 10 and 20 mg/ml) was mixed with 500 µl of 99.5% ethanol and 125 µl of 0.02% w/v DPPH prepared previously in 99.5% ethanol. The mixture was shaken vigorously then kept at room temperature in the dark. After 60 minutes, the colour reduction of the DPPH substrate was measured at an absorbance of 517 nm using a UV-visible spectrophotometer (Kontron Instrument UNIKON 860, UK). Trolox and BHT (both used at concentration 0.2 mg/ml) were also tested as positive controls for comparison, following the same method as above.

The DPPH radical scavenging activity was calculated as follows:

\[
\text{Radical scavenging activity(\%) = } \frac{A_c - A_s}{A_c} \times 100
\]

Where \( A_c \) is the absorbance for negative control samples measured using 500 µl deionized water instead of sample, \( A_s \) is the absorbance of the sample or positive controls (trolox/BHT). A lower absorbance of the reaction mixture indicated a higher DPPH radical-scavenging activity. The values are presented as the means of triplicate experiments.

### 3.2.2.4 Hydroxyl radical scavenging activity (HRSA) assay

In the HRSA, hydrogen peroxide was used to oxidize iron from the ferrous state (II) to the ferric state (III), and liberate the hydroxyl radical and a hydroxyl anion as follows
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 sodium phosphate buffer pH 7.4, and 0.2 ml of peptides at different concentrations (0.5, 1, 5, 10 and 20 mg/ml). All reagents were then thoroughly mixed in a tube and 0.2 ml of 10 mM hydrogen peroxide was added. The reaction mixture was then incubated at 37 °C for 1 hour. Following incubation, 1 ml of 2.8% TCA and 1 ml of 1.0% TBA were added to the test tubes, and the contents boiled for 15 minutes. After cooling at room temperature, the absorbance of the mixture was measured at 532 nm using a UV-visible spectrophotometer (Kontron Instrument UNIKON 860, UK). Trolox and BHT (both used at concentration 0.2 mg/ml) were also tested as positive controls for comparison, following the same method as above. The HRSA was evaluated as a percentage of α-deoxyribose oxidation inhibition by the hydroxyl radical and calculated as follows:

\[
\text{HRSA(\%)} = \frac{A_c - A_s}{A_c} \times 100
\]

Where \( A_c \) is the absorbance for negative control samples measured using 500 μl deionized water instead of sample, \( A_s \) is the absorbance of the sample or positive controls (trolox/BHT). The values presented are the means of triplicate experiments.

3.2.2.5 Superoxide anion scavenging activity (pyrogallol)

In this assay, the alkaline condition enhances the autoxidation of pyrogallol to produce a superoxide anion which, in turn, accelerates the autoxidation process by autocatalysis.
The autoxidation of a pyrogallol method described by Marklund and Marklund (1974) was applied to measure the superoxide anion scavenging power of EYGF-23 and EYGF-33. The reaction mixture consisted of 1.0 ml of peptides sample at different concentrations (0.5, 0.1, 5.0, 10 and 20 mg/ml) and 1.8 ml of 50 mM Tris-HCl buffer, previously adjusted to pH 8.2. The mixture was incubated at 25 °C for 20 minutes. Then, 40 µl of 45 mM pyrogallol (prepared previously in 10 mM HCl) was added. The absorbance of the reaction mixture was measured at 320 nm immediately after adding pyrogallol at 1 minute intervals up to 4 minutes using a UV-visible spectrophotometer (Kontron Instrument UNIKON 860, UK). Trolox and BHT (both used at a concentration of 0.2 mg/ml) were also tested as positive controls for comparison, following the same method as above.

The $O_2^{•−}$ scavenging activity was calculated as follows:

\[
\text{The } O_2^{•−} \text{-scavenging activity(%) } = \frac{\Delta A_c - \Delta A_s}{\Delta A_c} \times 100
\]

Where (ΔAc) is the autoxidation rate of pyrogallol in negative control samples, measured using 1.0 ml deionized water, (ΔAs) is the oxidation rate of pyrogallol for samples or positive controls (trolox/BHT). The values presented are the means of triplicate experiments.

3.2.2.6 Ferrous chelating activity assay

The activity of the peptide to chelate ferrous Fe$^{2+}$ ions was measured according to the method of Boyer and McCleary (1987). In this method, ferrozine was used to determine the chelating activity of EYGF-23 and EYGF-33. Ferrozine normally forms a water-soluble magenta-coloured complex with ferrous ions, which can be measured
Chapter 3: Antioxidant mechanisms of peptides derived from egg yolk protein

spectrophotometrically. In the presence of chelating agents (e.g. peptides), the complex
formation is interrupted and subsequently the intensity of the magenta colour is reduced,
as shown by reduced absorbance at 563 nm. Thus, lower absorbance indicates higher
chelating activity.

The reaction mixture contained 0.5 ml of isolated peptides at different concentrations
(0.5, 1, 5, 10 and 20 mg/ml), 1.6 ml deionized water and 0.05 ml of 2 mM FeCl₂. After
30 seconds, 0.1 ml of 5 mM ferrozine was added. After 10 minutes at room temperature,
the absorbance of the Fe²⁺-ferrozine complex was measured at 563 nm using a UV-
visible spectrophotometer (Kontron Instrument UNIKON 860, UK). EDTA (0.2 mg/ml)
was also tested as a positive control for comparison, following the same method as
above.

The activity of the peptide to chelate Fe²⁺ was calculated as:

\[
\text{Chelating ability (\%)} = \frac{A_c - A_s}{A_c} \times 100
\]

Where \(A_c\) is the absorbance of the control, using 0.5ml deionized water instead of
sample, \(A_s\) is the absorbance of the sample or positive control EDTA (0.2 mg/ml). The
values presented are the means of triplicate experiments.

3.2.3 Statistical analysis

All experimental procedures were performed with at least three independent repeats.
Results were analysed using GraphPad Prism version 6.0. Statistical analysis
comparisons were made by one-way analysis of variance (ANOVA) followed by
Dunnett’s multiple comparisons test. \( p < 0.05 \) was considered statistically significant. Data are presented as means ± SD.

### 3.3 Results

#### 3.3.1 Reducing power assay

The ability of EYGF-23 and EYGF-33 to reduce the ferricyanide complex to the ferrous form was measured in this study using a wide concentration range of peptide (0.5-20 mg/ml). Ferrous ion can, therefore, be monitored by measuring the Perl’s Prussian blue that formed at 700 nm. Table 3.1 shows that the reducing power of EYGF-23 and EYGF-33 increased with increased concentration in a dose-dependent manner. The absorbance reaches a maximum of 0.26 and 0.29 at a concentration of 20 mg/ml from EYGF-23 and EYGF-33, respectively.

<table>
<thead>
<tr>
<th>Sample concentration (mg/ml)</th>
<th>0.5</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYGF-23</td>
<td>0.06±0.01</td>
<td>0.07±0.01</td>
<td>0.13±0.01</td>
<td>0.18±0.01</td>
<td>0.26±0.01</td>
</tr>
<tr>
<td>EYGF-33</td>
<td>0.04±0.01</td>
<td>0.10±0.02</td>
<td>0.15±0.01</td>
<td>0.23±0.01</td>
<td>0.29±0.01</td>
</tr>
</tbody>
</table>

Table 3.1. Reducing power of EYGF-23 and EYGF-33. Reducing power was measured at a concentration range of 0.5 to 20 mg/ml. The activity was measured at 700 nm. Data correspond to the means ± SD of three independent experiments. Analysis was performed in Graphpad Prism version 6.0.
3.3.2 DPPH radical-scavenging activity

DPPH radical scavenging activity of a range of concentrations of EYGF-23 and EYGF-33 was measured and the results were compared with trolox and BHT (positive controls) (table 3.2). The scavenging activity of EYGF-23 and EYGF-33 increased with the concentration in a dose-dependent manner. The radical scavenging activity exhibited by 0.5, 1, 5, 10, 20 mg/ml of EYGF-23 were 8.10, 11.32, 15.61, 19.40, and 28.46 % respectively. On the other hand, the activity shown by EYGF-33 for the same concentrations was 6.15, 10.99, 13.58, 16.47 and 30.65% respectively. When comparing the scavenging activity of EYGF-23 and EYGF-33 with trolox, all the concentrations used from both fractions were significantly lower than trolox (38.46%, $p <0.05$). In contrast, only the highest concentration of both fractions (20 mg/ml) exhibited significantly higher scavenging activity when compared with BHT (23.60%, $p <0.05$).

<table>
<thead>
<tr>
<th>Sample concentration (mg/ml)</th>
<th>Trolox$^a$</th>
<th>BHT$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2mg/ml</td>
<td>0.2mg/ml</td>
</tr>
<tr>
<td>0.5</td>
<td>8.10$^{ab}$</td>
<td>38.46%</td>
</tr>
<tr>
<td>1</td>
<td>11.32$^{ab}$</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>15.61$^{ab}$</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>19.40$^{a}$</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>28.46$^{ab}$</td>
<td></td>
</tr>
<tr>
<td>EYGF-23</td>
<td></td>
<td>23.60%</td>
</tr>
<tr>
<td></td>
<td>±1.43$^{ab}$</td>
<td>±1.66</td>
</tr>
<tr>
<td>0.5</td>
<td>6.15$^{ab}$</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10.99$^{ab}$</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>13.58$^{ab}$</td>
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<td>10</td>
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<td>20</td>
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<tr>
<td>EYGF-33</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>±1.15$^{ab}$</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>±1.15$^{ab}$</td>
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</tr>
<tr>
<td>1</td>
<td>±1.90$^{ab}$</td>
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<tr>
<td>5</td>
<td>±1.16$^{ab}$</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>±1.49$^{ab}$</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>±2.86$^{ab}$</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. Comparative DPPH radical scavenging activity of EYGF-23 and EYGF-33. The scavenging activity was measured over a concentration range of 0.5 to 20 mg/ml from each isolated fraction using DPPH radical scavenging. The activity of both fractions was measured at 517 nm and were compared to BHT as a synthetic antioxidant standard and trolox as a natural antioxidant standard (both 0.2 mg/ml). Data correspond to the mean ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Dunnett’s multiple comparisons test. Values with subscript $a$ to present significant differences with trolox, and subscript $b$ to present significant differences with BHT $p <0.05$. 

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3.3.3 The hydroxyl radical-scavenging activity

A comparison of hydroxyl radical scavenging activity of EYGF-23, EYGF-33, trolox and BHT is presented in table 3.3. The scavenging activity of EYGF-23 and EYGF-33 increased with the concentration in a dose-dependent manner. From the table 3.3, it can be seen that all peptide concentrations from EYGF-23 were significantly less effective at scavenging hydroxyl radicals in comparison with trolox (70.03%, $p < 0.05$). The same pattern was observed with EYGF-33 with the exception of the highest concentrations (20 mg/ml) which exhibited a non-significant effect compared to trolox. Comparative activity was also done between all concentrations and BHT. The results show that all concentrations less than 20 mg/ml had a significantly lower effect than BHT (58.97%, $p < 0.05$). While there was no significant difference between EYGF-23 and BHT at 20 mg/ml, the same concentration of EYGF-33 was significantly more effective as a scavenger compared with BHT.

<table>
<thead>
<tr>
<th>Sample concentration (mg/ml)</th>
<th>Trolox$^a$</th>
<th>BHT$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>EYGF-23</td>
<td>5.62%±2.33$^a$</td>
<td>10.575±1.36$^a$</td>
</tr>
<tr>
<td>EYGF-33</td>
<td>5.08%±1.44$^a$</td>
<td>12.66%±1.74$^a$</td>
</tr>
</tbody>
</table>

Table 3.3. Comparative hydroxyl radical scavenging activity assay (HRSA) of EYGF-23 and EYGF-33. The scavenging activity was measured over a concentration range of 0.5 to 20 mg/ml from each isolated fraction using HRSA assay. The activity of both fractions was measured at 532 nm and compared to BHT as a synthetic antioxidant standard and trolox as a natural antioxidant standard (both 0.2 mg/ml). Data correspond to the mean ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. Values with subscript a to present significant differences with trolox, and subscript b to present significant differences with BHT $p < 0.05$. 

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3.3.4 Superoxide anion scavenging activity

Table 3.4 presents percentage of radical scavenging activity exhibited by EYGF-23, EYGF-33, trolox and BHT. The scavenging activity of EYGF-23 and EYGF-33 increased with the concentration in a dose-dependent manner. From the table 3.4, it can be seen that all peptide concentrations less than 20 mg/ml from both fractions EYGF-23 and EYGF-33 were significantly less effective as superoxide scavengers in comparison with trolox (63.48%, \( p < 0.05 \)) as well as BHT (67.45%, \( p < 0.05 \)). At 20 mg/ml EYGF-23, there was no significant difference between the activities shown by this fraction (67.66%) compared to trolox or BHT. In contrast, when the same concentration used for EYGF-33, the scavenging effect was significantly higher than trolox as well as BHT.

<table>
<thead>
<tr>
<th>Sample concentration (mg/ml)</th>
<th>0.5</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>Trolox (^a)</th>
<th>BHT (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYGF-23</td>
<td>11.63%</td>
<td>18.12%</td>
<td>30.62%</td>
<td>41.01%</td>
<td>67.66%</td>
<td>63.48%</td>
<td>67.45%</td>
</tr>
<tr>
<td></td>
<td>±2.32(^ab)</td>
<td>±0.51(^ab)</td>
<td>±1.86(^ab)</td>
<td>±1.70(^ab)</td>
<td>±2.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EYGF-33</td>
<td>11.59%</td>
<td>28.13%</td>
<td>35.43%</td>
<td>48.85%</td>
<td>82.88%</td>
<td>±1.63</td>
<td>±1.67</td>
</tr>
<tr>
<td></td>
<td>±1.70(^ab)</td>
<td>±2.77(^ab)</td>
<td>±3.90(^ab)</td>
<td>±3.14(^ab)</td>
<td>±2.49(^ab)</td>
<td></td>
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</tr>
</tbody>
</table>

Table 3.4. Comparative superoxide anion scavenging activity assay of EYGF-23 and EYGF-33. The scavenging activity was measured over a concentration range of 0.5 to 20 mg/ml from each isolated fraction using superoxide anion scavenging activity assay. The activity of both fractions was measured at 320 nm and were compared to BHT as a synthetic antioxidant standard and trolox as a natural antioxidant standard (both 0.2 mg/ml). Data correspond to the mean ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Dunnett’s multiple comparisons test. Values with subscript a present significant differences with trolox, and subscript b present significant differences with BHT \( p < 0.05 \).
3.3.5 \( \text{Fe}^{2+} \) chelating activity assay

Table 3.5 shows that the chelating activity of EYGF-23 and EYGF-33 increased with the concentration in a dose-dependent manner. From the table, it can be seen that all peptide concentrations from both fractions EYGF-23 and EYGF-33 were significantly less effective as chelating agent in comparison with EDTA (97.54%, \( p < 0.05 \)).

<table>
<thead>
<tr>
<th>Sample concentration (mg/ml)</th>
<th>EDTA* (0.2mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>EYGF-23</td>
<td>12.69%</td>
</tr>
<tr>
<td>±1.15*</td>
<td>±1.79*</td>
</tr>
<tr>
<td>EYGF-33</td>
<td>10.24%</td>
</tr>
<tr>
<td>±0.99*</td>
<td>±0.94*</td>
</tr>
</tbody>
</table>

Table 3.5. Comparative chelating activity assay of EYGF-23 and EYGF-33. Chelation activity was measured over a concentration range of 0.5 to 20 mg/ml from each fraction using \( \text{Fe}^{2+} \) chelating activity assay. The activity was measured at 563 nm and results were compared to EDTA (0.2 mg/ml). Data correspond to the mean ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Dunnett’s multiple comparisons test. Values with subscript a present significant differences with EDTA \( p < 0.05 \).

3.4 Discussion

In order to understand the action of EYGF-23 and EYGF-33 as antioxidants, many assays were applied to illustrate the antioxidant mechanism. The results obtained in this study from reducing power assay (table 3.1) suggested that EYGF-23 and EYGF-33 possibly contained amino acids that functioned as electron donors and could react with free radicals to form more stable products. This result is in agreement with other
researchers who concluded that the antioxidant activity of isolated peptides related to their abilities to donate electrons (Wu et al., 2003; Zhu et al., 2006; He et al., 2013).

According to the amino acid profile of EYGF-23, the presence of proline (8.01%), tyrosine (12.40%), tryptophan (16.40%) and lysine (11.03%) residues could explain its activity as a scavenger. The monophenolic structure of tyrosine and the presence of an indole ring in tryptophan can both increase stability of the residues and increase their reducing power, hereby enabling the isolated fraction to act as a radical scavenger. Moreover the presence of proline with its unique cyclic structure can also enhance the scavenging properties of the peptide. EYGF-33 also contains leucine (13.82%) and tryptophan (9.44%) that might contribute to the scavenging activity. The results presented herein concur with previous studies where the researchers report that the effect of peptides as antioxidant scavengers is related to the nature and composition of amino acids present in this peptide (Je et al., 2005; Kong and Xiong, 2006; Elias et al., 2008). Many studies have also shown that the hydroxyl radical scavenging activity demonstrated by isolated peptides is related to presence of specific amino acids such as tyrosine and tryptophan (Mendis et al., 2005; Pihlanto, 2006; Hernández-Ledesma et al., 2007; Kim et al., 2007; Zhuang and Sun, 2011).

Scientists have also reported that the radical scavenging activity of the isolated peptide is not only related to the composition of amino acids and their nature but also to their sequence in the peptide. Suetsuna et al. (2000) found that the presence of a glutamate-leucine sequence in an isolated peptide from casein is responsible for its antioxidant activity. In other research, Park et al. (2001) suggested that the presence of leucine at the N-terminus of a peptide isolated from lecithin-free egg yolk protein was mainly responsible for the scavenging power. Similarly, Ma et al. (2010) demonstrated that the
presence of a tryptophan at the C-terminus of isolated buckwheat peptides may be responsible for its radical scavenging activity. Luo et al. (2013) isolated a peptide with the sequence leucine-aspartate-lysine from Sphyrna lewini muscle protein, which exhibited powerful scavenging activity.

In the current study, the chelating of ferrous ions was used to evaluate the activity of EYGF-23 and EYGF-33 as a ferrous chelator. The result obtained revealed that both fractions have the ability to act as chelating agents although the activity was significantly lower in comparison with EDTA at the concentrations used (table 3.5). Ferrous ions can act as catalysts which enhance the generation of free radicals and subsequently initiate the oxidative chain reactions. Therefore, chelating agents may result in reduced availability of these ions and inhibit the oxidative chain reactions (Pokorný et al., 2001). Chen et al. (1995) demonstrated that the sequence of amino acids also plays an important role in the metal chelating activity of isolated peptides from soybean. The degree of peptide hydrolysis, the type of amino acid and their sequence in the isolated peptides have all been shown to alter chelation activity (Thiansilakul et al., 2007; Samaranayaka and Li-Chan, 2008; Jamdar et al., 2010; Torres-Fuentes et al., 2012). It was noted that, in all methods conducted in this study, the efficiency of the peptide as a reductant, scavenger and chelator was dose-dependent.

3.5 Conclusion

In conclusion, the results of the current study show that EYGF-23 and EYGF-33 possessed notable antioxidant activity. The isolated fraction can act as a reductant and quench three different radicals; DPPH, the superoxide anion and the hydroxyl radical. It can also exhibit chelating behaviour. In all assays, a good correlation existed between
the activity shown and the amount of peptide used. According to the amino acid analysis presented in the previous chapter (chapter 2), the activities shown in this study could be related to the high content of specific amino acids such as tryptophan, and the hydrophobic moieties present in the peptide. The variety of mechanisms by which EYGF-23 and EYGF-33 exerts their antioxidant activity is indicative of their potential to function in diverse oxidising environments.
Chapter 4
4 Antioxidant effect of egg yolk peptide against t-BHP-induced oxidative stress in Caco-2 cells

4.1 Introduction

Reactive oxygen species (ROS) are produced in living cells as a result of normal cell metabolism and xenobiotic detoxification. Oxidative stress is a consequence of an excess of ROS, which participate in cellular damage by reacting with lipid membranes, proteins, and DNA. The effects of oxidative stress in mammalian cells range from rapid cell mortality to disturbance of many cell signalling processes (Klotz et al., 2003). As a result, ROS are implicated in the pathogenesis of many diseases that include cardiovascular diseases (Cai and Harrison, 2000), certain cancers (Valko et al., 2004) and Alzheimer’s disease (Christen, 2000).

In response to these threats, aerobic cells utilize a wide array of enzymatic and non-enzymatic mechanisms that tightly control unwanted ROS accumulation. Cellular antioxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) can protect cell membranes and cellular content by scavenging ROS. Under certain conditions, excess ROS formation overwhelms the prooxidant/antioxidant balance despite the presence of the antioxidant defence system (Kehrer, 1993; Cimen, 2008).

Therefore, there is growing interest to find food-derived antioxidants that can protect tissues from free radical-mediated oxidative stress. Natural compounds that have antioxidative properties have been identified from plant include polyphenols and some dietary protein compounds. Curtin et al. (2002) found that the antioxidant activity of
these natural products could be mediated by upregulating intracellular defence systems or intercepting ROS formation.

The human colon carcinoma (Caco-2) cell line was applied in this research because it displays morphological and physiological characteristics that are similar to intestinal epithelial cells under standard culture conditions (Meunier et al., 1995). The purpose of using the organic hydroperoxide, tert-butylhydroperoxide (t-BHP), was to generate oxidative stress that results in cell injury which initiates intestinal degenerative processes (Parks et al., 1983). The resulting impact of ROS generated by t-BHP leads to a depletion of GSH and protein thiols, peroxidation of membrane lipids, DNA damage and a loss of mitochondrial membrane potential (Ochi and Cerutti, 1989; Lapshina et al., 2005).

Previous studies indicated that a phosvitin oligophosphopeptide, isolated from egg yolk protein, protected intestinal cells from oxidative stress induced by hydrogen peroxide by suppressing lipid oxidation, enhancing the synthesis of GSH and increasing its level (Katayama et al., 2006). Prevention of the deleterious effects of excess ROS is beneficial to protect cellular membranes and their components. Therefore, the aim of this study was to use cultured Caco-2 cells as an epithelial model to investigate the antioxidant behaviour of egg yolk peptide in cells exposed to pro-oxidant t-BHP. To meet this aim, the change in protective cellular antioxidant defence systems in response to oxidative stress was evaluated.
# 4.2 Materials and methods

## 4.2.1 Materials

<table>
<thead>
<tr>
<th>Materials</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human colon epithelial carcinoma cell line (Caco-2).</td>
<td>European Collection of Cell Cultures (ECACC).</td>
</tr>
<tr>
<td>Pencillin/ streptomycin (antibiotics solution at 50 U/ml)</td>
<td></td>
</tr>
<tr>
<td>3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl-tetrazolium bromide (MTT) dye</td>
<td></td>
</tr>
<tr>
<td>Dimethyl sulphoxide (DMSO)</td>
<td>Sigma-Aldrich, Dorset, UK.</td>
</tr>
<tr>
<td><em>Tert</em>-butyl hydroperoxide (<em>t</em>-BHP)</td>
<td></td>
</tr>
<tr>
<td>Lysis buffer</td>
<td></td>
</tr>
<tr>
<td>Dulbecco’s modified Eagle’s medium (DMEM, high glucose without pyruvate)</td>
<td></td>
</tr>
<tr>
<td>Foetal bovine serum (FBS)</td>
<td></td>
</tr>
<tr>
<td>5-(and-6)chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate acetyl ester (CM-H$_2$DCFDA)</td>
<td>Invitrogen, Paisley, UK.</td>
</tr>
<tr>
<td>Hanks buffer saline solution (HBSS)</td>
<td></td>
</tr>
<tr>
<td>Trypsin-EDTA solution (0.03%)</td>
<td></td>
</tr>
<tr>
<td>L-glutamine (200 mM)</td>
<td></td>
</tr>
<tr>
<td>Non-essential amino acids (NEAA)</td>
<td></td>
</tr>
<tr>
<td>Phosphate buffered saline tablet (PBS)</td>
<td>Oxoid, Hampshire, UK.</td>
</tr>
<tr>
<td>Caspase-Glo® 3/7 Assay.</td>
<td>Promega, Southampton, UK.</td>
</tr>
</tbody>
</table>
Chapter 4: Antioxidant effect of egg yolk peptide against t-BHP-induced oxidative stress in Caco-2 cells

<table>
<thead>
<tr>
<th>Superoxide Dismutase Activity Assay Kit (Colorimetric) catalog number ab65354</th>
<th>Abcam®, Cambridge, UK.</th>
</tr>
</thead>
<tbody>
<tr>
<td>OxiSelect™ Catalase Activity Assay Kit (Colorimetric) catalog number STA-341</td>
<td>Cell Biolabs, INC., Cambridge, UK.</td>
</tr>
<tr>
<td>OxiSelect™ Total Glutathione (GSSG/GSH) Assay Kit, catalog number STA-312</td>
<td></td>
</tr>
</tbody>
</table>

4.2.2 Experimental methods

4.2.2.1 Isolation and purification of peptide

The peptides (EYGF-23) and (EYGF-33) were isolated as described in chapter 2, section 2.2.

4.2.2.2 Cell culture and treatment

Caco-2 cells were grown in DMEM cell culture medium supplemented with 20% FBS, 1% glutamine, 1% NEAA, and 1% penicillin/streptomycin. Cells were seeded in 25cm² tissue culture flasks at a concentration of $1 \times 10^6$ cells/ml and were incubated at 37 °C in a 5% CO₂ humidified incubator. Cells were sub-cultured when they reached 80% confluence and were exposed to treatment when at 50% confluence. Confluent cells were harvested using 1 ml trypsin/EDTA after washing with 3 ml PBS.

4.2.2.3 Cell viability (MTT assay)

4.2.2.3.1 Effect of antioxidant egg yolk peptide on cell viability

To determine the effect of EYGF-23 and EYGF-33 on cell proliferation and viability, the MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl-tetrazolium bromide) assay was conducted. The MTT assay is an *in vitro* assay for the measurement of cell proliferation.
Chapter 4: Antioxidant effect of egg yolk peptide against t-BHP-induced oxidative stress in Caco-2 cells

and reduction of cell viability. The principle of the assay is based on the reduction of tetrazolium MTT salt (yellow colour) to intracellular insoluble formazan crystals (purple colour). The reduction is carried out by dehydrogenase enzymes produced by metabolically active cells. Formazan crystals are solubilized with dimethyl sulphoxide (DMSO) and quantified by spectrophotometry at a wavelength of 492 nm (Roehm et al., 1991).

In order to conduct the assay, cells were cultured in 96-well tissue culture plates by adding 200 µl/well of a cell suspension at a concentration of $1\times10^4$ cells/200 µl. After 24 hours, culture medium was replaced with 200 µl fresh medium containing different concentrations of EYGF-23 or EYGF-33 ranging from 0.2 to 4 mg/ml for 24 or 48 hours. Dose ranges and exposure times were selected to determine the effect of peptides on viability in a concentration- and time-dependent manner. Following the designated treatment exposure period, 20 µl of 5 mg/ml MTT dye (prepared in PBS) were added to each well and incubated at 37°C in a humidified atmosphere with 5% CO$_2$. After the 4 hour incubation period, the culture medium was aspirated and 200 µl of DMSO were added to solubilize the purple formazan crystals formed. Cell culture plates were shaken for 5 minutes at room temperature and the colour intensity was measured at 492 nm using a plate reader (Behring Co, Marburg, Germany). A column of wells containing untreated cells was used as a negative control at each time point. Each experimental condition was tested in triplicate and the experiment repeated three times to ensure reproducibility.
4.2.2.3.2 Induction of oxidative stress by t-BHP

To determine the minimum concentration of t-BHP to induce oxidative stress, different concentrations were tested using the MTT assay to assess cell viability. Cells were cultured in 96-well tissue culture plates by adding 200 µl/ well of cells at a concentration of $1 \times 10^4$ cells/200 µl. After 24 hours, culture medium was replaced with 200 µl fresh medium containing different concentrations of t-BHP (1, 3, 5, 10 mM). After 2 hours incubation period, MTT assay was carried out as described before (section 4.2.2.3.1).

4.2.2.3.3 Treatment of Caco-2 cells with EYGF-23 or EYGF-33 peptides and t-BHP

The effect of antioxidant peptides on cells stressed with t-BHP was assessed by MTT assay. After 24 hours, the culture medium was replaced with 200 µl fresh medium containing different concentrations of peptides ranging from 0.2 to 4 mg/ml. Following 24 hours of incubation, the pre-determined concentration of t-BHP was added for an additional 2 hours. At the end of the incubation time, the MTT assay was carried out as described before (section 4.2.2.3.1).

4.2.2.4 Cell morphology

Caco-2 cells were seeded at a density of $1 \times 10^6$ cells/ml in 25cm² cell culture flasks. After reaching 50% confluence, cells were treated with 1.0 mg/ml egg yolk peptide. After 24 hours of incubation, 10 µl of 3 mM t-BHP was added and incubated for another 2 hours. Changes in cell morphology between untreated control, cells treated with t-BHP alone, cells treated with peptide alone and cells treated with t-BHP and
peptide were investigated using a phase contrast microscope fitted with a camera (Nikon Eclipse TS100). A 10 x magnification was used to image all cells.

4.2.2.5 Measurement of intracellular ROS

The intracellular ROS level in Caco-2 cells was evaluated by flow cytometry with the use of a non-fluorescent cell permeable probe (CM-H$_2$DCFDA). This probe was used as an intracellular precursor of the fluorescent compound 2',7'-dichlorofluorescein (DCF) for assessment of oxidative stress levels. Once the non-fluorescent probe penetrates the cell membrane, the ester group is removed by intracellular esterases and the product is oxidised by cellular ROS to produce highly fluorescent DCF. Fluorescence intensity is then measured and analysed at excitation and emission wavelengths of 490 nm and 520 nm, respectively, using a flow cytometer. Fluorescence is assumed to be directly proportional to the concentration of ROS in the cells (Bass et al., 1983).

Briefly, Caco-2 cells were seeded in 25cm$^2$ flasks at a concentration $1 \times 10^6$ cells/ml. When cells reached 50% confluence, the medium was replaced with fresh medium containing samples of egg yolk peptides. After a 24 hour incubation period at 37°C in a humidified atmosphere with 5% CO$_2$, medium from each flask was collected in 15 ml centrifuge tubes and attached cells were washed with 1 ml PBS. After washing, cells were harvested using 3 ml trypsin. Trypsinised cells were re-suspended in their corresponding collected medium and centrifuged for 3 minutes at 150 x g. After media aspiration, cells were resuspended in 500 μl PBS and loaded with 5 μM DCFDA (prepared freshly in DMSO). Loaded cells were incubated for 30 minutes at 37°C in a humidified atmosphere with 5% CO$_2$. After incubation, cells were kept on ice and in the dark until the time of analysis to minimise the photo-oxidation of DCFDA by light.
Chapter 4: Antioxidant effect of egg yolk peptide against t-BHP-induced oxidative stress in Caco-2 cells

Cellular ROS levels were measured using a BD FACSCanto flow cytometer (BD Biosciences, California, USA). Forward and side scatters were gated to exclude cell debris. At least 10,000 events were acquired in list mode.

4.2.2.6 TBARS formation in Caco-2 cells

MDA formed as a secondary product of lipid oxidation was measured using TBARS assay according to the method of Ohkawa et al. (1979). Briefly, Caco-2 cells were seeded in 25 cm² flasks at a concentration $1 \times 10^6$ cells/ml. Cells were treated with 1.0 mg/ml peptide at 50% confluence. After a 24 hour incubation period at 37°C in a humidified atmosphere with 5% CO$_2$, cells were washed with 1 ml PBS. After washing, cells were treated with 10 µl of 3 mM t-BHP and incubated for 2 hours. Cells were then washed with PBS and were removed from the flask by scraping and lysing with 20% trichloroacetic acid (TCA). After lysing, 2 ml of 0.7% TBA was added and the cell solution was heated at 100 °C for one hour. After cooling, the solution was centrifuged for 10 minutes at 150 x g. The absorbance was measured at 532 nm using a spectrophotometer (UVIKON 860, Kontron instrument, UK). A standard curve of 1,1,3,3-tetramethoxypropane (TEP) was prepared from a 200 µg/ml stock solution (curve concentration range: 10, 50, 100, 150 and 200 µg/ml) to estimate the concentration of MDA in the sample (Appendix 2).

4.2.2.7 The Caspase-Glo 3/7 assay

The Caspase-Glo 3/7 is a homogeneous, luminescent, assay used in this study to measure the activities of caspase-3 and caspase-7. Caspase-3 and -7 are members of the caspase family (cysteine aspartic acid-specific protease) that play a key role in apoptosis (programmed cell death) in mammalian cells.
The assay contains a luminogenic caspase-3/7 substrate that contains a tetrapeptide sequence (DEVD) in its structure. By adding Caspase-GloR 3/7 Reagent to cultured cells, cell lysis will occur followed by caspase cleavage of the substrate. Following caspase cleavage, aminoluciferin (a substrate for luciferase) is released resulting in the luciferase reaction and generation of a glow type luminescent signal as depicted in figure 4.1. Generated luminescence is proportional to the amount of caspase activity present in the sample.

Figure 4.1. Schematic diagram to illustrate the principle of Caspase-Glo 3/7 assay. Caspase-3/7 cleavage of the luminogenic caspase substrate followed by luciferase reaction and generation of a glow-type luminescent signal.

The assay was conducted by culturing cells in white-bottomed 96-well tissue culture plates by adding 100 µl/ well of a cell suspension of 1×10^4 cells/100 µl. After 24 hours, culture medium was replaced with 100 µl fresh medium / well containing 1.0 mg/ml of tested peptide and incubated for another 24 hours. This was followed by 2 hours of 3 mM t-BHP exposure. In a new white-bottomed 96-well tissue culture plate, 100 µl of
oxidatively stressed cells and 100 μl Caspase-Glo 3/7 reagent were added and incubated for 30 minutes. Luminescence was measured using a luminometer (GloMax® 96 Luminometer, Promega, USA). The amount of luminescence generated is proportional to the amount of caspase activity present.

4.2.2.8 Effect of egg yolk peptide on endogenous antioxidant defence systems in Caco-2 cells under oxidative stress

4.2.2.8.1 Preparation of cell lysate

Caco-2 cells were seeded in 25cm² flasks at a concentration of 1×10⁶ cells/ml and incubated at 37°C in a humidified atmosphere with 5% CO₂. When cells reached 50% confluence, the medium was replaced with fresh medium containing samples of 1.0 mg/ml egg yolk peptide and incubated for a further 24 hours. Cells were then treated with 3 mM t-BHP to induce stress, and were incubated for 2 hours. Following the incubation, culture medium from each tested flask was collected in 15 ml centrifuge tubes and attached cells were washed with 1 ml PBS. After washing, cells were harvested using 3 ml trypsin. Trypsinised cells were re-suspended in their corresponding collected medium and centrifuged for 3 minutes at 150 x g. The supernatant was decanted and cell pellets were lysed using 300 μl lysis buffer (50 mM Tris HCL, 150 mM NaCl, 1% Nonidet P40 (NP-40), 0.2% SDS solution, 20 μM PMSF, 1 μg/ml Aprotinin, 1 μg/ml Leupeptin, and 1mM Na₃VO₄). After lysing, cells were kept in ice for 20 minutes and stored at – 80°C until used further.
4.2.2.8.2 Determination of total glutathione concentration

The total reduced and oxidized (GSH and GSSG respectively) glutathione concentration was measured quantitatively using the OxiSelect™ Total Glutathione Assay Kit according to the manufacturer’s instructions. The principle of this assay is based on the ability of the glutathione reductase enzyme to reduce GSSG to GSH in the presence of NADPH (reduced form of nicotinamide adenine dinucleotide phosphate). Consequently, the chromogen included in the kit reacts with the thiol group of GSH to produce a chromophore compound. The absorbance of the coloured compound was then read at a wavelength of 405 nm using a microplate reader (Behring Co, Marburg, Germany). Glutathione concentration in cell lysate is proportional to the amount of chromophore produced. The unknown concentration of GSH in the samples was calculated by generating a GSH standard curve supplied with the kit. Each experimental condition was tested in triplicate and the experiment repeated three times to ensure reproducibility.

4.2.2.8.3 Superoxide dismutase assay

Superoxide dismutase (SOD) activity was determined in Caco-2 cell lysate. SOD activity was measured using the SOD Activity Assay Colorimetric Kit according to the manufacturer’s instructions. The principle of the assay is based on the ability of SOD to catalyze the dismutation of the superoxide anion to H$_2$O$_2$ and molecular oxygen. In the kit, WST-1 was used to produce a water soluble formazan dye upon reduction with any superoxide anion present in the reaction mixture. The absorbance of formazan dye formed was measured at 450 nm using micro plate reader (Behring Co, Marburg, Germany). Reduced colour intensity indicated increase the activity of SOD enzyme.
Each experimental condition was tested in triplicate and the experiment repeated three times to ensure reproducibility.

4.2.2.8.4 Catalase assay

In order to measure the level of catalase in Caco-2 cell lysate, the OxiSelect™ Catalase Assay Colorimetric Kit was used according to the manufacturer’s instructions. The principle of the assay involves a two-step reaction. In the first step, hydrogen peroxide (H$_2$O$_2$) in the cell lysate is decomposed into water and oxygen by the action of catalase. The second step occurs when the remaining H$_2$O$_2$ facilitates the coupling of chromogenic reagent A and chromogenic reagent B in the presence of horseradish peroxide (HRP) as a catalyst. The quinoneimine dye formed is then measured at 520 nm using microplate reader (Behring Co, Marburg, Germany); the absorbance measured is correlated with the amount of remaining H$_2$O$_2$ in the reaction mixture. The unknown concentration of catalase in the samples was calculated by generating a catalase standard curve supplied with the kit. Each experimental condition was tested in triplicate and the experiment repeated three times to ensure reproducibility.

4.2.3 Statistical analysis

Each experimental condition was tested in triplicate and the experiment repeated three times on separate occasions to ensure reproducibility. Results were analysed using GraphPad Prism software version 6.0. Statistical analysis comparisons were made by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test or by two-way analysis of variance followed by Tukey’s multiple comparisons test when appropriate. Details of the statistical test used are provided in the figure legends of
presented results. $P < 0.05$ was considered statistically significant. Data were presented as means ± standard deviation (SD).

### 4.3 Results

#### 4.3.1 Cell viability (MTT assay)

##### 4.3.1.1 Cell viability of Caco-2 cells treated with peptides alone

The effect of EYGF-23 and EYGF-33 on the cell viability of Caco-2 cells was assessed by MTT assay. Caco-2 cells were cultured and treated with EYGF-23 and EYGF-33 then incubated for 24 and 48 hours. There was no significant difference in cell viability between any of the concentrations used (0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0 and 4.0 mg/ml) and the untreated cells after 24 hours in cells treated with either peptide fractions (figure 4.2).

![Figure 4.2. Cell viability of Caco-2 cells treated with EYGF-23 and EYGF-33 for 24 hours. Caco-2 cells were cultured and incubated with EYGF-23 and EYGF-33 individually over a concentration range of 0.2 to 4.0 mg/ml. The MTT assay was used to evaluate cell viability after 24 hours and the absorbance of solubilised formazan crystals was measured at 492 nm. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Tukey’s multiple comparisons test.](image-url)
At 48 hours, a marked reduction in cell viability was observed in those cells treated with EYGF-33 at a concentration of 0.8 mg/ml (71.51%, p < 0.01) (figure 4.3). Cell viability significantly decreased with increased EYGF-33 concentration starting from 1.0-4.0 mg/ml (p < 0.001) compared with untreated cells. At 48 hours, EYGF-23 did not lower cell viability at any concentration and actually looked to increase cell viability compared with the untreated cells. Based on the results obtained, only the EYGF-23 fraction was studied further in relation to antioxidant activity. Conversely, EYGF-33 was chosen for study as a potential anticancer agent.

Figure 4.3. Cell viability of Caco-2 cells treated with EYGF-23 and EYGF-33 for 48 hours. Caco-2 cells were cultured and incubated with EYGF-23 and EYGF-33 individually over a concentration range of 0.2 to 4.0 mg/ml. The MTT assay was used to evaluate cell viability after 48 hours and the absorbance of solubilised formazan crystals was measured at 492 nm. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Tukey’s multiple comparisons test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus untreated cells.
4.3.1.2 Cell viability of Caco-2 cells treated with t-BHP as oxidative inducer

In the current study, t-BHP was used to induce oxidative stress in Caco-2 cells. Figure 4.4 demonstrates the cytotoxic effect induced by different concentrations of t-BHP ranging from 1.0-10.0 mM when cells were treated for 2 hours. The toxicity of t-BHP occurred in a dose-dependent manner. All concentrations, including the lowest concentrations used, significantly inhibited cell viability ($p < 0.001$) compared with the untreated cells. The cytotoxic effect of 1.0, 3.0, 5.0 and 10.0 mM was 85.53, 66.18, 47.7 and 44.31 % respectively. Based on these results, 3 mM t-BHP was chosen to induce stress in Caco-2 cells in the following experiments because it reduces cell viability by almost 30 %. The following experiments were performed with the aim of elucidating the antioxidant activity of the isolated EYGF-23 peptide.

![Figure 4.4. Effect of t-BHP on Caco-2 cell viability. Cultured cells were incubated with a range of t-BHP concentrations (1.0 to 10.0 mM) for 2 hours. The MTT assay was used to evaluate cell viability, and the absorbance of solubilised formazan crystals was measured at 492 nm. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Dunnett’s multiple comparisons test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ versus untreated cells.](image-url)
4.3.1.3 Effect of EYGF-23 on Caco-2 cell viability under oxidative stress

To study the effect of EYGF-23 on Caco-2 cells stressed by t-BHP, Caco-2 cells were cultured and incubated with different concentrations of EYGF-23 (0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0 and 4.0 mg/ml) for 24 hours followed by a 2 hour incubation with 3mM t-BHP. As depicted in figure 4.5, cells treated with 0.2 - 0.8 mg/ml of EYGF-23 demonstrated cell viability levels comparable to cells treated with t-BHP alone. At concentrations of EYGF-23 ≥ 1.0 mg/ml, cell viability significantly increased compared with the t-BHP control (*p <0.01 at least). A cell viability of 87.31 % was achieved at 1.0 mg/ml (p <0.01), which continued to increase significantly when treated with 2.0, 3.0, and 4.0 mg/ml (93.70, 94.45, and 96.35 % respectively; all p <0.001). As concentrations of 1.0 mg/ml EYGF-23 and above significantly increased cell viability, the minimum dose of 1.0 mg/ml was used to conduct further experiments.

![Figure 4.5. Effect of EYGF-23 on Caco-2 cells stressed with t-BHP.](image)

Caco-2 cells were incubated with EYGF-23 using a concentration range of 0.2 to 4.0 mg/ml for 24 hours, followed by a 2 hour incubation with 3 mM t-BHP to induce stress. The MTT assay was used to assess cell viability, and the absorbance of solubilised formazan crystals was measured at 492 nm. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Dunnett’s multiple comparisons test versus treated cells with t-BHP alone * = p <0.05, ** = p <0.01, *** = p <0.001.
4.3.2 Effect of EYGF-23 on the morphology of Caco-2 cells under oxidative stress

The effect of the EYGF-23 peptide in protecting Caco-2 cells from the oxidative stress induced by t-BHP was observed by microscopy. Figure 4.6 illustrates the morphology of untreated Caco-2 cells (A), cells stressed with t-BHP alone (B), cells treated with EYGF-23 alone (C), and stressed cells treated with 1.0 mg/ml EYGF-23 (D). Stressed cells treated with egg yolk peptide appeared healthy compared with those treated with t-BHP alone.

![Figure 4.6. Morphological changes occurring in the Caco-2 cell line. Morphological changes occurring in the Caco-2 cell line in (A) untreated cells, (B) cells treated with t-BHP alone, (C) cells treated with 1.0 mg/ml EYGF-23 and (D) cells treated with 1.0 mg/ml EYGF-23 and stressed with t-BHP. Each image is magnified by 10x.](image)

4.3.3 Effect of EYGF-23 on intracellular ROS levels in Caco-2 cells under oxidative stress

The amount of ROS generated by untreated cells was significantly lower (69.00 %; $p < 0.001$) than that produced by cells stressed with t-BHP (75.27 %), as shown in figure...
4.7. ROS production levels in Caco-2 cells treated with 1.0 mg/ml EYGF-23 under oxidative conditions were markedly inhibited to 60.00 % \((p < 0.001)\) when compared with cells treated by \(t\)-BHP alone (75.27%).

![Graph](image)

**Figure 4.7. Measurement of intracellular ROS levels in Caco-2 cells.** ROS was measured using flow cytometry for untreated cells, cells treated with 1.0 mg/ml EYGF-23 alone for 24 hours, cells exposed to 3 mM \(t\)-BHP only for 2 hours or treated with \(t\)-BHP for two hours after the 24 hour exposure to EYGF-23. Cellular ROS levels were monitored by loading treated cells with CM-H\(_2\)DCFDA probe and measuring the intensity of DCF fluorescence at an excitation wavelength of 490 and an emission wavelength of 520 nm. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Dunnett’s multiple comparisons test. * = \(p < 0.05\), ** = \(p < 0.01\), *** = \(p < 0.001\) versus treated cells with \(t\)-BHP alone.

### 4.3.4 Effect of EYGF-23 on lipid peroxidation product (TBARS) in Caco-2 cells under oxidative stress

When Caco-2 cells were treated with 3 mM \(t\)-BHP for 2 hours, almost 2-fold increase in lipid peroxidation products was observed compared with untreated cells (32.19 \(\mu g/ml\) versus 19.85 \(\mu g/ml\), respectively) \((p <0.001)\) as measured by TBARS (figure 4.8). However, 1.0 mg/ml of EYGF-23 was able to significantly inhibit the formation of MDA to a concentration of 24.15 \(\mu g/ml\) \((p <0.05)\) in stressed cells when compared with cells treated by \(t\)-BHP alone (32.19).
Chapter 4: Antioxidant effect of egg yolk peptide against t-BHP-induced oxidative stress in Caco-2 cells

4.8 Measurement of lipid peroxidation product malondialdehyde (MDA) formed in Caco-2 cells. MDA level was measured using TBARS method for untreated cells; cells treated with 1.0 mg/ml EYGF-23 alone for 24 hours; cells exposed to 3 mM t-BHP only for 2 hours; or cells treated with t-BHP for two hours after the 24 hour exposure to EYGF-23. Harvested cells were treated with TCA and TBA followed by incubation at 100 °C for one hour. Absorbance was measured at 532 nm using a spectrophotometer. The amount of MDA in the sample was calculated using TEP as standard and was expressed as µg/ml of MDA. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus treated cells with t-BHP alone.

4.3.5 Caspase activity in Caco-2 cells under oxidative stress

Caspase 3/7 activity in Caco-2 cells under oxidative stress was assessed using a luminometer. As demonstrated in figure 4.9, Caco-2 cells exhibited high Caspase 3/7 activity when treated with the oxidant t-BHP alone (78.00 %). Caspase 3/7 activity produced under oxidative stress was significantly inhibited to a third of this level (26.85 %), when cells were pre-treated with 1.0 mg/ml EYGF-23 (p < 0.001). These levels were comparable to when cells were treated with EYGF-23 alone, in the absence of stress induced by t-BHP.
Figure 4.9. Measurement of caspase 3/7 activity in Caco-2 cells. Caco-2 cells were exposed to 3 mM t-BHP alone for 2 hours, or cells were treated with 1.0 mg/ml EYGF-23 in the presence and absence of t-BHP. Caspase 3/7 activity was measured using a luminometer. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Dunnett’s multiple comparisons test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus treated cells with t-BHP alone.

4.3.6 Influence of EYGF-23 on antioxidant defence systems

4.3.6.1 Effect of EYGF-23 on the amount of total glutathione (GSH)

There was a significant depletion of total glutathione in cells treated with t-BHP (glutathione 0.10 μM) compared with untreated cells (0.18 μM; p <0.01). Total glutathione level was significantly elevated in stressed cells pretreated with 1.0 mg/ml EYGF-23 (0.29 μM) in comparison to cells treated with t-BHP alone (p <0.001). These findings are represented in figure 4.10.
Figure 4.10. Effect of EYGF-23 on the amount of total glutathione (GSH) in Caco-2 cells. The amount of total glutathione was measured for untreated cells, cells treated with 1.0 mg/ml EYGF-23 alone for 24 hours, cells exposed to 3 mM t-BHP only for 2 hours or treated with t-BHP for two hours after the 24 hour exposure to EYGF-23. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus cells treated with t-BHP.

4.3.6.2 Effect of EYGF-23 on superoxide dismutase (SOD) activity

Figure 4.11 illustrates that SOD activity was significantly inhibited in cells stressed with t-BHP (44.22 %) compared with untreated cells (50.75%; p < 0.05). Cells treated with 1.0 mg/ml EYGF-23 prior to oxidative induction exhibited significantly higher SOD activity (55.10 %) when compared with cells treated with t-BHP (p < 0.01).
Figure 4.11. Effect of EYGF-23 on the percentage of superoxide dismutase (SOD) activity in Caco-2 cells. The percentage of SOD was measured for untreated cells, cells treated with 1.0 mg/ml EYGF-23 alone for 24 hours, cells exposed to 3 mM t-BHP only for 2 hours or treated with t-BHP for two hours after the 24 hour exposure to EYGF-23. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus cells treated with t-BHP.

4.3.6.3 Effect of EYGF-23 on the amount of catalase

There was no significant difference in the concentration of catalase between untreated cells (18.80 U/ml), cells treated with t-BHP alone (20.88 U/ml) and cells pretreated with 1.0 mg/ml EYGF-23 prior to their exposure to t-BHP (19.46 U/ml) (figure 4.12).
Figure 4.12. Effect of EYGF-23 on the level of catalase in Caco-2 cells. The amount of catalase in Caco-2 cells was measured for untreated cells, cells treated with 1.0 mg/ml EYGF-23 alone for 24 hours and cells exposed to 3mM t-BHP only for 2 hours or treated with t-BHP for two hours after the 24 hour exposure to EYGF-23. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus cells treated with t-BHP alone.

4.4 Discussion

The results obtained from cell viability assay when cells were treated with isolated peptide fractions indicated that EYGF-23 in the concentration range used (0.2 - 4.0 mg/ml) and under the study conditions, was not toxic to Caco-2 cells. However, at 48 hours, and at concentrations ≥ 0.8 mg/ml, a second peptide fraction EYGF-33 exhibited cytotoxicity (figure 4.3). It is well known that the nature and sequence of amino acids that comprise isolated peptides are important factors that dictate peptide activity (Elias et al., 2008; Samaranayaka and Li-Chan, 2011). The cytotoxic behaviour exhibited by EYGF-33 could be associated with these factors which are discussed further in chapter 5.
In this chapter only fraction EYGF-23 has been considered in relation to its effect on reducing oxidative stress in Caco-2 cells. Many studies have demonstrated that t-BHP exhibits oxidative stress in different mammalian cells (Heo and Lee, 2005; Shivananjappa et al., 2012; Kim et al., 2012). Once t-BHP penetrates the cell membrane, it can easily degrade to alkoxyl and peroxyl radicals to generate ROS. Generating an excess amount of ROS causes changes in membrane integrity and permeability, lipid oxidation, DNA damage, and glutathione depletion by alterations in intracellular calcium homeostasis (Domanski et al., 2005; Lapshina et al., 2005). Rao et al. (1997) demonstrated that oxidative stress induced in Caco-2 cells by peroxides was due to tyrosine phosphorylation of membrane proteins causing disruption of paracellular junctional complexes.

In this study, stressed Caco-2 cells were treated with different concentrations of EYGF-23. The results obtained in figure 4.5 indicated that the EYGF-23 egg yolk peptide had the ability to enhance cell viability and to protect Caco-2 cells from the oxidative toxicity induced by t-BHP. The protective effect of EYGF-23 could be related to its scavenging activity which was reported in chapter 3. Results obtained from the measurement of cellular ROS (figure 4.7) and MDA levels (figure 4.8) in Caco-2 cells treated with EYGF-23 under oxidative conditions confirmed the scavenging activity of this fraction. These results concur with those observed with peptides isolated from Alaska pollack (Je et al., 2005), hoki fish (Kim et al., 2007), giant squid (Mendis et al., 2005a) and silver carp (Zhong et al., 2011). The observations obtained from morphological images confirm the protective effect exhibited by EYGF-23 when Caco-2 cells were oxidatively stressed (figure 4.6).
Peptides with low molecular weight have the ability to easily permeate cell membranes. Moreover, the presence of specific amino acids with hydrogen donating ability increases the activity of peptides as scavengers. In the current study, EYGF-23 is not only characterised by low molecular weight (200-750 Da) as depicted in chapter 2 but also by the presence of tyrosine and tryptophan which have a high capacity as hydrogen donors.

Cells of the intestinal epithelium have a very rapid cell turnover rate because tissue homeostasis is balanced by cell proliferation and cell death. Any inducing shifts in the redox balance by oxidants such as t-BHP could lead to an enhancement in mutagenic and apoptotic responses. Subtoxic levels of hydroperoxide result in DNA damage and apoptosis, via the activation of the caspase family (Dandrea et al., 2004). Caspases 3 and 7 are members of this family and represent the downstream caspases. They are activated by the upstream proteases and act by themselves to cleave cellular targets (Danial and Korsmeyer, 2004). In the current study, the caspase 3/7 activity observed in stressed cells was related to the ability of t-BHP to permeate the cell membrane, and generate an array of ROS that were involved in a free radical chain reaction. These radicals attack vital components in the cell, causing membrane lipid oxidation, DNA damage, and apoptosis. The presence of EYGF-23 as an antioxidant could help intercept the free-radical chain reaction, preventing caspase activation and hence inhibiting cell apoptosis (figure 4.9).

In this research, the antioxidative activity of EYGF-23 as a radical scavenger and lipid peroxidation inhibitor in vitro was confirmed. Therefore, we investigated further whether the presence of this peptide in a cell model has potential impact on cellular
antioxidative enzyme levels. In this regard, Caco-2 cells were treated with EYGF-23, prior to inducing stress with t-BHP, in order to measure the activity of superoxide dismutase and also to measure the level of total glutathione and level of catalase.

Glutathione plays a major role in the elimination of hydroperoxide. In a healthy system, the high level of total glutathione plays a substantial role in protecting cells from unexpected attack by hydroxyl radicals. Under oxidative stress, the increase in concentration of peroxides leads to a shift in thiol redox status represented by a severe decrease in the reduced form of glutathione (GSH) and an increase in the level of the oxidised form (GSSG). In the current study, 3 mM of t-BHP caused significant depletion of total glutathione, but this was elevated back to basal levels when cells were treated with the isolated fraction prior to stress induction (figure 4.10). The findings related to the imbalance of reduced and oxidised forms of glutathione due to t-BHP, led to the further examination of other antioxidant enzymes namely SOD and catalase in Caco-2 cells when treated with EYGF-23 under oxidative stress.

SOD is responsible for the dismutation of the superoxide anion to hydrogen peroxide and a molecule of oxygen (Scandalios, 1993; Inoue et al., 2003). In this study (figure 4.11), cells treated with 1.0 mg/ml EYGF-23 prior to oxidative induction exhibited significantly higher SOD activity (55.10 %) as compared with cells treated with t-BHP ($p < 0.01$). These findings illustrate the effect of EYGF-23 on enhancing the activity of SOD under oxidative stress.

In contrast, there was no significant difference in catalase level between cells stressed with t-BHP and unstressed cells (figure 4.12); this may be explained as follows. The
role of catalase enzyme in the defence system is to catalyse the decomposition of $H_2O_2$

<ref>$H_2O_2$</ref> to water and oxygen; this action is similar to the role of glutathione peroxidase. Baud et al. (2004) measured the $K_m$ value (which represents the affinity between the enzyme and the substrate) of catalase and glutathione peroxidase. The above author found that the high $K_m$ of catalase in removing $H_2O_2$ (up to 25mM), was 4000-fold higher than the $K_m$ of glutathione peroxidase (6 $\mu$M); this indicated a lower level of activity for catalase as compared to that of glutathione peroxidase. The concentration level of $t$-BHP used in this study may, therefore, not be enough to activate catalase and may explain why there was no significant difference in the amount of catalase between cells stressed with $t$-BHP and unstressed cells (figure 4.12).

Although the molecular mechanism of EYGF-23 in the induction of antioxidative enzymes is not fully understood, similar up-regulation of antioxidative enzymes has been observed following the administration of antioxidants. For example, gelatin hydrolysate, obtained from hoki skin, significantly increased levels of superoxide dismutase, catalase and glutathione peroxidase in a hepatoma cell line (Mendis et al., 2005). Young et al. (2010) also prepared egg yolk peptides that have the ability to increase the glutathione levels in red blood cells by increasing its synthesis.

4.5 Conclusion

Although both isolated egg yolk protein fractions EYGF-23 and EYGF-33 exhibited antioxidant activity by FTC and TBARS methods in oxidising linoleic model systems (chapter 2), they exhibited different behaviour on cell viability. EYGF-23 possessed antioxidant activity in oxidatively stressed Caco-2 cells and protected them against
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programmed cell death. In contrast, EYGF-33 did not show any protection to Caco-2 cells at concentrations ≥ 0.8 mg/ml. The protective effect of EYGF-23 was demonstrated by the significant increase in Caco-2 cell viability under stressed conditions (3 mM t-BHP). Intracellular ROS and malondialdehyde levels were also reduced in stress-induced cells treated with EYGF-23. Moreover, EYGF-23 was able to inhibit the activity of caspase 3/7 in stressed cells and thus protected cells from induced caspase-dependent apoptosis. EYGF-23 was able to increase the level of total glutathione and SOD activity but had no significant effect on the level of catalase. All of these findings indicate that EYGF-23 causes favourable changes within the cell to act as an antioxidant, protecting cells from oxidative stress conditions.
Chapter 5
5 Antiproliferative activity of egg yolk protein hydrolysates in a human colon cancer cell line

5.1 Introduction

Cancer is the unrestrained growth of cells forming malignant tumours. Cells undergoing unregulated growth can easily invade surrounding tissue and can metastasise to nearby sites in the body (Surh, 2003). Due to its uncontrolled and rapid spread, cancer is considered a major cause of mortality all over the world. According to the latest cancer research UK statistics (2011), colorectal cancers are one of the top ten causes of cancer-related deaths and incidence in the UK. Colorectal cancers are the second most common cause of cancer mortality (10%) and the third most common cancer incidence in men and women (14%) (CRUK, 2011).

Although causes of cancer are diverse and their mechanisms are still not fully defined, a wealth of evidence points to lifestyle and diet as risk factors contributing to the cause and progression of some types of cancer (Anand et al., 2008). Lifestyle interventions and the development of preventive approaches to control the development of this disease have gained interest in recent years. One approach is to use natural bioactive compounds occurring in dietary substances as chemopreventative agents (Milner, 2004; Stan et al., 2008).

Bioactive compounds have a wide range of functional groups that allow them to exhibit many biological functions. In order to exhibit their biological activity, it is important that they are released or extracted from the parent food source and are resistant to degradation by gut enzymes (Kris-Etherton et al., 2002). Many studies have revealed
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that bioactive compounds exhibit anti-carcinogenic activity via different mechanisms. They may quench ROS and thereby protect cellular components from oxidative stress, inhibit cell growth, induce apoptosis, alter cell cycle kinetics and interfere with intracellular signal transduction events in cancer cells (Yun, 2003; Hou, 2003; Heber, 2004).

Bioactive peptides released from plant and animal protein have been the focus of increasing interest as anticancer agents due to their properties of multi-functionality and high stability (Lee et al., 2005; Leng et al., 2005). Several protein-rich sources including soy bean, milk, and cereal grains that can bear potential anticancer peptides have been reported (Kim et al., 2000; Xiao et al., 2004; Ward et al., 2005; Kannan et al., 2008).

Anticancer properties have also been documented in several egg proteins (Kovacs-Nolan et al., 2005). Mine and Kovacs-Nolan (2006) reported that ovomucin, an egg white protein, has the potential to inhibit tumour growth by limiting angiogenesis in cells. The role of the egg white protein avidin as an anticancer agent may involve changes in the host–tumour relationship or the host–mediated antitumor response (Gasparri et al., 1999). Egg white cystatin was also reported as a potential novel anticancer agent through its suppression of cysteine proteases. These proteases, when tested in cancer cells, are implicated in multiple steps of cancer progression, for example in the early stages of immortalization and transformation, in the intermediate steps of tumour invasion and angiogenesis, and in the late steps of metastasis and drug resistance (Keppler, 2006).
Colon cancer is second to lung cancer as a leading cause of cancer-related deaths in men and women in the UK (CRUK, 2011). Thus, there is an urgent need to intensify efforts to identify novel agents that could delay or prevent the development of colon cancer. The potential of egg yolk peptides to contribute to anticancer effects has not been previously investigated. Therefore, the aim of this study was to examine the effects of the isolated egg yolk peptide EYGF-33 on apoptosis, and the generation of ROS in a Caco-2 colon carcinoma cell culture model. To achieve this aim, the effect of EYGF-33 on cell viability and morphology of Caco-2 cells and normal colon cells (HCEC) was studied, along with cellular and mitochondrial ROS levels and cell death mechanisms induced by EYGF-33.

5.2 Materials and methods

5.2.1 Materials

<table>
<thead>
<tr>
<th>Materials</th>
<th>Suppliers</th>
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<tbody>
<tr>
<td>Human colon epithelial normal cells</td>
<td>Gratefully gifted from Dr. Karen Brown,</td>
</tr>
<tr>
<td>(HCEC)</td>
<td>University of Leicester, UK.</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
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</tr>
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<td>Propidium iodide (PI)</td>
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<tr>
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<tr>
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<tr>
<td>WesternBreeze® Chemiluminescent Kit–</td>
<td>Invitrogen, Paisley, UK.</td>
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<tr>
<td>Anti-Rabbit</td>
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<tr>
<td>WesternBreeze® Chemiluminescent Kit–</td>
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<td>Anti-Mouse</td>
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5.2.2 Experimental methods

5.2.2.1 Isolation and purification of EYGF-33

EYGF-33 was isolated and purified as explained previously in chapter 2, sections 2.2.2.1, 2.2.2.2, 2.2.2.3 and 2.2.2.4.

5.2.2.2 Cell culture

Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% heat-inactivated foetal bovine serum (FBS), 1% pencillin/
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streptomycin, 1% glutamine, and 1% non-essential amino acids. Cells were seeded at concentrations of \(1 \times 10^6\) cells/ml in 25 cm\(^2\) tissue culture flasks (Corning Co., Cambridge, MA). Cells were then incubated at 37 °C in a humidified atmosphere with 5% CO\(_2\). When cells reached 80% confluence, 5 ml phosphate buffered saline solution (PBS) was used for cell washing following by 3 ml trypsin/EDTA for cell detaching. Fresh medium was added and cells were counted as required in each cell experiment.

HCEC (human colon epithelial normal cells) were cultured in the same way but a coating solution was used before the seeding step. Flasks and plates used for culturing HCEC cells were coated for at least 10 minutes. Coating solution consisting of 50 ml culture medium without FBS, 65 μl of 5% BSA, 125 μl fibronectin, and 500 μl of 2.7% collagen was freshly prepared for each coat.

5.2.2.3 Cell viability (MTT assay)

In order to determine the effect of EYGF-33 on cell proliferation and viability, the MTT assay was performed on Caco-2 and HCEC cells according to the method described by Roehm et al. (1991). The principle of the assay is the reduction of the yellow tetrazolium MTT salt to a purple intracellular insoluble formazan crystal, the formation of which is quantified by spectrophotometry. The reduction is carried out by dehydrogenase enzymes produced by metabolically active cells. The formazan crystals formed were solubilized in DMSO and quantified by spectrophotometry at 492 nm.

Briefly, cells were cultured in 96-well tissue culture plates by adding 200 μl/well of a cell suspension at a concentration of \(1\times10^4\) cells/well. After 24 hours, culture medium was replaced with fresh medium containing different concentrations of EYGF-33 ranging from 0.2 to 4 mg/ml, to determine the time- and concentration-dependent
effects of EYGF-33 on growth. After incubation for the indicated time point (24 or 48 hours), 20 \mu l of 12 mM MTT dye was added to each well and incubated at 37 °C in a humidified atmosphere with 5% CO₂. Following 4 hours incubation, culture medium was aspirated and 200 \mu l of DMSO was added to solubilize the purple formazan crystals formed. Colour intensity was measured at 492 nm using a plate reader (Behring Co, Marburg, Germany). Untreated cells at each time point were used as a control.

### 5.2.2.4 Cell morphology

Caco-2 and HCEC cells were seeded at 1×10^6 cells/ml in 25 cm² cell culture flasks. After reaching 50 % confluence, cells were treated with EYGF-33 (0, 0.4, and 1.0 mg/ml) and incubated for 24 or 48 hours. Changes in cell morphology were investigated using phase contrast microscope fitted with a camera (Nikon Eclipse TS100). A 10x magnification was used to image all cells.

### 5.2.2.5 Measurement of intracellular reactive oxygen species (cellular ROS)

The intracellular ROS level in Caco-2 cells was evaluated by flow cytometry with the use of a non-fluorescent cell permeable probe (CM-H₂DCFDA). Once the non-fluorescent probe penetrates the cell membrane, the ester group is removed by intracellular esterases and the product is oxidised by cellular ROS to produce highly fluorescent 2’, 7’-dichlorofluorescein (DCF). Fluorescence intensity is then measured and analysed at excitation and emission wavelengths of 490 nm and 520 nm, respectively, using a flow cytometer. Fluorescence is assumed to be directly proportional to the concentration of ROS in the cells (Bass et al., 1983).

Briefly, Caco-2 cells were seeded in 25 cm² flasks at a concentration 1×10^6 cells/ml. When cells reached 50% confluence, the medium was replaced with fresh medium
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containing samples of EYGF-33 (0, 0.4 and 1.0 mg/ml). After a 48-hour incubation period at 37 °C in a humidified atmosphere with 5% CO₂, the medium from each flask was collected in 15 ml centrifuge tubes and attached cells were washed with 1 ml PBS. After washing, cells were harvested using 3 ml trypsin (0.03 % in EDTA). Trypsinised cells were re-suspended in their corresponding collected medium and centrifuged for 3 minutes at 150 x g. After media aspiration, cells were re-suspended in 500 μl PBS and loaded with 5 μM DCFDA (prepared freshly in DMSO). Loaded cells were incubated for 30 minutes at 37 °C in a humidified atmosphere with 5% CO₂. After incubation, cells were kept on ice and in the dark until the time of analysis to minimise the photo-oxidation of DCFDA by light. Cellular ROS levels were measured using a BD FACSCanto flow cytometer (BD Biosciences, California, USA). Forward and side scatters were gated to exclude cell debris. At least 10,000 events were acquired in list mode.

5.2.2.6 Measurement of mitochondrial superoxide production (Mitosox ROS)

Mitochondrial ROS were evaluated by flow cytometry using MitoSOX dye. MitoSOX is selectively oxidised by superoxide occurring in the mitochondria in living cells to produce red fluorescence upon binding to nucleic acids (Robinson et al., 2008). This fluorescence can be detected by flow cytometry at an excitation wavelength of 400 nm and an emission wavelength of 590 nm.

For this assay, Caco-2 cells were seeded in 25 cm² flasks at a concentration 1×10⁶ cells/ml. Cells were treated with samples of EYGF-33 (0, 0.4 and 1.0 mg/ml) when at 50 % confluence. After 48 hours incubation at 37 °C in a humidified atmosphere with 5% CO₂, medium from each tested flask was collected in 15 ml
centrifuge tubes and attached cells were washed with 1 ml PBS. After washing, cells were harvested using 3 ml trypsin (0.03 % in EDTA). Trypsinised cells were re-suspended in their corresponding collected media and centrifuged for 3 minutes at 150 x g. After media inspiration, cells were re-suspended in 1000 µl HBSS containing calcium and magnesium and then loaded with 5 µM of fresh stock solution of MitoSOX prepared in DMSO. MitoSOX dye consists of (3,8-phenanthridinediamine, 5-6'-triphenylphosphoniumhexyl and 5,6-dihydro-6-phenyl). Loaded cells were incubated for 10 minutes at 37 °C in a humidified atmosphere with 5% CO₂. After incubation, cells were kept on ice and in the dark until analysis to minimise the photo-oxidation of MitoSOX by light. Mitochondrial ROS levels were measured with a BD FACSCanto flow cytometer. Forward and side scatters were gated to exclude cell debris with 10,000 events acquired in list mode.

5.2.2.7 Western blotting

5.2.2.7.1 Preparation of cell lysate

Caco-2 cells were seeded in 25 cm² flasks at a concentration 1×10⁶ cells/ml and incubated at 37 °C in a humidified atmosphere with 5% CO₂. When cells reached 50 % confluence, the medium was replaced with fresh medium containing samples of EYGF-33 (0, 0.4, 1.0 mg/ml) and incubated at 37 °C in a humidified atmosphere with 5% CO₂. After 48 hours, culture medium from each tested flask was collected in a 15 ml centrifuge tube and attached cells were washed with 1 ml PBS. After washing, cells were harvested using 3 ml trypsin (0.03 % in EDTA). Trypsinised cells were re-suspended in their corresponding collected media and centrifuged for 3 minutes at 150 x g. The supernatant was decanted and cell pellets were lysed using 300 µl lysis buffer (50 mM Tris HCL, 150 mM NaCl, 1 % Nonidet P40 [NP-40], 0.2 % SDS solution, 20
µM PMSF, 1 µg/ml Aprotinin, 1 µg/ml Leupeptin, and 1 mM Na₃VO₄). After lysis, cells were incubated on ice for 20 minutes and stored at –80 °C until required.

5.2.2.7.2 Quantification of cellular protein concentration

Protein quantification was carried out according to Bradford (1976), using a BioRad assay kit. In this assay, bovine serum albumin (BSA; stock concentration 1.5 mg/ml) was used to generate a protein standard curve ranging from 0.2-1.5 mg/ml BSA.

Samples were prepared in 96-well plates. A 50 µl aliquot of each standard solution or cell lysate (diluted 1:10 in milli-Q water) was added per well. To each well, 25 µl working solution (20 µl reagent S / 1000 µl reagent A) was added, followed by 200 µl of reagent B. The absorbance of the resulting solution was measured at 690 nm using a plate reader (Behring Co, Marburg, Germany). The protein concentration of each sample was calculated from the BSA standard curve.

5.2.2.7.3 Gel electrophoresis

Gel electrophoresis was used for the separation of proteins by molecular weight. Briefly, 4 µl of sample buffer was added to a micro centrifuge tube containing 20 µg protein sample. Then 2 µl of reducing agent was added per tube and made up to a final volume of 20 µl with distilled water. Samples were heated at 70 °C for 10 minutes and placed immediately in ice for a few minutes. Invitrogen NuPAGE 4-12 % Bis-Tris gel was used for electrophoretic separation. An Invitrogen Xcell SureLock Mini-cell chamber was filled with NuPAGE® SDS Running Buffer (50 ml MOPS and 950 ml distilled water). Each sample (20 µl) was loaded per well into the gel. One well was
used for loading 20 μl of a pre-stained ladder (Novex Sharp, Invitrogen, Paisley, UK).

The loaded gel was then run at room temperature for 2.5 hours at 120 V.

5.2.2.7.4 Blotting and developing

After electrophoresis, the gel cassette was removed, washed with distilled water and opened with a gel knife. The removed gel and transfer PVDF membrane were soaked in methanol and placed into an Invitrogen transfer chamber. The chambers were filled with buffer consisting of 50 ml of transfer buffer + 100 ml methanol to a final volume of 1 L with distilled water. The transfer step was carried out at room temperature for 2 hours at 30 V. At the end of transfer period, the transfer membrane was washed three times with 20 ml distilled water for 5 minutes per wash.

After washing with water, the membrane was soaked in 10 ml blocking solution (0.5 g BSA, 2 ml blocker/diluent (Part A), 3 ml blocker/diluent (Part B) and 5 ml distilled water) and placed on a rotary mixer at 4 °C to saturate the membrane and reduce non-specific binding. After one hour, the membrane was exposed to 10 ml primary antibody solution (0.5 g BSA, 7 ml distilled water, 2 ml blocker/diluent (Part A) and 1 ml blocker/diluent (Part B) and 10 μl primary antibody) and incubated overnight at 4 °C on a rotary mixer. The membrane was then washed three times with 20 ml antibody washing solution (10 ml antibody wash solution and 150 ml distilled water). After the washing step, the membrane was exposed to 10 ml of secondary antibody solution (anti-mouse or anti-rabbit) for 1 hour on a rotary mixer to allow binding of the secondary antibody to the primary antibody.
For developing the protein band on the membrane, the Chemiluminescent Western Blot Immunodetection kit was used (Invitrogen, Paisley, UK). In the dark, 2.5 ml chemiluminescent substrate was applied to the membrane surface for 5 minutes. Excess chemiluminescent reagent was removed using filter paper, the membrane was covered with a sheet of cling film and inserted into a cassette. A piece of Amersham development film was placed on top of the membrane and the cassette was closed securely. The exposure time varied from 30 seconds to 10 minutes depending on the type of antibody used (table 5.1). After the indicated time, the films were developed and fixed using 1 litre of champion amfix developing and fixing solution, respectively (First call photography, West Monkton, UK).

<table>
<thead>
<tr>
<th>Type of antibody</th>
<th>Host</th>
<th>Concentration (Antibody:solution)</th>
<th>Exposure time</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARP</td>
<td>Rabbit</td>
<td>1:1000 (v/v)</td>
<td>10 minutes</td>
</tr>
<tr>
<td>β-actin</td>
<td>Mouse</td>
<td>1:1000 (v/v)</td>
<td>1 minute</td>
</tr>
</tbody>
</table>

Table 5.1. Type, concentration and exposure time of antibodies used in Western blotting.

5.2.2.8 Apoptosis assay (Annexin V-FITC)

In this study, the occurrence of apoptosis was evaluated using the Annexin V-FITC staining technique. In healthy viable cells, phosphatidylserine (PS) is located in the inner layer of the plasma membrane bilayer. When apoptosis is induced, the cell membrane loses its integrity, which permits PS to move and relocate to the outer membrane layer. Externalised PS can be detected using Annexin V-FITC. To
discriminate living cells from apoptotic, necrotic, and dead cells, the combination of Annexin V-FITC and propidium iodide (PI) staining was used. In healthy cells with an uncompromised cell membrane, cells cannot be stained with either Annexin V-FITC or PI. In early apoptosis, cells are stained only with Annexin V-FITC but not PI. As these cells began to die (late apoptosis or necrosis), they are stained with Annexin V-FITC and PI (double positive). Cells stained only with PI are dead (figure 5.1).
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Figure 5.1. A typical histogram obtained by flow cytometry for cells treated with Annexin. (Q1) represents healthy non-stained cells (AV-ve/PI-ve); (Q2) represents cells in early apoptotic stage conjugated with AV (AV+ve/PI-ve); (Q3) represents cells in late apoptotic stage conjugated with AV and stained with PI (AV+ve/PI+ve); and (Q4) represents dead cells stained with PI (AV-ve/PI+ve).

The procedure was carried out according to methods described in the Annexin V-FITC apoptosis detection kit (Merck Millipore, Nottingham, UK). Briefly, Caco-2 cells were seeded in 25 cm² flasks at a density of 1×10⁶ cells/ml and incubated at 37 °C in a humidified atmosphere with 5% CO₂. When cells reached 50% confluence, they were treated with EYGF-33 (0, 0.4, and 1.0 mg/ml) and maintained for 48 hours. Growth medium was collected from each flask before trypsinisation to collect cells detached during cell death process. After washing with PBS, cells were trypsinised and re-suspended in this media. The suspended cells were centrifuged for 5 minutes at 150 x g.

The cell pellet was then re-suspended in 500 µl ice-cold binding buffer followed by the addition of 25 µl conjugated Annexin V-FITC. After 15 minutes incubation in the dark at room temperature, the suspension was centrifuged, the supernatant was discarded, and the cell pellet was suspended in 500 µl binding buffer. The addition and gentle mixing of 10 µl (1 mg/ml) PI to the final volume was performed to identify cells with diminished membrane integrity. The cells were kept on ice until analysed by BD
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FACSCanto flow cytometer (BD Biosciences, San Jose, USA) to quantitatively measure cells undergoing cell death. Forward and side scatters were used for gating to exclude cell debris. Fluorescence emission was detected in the FL-1 channel (519 nm) for cells labelled with Annexin V-FITC and in the FL-2 channel (617 nm) for cells labelled with PI. For each experimental sample, a total of 10,000 events were acquired for Annexin V-FITC and PI stain.

5.2.2.9 Cell cycle assessment

Caco-2 cells were seeded in 25 cm² flasks at a density of 1×10⁶ cells/ml and incubated at 37 °C in a humidified atmosphere with 5% CO₂. EYGF-33 (0, 0.4, and 1.0 mg/ml) was added when cells reached 50 % confluence. Treated cells were then maintained for 48 hours. The media was collected from each flask before trypsinisation and retained so that cells could be re-suspended in this media after washing with PBS. The suspended cells were then centrifuged for 5 minutes at 150 x g. The pellet was then re-suspended with 200 µl cold PBS followed by 1 ml cold fixing buffer (70% ethanol in PBS). Fixing buffer was added drop-wise while vortexing to achieve full fixation and full clump separation. Fixed cells were spun in order to remove ethanol fixing buffer after overnight incubation at 4 °C. PBS was used for cell washing followed by the addition of 10 μM of RNase. After 30 minutes incubation at 37°C, 10 μl (1 mg/ml) PI staining dye was added and cells were kept on ice until analysed using a BD FACSCanto flow cytometer. A minimum of 10,000 events was acquired in list mode and analysed at excitation and emission wavelengths of 535 nm and 617 nm, respectively.
5.2.3 Statistical analysis

Each experimental condition was tested in triplicate and the experiment repeated three times on separate occasions to ensure reproducibility. Results were analysed using GraphPad Prism software version 6.0. Statistical analysis comparisons were made by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test or by two-way analysis of variance followed by Tukey's multiple comparisons test when appropriate. Details of the statistical test used are provided in the figure legends of presented results. \( p < 0.05 \) was considered statistically significant. Data are presented as means \( \pm \) standard deviation (SD).

5.3 Results

5.3.1 Effect of EYGF-33 on cell viability and morphology

The MTT assay evaluated the number of living cells that remained after exposure of EYGF-33 to Caco-2 and HCEC cells. After 24 hours incubation (figure 5.2), cell viability of Caco-2 cells was similar to untreated cells with no significant difference among all concentrations used. After the same incubation period, there was a significant increased in HCEC cell viability \( (p < 0.001) \) when a higher concentration range of EYGF-33 (from 0.6 to 4.0 mg/ml) was used.
Figure 5.2. Cell viability of Caco-2 cells and HCEC treated with EYGF-33 for 24 hours using the MTT assay. Cells were treated with different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0 and 4.0 mg/ml) of EYGF-33. Cell viability was measured after 24 hours incubation with the peptide fraction. The absorbance of formazan crystals was monitored at 492 nm using a spectrophotometer. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Tukey’s multiple comparisons test. The result was considered statistically significant versus the control, untreated cells, (\(* = p < 0.05, ** = p < 0.01, *** = p < 0.001\)).

After 48 hours, cell viability of Caco-2 cells was significantly reduced (\(p < 0.01\)) when treated with 0.8 mg/ml EYGF-33 compared with untreated cells (71.51 % and 100 % viability respectively; figure 5.3). Cell viability reduced to 54.26%, 45.15% and 43.10% when cells were treated with 1.0, 2.0 and 3.0 mg/ml EYGF-33, respectively. The largest reduction in Caco-2 cell viability (34.36 %, \(p < 0.001\)) was observed in the presence of 4.0 mg/ml EYGF-33. In contrast, HCEC cell viability was not significantly affected by any concentration of EYGF-33 (figure 5.3). This result indicates that EYGF-33 peptide significantly reduced the viability of cancer cells after 48 hours, whilst causing no toxicity to normal colon cells. According to the results obtained in figure 5.3, the IC\(_{50}\) was estimated to be 1.0 mg/ml when using a 48-hours incubation time with EYGF-33.
Figure 5.3. Cell viability of Caco-2 cells and HCEC treated with EYGF-33 for 48 hours using the MTT assay. Cells were treated with different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0 and 4.0 mg/ml) of EYGF-33. Cell viability was measured after 48 hours incubation with the peptide fraction. The absorbance of formazan crystals was monitored at 492 nm using a spectrophotometer. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Tukey’s multiple comparisons test. The result was considered statistically significant versus the control, untreated cells, (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

The effect of EYGF-33 on the morphology of both cell types was monitored for 24 and 48 hours. Cells were treated with 1.0 mg/ml and 0.4 mg/ml EYGF-33 and the morphology was compared with untreated cells. After 24 hours incubation, the morphology of Caco-2 cells as well as HCEC cells did not show noticeable difference between untreated and treated cells. The same observation was noticed in cell growth when both types of cells were treated with 0.4 mg/ml EYGF-33 and incubated for 48 hours. At 1.0 mg/ml EYGF-33, Caco-2 cell growth was considerably inhibited after 48 hours (figure 5.4) while HCEC cells treated with same concentration, exhibited no noticeable change in cell morphology (figure 5.5). These observations confirmed the results obtained from cell viability assays, which indicated that EYGF-33 reduces cell viability of colon cancer cells without cytotoxic effects in normal colon cells, at the
concentrations used, after 48 hours incubation. Therefore, based on both MTT results and morphological observations, subsequent experiments used EYGF-33 concentrations of 1.0 and 0.4 mg/ml with an incubation period of 48 hours.

![Figure 5.4. Morphological characteristics of the Caco-2 cell line after 24 and 48 hours. Caco-2 cells were treated with 0.4 and 1.0 mg/ml EYGF-33. Images were taken after 24 and 48 hours incubation using light microscopy at 10x magnification.](image)

![Figure 5.5. Morphological characteristics of the HCEC cell line after 24 and 48 hours. HCEC cells were treated with 0.4 and 1.0 mg/ml EYGF-33. Images were taken after 24 and 48 hours incubation using light microscopy at 10x magnification.](image)
5.3.2 Effect of EYGF-33 on cellular and mitochondrial ROS

Changes in the cellular and mitochondrial redox status of Caco-2 cells, when treated with EYGF-33, were monitored by flow cytometry using a fluorescent probe. In figure 5.6-A, cellular ROS levels in untreated cells (55.2%) were similar to levels in cells treated with 0.4 mg/ml (58.8%) and 1.0 mg/ml EYGF-33 (47.4%; figure 5.6-A).

Although there was no significant difference in intracellular ROS levels between untreated and treated cells, mitochondrial ROS levels in cells treated with 1.0 mg/ml EYGF-33 were significantly higher than untreated cells ($p < 0.05$). In figure 5.6-B, mitochondrial ROS levels in untreated cells and in cells treated with 0.4 mg/ml EYGF-33 were 53.8% and 62.9% respectively. In cells treated with 1.0 mg/ml EYGF-33, mitochondrial ROS levels were 67.0% which is significantly higher than the mitochondrial ROS level in untreated cells (53.8%; $p < 0.05$).

![Figure 5.6. Effect of EYGF-33 on cellular and mitochondrial ROS levels using flow cytometry.](image)

Figure 5.6. Effect of EYGF-33 on cellular and mitochondrial ROS levels using flow cytometry. Caco-2 cells were treated with 0.4 and 1.0 mg/ml EYGF-33 for 48 hours. (A) Cellular ROS were monitored by loading treated cells with CM-H$_2$DCFDA probe and measuring the intensity of DCF fluorescence at 490 nm excitation and 520 nm emission. (B) Mitochondrial superoxide was monitored by loading treated cells with MitoSOX dye and measuring the intensity of fluorescence at 400 nm excitation and 590 nm emission. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Dunnett’s multiple comparisons test. The result was considered statistically significant versus the control, untreated cells, ($* = p < 0.05$, **$* = p < 0.01$, ***$* = p < 0.001$).
5.3.3 Effect of EYGF-33 on Poly ADP-ribose polymerase (PARP) expression

Western blotting was performed to determine if PARP protein expression was affected by EYGF-33 treatment, with the intention of elucidating the mechanism of cell death. PARP cleavage was detected when cells were treated with 1.0 mg/ml EYGF-33 only, as shown in (figure 5.7). PARP is an important downstream biomarker of apoptosis; therefore, its cleavage indicated programmed cell death (apoptosis).

![Figure 5.7. Western blot of PARP expression in Caco-2 cells treated with EYGF-33.](image)

Figure 5.7. Western blot of PARP expression in Caco-2 cells treated with EYGF-33. Cellular protein was extracted from Caco-2 cells after treatment with 0.4 mg/ml or 1.0 mg/ml EYGF-33 for 48 hours. Gel electrophoresis was used for protein separation and Western blot analysis, accompanied with chemiluminescence detection when a PARP-specific antibody was applied to develop protein bands. Equal protein loading (20 µg/well) was confirmed using β-actin expression.

5.3.4 Effect of EYGF-33 on phosphatidylserine (PS) externalization

The initiation of cell death by apoptosis was also investigated by examining the effect of EYGF-33 on PS externalization in Caco-2 cells using flow cytometry. The images (figure 5.8 A) represent the flow cytometry results obtained for untreated cells (control),
cells treated with 0.4 mg/ml EYGF-33 and cells treated with 1.0 mg/ml EYGF-33 respectively. Apoptosis was significantly induced in Caco-2 cells when treated with 1.0 mg/ml EYGF-33 \( (p < 0.001; \text{figure 5.8}) \), while there was no significant difference between untreated cells and cells treated with 0.4 mg/ml EYGF-33 in early apoptotic (Q2), late apoptotic (Q3) and dead cells (Q4).

Figure 5.8-B, represents the percentage of cells in each quadrant (Q). In early apoptotic (Q2), the percentage of cells was 3.3%, 5.7% and 14.9% for untreated cells, cells treated with 0.4 mg/ml EYGF-33 and cells treated with 1.0 mg/ml EYGF-33, respectively. In addition, cell percentage in late apoptotic (Q3) was 1.4%, 2.7% and 30.6% for untreated cells, cells treated with 0.4 mg/ml EYGF-33 and cells treated with 1.0 mg/ml EYGF-33, respectively. The percentage of dead cells (Q4) was elevated significantly when cells were treated with 1.0 mg/ml EYGF-33 (4.8%, \( p < 0.001 \)) in comparison to untreated cells (0.8%).
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Figure 5.8. Flow cytometry images of cell death associated with phosphatidylserine (PS) externalization. Caco-2 cells were untreated (control) or treated with 0.4 mg/ml or 1.0 mg/ml EYGF-33 followed by incubation for 48 hours. Cells were trypsinized and labelled with Annexin V-FITC (AV) and propidium iodide (PI). PS externalization was detected using a FACScanto flow cytometer. (A) flow cytometry images of untreated cells, cells treated with 0.4 mg/ml, and cells treated with 1.0 mg/ml EYGF-33. Data presented in (table B) correspond to the means ± SD of three independent experiments. (Q1) represents healthy non-stained cells (-ve/-ve); (Q2) represents cells in early apoptotic stage conjugated with AV (+ve/-ve); (Q3) represents cells in late apoptotic stage conjugated with AV and stained with PI (+ve/+ve); and (Q4) represents dead cells stained with PI (-ve/+ve). A total of 10,000 cells were counted for each experiment. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. The result was considered statistically significant versus the control, untreated cells, (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

<table>
<thead>
<tr>
<th></th>
<th>Untreated cells</th>
<th>EYGF-33 (0.4 mg/ml)</th>
<th>EYGF-33 (1.0 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1:AV(-ve),PI(-ve)</td>
<td>93.4 ± 0.8</td>
<td>90.3 ± 1.3</td>
<td>46.4 ± 2.1(***))</td>
</tr>
<tr>
<td>Q2:AV(+ve),PI(-ve)</td>
<td>3.3 ± 0.5</td>
<td>5.7 ± 0.9</td>
<td>14.9 ± 1.3(***))</td>
</tr>
<tr>
<td>Q3:AV(+ve),PI(+ve)</td>
<td>1.4 ± 3.1</td>
<td>2.7 ± 0.7</td>
<td>30.6 ± 0.7(***))</td>
</tr>
<tr>
<td>Q4:AV(-ve),PI(+ve)</td>
<td>0.8 ± 0.3</td>
<td>0.9 ± 0.4</td>
<td>4.8 ± 0.5(***))</td>
</tr>
</tbody>
</table>
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5.3.5 Cell cycle assessment

In this assay PI stain was used due to its ability to permeate the cell membrane and chelate cellular DNA. The intensity of the PI signal is assumed to be directly proportional to DNA content. Figure 5.9 demonstrates the effect of 0.4 and 1.0 mg/ml EYGF-33 on Caco-2 cell cycle phases.

The cell population of untreated cells was distributed as follows: 74.1% in G1 phase (growth phase), 18.5% in S phase (DNA synthesis phase), and 7.4% in G2 phase (growth 2 phase). After treatment with 0.4 mg/ml EYGF-33, there was no significant change in the distribution of cells in the G1, S, and G2 phases of the cell cycle (72.1%, 21.4% and 6.6%, respectively) compared with untreated cells. When Caco-2 cells were treated with 1.0 mg/ml EYGF-33, there was a significant reduction in the cell population in the G1 phase (34.6%, \( p < 0.01 \)) whereas a significant elevation was noticed in the cell population in the S phase (55.3 %, \( p < 0.05 \)) when compared with untreated cells (18.5%). However, treatment with 1.0 mg/ml EYGF-33 caused no significant change in the proportion of cells in the G2 phase (9.9%) compared with control cells (7.4%).
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Figure 5.9. Effect of EYGF-33 on Caco-2 cell cycle distribution using flow cytometry. Caco-2 cells were treated with 0.4 and 1.0 mg/ml of EYGF-33 and compared with untreated cells. Cells were fixed in 70% ethanol after 48 hours, followed by staining with 10 µl of propidium iodide (PI, 1 mg/ml stock). Flow cytometer images represent cell cycle distribution, with 10,000 cells counted for each experiment. G1 phase represents growth phase, S phase represents DNA synthesis phase, and G2 phase represents growth 2 phase. The bar graph represents mean ± SD of three independent flow cytometry experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Tukey’s multiple comparisons test. The result was considered statistically significant versus the control, untreated cells, (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
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5.4 Discussion

In the last 10 years, the chemotherapeutic effects of bioactive peptides on different cancer cells have gained considerable attention (Picot et al., 2006; Kannan et al., 2008; Hsu et al., 2011; You et al., 2011). However, the relationship between the molecular mechanisms and the chemotherapeutic effects of bioactive peptides needs more investigation. The present study was designed to investigate the possible molecular mechanisms responsible for the cytotoxic effect exerted by EYGF-33 on Caco-2 cells, as presented in chapter 4.

The cell viability results (figure 5.2) and morphological observations (figure 5.4) indicated that EYGF-33 exerts its toxic effects specifically on colon cancer cells after 48 hours. At the same time and dose, no harmful effects on normal HCEC cells were observed (figure 5.3 and figure 5.5). The mechanism of cell death appears to be due to the ability of EYGF-33 to significantly enhance the production of the superoxide anion in the mitochondria of cancer colon cells, leading to cell death by apoptosis.

Mitochondria are critical for cellular regulatory processes and hence cell survival, therefore mitochondrial dysfunction can be a key determinant of cell death (Schulze-Osthoff et al., 1992; Kamp et al., 2011). The respiratory chain reaction that occurs in mitochondria is considered to be one of the major sources of free radicals, namely the superoxide anion. Although this radical is not a strong oxidant, it is considered to be a precursor of other ROS and thus becomes involved in the propagation of the oxidative chain reaction (Turrens, 2003). There is now mounting evidence that excess ROS are not only the source of direct damage to proteins, lipids, and DNA but are also
considered to be major mediators in cell death pathways (Fleury et al., 2002; Lovrić et al., 2005; Circu and Aw, 2010).

Some researchers have suggested that damage to mitochondrial function occurs due to the accumulation of mitochondrial ROS leading to cell death (Kowaltowski and Vercesi, 1999; Fleury et al., 2002; Kroemer et al., 2007). Therefore, in our current study, the significant increase in superoxide anion in cells treated with 1.0 mg/ml EYGF-33 (figure 5.6-B) may lead to the activation of cellular death pathways.

EYGF-33 could disrupt the functions of mitochondria by the elevation of ROS levels, in particular the superoxide anion. This could subsequently cause PARP cleavage as observed in Western blot result (figure 5.7). PARP has the ability to induce programmed cell death (apoptosis) as a result of stimulating mitochondria to release the apoptosis inducing factor (AIF) (Green and Reed, 1998; Yu et al., 2006). AIF is involved in initiating a caspase-independent pathway of apoptosis by causing DNA fragmentation and chromatin condensation in the absence of caspase cascade activation (Candé et al., 2002). Therefore, the results observed in this study suggest that EYGF-33 causes activation of a mitochondrial apoptotic pathway, by increases mitochondrial ROS leading to typical PARP degradation.

The induction of apoptotic cell death by EYGF-33 was supported by the externalisation of PS (figure 5.8). Kaufmann and Earnshaw (2000) suggested that chemotherapeutic agents trigger cell apoptosis by two pathways; an extrinsic pathway that involves the ligation of cell-surface death receptors as TNF-α, and an intrinsic pathway that involves mitochondrial release of cytochrome C and caspase activation.
Induction of apoptosis is considered to be an important mechanism of cancer prevention treatment by chemo-preventive agents. Many researchers have reported that excess ROS generation causes cell cycle arrest (Shackelford et al., 2000; Burhans and Heintz, 2009). In agreement with this, our current research also suggested that oxidative stress induced in mitochondria caused cell death via arrest of the Caco-2 cell-cycle in S-phase. Accumulation of cells in S-phase as a result of EYGF-33 treatment suggests a block in the transition from S phase to G2 phase. Alternatively, the cyclin A/CDK2 protein complex, which controls the progression of cell cycle in S phase, may have been altered. Disruption of the A/CDK2 complex would interfere with the DNA replication process and subsequently arrest cell cycle progression. This is suggested by the findings of the cell viability assay and morphological observations. Further elucidation of the mechanism of EYGF-33-mediated apoptosis, as suggested above, would provide further support for its use as a potential therapeutic and chemo-preventive agent.

5.5 Conclusion

The present results suggest that EYGF-33 is a promising peptide candidate for reducing cancer due to its anti-proliferative potential. Cytotoxic effects of EYGF-33 were observed in vitro in colon cancer cells without any toxicity in normal colon cells. Apoptosis was mediated by mitochondrial ROS generation and PARP cleavage, which was confirmed by Western blotting and Annexin-V conjugation. Reduced cell viability can be explained by cell cycle arrest in S-phase in which DNA replication normally takes place. However, preclinical efficacy and safety studies using animal models, as well as carefully designed pharmacokinetics studies are needed before clinical trials of peptides as cancer preventive or therapeutic agents can be initiated.
6 Isolation and identification of ACE inhibitory peptides

6.1 Introduction

High blood pressure is a chronic medical symptom leading to worldwide health problems because of its ability to trigger cardiovascular complications. About one billion people suffer from hypertension, resulting in over 7.1 million deaths per year globally (WHO, 2012). Approximately 20% of adults in the world are estimated to have hypertension, especially patients over 60 years of age. In the UK, the number of people diagnosed with hypertension rose by 2.7% from 2004 to 2008 (WHO, 2012).

Regulation of arterial blood pressure in the human body is mainly achieved through diverse physiological systems including the kinin-nitric oxide system (KNOS), the neutral endopeptidase system (NEPS), the renin-chymase system (RCS) and the renin-angiotensin system (RAS). Any disturbance in the blood pressure by increase (hypertension) or decrease (hypotension) will trigger a number of chronic diseases, such as cardiovascular diseases, peripheral vascular disease and renal dysfunction.

It has been recognized that the renin-angiotensin system exists in various human body organs, such as the brain, kidneys and lungs, as well as in the circulatory blood system (Cholewa et al., 2005). One of the key elements constituting the renin-angiotensin system is the angiotensin converting enzyme (ACE; EC3.4.15.1, dicarboxy peptidase). ACE hydrolyses inactive decapeptide (angiotensin-I), through the removal of the dipeptide His-Leu from the C-terminus, to produce the potent vasoconstrictor octapeptide (Angiotensin-II) (Johnston, 1990; Crowley et al., 2006).
Angiotensin-I results from the cleavage of angiotensinogen (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Glu-Ser) from the liver by the action of renal renin.

It is noteworthy that ACE enhances the reabsorption of renal tubular sodium by increasing the release of adrenal aldosterone. ACE also plays an important role in the depressor hormonal (kinin-kallikrein) system. In this system, ACE hydrolyses vasodilatory bradykinin to inactive metabolites (Tschope et al., 2002; Zhao and Xu, 2008). Therefore, the utilisation of ACE inhibitors blocks the generation of the vasoconstrictor angiotensin II and potentiates the action of the vasodilator bradykinin.

Although potent synthetic ACE inhibitors namely captopril, lisinopril, fosinopril, and enalapril are used extensively in the clinical treatment of hypertension, they have significant adverse effects on health such as a dry cough, skin rashes, and headache (Morimoto et al., 2004; Coleman and Cox, 2011). On the other hand, the most recent studies on food-derived ACE inhibitors such as peptides from food proteins, such as whey and soy protein, have so far indicated that these inhibitors do not have known side effects on the human body (Hong et al., 2008; Kim and Wijesekara, 2010; Norris and FitzGerald, 2013). Accordingly, more studies are now directed towards understanding how to produce food-derived peptides that can act as ACE inhibitors for the clinical treatment of hypertension.

ACE is a dipeptide carboxy peptidase which characterised by its three dimensional (3D) structure that consists of α-helices, chloride and zinc ions. The active site is composed of two subdomains (N-domain and C-domain), which are separated by a deep, narrow channel. Although there is some sequence similarity between these subdomains, they differ in their affinity for the substrate and inhibitors, as well as
their extent of activation by chlorine ions. There is also an amino terminal (lid) on the
top of the active site that permits the small peptide substrate to access the active site
cleft (Natesh et al., 2003; Natesh et al., 2004).

Researchers have purified bioactive peptides with ACE inhibitory activity from
enzymatic hydrolysates of various food proteins (Brian et al., 2005). These food
proteins are contained in whey (FitzGerald and Meisel, 1999), egg (Miguel and
Aleixandre, 2006b), soybean (Lo and Li-Chan, 2005) and chicken breast muscle
(Saiga et al., 2006). Some peptides were also tested for their activity as
antihypertensives in a spontaneous hypertensive rat (SHR) model (Yamamoto et al.,
1994; Miguel et al., 2005; Nakano et al., 2006; Miguel et al., 2009; Thewissen et al.,
2011).

The aims of this study were to isolate and characterize peptides from hydrolyzed egg
yolk protein, with respect to ACE inhibitory activity and to examine whether
additional purification improves the activity of the inhibitor. Furthermore, this study
aimed to identify the amino acid composition of the isolated peptide and to investigate
the mechanism of ACE inhibition.
6.2 Materials and methods

6.2.1 Materials

<table>
<thead>
<tr>
<th>Materials</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile HPLC grade</td>
<td>Fisher Scientific, Loughborough, UK.</td>
</tr>
<tr>
<td>ACE (Angiotensin-I-converting enzyme, 1 unit/mg) from rabbit lung</td>
<td>Sigma-Aldrich Ltd, Dorset, UK</td>
</tr>
<tr>
<td>Trifluoroacetic acid (TFA)</td>
<td></td>
</tr>
<tr>
<td>Captopril</td>
<td>Sigma-Aldrich Ltd, Dorset, UK</td>
</tr>
<tr>
<td>Hippuryl-histidyl-leucine (HHL)</td>
<td></td>
</tr>
<tr>
<td>Hippuric acid (HA)</td>
<td></td>
</tr>
<tr>
<td>Histidyl-leucine (HL)</td>
<td></td>
</tr>
</tbody>
</table>

6.2.2 Experimental methods

6.2.2.1 Protein extraction and hydrolysate preparation

As described previously in chapter 2, sections 2.2.2.1 and 2.2.2.2.

6.2.2.2 Fractionation of egg yolk hydrolysate by ultrafiltration

As described previously in chapter 2, section 2.2.2.3.

6.2.2.3 Determination of angiotensin converting enzyme inhibitory activity

The measurement of ACE inhibitory activity was conducted using HPLC in accordance with the method of Wu et al. (2002), modified to improve the method of Cushman and Cheung (1971).
All samples were prepared with 100 mM borate buffer, containing 300 mM NaCl at pH=8.3. The total reaction volume was 70 µl, consisting of 50 µl of 2.17 mM hippuryl-histidyl-leucine (HHL), 10 µl of peptide fractions and 10 µl of 2 mU angiotensin converting enzyme (ACE). The peptide fractions and HHL were mixed and maintained at 37 °C for 10 minutes in 2 ml polyethylene microcentrifuge tubes. ACE was maintained at 37 °C for 10 minutes before the two solutions were combined. The combined mixture was incubated at 37 °C for 30 minutes with continuous agitation. In order to terminate the reaction, 85 µl of 1 M HCl was added. Samples were analyzed on a C18 column (3.0×150 mm, 5 µm, phenomenex) and hippuric acid (HA) and HHL were detected at 228 nm using HPLC system (Ultimate 3000 HPLC, Thermo Fisher Scientific Inc., Loughborough, UK). Data were analysed using integration software (Chromeleon).

The column was eluted at a rate of 1 ml/min with a two solvent system: (A) 0.05% trifluoroacetic acid (TFA) in water and (B) 0.05% TFA in acetonitrile, with a 5-60% acetonitrile gradient for the first 10 minutes, maintained for 2 min at 60% acetonitrile, then returned to 5% acetonitrile for 1 minute. This was followed by isocratic elution for 4 min at the constant flow rate of 1 ml/min. The ACE inhibitory activity was calculated as follows:

\[
ACE \text{ inhibitory activity (\%)} = \frac{C - S}{C - B} \times 100
\]

Where C is the peak area of control (buffer added instead of test sample), B the peak area of the reaction blank (without ACE and sample), and S is the peak area in the presence of a sample or captopril.
6.2.2.4 Gelfiltration chromatography

As described previously in chapter 2, section 2.2.2.4.

6.2.2.5 Reversed-phase high performance liquid chromatography (RP-HPLC)

The egg yolk fraction with the highest ACE inhibitory activity was injected on to a reversed-phase high performance liquid chromatography (RP-HPLC) column for further purification. The sample was loaded into a Partisil 10 ODS-1 column (9.5 mm x 500 mm) (Whatman plc, UK). The elution was a linear gradient of acetonitrile (0-70 % containing 0.1 % TFA) with flow rate of 1 ml/min for 50 minutes. Peaks detected at 215 nm or 250 nm were collected and lyophilised at -80 °C for further study.

6.2.2.6 Determination of IC\textsubscript{50}

In order to determine IC\textsubscript{50}, a wide range of isolated peptide concentrations (2, 4, 6, 8 and 10 mg/ml) was prepared as mentioned previously in section 6.2.2.3 and analysed using HPLC.

6.2.2.7 Amino acid analysis

As described previously in chapter 2, section 2.2.2.6.

6.2.2.8 ACE inhibition mechanism

In order to determine the mechanism of the ACE inhibition, different concentrations of ACE inhibitory peptide (0, 4, and 10 mg/ml) and HHL substrate (1, 2.17, 5.5, and 11 mM) were used. Resultant mixtures were analysed by measuring ACE inhibition...
activity using HPLC as described in the previous section 6.2.2.3. Enzyme kinetics in the presence of the peptide was determined using Lineweaver-Burk plots.

6.2.3 Statistical analysis

All experimental procedures were conducted with at least three independent replicates on three separate occasions. Results were analysed using GraphPad Prism software version 6.0. Statistical analysis comparisons were made by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test. \( P < 0.05 \) was considered statistically significant. Data are presented as means ± standard deviation (SD).

6.3 Results

6.3.1 Purification of ACE peptide

ACE inhibition was measured by quantification of HA which appears at 228 nm as a reaction product. The ACE inhibitory activity exhibited by 10 mg/ml of ultrafiltration fractions obtained after hydrolysis (EYUF-10, EYUF-5 and EYUF-2) was compared with that of 10 mg/ml captopril (figure 6.1). Among the fractions, the highest inhibitory activity was observed in the EYUF-2 sample, followed by EYUF-5, then EYUF-10 (49.7 %, 42.2 %, and 33.1 % inhibition, respectively). Inhibitory activity increased with decreasing fraction molecular weight, although none was as potent as captopril (98.8 %; \( p < 0.001 \)).
Chapter 6: Isolation and identification of ACE inhibitory peptides

Figure 6.1. ACE inhibitory activity of fractionated egg yolk protein hydrolysates. The activity of 10 mg/ml EYUF-10, EYUF-5 and EYUF-2 to inhibit ACE was measured by HPLC. Captopril (10 mg/ml) was used as a positive control. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. The result was considered statistically significant versus captopril (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

The EYUF-2 fraction with the highest ACE inhibitory activity was collected from the ultrafiltration process and separated by gel filtration chromatography using a Sephadex G-25 column to further purify the peptides.

Fractions obtained from different runs of gel filtration chromatography were pooled and lyophilized before their ACE inhibition activity was assayed. The method was conducted for all fractions to test their ability to inhibit the activity of the enzyme when used at a concentration of 10 mg/ml. Figure 6.2 shows that ACE inhibition activity was widely observed for all fractions, but the most effective fraction (depicted by a shaded bar) was EYGF-56 (69.2% inhibition) compared with 10 mg/ml captopril. EYGF-56 fraction was, therefore, collected for further study.
Figure 6.2. ACE inhibitory activity (%) of egg yolk protein fractions separated on a Sephadex G-25 column. The activity of 10 mg/ml for each gel filtration fraction to inhibit ACE was measured by HPLC. Captopril (10 mg/ml) was used as a positive control. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. The result was considered statistically significant versus captopril (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

The EYGF-56 fraction with the highest ACE inhibitory activity (as identified in figure 6.2 above) was pooled and collected from the gel filtration process and separated by RP-HPLC using a Partisil 10 ODS-1 column to obtain peptides of greater purity. The elution profile generated with a flow rate of 1 ml/min is shown in figure 6.3. One prominent peak appeared in the chromatogram labelled by EYLCF-16, which corresponded to the presence of the peptide.
Figure 6.3. Elution profile of EYGF-56 separated by reverse-phase high performance liquid chromatography. Separation was achieved using a Partisil 10 ODS-1 column and peaks were detected at 215 nm with flow rate of 1 ml/min.

As before, the fractions obtained from different RP-HPLC runs were pooled and lyophilized prior to assaying ACE inhibition activity. The purified EYLCF-16 fraction (10 mg/ml) achieved 91.8% ACE inhibition compared with the same concentration used by captopril (figure 6.4). The activity exhibited by EYLCF-16 was significantly lower than that of captopril (98.8%; $p < 0.05$), which was used as a positive control in the experiment. The ACE inhibition activity shown by EYLCF-16 was more effective than the ultrafiltered EYUF-2 and gel filtration fraction EYGF-56, which inhibited the activity of ACE by only 49.7% and 69.2%, respectively.
Figure 6.4. ACE inhibitory activity of EYUF-2 obtained after ultrafiltration, EYGF-56 obtained by gel filtration and EYLCF-16 obtained by RP-HPLC. The activity of 10 mg/ml of each fraction to inhibit ACE was measured by HPLC. Captopril (10 mg/ml) was used as a positive control. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Dunnett’s multiple comparisons test. The result was considered statistically significant versus captopril (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

The 50% enzyme inhibitory concentration (IC50) of the fractions with the highest ACE inhibition activity obtained from each purification step was calculated. As observed in table 6.1., ACE inhibition activity was enhanced with further purification; EYUF-2 exhibited the lowest activity (49.7%) with an IC50 of 5.44 mg/ml, followed by EYGF-56 with 69.2% activity and IC50 of 3.35 mg/ml. In contrast, EYLCF-16, obtained from the final purification step exhibited the highest activity (91.8%) with the lowest IC50 concentration (1.53 mg/ml). The IC50 values among the three fractions were significantly different (p <0.001).
Table 6.1. IC$_{50}$ values for of EYUF-2 obtained from ultrafiltration, EYGF-56 obtained from gel filtration and EYLCF-16 obtained from RP-HPLC. The activity of different concentrations of each fraction to inhibit ACE, was measured by HPLC. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0, followed by Tukey's multiple comparisons test. The result was considered statistically significant between all fractions (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

<table>
<thead>
<tr>
<th>Fractions</th>
<th>ACE inhibitory activity (%)</th>
<th>IC$_{50}$ (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYUF-2</td>
<td>49.7</td>
<td>5.44 ± 0.02***</td>
</tr>
<tr>
<td>EYGF-56</td>
<td>69.2</td>
<td>3.35 ± 0.05***</td>
</tr>
<tr>
<td>EYLCF-16</td>
<td>91.8</td>
<td>1.53 ± 0.13***</td>
</tr>
</tbody>
</table>

6.3.2 Amino acid analysis

Given the substantial inhibitory effect of EYLCF-16 on ACE activity, the fraction was subjected to amino acid analysis in order to elucidate the possible effect of amino acid composition on ACE inhibitory activity. The chromatogram of amino acids and calculated composition of the selected fractions after RP-HPLC is presented in figure 6.5 and table 6.2, respectively. EYLCF-16 contained a high proportion of leucine (20.26%), electrically charged arginine (14.69%) and lysine (10.90%), aromatic tryptophan (12.49%) and hydrophilic glutamic acid (8.42%).
Figure 6.5. Amino acid chromatograms of (A) amino acid standards, (B) EYLCF-16 after hydrolysis with 6 N HCl. Amino acids were separated on a C18 reverse-phase column and detected at 254 nm. Gradient mobile phase was used consisting of two eluents; A and B. Eluent A was composed of 0.22 M sodium acetate buffer containing 0.05 % (v/v) TEA, pH 6.2 (adjusted using glacial acetic acid). Eluent B was composed of 60:40 acetonitrile: water.
<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Amino acid residues (%) in EYLCF-16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>4.06 ± 0.27</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>8.42 ± 0.26</td>
</tr>
<tr>
<td>Serine</td>
<td>4.80 ± 0.13</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.02 ± 0.11</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.96 ± 0.26</td>
</tr>
<tr>
<td>Arginine</td>
<td>14.69 ± 0.12</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.77 ± 0.04</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.55 ± 0.14</td>
</tr>
<tr>
<td>Proline</td>
<td>2.99 ± 0.07</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.10 ± 0.16</td>
</tr>
<tr>
<td>Valine</td>
<td>3.90 ± 0.23</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.82 ± 0.17</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1.03 ± 0.17</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.09 ± 0.05</td>
</tr>
<tr>
<td>Leucine</td>
<td>20.26 ± 0.39</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.10 ± 0.10</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>12.49 ± 0.07</td>
</tr>
<tr>
<td>Lysine</td>
<td>10.90 ± 0.02</td>
</tr>
</tbody>
</table>

Table 6.2. Amino acid composition of egg yolk fraction EYLCF-16. Data correspond to the means ± SD of three independent experiments.
6.3.3 Inhibition kinetics

In order to elucidate how the isolated peptides bind to ACE and inhibit its activity, ACE inhibition kinetics were studied using Lineweaver-Burk plots. Enzyme kinetics were investigated for uninhibited ACE (control) and ACE in the presence of two different concentrations of EYLCF-16 (4 mg/ml and 10 mg/ml) (figure 6.6). The Michaelis-Menten constant ($K_m$) in the absence of EYLCF-16 (control) was 0.53 mM, whereas the $K_m$ in the presence of two different concentrations (4 and 10 mg/ml) of EYLCF-16 was 0.99 and 1.75 mM, respectively. Furthermore, the maximum velocity ($V_{max}$) values for the control, 4 mg/ml, and 10 mg/ml of EYLCF-16 were 1.05, 1.15 and 1.19 mM/min, respectively (table 6.3). As shown in figure 6.6, there is no significant difference between the y-axis intercept of the control and the two concentrations of tested peptide, but there is a significant difference in the x-axis intercepts. According to these results, EYLCF-16 is a competitive inhibitor of ACE because the $K_m$ changed while $V_{max}$ remained the same.

<table>
<thead>
<tr>
<th></th>
<th>Control (no inhibitor)</th>
<th>EYLCF-16 (4 mg/ml)</th>
<th>EYLCF-16 (10 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (mM/min)</td>
<td>1.05</td>
<td>1.15</td>
<td>1.19</td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>0.53</td>
<td>0.99</td>
<td>1.75</td>
</tr>
</tbody>
</table>

Table 6.3. $V_{max}$ and $K_m$ values of ACE control (no inhibitor) and ACE with two different concentrations of EYLCF-16 (4 and 10 mg/ml).
Chapter 6: Isolation and identification of ACE inhibitory peptides

Figure 6.6. Lineweaver-Burk plots for determination the inhibition pattern of EYLCF-16 on ACE. ACE inhibition activity was determined in the absence of the peptide (control) and in the presence of the peptide (4 mg/ml and 10 mg/ml).

6.4 Discussion

ACE inhibitory peptides isolated from different sources of protein have potential as antihypertensive compounds in nutraceutical applications and functional foods (Meisel, 1997; Holdt and Kraan, 2011). Historically, these peptides have been isolated from both plant and animal proteins. In this study, ACE inhibitory peptides were isolated from egg yolk protein and purified using a sequence of chromatographic techniques. In the current study, the aim of using an ultrafiltration process was to separate the hydrolysed protein into different molecular weight fractions. As observed from the result depicted in figure 6.1, ACE inhibition activity was inversely proportional to peptide molecular weight. EYUF-2, which is the lowest molecular weight fraction separated by ultrafiltration, exhibited the highest activity (49.7% inhibition) compared with EYUF-5 (42.2%) and EYUF-10 (33.1%). The observation that ACE inhibitory activity increases with lower molecular weight concurs with many studies in this field (Howell and Kasase, 2010; Balti et al., 2010; Dai et al., 2013).
In order to separate the most effective fraction, further purification steps were conducted by gel filtration chromatography followed by RP-HPLC. ACE inhibitory activity increased after each purification step with a concomitant decrease in IC\textsubscript{50} values (table 6.1). Thus, the highest ACE inhibitory activity (91.8\%) and lowest IC\textsubscript{50} value (1.53 mg/ml) was exhibited by the EYLCF-16 fraction obtained from the final purification step of this study. This observation is in agreement with previous studies reporting that purification decreased IC\textsubscript{50} values (Pihlanto-Leppälä, 2000; Jung \textit{et al.}, 2006; Dai \textit{et al.}, 2013). IC\textsubscript{50} values obtained in the present study were in the same range (1.18-1.86 mg/ml) as those reported in earlier studies on egg proteins (Yoshii \textit{et al.}, 2001; Miguel and Aleixandre, 2006b).

The structure-activity relationships of ACE inhibitory peptides is highly influenced by the sequence of the tripeptide positioned in the C-terminal (Li \textit{et al.}, 2004). In this research, the presence of positively charged arginine (14.69\%) and lysine (10.90\%) in EYCLF-16, as well as the presence of hydrophobic tryptophan (12.49\%) and leucine (20.26\%) may be responsible for the high activity shown by the fraction. Li \textit{et al.} (2004) revealed that the presence of hydrophobic amino acids increased ACE inhibitory activity. The hydrophobic and hydrophilic properties of the isolated peptides enhanced the stabilisation of complex formed between the peptide and the active site of the enzyme (Fernandez \textit{et al.}, 2003; Andújar-Sánchez \textit{et al.}, 2004; Masuyer \textit{et al.}, 2012; Pihlanto and Mäkinen, 2013). This stabilization is achieved by hydrogen bonding and electrostatic interaction and, hence, enhances the inhibition activity (Pan \textit{et al.}, 2012).

Zhao and Xu (2008) explained that the interaction between ACE inhibitors and the Zn\textsuperscript{2+} in the active site of the enzyme plays a crucial role in enzyme inhibition. Pan \textit{et
al. (2012) suggested that leucine and the Zn\(^{2+}\) ion are linked via the carbonyl group of leucine and this coordination is responsible for the enzyme inhibition activity. The presence of leucine in the composition of EYCLF-16 may explain the inhibition activity shown in the current study. Many studies have suggested that the ACE inhibitory activity of peptides may be due to the presence of a positive charge on ε-amino of lysine, or the guanidino group of arginine at the C-terminal of the isolated peptide (Cheung et al., 1980; Murray and FitzGerald, 2007).

The mechanism of ACE inhibition by EYLCF-16 was identified by the use of Lineweaver-Burk plots. The results of the kinetics study showed that EYLCF-16 is a competitive inhibitor of ACE (figure 6.6), that is, it competes with the substrate (HHL) at the active site of the enzyme. The inhibitory mechanism of the isolated peptide is similar to that of Captopril, which is widely used as an antihypertensive drug (Natesh et al., 2004; Dalkas et al., 2010). Due to the various amino acid compositions of different isolated peptides, some exhibit competitive inhibition (Yokoyama et al., 1992; Fujita and Yoshikawa, 1999; Choi et al., 2001; Hyoung Lee et al., 2004) while others demonstrate non-competitive action (Jung et al., 2006; Ono et al., 2006; Jang et al., 2011; Asoodeh et al., 2012).

6.5 Conclusion

A peptide fraction with high ACE inhibitory activity was isolated successfully from egg yolk protein. The isolated peptide EYLCF-16, obtained after a combination of chromatographic techniques, exhibited the highest ACE inhibition activity (91.8%) at 10 mg/ml and IC\(_{50}\) value =1.53. The presence of predominantly leucine, which can bind to Zn\(^{2+}\) at the enzyme active site, as well as positively charged lysine and
arginine and hydrophobic tryptophan in this fraction maybe responsible for its activity. The isolated fraction EYLCF-16 exhibited competitive inhibition towards the enzyme. Although these findings indicate a huge potential for producing peptides with ACE inhibitory activity from egg yolk, clinical studies are needed before they can be used for pharmaceutical applications and functional foods.
Chapter 7
Chapter 7: General discussion and conclusions

7 General discussion and conclusions

7.1 General discussion

7.1.1 Egg yolk protein fractionation and peptide purification

Nowadays, people are becoming increasingly aware about the benefits of healthy foods and, therefore, scientists have been working on the identification of food-derived bioactive compounds that maintain health and reduce the risk of chronic disease. There is wide consensus among scientists that there are other uses of bioactive peptides yet to be unraveled and more studies are needed in order to examine the efficacy of bioactive peptides on human health prior to their application as ingredients of functional foods. From this point of view, the current research is built on the investigation of bioactive properties exhibited by peptides isolated from egg yolk protein.

Although several studies have focused on egg albumen proteins, few have examined the nutritional value of egg yolk proteins, mainly due to their lipid content that negatively affects human health. The main components of egg yolk are phospholipids, triacylglycerol and proteins. The first two components are used as lecithin in food or cosmetics. The third component of egg yolk is protein that represents approximately 30% of dried egg yolk. Therefore, this research aimed to investigate the possibility of producing bioactive peptides from egg yolk protein using physiological proteases, and to identify the bioactive properties of isolated peptides.

Enzyme specificity and degree of hydrolysis are some of the important factors that affect peptide activity when prepared *in vitro*. In the current study, gasterointestinal
proteases were used to achieve a high degree of hydrolysis. Therefore, a combination of pepsin and pancreatin was used in the hydrolysis step. The resultant hydrolysates were subjected to fractionation, as the first purification step, using 10, 5 and 2 kDa molecular weight cut off (MWCO) ultrafiltration membranes and tested for their activities as antioxidants, anti-cancer agents and ACE inhibitors. The high yield (70%) of the lower molecular weight (EYUF-2) fractions obtained from the ultrafiltration process indicated that EYP had been hydrolysed extensively. Because the highest activity as an antioxidant or ACE inhibitor was exhibited by EYUF-2, this fraction was collected and purified further using gel filtration and high performance liquid chromatography. After purification, the amino acid composition of the most effective antioxidant and ACE inhibitory fraction was analysed by HPLC. The following diagram demonstrates the experimental design applied in this study.
7.1.2 Antioxidant activity and mechanism of isolated peptides from egg yolk protein

Lipid peroxidation is a free radical chain reaction; radicals formed in the first stage (initiation) of lipid oxidation are primary oxidation products such as peroxides and hydroperoxides. In the second stage (propagation) of oxidation, further degradation of primary oxidation products occurs and produces secondary oxidation products such as carbonyl compounds. In this study the antioxidant activity of peptides obtained from...
Chapter 7: General discussion and conclusions

Egg yolk protein was measured in an oxidizing linoleic acid model system and the oxidation process was monitored for 7 days. Two methods were used to monitor the oxidation inhibition activity: the ferric thiocyanate method (FTC) was used to monitor the primary oxidation products and the thiobarbituric reactive substances (TBARS) test was used to monitor the secondary oxidation products which include malondialdehyde (MDA). Hydroperoxides and malodialdehyde are considered as biomarkers of lipid oxidation.

The results obtained from both tests indicated that egg yolk hydrolysate (EYH) contained peptides with antioxidant activity. Among the fractions, the highest activity was highlighted by two fractions that were obtained after gel filtration purification namely EYGF-23 and EYGF-33. Purified peptides were effective antioxidants; at 80 mg/ml, both fractions EYGF-23 and EYGF-33 were comparable to the positive controls trolox and BHT.

The amino acid content and sequence in the isolated peptide and peptide size, together play an important role in determining the activity of a peptide (Elias et al., 2008; Bougatef et al., 2009). The amino acids profile of EYGF-23 indicated that this fraction contained tryptophan (16.40%), tyrosine (12.40%), lysine (11.03%) and proline (8.01%) while EYGF-33 contained leucine (13.82%), arginine (11.80%), tryptophan (9.44%), lysine (8.27%), valine (6.71%) and alanine (7.80%). The result obtained from amino acid residue analysis of both EYGF-23 and EYGF-33 demonstrated that both of the isolated fractions contained considerable amounts of hydrophobic amino acid residues such as valine, tryptophan, leucine and tyrosine. It is well known that the presence of hydrophobic amino acid residues in protein hydrolysates or peptides elevates their antioxidant activity by increasing peptide
solubility in a lipid system (Rajapakse et al., 2005; Wang et al., 2007). Chen et al. (1995) isolated antioxidative peptides that contained hydrophobic amino acid residues, valine and leucine at the N-terminus and demonstrated that the presence of hydrophobic moieties in an antioxidant peptide was important to obtain access to hydrophobic groups in membranes and lipids. Tryptophan is also an important amino acid and removal of the labile hydrogen attached to the nitrogen of its indole ring produces a free radical that is easily stabilised due to electron delocalization, thereby allowing tryptophan to break free radical chain reactions and stop the oxidation process (Tsopmo et al., 2011).

Tyrosine-containing peptides can also scavenge radicals as shown by peptides derived from pepsin-digested cow casein (Suetsuna et al., 2000), and hydrolysed beta-lactoglobulin produced both tyrosine and tryptophan that could quench radicals (Hernández-Ledesma et al., 2008). Saeed et al. (2006) suggested that, due to the ability of tyrosine to donate hydrogen, protein containing tyrosine was preferentially oxidised by radicals to protect lipid systems from oxidation. Tsopmo et al. (2011) also found that the presence of tryptophan in human milk hydrolysates was essential to maintain scavenging activity; activity was markedly decreased upon tryptophan deletion. As a result, antioxidant activities of EYGF-23 and EYGF-33 are thought to be related to their amino acid compositions.

In order to understand the action of EYGF-23 and EYGF-33 as an antioxidant, many assays were applied to illustrate the antioxidant mechanism. The results obtained from measuring the ability of EYGF-23 and EYGF-33 to react as a reductant illustrates how this peptide reacts with free radicals to convert them to a more stable form. Moreover, the ability of EYGF-23 and EYGF-33 to quench DPPH, superoxide anions...
and hydroxyl radicals indicated that the isolated fractions act as good scavengers. EYGF-23 and EYGF-33 were also able to chelate transition metals ions (Fe\(^{2+}\)) which act as catalysts that initiate and accelerate free radical chain reaction processes. The results revealed that, although both fractions have the ability to act as chelating agents, the activity was significantly lower in comparison with EDTA at the concentrations used. However, similar results to EDTA could be obtained by using concentrations greater than 20 mg/ml. Therefore, using chelating agents may reduce the availability of these ions and inhibit the oxidative chain reactions in food systems and, consequently, improve food stability (Pokorny et al., 2001). It was noted that, in all methods conducted in this study, the efficacy of the peptide as a reductant, scavenger and chelator was dose-dependent. Because of the good antioxidant properties demonstrated by the peptides in an oxidising linoleic model system, further antioxidant studies were conducted in human cells.

### 7.1.3 Antioxidant activity of isolated peptides from egg yolk protein on epithelial colon cells under oxidative stress

Reactive oxygen species (ROS) are produced in living cells as a result of normal cell metabolism and xenobiotic detoxification. Oxidative stress is a consequence of an excess of ROS, which participate in cellular damage by reacting with lipid membranes, proteins, and DNA. Therefore, the aim of this study was to use cultured Caco-2 cells as an epithelial model to investigate the antioxidant behaviour of egg yolk peptide in cells exposed to the pro-oxidant t-BHP.

The results obtained from cell viability assays, in which cells were treated with isolated peptide fractions, indicated that EYGF-23 in the concentration range used (0.2 - 4.0 mg/ml) was not toxic to Caco-2 cells. However, at 48 hours, and at
concentrations ≥ 0.8 mg/ml, a second peptide fraction EYGF-33 exhibited cytotoxicity (figure 4.3). The cytotoxic behaviour exhibited by EYGF-33 could be associated with the nature and sequence of amino acids that comprise isolated peptides. Based on the results obtained, only the EYGF-23 fraction was studied further in relation to antioxidant activity. Conversely, EYGF-33 was chosen for study as a potential anticancer agent.

To investigate the ability of EYGF-23 to protect oxidatively stressed cells, stressed Caco-2 cells were treated with different concentrations of EYGF-23. The results (figure 4.5) indicated that the EYGF-23 egg yolk peptide enhanced cell viability and protected Caco-2 cells from oxidative toxicity induced by t-BHP. The protective effect of EYGF-23 could be related to its scavenging activity as, once t-BHP penetrates the cell membrane, it can easily degrade to alkoxyl and peroxyl radicals to generate ROS. The scavenging activity of EYGF-23 was confirmed by the significant reduction in cellular ROS (figure 4.7) and MDA levels (figure 4.8) in Caco-2 cells treated with the fraction under oxidative conditions. Subtoxic levels of hydroperoxide result in DNA damage and apoptosis, via the activation of the caspase family of enzymes (Dandrea et al., 2004). In this study, caspase 3/7 activity observed in stressed cells was related to the ability of t-BHP to permeate the cell membrane and generate an array of ROS that were involved in a free radical chain reaction. These radicals attack vital components in the cell, causing membrane lipid oxidation, DNA damage and apoptosis. In the current study, EYGF-23 protected stressed cells from programmed cell death (apoptosis); this protection was evident by the significant reduction in caspase 3/7 activity in cells treated with EYGF-23 prior to stress induction. The low molecular weight (200-750 Da) of the EYGF-23 fraction and
presence of hydrophobic amino acid residues in its composition probably helped the fraction to permeate cell membranes and intercept the free-radical chain reaction by scavenging free radicals.

In a healthy system, the high level of total glutathione plays a substantial role in protecting cells from unexpected attack by hydroxyl radicals. Under oxidative stress, the increase in concentration of peroxides leads to a shift in thiol redox status, as represented by a severe decrease in the reduced form of glutathione (GSH) and an increase in the level of the oxidised form (GSSG). In the current study, 3 mM of t-BHP caused significant depletion of total glutathione, but this was elevated back to basal levels when cells were treated with the isolated fraction prior to stress induction (figure 4.10). The findings, related to the imbalance of reduced and oxidised forms of glutathione due to t-BHP, led to further examination of other antioxidant enzymes namely SOD and catalase in Caco-2 cells when treated with EYGF-23 under conditions of oxidative stress.

SOD is responsible for the dismutation of the superoxide anion to hydrogen peroxide and molecule of oxygen (Scandalios, 1993; Inoue et al., 2003). In the current study (figure 4.11), cells treated with 1.0 mg/ml EYGF-23 prior to oxidative induction exhibited significantly higher SOD activity (55.10 %) as compared with cells treated with t-BHP (p <0.01). These findings illustrate the effect of EYGF-23 on enhancing the activity of SOD under oxidative stress.

In contrast, there was no significant difference in catalase activity between cells stressed with t-BHP and unstressed cells (figure 4.12); this may be explained as follows. The role of catalase enzyme in the defence system is to catalyse the
decomposition of $H_2O_2$ to water and oxygen; this action is similar to the role of glutathione peroxidase. Baud et al. (2004) measured the $K_m$ value (which represents the affinity between the enzyme and the substrate) of catalase and glutathione peroxidase. The high $K_m$ of catalase in removing $H_2O_2$ (up to $25 \text{ mM}$), was 4000-fold higher than the $K_m$ of glutathione peroxidase (6 $\mu$M); this indicated a lower level of activity for catalase as compared to that of glutathione peroxidase. The concentration of $t$-BHP used in this study may, therefore, not be enough to activate catalase and may explain why there was no significant difference in the amount of catalase between cells stressed with $t$-BHP and unstressed cells (figure 4.12).

7.1.4 Antiproliferative effect of isolated peptide on human colon cancer cells

The highlight of this study was that the peptide EYGF-33 inhibits the proliferation of Caco-2 colon cancer cells but not normal HCEC cells. The cell viability results (figure 6.1) and morphological observations (figure 6.3) indicated that EYGF-33 ($IC_{50}=1.0 \text{ mg/ml}$) exerts its toxic effects specifically on colon cancer cells after 48 hours. At the same time and dose, no harmful effects on normal HCEC cells were observed. The mechanism of cell death appears to be due to the ability of EYGF-33 to significantly enhance the production of the superoxide anion in the mitochondria of cancer colon cells, leading to cell death by apoptosis.

Mitochondria are critical for cellular regulatory processes and hence cell survival; therefore, mitochondrial dysfunction can be a key determinant of cell death (Schulze-Osthoff et al., 1992). The respiratory chain reaction that occurs in mitochondria is considered to be one of the major sources of free radicals, namely the superoxide anion. Although this radical is not a strong oxidant, it is considered to be a precursor
of other ROS and, thus, becomes involved in the propagation of the oxidative chain reaction (Turrens, 2003). Therefore, the significant increase in superoxide anion in cells treated with 1.0 mg/ml EYGF-33 (figure 5.5-B) may lead to the activation of cellular death pathways.

EYGF-33 could disrupt the functions of mitochondria by the elevation of ROS levels, in particular the superoxide anion. This could subsequently cause PARP cleavage as observed in the Western blot result (figure 5.6). PARP has the ability to induce programmed cell death (apoptosis) as a result of stimulating mitochondria to release the apoptosis inducing factor (AIF) (Green and Reed, 1998; Yu et al., 2006). AIF is involved in initiating a caspase-independent pathway of apoptosis by causing DNA fragmentation and chromatin condensation in the absence of caspase cascade activation (Candé et al., 2002). Therefore, the results observed in this study suggest that EYGF-33 causes activation of a mitochondrial apoptotic pathway, by increases mitochondrial ROS leading to typical PARP degradation. The induction of apoptotic cell death by EYGF-33 was supported by the externalisation of PS (figure 5.7). Induction of apoptosis is considered to be an important mechanism of cancer prevention treatment by chemo-preventive agents. Many researchers have reported that excess ROS generation causes cell cycle arrest (Shackelford et al., 2000; Burhans and Heintz, 2009). In agreement with this, our current research also suggested that oxidative stress, induced in mitochondria, caused cell death via arrest of the Caco-2 cell-cycle in the S-phase. Accumulation of cells in the S-phase as a result of EYGF-33 treatment suggests a block in the transition from S-phase to G2-phase. However, further elucidation of the mechanism of EYGF-33-mediated apoptosis would provide further support for its use as a potential therapeutic and chemo-preventive agent.
7.1.5 Angiotensin converting enzyme inhibitory activity of isolated peptide from egg yolk protein

ACE inhibitory peptides isolated from different sources of protein have potential as antihypertensive compounds in nutraceutical applications and functional foods (Mine and Shahidi, 2005). Historically, these peptides have been isolated from both plant and animal proteins. In this study, ACE inhibitory peptides were isolated from egg yolk protein and purified using a sequence of chromatographic techniques. The isolated peptide fraction EYLCF-16 comprised leucine, arginine, lysine, tryptophan, and glutamine residues. EYLCF-16 exhibited competitive ACE inhibition and inhibited the enzyme activity by 91.8% at 10 mg/ml.

In order to separate the most effective fraction, further purification steps were conducted by gel filtration chromatography followed by RP-HPLC. ACE inhibitory activity increased after each purification step with a concomitant decrease in IC\textsubscript{50} values (table 6.1). Thus the highest ACE inhibitory activity (91.8%) and lowest IC\textsubscript{50} value (1.53 mg/ml) was exhibited by the EYLCF-16 fraction obtained from the final purification step of this study. This observation is in agreement with previous studies reporting that purification decreased IC\textsubscript{50} values (Pihlanto-Leppälä, 2000; Jung \textit{et al.}, 2006; Dai \textit{et al.}, 2013).

The structure-activity relationships of ACE inhibitory peptides are highly influenced by the sequence of the tripeptide positioned in the C-terminus (Li \textit{et al.}, 2004). In this research, the presence of positively charged arginine (14.69%) and lysine (10.90%) residues in EYCLF-16, as well as the presence of hydrophobic tryptophan (12.49%) and leucine residues (20.26%) may be responsible for the high activity shown by the fraction. The hydrophobic and hydrophilic properties of the isolated peptides enhance
the stabilisation of the complex formed between the peptide and the active site of the
enzyme. This stabilization is achieved by hydrogen bonding and electrostatic
interaction and hence, enhances the inhibition activity (Pan et al., 2012). Zhao and Xu
(2008) explained that the interaction between ACE inhibitors and the Zn\(^{2+}\) in the
active site of the enzyme plays a crucial role in enzyme inhibition. Pan et al. (2012)
suggested that leucine and the Zn\(^{2+}\) ion are linked via the carboxyl group of leucine
and this coordination is responsible for the enzyme inhibition activity. Therefore, the
presence of leucine residues in the composition of EYCLF-16 may explain the
inhibition activity shown in the current study. Many studies have suggested the ACE
inhibitory activity of peptides may be due to the presence of the positive charge on ε-
amino group of lysine residues, or the guanidino group of arginine residues, in the C-
terminus of the isolated peptide (Cheung et al., 1980; Murray and FitzGerald, 2007).
The mechanism of ACE inhibition by EYLCF-16 was identified with the use of
Lineweaver-Burk plots. The results of the kinetics study showed that EYLCF-16 is a
competitive inhibitor of ACE (figure 6.6), that is, it competes with the substrate
(HHL) at the active site of the enzyme.

7.2 General conclusions

- The *in vitro* digestion of egg yolk protein using pepsin and pancreatin
produced several peptides that exhibited antioxidant properties; the most
effective fractions were EYGF-23 and EYGF-33.
- EYGF-23 and EYGF-33 inhibited the formation of peroxides and
malondialdehyde in a linoleic acid oxidising system in a dose-dependent
manner. Both fractions (80 and 100 mg/ml) exhibited antioxidant activity comparable to trolox and BHT.

- Amino acid residue composition and the low molecular weight of EYGF-23 and EYGF-33 may help to explain the antioxidant activity possessed by these fractions. In particular the EYGF-23 fraction, with a molecular weight of 200-750 Da contained tryptophan, tyrosine, lysine and proline residues, while the EYGF-33, with a molecular weight of 250-800 Da, contained leucine, tryptophan, alanine, valine, arginine, lysine residues.

- The variety of mechanisms: reducing power, radical scavenging and ferrous chelating, by which EYGF-23 and EYGF-33 exerted their antioxidant activity, is indicative of their potential to function in diverse oxidising environments.

- While EYGF-23 possessed antioxidant activity in oxidatively stressed Caco-2 cells and protected them against programmed cell death, EYGF-33 exhibited a cytotoxic effect on the same cell line. Based on the results obtained, only the EYGF-23 fraction was studied further in relation to antioxidant activity. Conversely, EYGF-33 was chosen for study as a potential anticancer agent.

- Under stressed conditions, EYGF-23 enhanced Caco-2 cell viability, reduced ROS as well as malondialdehyde levels and inhibited caspase 3/7 activity.

- EYGF-23 increased the level of total glutathione and SOD activity without affecting catalase levels.

- EYGF-33 exhibited in vitro cytotoxic effects in colon cancer cells without any toxicity in normal colon cells. Apoptosis in cancer cells was mediated by mitochondrial ROS generation and PARP cleavage.

- EYGF-33 is a promising peptide candidate for reducing cancer due to its anti-proliferative potential. However, preclinical efficacy and safety studies using
animal models, as well as carefully designed pharmacokinetics studies are needed before clinical trials of peptides as cancer preventive or therapeutic agents can be initiated.

- The *in vitro* digestion of egg yolk protein using pepsin and pancreatin produced several peptides that exhibited ACE inhibitory activity; the most effective fraction, named EYLCF-16, showed 91.8% inhibition activity with an IC$_{50}$ value of 1.53.

- EYLCF-16 comprising leucine, lysine, arginine and tryptophan residues is a competitive inhibitor for ACE.

### 7.3 Future work

- To investigate the mechanism of ACE inhibition activity of egg yolk protein in *in vitro* using endothelial Eahy 962 cell line.
- To study the antihypertensive effect of egg yolk peptides in spontaneously hypertensive rats (SHR).
- To investigate the effect of egg yolk peptides as a colon cancer treatment agent *in vivo* in rats.
- To investigate the effect of egg yolk peptides on liver and breast cancer cell lines.
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Appendices
Appendices

Appendix 1: Dose response curve of ferrous chloride (FeCl₂)

\[
Y = 1.40X + 0.006
\]

\[
R^2 = 9845
\]
Appendix 2: Dose response curve of Malondialdehyde (MDA)

\[ Y = 0.008314X + 0.02152 \]
\[ R^2 = 0.9985 \]
Appendix 3: Conferences and presentation

Posters presentations


Oral Presentations
