Elucidation of bioactive properties of salmon skin proteins

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ABSTRACT

Bioactive peptides are peptides that are able to exert beneficial health effects apart from providing the basic nutritional needs. In this study, bioactive peptides were produced and purified from alcalase hydrolysed salmon skin gelatin. A gel filtration fraction, GF28, was identified as the most potent fraction. A further aim of this study was to investigate the bioactive properties including angiotensin converting enzyme (ACE) inhibition, antioxidant and anti-proliferative activities of the peptide. GF28 (5mg/mL) exhibited ACE inhibition activity of 46.5 %. Further studies elucidated that GF28 acts as a competitive inhibitor of ACE. GF28 also exhibited comparable peroxide formation inhibition activity to the positive control trolox (67.1 % and 67.3 % respectively). The antioxidant mechanism of GF28 mainly involved transition metal ion chelating activity and less reducing power activity. Apart from that, GF28 had poor radical scavenging activity in both hydrophobic and hydrophilic environments. GF28 also showed cytotoxic effects in hepatocellular carcinoma, HepG2 cells (IC\textsubscript{50}=0.154 mg/mL). In addition, GF28 also affected the endogenous antioxidant defence system in HepG2 cells by decreasing superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities as well as the amount of total glutathione. Catalase activity also significantly increased in 0.150 and 0.175 mg/mL GF28-treated HepG2 cells (70.5 mU and 77.0 mU, respectively) compared to the control (47.9 mU). Anti-proliferative effects of GF28 were also observed in Caco-2 cells (IC\textsubscript{50}=0.16 mg/mL). In 0.15 mg/mL GF28-treated Caco-2 cells, intracellular and mitochondrial reactive oxygen species (ROS) (15.3 % and 7.8 % respectively) were significantly lower than untreated cells (32.9 % and 25.2 % respectively). Caco-2 cells treated with GF28 showed cell cycle arrest in the G1 phase as well as apoptosis. The extrinsic and intrinsic apoptosis pathways were activated with GF28. Caspase-8, caspase-
3/7 and poly ADP ribose polymerase (PARP) were activated in the extrinsic pathway whereas caspase-9 and LaminA/C were activated in the intrinsic pathway.
ACKNOWLEDGEMENTS

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<th>Description</th>
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<tbody>
<tr>
<td>ABTS</td>
<td>2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ADI</td>
<td>Acceptable daily intake</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
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<td>Angiotensin I</td>
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<td>Angiotensin II</td>
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<td>ANOVA</td>
<td>One way analysis of variance</td>
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<td>Base excision repair</td>
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<tr>
<td>BHA</td>
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<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
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<td>Cyclin dependant kinase</td>
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<td>CM-H₂DCFDA</td>
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<td>Cysteine</td>
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<tr>
<td>DCF</td>
<td>2’,7’-dichloroflurescein</td>
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<tr>
<td>DHA</td>
<td>Docosahexanoic acid</td>
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<tr>
<td>DMEM</td>
<td>Dolbecco’s Modified Eagle Medium</td>
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<td>DPPH</td>
<td>1,1-diphenyl-2-picrylhydrazyl</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
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<td>Eicosapentaenoic acid</td>
</tr>
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<td>ER</td>
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<td>FTC</td>
<td>Ferric thiocyanate</td>
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<td>GR</td>
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</tr>
<tr>
<td>GRAS</td>
<td>Generally regarded as safe</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
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<td>GSSG</td>
<td>Oxidised glutathione</td>
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<tr>
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<td>Hydrogen peroxide</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Ile</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>JECFA</td>
<td>Joint FAO/WHO Expert Committee on Food Additives</td>
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</table>
Km  Half of maximum velocity
KNOS  Kinin-nitric oxide system
Leu  Leucine
LO'  Lipid radical
LOO'  Alkyl radical
LOOH  Peroxides
LOX  Lipoxygenase
Lys  Lysine
MCM complex  Minichromosome maintenance complex
Met  Methionine
mt  Metric tonnes
MTT  Thiazolyl blue tetrazolium bromide
MW  Molecular weight
NEPS  Neutral endopeptidase system
NER  Nucleotide excision repair
O₂⁻  Superoxide radicals
PARP  Poly ADP ribose polymerase
PBS  Phosphate buffer saline
PCB  Polychlorinated biphenyl
Phe  Phenylalanine
PI  Propidium iodide
PITC  Phenylisothiocyanate
PMSF  Phenylmethanesulfonylfluoride
Pro  Proline
PS  Phosphatidylserine
<table>
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<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>RCS</td>
<td>Renin-chymase system</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative luminescence unit</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S phase</td>
<td>DNA synthesis phase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SCF</td>
<td>Scientific Committee for Food</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
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</tr>
<tr>
<td>TFA</td>
<td>Triflouroacetic acid</td>
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<td>Thr</td>
<td>Threonine</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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Chapter 1
1 GENERAL INTRODUCTION

1.1 Amino acids

Twenty naturally occurring amino acids are the essential building blocks of proteins. All amino acids have a common structure, depicted in Figure 1.1, which includes an α-carbon attached to an amino and a carboxylic acid group. The different chemical properties of amino acids are attributed to their unique side chains. Peptide bonds are covalent bonds formed between two amino acids where condensation will occur between the amino group of an amino acid with the carboxylic group of another amino acid. Two amino acids link to form a dipeptide and more amino acids are linked in the same manner, forming polypeptides that form proteins.

Amino acids exist as zwitterions with the amino group in its conjugate acid form (NH$_3^+$) and the carboxyl group at a conjugate base form (-COO$^-$). Individual amino acids have their own characteristics due to their R-group (Fennema et al., 2008). In general, basic and acidic amino acids are hydrophilic due to their charged side chains which enable them to form hydrogen bonds with water molecules. Amino acids can be classified via their characteristic ability to react with water which are: (1) acidic, (2) basic, (3) non-polar, uncharged and (4) polar, uncharged respectively.
1.1.1 Acidic amino acids

Both glutamic (Glu) and aspartic acid (Asp) belong to the acidic group of amino acids. The amino acids only differ in the number of methylene groups they possess where Glu has two methylene groups compared to one in Asp. Acidic amino acids are found to play a structural role in proteins where the carboxyl groups are able to form salt bridges within polypeptide chains in close proximity to one another.

1.1.2 Basic amino acids

Histidine (His), lysine (Lys) and arginine (Arg) are basic amino acids. In contrast to negatively charged acidic amino acids, basic amino acids are positively charged molecules. Basic amino acids can associate with acidic amino acids to form both salt bridges and ionic linkages. The opposing charges form salt bridges which are found in the centre of helical structures, which in turn stabilizes the tertiary structure and provides protein stabilization (Sundaralingam et al., 1985).

1.1.3 Non-polar, uncharged amino acids

Aliphatic and aromatic amino acids possess non-polar side chains that enable the amino acids to take part in hydrophobic interactions. The non-polar uncharged amino acids are normally found in the apolar face of α-helix and β-pleated sheets at the core of tertiary protein structures. Tryptophan (Trp) and phenylalanine (Phe) have benzene rings as side chains; thus, they are referred to as aromatic amino acids. The bulky aromatic rings also generally confer hydrophobicity (Golan et al., 2012).
Proline (Pro), isoleucine (Ile), leucine (Leu), valine (Val), alanine (Ala) and glycine (Gly) are aliphatic amino acids. Aliphatic amino acids possess hydrocarbon side chains with the exception of Pro where the side chain is covalently bonded to the amide backbone. As the aliphatic side chains increase in their bulk, the hydrophobicity increases due to the increase in the restriction of the hydrocarbon backbone flexibility (Huang and Nau, 2003). Gly has only one hydrogen atom as its side chain which enables more molecular flexibility than other aliphatic amino acids. Cysteine (Cys) and methionine (Met) have sulphur containing aliphatic side chains. Similar to the other aliphatic amino acids, they are uncharged and hydrophobic.

1.1.4 Polar, uncharged amino acids

Serine (Ser) and threonine (Thr) have hydroxyl aliphatic R-groups whilst tyrosine (Tyr) has a hydroxyl aromatic R-group. The –OH group enables the amino acids to participate in hydrogen bonding, thus their hydrophilic properties. In addition, the hydroxyl groups in Ser and Thr present as sites for carbohydrate attachment in the amino acids (Lodish et al., 2000).
1.2 Protein structure and stability

Protein structure can be categorised into four different levels which will be further discussed below (Fennema et al., 2008). The conformation and stability of proteins are governed by covalent and non-covalent bonds. Covalent bonds such as intra or interchain disulphide bonds are normally formed by the coupling of two thiol molecules and have a major influence on the stability of protein structures. In addition, peptide bonds, as described previously, are also covalent bonds.

In contrast to covalent bonds, non-covalent bonds are less stable (Lodish et al., 2000). Hydrogen bonds are the sharing of the proton (H\(^+\)) between two electronegative atoms. Electrostatic interactions can either be repulsive or attractive which can occur between any two charged particles. Van der Waals interactions are weak non-covalent bonds formed by the attractive interactions between dipoles. The packing of amino acid molecules in the interior of proteins illustrates the importance of van der Waals forces. Repulsive forces between non-polar molecules and water results in the interaction with other non-polar molecules. This preferred non-polar-non-polar molecule bond is referred to as hydrophobic forces. Hydrophobic forces are the main interactions that dictate both the folding and stability of polypeptides or protein molecules in an aqueous environment.

1.2.1 Primary structure

The primary structure of protein is basically a linear chain of different amino acids linked by peptide bonds. In other words, the sequence of different individual amino acids along a peptide backbone constitutes the basic primary structure of protein.

1.2.2 Secondary structure
Formation of hydrogen bonds locally within the polypeptide chain yields the secondary structure of protein. The most commonly observed secondary structures are α-helix and β-pleated sheet (Fennema et al., 2008).

In an α-helix, hydrogen bonds are formed between the oxygen atom in the carbonyl residue of an amino acid residue with the amide hydrogen residue of the fourth subsequent amino acid which creates a rigid and helical structure. Beta-pleated sheets are regular structures composed of β-strands linked by hydrogen bonds between the peptide backbones of adjacent strands. Beta-sheets are separated into parallel and anti-parallel conformations. Anti-parallel β-sheets are generally more stable than the parallel form as the co-linear hydrogen bond is the strongest form (Creighton, 1993).

1.2.3 Tertiary structure

Polypeptide chains assume their three-dimensional structure as they fold and twist into a range of complex, tangled shapes (Fennema et al., 2008). The folding into tertiary structures is achieved via the interaction between side chains of amino acids in the primary structure. These are mostly linked by non-covalent bonds with some covalent bonds which are mentioned and described previously. Tertiary structure of proteins can be divided to either globular or fibrous proteins.

Globular proteins are densely packed structures of polypeptides. In general, hydrophilic amino acids are found on the surface whilst hydrophobic amino acids form the globular protein core. On the other hand, fibrous proteins are polypeptides arranged and packed as a single regular structure. Collagen is a fibrous protein with a repeating sequence of Gly-X-Y.
1.2.4 Quaternary structure

Multiple polypeptide chains (subunits) are linked via non covalent bonds to form a single large protein complex. In the interior of the complex, the interaction between the interface of subunits is similar to those amongst residues in globular proteins. Thus the role of hydrophobic interactions is critical in the stabilization of the quaternary structure. Although covalent bonds are found in low amounts, these strong linkages are vital to stabilize proteins with multiple subunits (Fennema et al., 2008).
1.3 Salmon

The term “salmon” is derived from the Latin word *Salmo*, meaning “to leap”. Belonging to the family Salmonidae, salmon can be divided into two main genera *Salmo* and *Onchorhyncus*. Atlantic salmon are found in the North Atlantic whereas Pacific salmon are found in the North Pacific. The major difference between genera is that Pacific salmon die after spawning once, whilst Atlantic salmon can spawn multiple times, returning to the ocean in between.

From raw to smoked salmon, salmon is enjoyed in different cuisines around the world in different ways. Consumption of salmon worldwide has increased significantly over the past decades; the total volume of world salmon supply increased from 550,000 metric tonnes (mt) in the 1980s to almost 2.5 million mt in 2004 (Knapp et al., 2007). A report by Marine Harvest (2010) showed that Atlantic salmon (*Salmo salar*) is the most widely farmed and sold species in the world.

The drastic increase in salmon consumption is largely due to the highly desirable high omega-3 fatty acid content and lean protein content in salmon. It is well documented that both docosahexanoic acid (DHA) and eicosapentaenoic acid (EPA) promote health benefits such as improving childhood and adult brain development and performance, as well as reducing cognitive decline in the elderly (Kidd, 2007). Current investigations also show that both fatty acids are able to improve the lipid metabolism in obese non-diabetic patients (Itariu et al., 2012) and reduce inflammation (Calder, 2012) which is linked to diseases such as diabetes, arteriosclerosis and even hypertension. Apart from the health benefits, statements regarding the higher saturated fat and cholesterol content in other meat sources also makes salmon a preference.
1.4 Fish protein

Fish is an attractive source of protein for an increasing number of health conscious individuals as it provides a very high quality protein with lower amounts of total fat and calories when compared to other meat protein sources. Although there are concerns about the mercury and polychlorinated biphenyl (PCB) levels, known immunotoxins (Lauriano et al., 2012) and carcinogens, fish remains one of the most popular protein sources worldwide.

1.4.1 Myofibrillar protein

Accounting for up to 65-80% of the total muscle protein, myofibrillar protein is the largest component (Gudmundsson and Hafsteinsson, 2002). Although the myofibrillar protein is made up of a further 20 types of proteins, myosin and actin (70% of the total protein) are the major component proteins.

1.4.2 Sarcoplasmic protein

Sarcoplasmic proteins are low molecular weight, water soluble, non-structural proteins. These proteins are found in the sarcoplasm bathing the myofibril proteins. Sarcoplasmic proteins can form protein films. Even when used in very low quantities (0.2%), sarcoplasmic proteins exposed to moderate pH (pH 5-9) have high emulsification properties (Yongsawatdigul and Hemung, 2010).
1.4.3 Non-soluble protein

Non-soluble proteins of fish include all connective tissues which are insoluble in both water and low ionic solutions. Connective tissue plays a principle role in mechanical and structural support. However, mast cells and macrophages in connective tissues play a role in the immunological defence during inflammation. The low amount of connective tissue in fish (1.3%) makes the flesh more tender than the flesh of other animals. Collagen constitutes 90% of non-soluble fish proteins whereas the remainder is elastin (Gudmundsson and Hafsteinsson, 2002).

1.4.3.1 Elastin

Connective tissues with a high concentration of elastin are a yellowish colour. Elastin, a fibrous protein, is produced by most smooth muscle cells and fibroblasts which enable tissues and organs the ability to recoil when subject to pressure or stretching. Elastin is made up of randomly coiled polypeptides with irregular amino acid composition. The bond formed from the cross linking of the side chains of four lysine molecules is referred to as desmosine (Bhagavan, 2002). In the skin, elastin works with collagen to provide firmness and elasticity.

1.4.3.2 Collagen

Collagen is the major insoluble fibrous protein found in both connective tissues and the extracellular matrix such as skin and bone. It can also be found in small amounts in the intracellular matrix. In general, collagen is low in aromatic and sulphur amino acids such as Trp and Cys. Unlike elastin, it has a repeating motif of Gly-X-Y. Although X and Y residues can be any amino acids, mostly Pro and hydroxyproline (Hyp). Each amino acid
plays a role in the triple helix where only the side chain of Gly, a H molecule, can fit into the centre to hold the helix by forming a peptide bond with the adjacent polypeptide.

In general, Type I, III and V collagens are found in the skin of animals. However, Type III collagen is absent in fish skin. Apart from that, composition of Type I collagen differs in fish where one of the two α1 chains is replaced by an α3 chain. The α3 chain in Type I collagen is found in most bony fishes and is characteristic to skin collagen only. In Alaskan Pollock, swim bladder Type I collagen was the typical [(α1)2(α2)] heterotrimer whilst skin collagen [(α1)(α2)(α3)] (Kimura and Ohno, 1987). Phylogenetics also proved that the α3 chain is a product of divergence from the duplication of α1 gene (Saito et al., 2001). Saito et al. (2001) also gave evidence that the lower stability of skin collagen is contributed by the loosened triple helical structure due to a higher amount of Gly-Gly doublets of the collagen molecule compared to α1.
1.5 Bioactive peptides

Bioactive compounds are essential (e.g. proteins) and non-essential (e.g. polyphenols) components derived from food compounds that have an effect on human health (Biesalski et al., 2009). Inactive within the parent protein, bioactive peptides have to be released via enzymatic hydrolysis, food processing, microbial fermentation or gastrointestinal digestion (Bhat et al., 2015).

Bioactive peptides are typically 2-20 amino acids long. Once liberated from the parent protein, bioactive peptides have functional properties apart from the nutritional properties (Korhonen and Pihlanto, 2003). Bioactive peptides can derive from a wide range of sources such as grains, plants, animals, dairy and marine sources. For example, wheat germ (Huang et al., 2014), hemp seed (Girgih et al., 2014), dry-cured ham (Gallego et al., 2014), milk (Gobba et al., 2014), fish scale (Azuma et al., 2014) and red seaweed (Fitzgerald et al., 2014).

1.5.1 Absorption

The bioactivity of bioactive peptides is dependent on several factors including the absorption and bioavailability of the peptide itself. The peptide sequences of bioactive peptides have to be maintained during digestion so that they can act on the target cellular site (Shahidi and Li, 2015).

Wada and Lönnerdal (2015) showed that bioactive peptides produced from human milk were resistant to in vitro digestion. Bioactive peptides can also be absorbed intact through the intestines intact (Gardner, 1988; Vermeirssen et al., 2002). However, the absorption
rate and kinetics of peptides are highly associated with physiological conditions such as the presence of carbohydrate or fibre in the gut (Ten Have et al., 2015).

1.5.2 Production

Hydrolysis of precursor proteins with digestive enzymes, microbial fermentation and digestion with bacterial or fungal derived proteases are the common methods for producing biologically active peptides. If the desired peptide sequence is known, peptides can also be chemically synthesised.

Many of the bioactive peptides are produced by the digestion of gastrointestinal enzymes such as pepsin, trypsin and chymotrypsin. Apart from that, bacterial or fungal derived enzymes such as alcalase and thermolysin are commonly used to produce bioactive peptides with functions such as anticancer (Kim et al., 2013), antioxidant and ACE inhibitory activities (Ambigaipalan et al., 2015).

The use of highly proteolytic starter cultures in the production of fermented dairy products also leads to the production of bioactive peptides. Val-Pro-Pro and Ile-Pro-Pro are well-known ACE inhibitory peptides which are produced from fermented milk products using Lactobacillus helveticus as a dairy starter (Sipola et al., 2001; Korhonen, 2009). Bioactive peptides were also produced in milk using yoghurt and cheese starters (Korhonen, 2009).
1.5.2 Factors influencing bioactivity of peptides

Different methods can be used on various sources of parent proteins, which would produce many different bioactive peptides. The activities of these peptides are influenced by factors such as amino acid composition and sequence, peptide length, terminal amino acid, and electrical charge.

1.5.2.1 Molecular weight (MW)

Bioactive peptides are typically 2-20 amino acids long and less than 6 kDa (Sun et al., 2004). Separation methods which are dependent on MW exclusion, such as ultrafiltration and gel filtration chromatography, are able to produce bioactive peptides with different MW ranges. However, small peptides were shown to have better anti-proliferative activities in cancer cells, antioxidant, ACE inhibition and antimicrobial activity when compared to larger MW peptides (Bhutia and Maiti, 2008; Khiari et al., 2014; Mosquera et al., 2014).

1.5.2.2 Amino acid composition and sequence

The different amino acids present in the bioactive peptides also play a major role in its activity. Aliphatic amino acids at the N-terminal, and aromatic amino acids at the C-terminal were reported to act as the binding sites for ACE as competitive inhibitors (Byun and Kim, 2001). The presence of proline in the penultimate position of peptides also contributes to ACE inhibition via the increase of enzyme binding (Contreras et al., 2009). Tryptophan and arginine rich peptides are also known for their antimicrobial and antifungal activities (Chan et al., 2006; Gopal et al., 2012). Hydrophobic amino acids also contribute to the antioxidant activity of bioactive peptides (Chi et al., 2015; Najafian and
Babji, 2015). This may be due to the fact that hydrophobic amino acids are more soluble in lipids.

1.5.2.3 Electric charge

Antimicrobial peptides tend to be cationic and amphiphatic. The cationic structure may interact with the outer bacterial membrane/cell wall which is negatively charged (Teixeira et al., 2012). Conjugation of nanoparticles to a cationic peptide also improved anti-inflammatory drug delivery to the corneal epithelium (Vasconcelos et al., 2015).
1.6 Functions of food derived bioactive peptides

As discussed previously, food derived bioactive peptides may aid in the prevention or reduction of chronic diseases. For example, sea bream bone and scale derived bioactive peptides showed good ACE inhibition activity (Akagündüz et al., 2014). Multiple bioactive peptides derived from skate (Ngo et al., 2015), cuttlefish (Balti et al., 2015) and red seaweed (Fitzgerald et al., 2014) also exerted hypotensive effects in spontaneously hypertensive rats. These may play a role in reducing the risk of hypertension or cardiovascular diseases. Although more human studies are needed to confirm the activity of bioactive peptides in vivo, bioactive peptides may play an important role in the future as an alternative source of functional food or nutraceutical products. The potential uses of bioactive peptides on hypertension, in vitro and in vivo lipid oxidation as well as cancer will be further discussed below.

1.6.1 Blood pressure and hypertension

Blood pressure is the pressure exerted on the walls of blood vessels when blood is pumped throughout the body by the heart. Hypertension is a condition where systolic blood pressure $\geq 140$ mmHG and/ or the diastolic pressure $\geq 90$mmHG. According to the NHS, 30% of the England’s population suffers from hypertension (NHS, 2014). Hypertension can also be linked to the increment of arteriosclerosis, stroke and renal failure (Kim and Iwao, 2000; Ishizaka et al., 2005).

Blood pressure can be controlled by several mechanisms, divided into the long or short-term control of blood pressure.
1.6.1.1 Short-term control: baroreceptors and chemoreceptors

The autonomic nervous system affects blood pressure most rapidly. Baroreceptors located at the carotid sinus and aortic arch are mechanoreceptors which monitor both mean arterial pressure and pulse pressure in the arteries (Guyenet, 2006). The cardio-regulatory centre located at the medulla oblongata within the brain stem, acts as the integration centre for the afferent impulses from the baroreceptors. An increase in blood pressure will lead to the activation of the parasympathetic nervous system which leads to vasodilation and hence a decrease in blood pressure.

Apart from baroreceptors, chemoreceptors are sensory neurons that are also located at the carotid sinus and aortic arch. They monitor the oxygen and carbon dioxide levels in the blood. The afferent signals from the chemoreceptors are conducted via the glossopharyngeal and vagus nerves separately to the cardio regulatory center (Dampney et al., 2001). A decrease in blood oxygen and increase in carbon dioxide results in the decrease of blood pH. This causes the activation of sympathetic nervous system which increases heart rate and stroke volume. The increased in sympathetic stimulation over blood vessels causes an increase in vasoconstriction which increases blood pressure.
1.6.1.2 Long-term control: renin-angiotensin-aldosterone system

The kinin-nitric oxide system (KNOS), neutral endopeptidase system (NEPS) and the renin-angiotensin-aldosterone system (RAAS) are physiological systems responsible for the long term control of blood pressure.

Kidneys help regulate blood pressure via the RAAS by affecting the blood volume. RAAS is regarded as the most important long-term control of blood pressure via vasoconstriction and vasodilation. The cascade process of RAAS is illustrated in Figure 1.2 below. Renin is an aspartyl protease released from the juxtaglomerular cells in the kidneys in response to stimuli such as renal arteriole hypotension, sympathetic nerve stimulation via β1-adrenoceptors and decreased Na+ delivery to the renal tubule.

Angiotensinogen is a glycoprotein mainly produced by hepatic cells with molecular weight varying from 55-60kDa depending on the extent of glycosylation (Abramowitz et al., 2010). Angiotensinogen acts as the only substrate for renin where the peptide bond between Leu and Val would be cleaved splitting off the decapeptide angiotensin I (Ang I). Angiotensin-converting-enzyme (ACE) is a zinc-containing metalloprotease that cleaves the dipeptide His-Leu at the carboxyl terminal of Ang I which results in the formation of angiotensin II (Ang II). ACE is produced in the vascular endothelium of different tissues such as testis, brain and kidneys. Apart from Ang II production, ACE is able to degrade bradykinin, thus inhibiting vasodilation (Tschope et al., 2002).

AT1 and AT2 are the subtypes of receptor for Ang II where AT1 is expressed predominantly in adults whilst AT2 is expressed in the foetus (Ardaillou, 1999). The binding of Ang II to AT1 will lead to the release of aldosterone from the adrenal cortex, resulting in sodium and water retention as well as potassium excretion in the kidneys. The
octapeptide Ang II is also a potent vasoconstrictor which acts via the stimulation of vascular smooth muscle contraction and release of noradrenalin. The cascade of events would lead to the elevation of blood pressure.
Angiotensinogen

N (1 2 3 4 5 6 7 8 9 10 11 12 ......) C
Asp – Arg – Val – Tyr – Ile – His – Pro – Phe – His – Leu – Val – Ile

Figure 1.2 The renin-angiotensin-aldosterone system.
1.6.1.3 Treatment of hypertension

Generally, lifestyle changes such as lowering salt consumption and eating a healthy, balanced diet are encouraged for hypertension patients. However, medication will be offered to patients with existing or high risk of cardiovascular disease and a consistent blood pressure above 140/90 mmHg or patients with a consistent 160/100 mmHg blood pressure (NHS, 2014).

Different types of medications which act on different pathways and mechanisms of blood pressure control can be used in controlling hypertension. For example, ACE inhibitors, as suggested by the name, are used to inhibit the enzyme ACE and thus preventing the formation of the potent vasoconstrictor Ang II. Angiotensin receptor blockers target the AT₁ receptors preventing the binding of Ang II to the receptors.

However, blood pressure will be dependent on salt intake when the RAAS system is blocked (Burnier and Brunner, 2000). Thus, diuretics are often used together with ACE inhibitors. Different types of diuretics target different parts of the kidney nephron, reducing the reabsorption of sodium and water. Blood pressure is then reduced by the natriuretic effect which decreases extracellular volume.

In addition, other drugs such as beta-blockers and calcium channel blockers are also used to treat hypertension. However, these synthetic drugs come with side effects such as headache, hypokalemia and dry cough as well as being teratogenic (Morimoto et al., 2004; Chu et al., 2014).

On the other hand, recent studies showed that food derived bioactive peptides have no known side effects (Wijesekara and Kim, 2010; Sarmadi and Ismail, 2010). Balti et al.
(2015) identified bioactive peptides that are effective ACE inhibitors from cuttlefish muscle. Activity has been shown in spontaneously hypertensive rats and the peptides although less potent than the synthetic drugs, do not possess any of the side effects of the synthetic drugs. Buttermilk is rich in milk fat globule membranes which have different unique bioactive peptides. Consumption of buttermilk was shown to reduce blood pressure in men and women and there were no associated side effects (Conway et al., 2014).

More studies need to be undertaken to evaluate the safety and efficacy of bioactive peptides as an alternative means of hypertensive treatment. Apart from that, bioactive peptides can also be used with the synthetic drugs to minimize the dose used, thus reducing the side effects of the synthetic drugs.
1.6.2 Lipid oxidation

Lipid peroxidation refers to the degradation of lipids. It is well known that oxidation of lipids, both in vitro and in vivo, has undesirable effects on both food and human health. Foods that are high in fat have a shorter shelf life compared to those with lower fat content as the oxidation of lipid can cause off flavours, colours and changes in texture, which affect consumer acceptance. Lipid oxidation also causes the loss of essential fatty acids and vitamins. Oxidised phospholipids can be found in hyperlipidaemic plasma and also atherosclerotic regions which are considered to be pro-atherogenic (Lee et al., 2012). The extent to which lipid is degraded is mainly dependent on the presence of sunlight, metal ions and the nature of the lipid itself. Double bonds make lipid molecules more prone to peroxidation. Thus, the higher degree of unsaturation of the lipid molecule makes it more susceptible to peroxidation.
1.6.2.1 Types of lipid peroxidation

Lipid peroxidation can be classified into enzymatic, autoxidation and photoxidation types.

**Enzymatic oxidation:** Enzymatic lipid oxidation involves an enzyme as a catalyst. For example, the enzyme lipoxygenase (LOX) is found in many oil-containing seeds such as soybeans. The Fe$^{2+}$ of LOX needs to be oxidised to Fe$^{3+}$ before LOX can catalyse the oxygenation of polyunsaturated fatty acids to form hydroperoxides (Baysal and Demirdöven, 2006). Blanching inactivates LOX and may therefore prevent enzymatic peroxidation.

**Autoxidation:** Autoxidation is the major process of lipid peroxidation. It is also spontaneous oxidation which involves unsaturated lipids and oxygen in the absence of light and catalyst (Sun et al., 2011). Free radicals are normally involved in the initiation of autoxidation where the lipid reacts non-enzymatically with the oxygen.

**Photoxidation:** Photosensitization is the process where a light absorbing molecule initiates a reaction without changing itself. In photoxidation, ultraviolet light excites stable ground state oxygen excited to singlet oxygen (${}^{1}O_2$) (Yousif and Haddad, 2013). Chlorophyll is an example of those molecules that absorb light and transfer the energy to oxygen. These compounds are referred to as photosensitizers.
1.6.2.2 Lipid oxidation mechanism

The mechanism of lipid peroxidation involves: initiation, propagation and termination.

**Initiation:** In general, the initiation of lipid peroxidation, the H of the α-methylene group in the double bond is removed to form a lipid radical (LO) as well as a hydrogen radical (H). The LO then reacts with oxygen to form an active alkyl radical (LOO).

**Propagation:** The LOO formed will then propagate lipid peroxidation via the further abstraction of H from other PUFA in proximity. The reduction of LOO causes the formation of peroxides (LOOH) which are odourless and colourless. LOOH is the major initial lipid peroxidation product which is an unstable compound and would breakdown over time. LOOH often decompose via dismutation which could either form more free radicals that further propagate the lipid peroxidation or volatile components such as aldehydes and ketones that contribute to the off flavours and rancidity of lipids. Trace quantities of metal ions such as copper ions (Cu²⁺) also act as a catalyst in the breakdown of LOOH.

**Termination:** Termination occurs when two free radicals combine to form a non-reactive stable product. This process is referred to as radical recombination and the number of variation of possible recombination is limitless. However, the term termination can only be used on the free radical that is stabilised but not the entire lipid peroxidation itself. This is because if stabilization is achieved by hydrogen abstraction or rearrangement, lipid peroxidation is still continued and propagated.
1.6.2.3 Free radicals and reactive oxygen species (ROS)

Free radicals are molecules with an unpaired electron but able to exist independently. As free radicals have an unpaired electron, they are relatively unstable and readily react with other molecules. Reactive oxygen species (ROS) are free radicals containing oxygen. Hydrogen peroxide, superoxide anion, hydroxyl radical are commonly found ROS.

In food products, free radicals take part in lipid peroxidation which would cause off flavours and rancidity. Biologically, free radicals react with DNA, lipids and proteins which would lead to tissue damage (Young and Woodside, 2001). ROS are also involved in the initiation and progression of diabetes mellitus, atherosclerosis and neurodegenerative disease (Uttara et al., 2009; Kaneto et al., 2010).

As the excess of lipid peroxidation and free radicals show undesirable effects in both food products as well as human health, it is vital to control lipid peroxidation.

1.6.2.4 Antioxidants

Antioxidants are compounds with the function of retarding or preventing lipid peroxidation. This is achieved either by preventing the initiation or quenching the free radical to prevent further propagation of lipid peroxidation.

Antioxidants used in food products can be mainly divided into synthetic or natural antioxidants. The ideal antioxidants used as an additive in food must show no toxicity including any adverse physiological effect, not be absorbed by the body, be stable in treated food even after 1 year of storage, economical, efficient at low doses and must not affect the properties of the food by being odourless and colourless.
Both types of antioxidants have to go through stringent tests by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Community’s Scientific Committee for Food (SCF) to be approved as “generally regarded as safe” (GRAS) to be used in the food industry. Toxicology of the compound is also evaluated using the acceptable daily intake (ADI).

Apart from antioxidants in food products, antioxidants such as superoxide dismutase and glutathione are also found in biological systems. They are also referred to as the biological antioxidant defence system. The antioxidant defence system prevents the excess of free radicals and ROS in the body.

1.6.2.4.1 Synthetic antioxidants

Butylated phenols and polyphenols mainly constitute the main components of synthetic antioxidants. Artificial antioxidants are generally cheaper and more easily obtained compared to natural antioxidants. The efficacy of synthetic antioxidants is mainly dependent on the oxidation-reduction potential. Resonance delocalization and susceptibility to auto oxidation are also important. The maximum amount of synthetic antioxidant allowed in food products is 0.02%.

Butylated hydroxytoluene (BHT) is a monohydroxyphenol compound and most commonly used as a multi-use antioxidant including preservation of petrochemicals and animal feed. BHT acts as a chain breaking antioxidant where it accepts or donates a proton to the free radical to form a stable end product that prevents initiation or further propagation of auto oxidation.
However, strong evidence shows that BHT induces carcinogenic or mutagenic effects; 0.2M BHT causes aneugenic effects via the increase in proliferation and aneuploidy after 24 hours exposure to the macronuclei (Hatzi et al., 2011). Thus, BHT has an ADI of 0.125mg/kg body weight and is only allowed as an antioxidant in restricted foods such as oil as it is very thermostable.

Therefore, there has been an increased of demand for naturally sourced antioxidants over the years due to the potential or known adverse effects of synthetic antioxidants.

### 1.6.2.4.2 Natural antioxidants

Currently, there is intensive research on antioxidant compounds from naturally occurring sources. This is due to the increased concerns over the adverse toxicology reports on synthetic antioxidants and the consumers’ preference for “natural sources” of antioxidants. Natural antioxidants, with the exception of carotenes and tocopherols, are generally more polar compared to synthetic ones (Pokorný, 2007). This would decrease the solubility of natural antioxidants in the lipid phase which would affect their efficiency in retarding lipid oxidation.

The antioxidant effects of bioactive peptides *in vivo* are well established. Bioactive peptides derived from tuna backbone protein showed dose-dependent scavenging effects of several free radicals including DPPH, superoxide and hydroxyl radicals (Je et al., 2007). Bioactive peptides derived from various sources also exhibited metal chelation effects of iron (Yan et al., 2015), zinc (Xie et al., 2015) and copper (Torres-Fuentes et al., 2014).
1.6.2.4.3 Biological antioxidant defense system

Free radicals are produced naturally in the body via normal metabolic processes. However, external factors such as smoking and exposure to X-ray will also increase free radical levels in the body. Although a high concentration of free radicals is harmful, low to moderate levels of free radicals are essential (Valko et al., 2007). Excess free radicals in the body are removed or neutralised by the biological antioxidant defense system.

**Superoxide dismutase:** Superoxide dismutase (SOD) is the only eukaryotic enzyme capable of detoxifying superoxide (O$_2^-$) (Van Raamsdock and Hekimi, 2012). It catalyzes the dismutation of the highly reactive O$_2^-$ to the less reactive H$_2$O$_2$. H$_2$O$_2$ is further neutralised by either glutathione peroxidase (GPx) or catalase. The dismutation of O$_2^-$ to H$_2$O$_2$ is as follows:

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

Three forms of SOD exist in humans: extracellular SOD, cytosolic Cu/Zn-SOD and mitochondrial Mn-SOD (Matés et al., 1999). Apart from that, differential Mn-SOD expression in cancer cells may be linked to the different stages and types of cancer, which may be use as a target in cancer therapy (Dhar and St. Clair, 2012).

**Glutathione system:** The glutathione system includes glutathione peroxidase (GPx) and glutathione reductase (GR). GPx is a selenium containing enzyme found in plants and microorganisms as well as mammals. GPx catalyze the reduction of H$_2$O$_2$ or hydroperoxides using reduced glutathione (GSH) to alcohols, water and oxidised
glutathione (GSSG). Seven GPx isoforms exists where GPx1, GPx2, GPx3 and GPx4 are the major isoforms (Margis et al., 2008).

GR is responsible for the conversion of GSSG back to GSH. GR can be found in the cytosol as well as mitochondrial compartments of eukaryotic cells (Couto et al., 2013). A high level of GR is important to ensure that adequate GSH is available to prevent oxidative stress.

The roles of GPx and GR in the antioxidant defense system are shown in the following reaction.

\[
2 \text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{GPx}} \text{GSSG} + 2 \text{H}_2\text{O} \\
2 \text{GSH} + \text{ROOH} \xrightarrow{\text{GPx}} \text{GSSG} + \text{H}_2\text{O} + \text{ROH} \\
\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} 2 \text{GSH} + \text{NADP}^+ 
\]

Catalase: Catalase catalyses the conversion of H\textsubscript{2}O\textsubscript{2} to oxygen and water shown below. Although CAT is not essential for survival in some cell types under normal conditions, it is important during oxidative stress conditions (Matés et al., 1999).

\[
2 \text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} 2 \text{H}_2\text{O} + \text{O}_2
\]
1.6.3 Cancer

Cancer is defined as the condition where cells in a certain part of the body divide and grow uncontrollably (NHS, 2014). The uncontrollable cell growth will lead to the formation of either benign tumors or malignant tumors. Benign tumors are localised; they remain at their original site and do not invade or spread to other sites in the body. On the other hand, malignant tumors invade surrounding tissues, as well as spread to other sites using the circulatory or lymphatic system. The spreading of cancer cells is referred to as metastasis.

In 2012, 14.1 million new cancer cases were diagnosed worldwide and 311,000 cases were diagnosed in the UK in 2011 (Cancer Research UK, 2015). Breast, bowel, lung and prostate cancer accounts for more than half of all cancer cases. Breast cancer accounts for 15 % of all cancer cases but 7 % of mortality whereas lung cancer accounts for 13 % of all cancer cases with the highest mortality rate of 22 % amongst all cancers (Cancer Research UK, 2015).
1.6.3.1 Cell cycle and control of cell division

Cell division consists of four major processes consisting of cell growth, replication of DNA, chromosome replication and cell division. Growth 1 phase (G1 phase) is the gap between mitosis and initiation of DNA replication. Cells are metabolically active and continuously grow during the G1 phase. However, DNA replication does not occur during G1 phase. When conditions are unfavourable for cell division, the G1 phase can be delayed and cells enter a resting state known as the G0 phase (Cooper, 2000).

The G1 phase is then followed by the S phase. DNA replication and centrosome duplication occur during the S phase. Growth 2 phase (G2 phase) then occurs where cell growth continues. This phase is followed by mitosis phase (M phase) where cell growth stops and the cell divides into two daughter cells.

G1 and G2 phases are gap phases which allow sufficient time for the cell to grow before committing to the S phase or M phase. Checkpoints also exist at different phases as quality controls. G1/S DNA damage checkpoint ensures the quality of DNA prior to DNA replication in S phase. Cyclin dependent kinase (CDK) activity is inhibited when DNA damage is detected, prevents progression to S phase and thus leads to cell cycle arrest and DNA damage or cell death.

The minichromosome maintenance complex (MCM complex) is vital to ensure that DNA replication occurs precisely once. The MCM complex are licensing factors which bind to replication origins during the G1 phase only. Once activated, the activated complex moves along the chromatin and acts as a replication helicase initiating DNA replication as well as making the replication origin in an unlicensed state (Nishitani and Lygerou, 2002). Restriction on licensing is only relieved after the completion of mitosis which then enables
another round of DNA replication. Apart from that, spindle checkpoint ensures chromatids are properly attached prior to progression from metaphase to anaphase during the M phase (Lara-Gonzalez et al., 2012).

1.6.3.2 Gene mutations

Gene mutations can be permanent alterations of genes which can trigger the uncontrollable cell division leading to cancer. These gene mutations can be caused by hereditary mutations (Shlien et al., 2015) or external factors such as tobacco smoke carcinogens (Pfeifer et al., 2002). Few classes of genes are identified as important factors in controlling cell division and growth as well as cancer development.

**DNA repair genes:** DNA repair can be divided into two major types: (1) DNA damage caused by external factors such as UV light exposure and endogenous factors such as oxidative stress DNA damage; (2) DNA damage caused during DNA replication (Lahtz and Pfeifer, 2011). Base excision repair (BER) and nucleotide excision repair (NER) are amongst the DNA repair pathways involved in the first type of DNA damage whereas the latter type of DNA damage follows the mismatch repair (MMR) pathway.

In general, BER repairs small regions whereas NER repairs large regions of the DNA. On the other hand, MMR corrects errors of DNA replications such as DNA mismatch. Polymorphisms in DNA repair genes such as *OGG1 S326C, XRCC1 R194W* involved in the DNA repair pathways above were suggested to be correlated to carcinogenesis (Goode et al., 2002).
Proto-oncogenes and oncogenes: Proto-oncogenes are genes capable of causing cancer when mutated. The mutated proto-oncogenes are referred to as oncogenes. Chromosomal rearrangements, mutations and gene amplifications are mechanisms involved in oncogene activation (Croce, 2008).

Cyclin D1 (CCND1) is a vital regulator in the cell cycle progression from G1 phase to S phase (Alao, 2007). CCND1 is also proven to be an oncogene where the deregulation of CCND1 promotes mitogen-independent proliferation as well as affecting other oncogenic effects such as angiogenesis (Musgrove et al., 2011). CCND1 mutations or overexpression were detected in several cancers including endometrial cancer (Moreno-Bueno et al., 2003), breast cancer (Yu et al., 2013) and bladder cancer (Seiler et al., 2014).

Tumor suppressor genes: Oncogenes drive abnormal cell proliferation and growth whereas tumor suppressor genes have the opposite effect of inhibiting cell proliferation and survival. Therefore, the absence of negative regulatory proteins due to the inactivation of tumor suppressor genes can lead to tumor formation (Cooper, 2000).

For example, p53 tumor suppressor gene is involved in apoptosis induced by DNA damage. p53 mutations are a common feature of cancer. Mutations in p53 are found at different stages of cancer development including tumor initiation, tumor development and metastasis (Rivlin et al., 2011). Mutated p53 also showed oncogenic functions that are totally independent of wild-type p53 and exerts a dominant-negative characteristic (Muller and Vousden, 2013).
1.6.3.3 Cell death mechanisms

Different cell death pathways exist where apoptosis and necrosis are the most common. However, other novel cell death pathways investigated include autophagy and necroptosis.

**Apoptosis**: Termed as “programmed cell death”, apoptosis is required during foetal development. Apoptosis is required to remove the interdigital web of the limbs to sculpture individual embryonic fingers and toes (Díaz-Mendoza et al., 2013).

Apoptosis can be initiated by either the external pathway or internal pathway. The external pathway involves the activation of death receptors of the tumor necrosis factor (TNF) superfamily which causes the propagation of apoptotic signals via caspase-8 cleavage (Fulda and Debatin, 2006). In the internal pathway, caspases are activated via the permeabilization of the mitochondrial outer membrane by apoptogenic factors such as cytochrome c (Fulda and Debatin, 2006).

Activation of apoptosis can be characterised by several morphological features: nuclear and cytoplasmic condensation, blebbing, formation of apoptotic bodies and maintenance of membrane integrity (Fink and Cookson, 2005).

**Necrosis**: Necrosis was initially thought to be a form of unprogrammed cell death but recent studies suggested that its occurrence and process might be tightly regulated (Golstein and Kroemer, 2007). Typical morphological characteristics of necrosis are cytoplasm vacuolation, plasma membrane breakdown and inflammation around the necrotic site (Edinger and Thompson, 2004). However, necrotic cells do not undergo chromatin condensation and DNA fragmentation which are characteristics of apoptosis.
**Necroptosis:** Cells undergoing necroptosis are morphologically similar to necrotic cells but the process is a regulated active type of cell death (Green et al., 2011). Unlike apoptosis, necroptosis does not require caspase activation. Activation of T-cell receptor (TCR), Toll-like receptor (TLR) and in particular TNF, induces necroptosis (Tait et al., 2014). Caspase-8/FADD mediated apoptosis is able to inhibit necroptosis (Zhou and Yuan, 2014).

### 1.6.3.4 Anticancer agents

Conventional anticancer agents are cytotoxic compounds; those with significant cytotoxicity on cancer/tumor cell lines and which also caused tumor regression in murine tumor xenografts or allografts were normally selected (Narang and Desai, 2009). However, recent advances in molecular biology as well as a more in-depth knowledge of cancer have enabled the development of target-based anticancer agents. These target-based anticancer agents are designed to inhibit and/or modify molecular markers vital in cancer growth, development and metastasis.

Natural alternatives pose as an attractive proposition for anticancer agents. Bioactive compounds, as explained previously, are naturally derived compounds which are able to exert health effects. Studies show that bioactive compounds are able to exert anti-proliferative effects in different types of cancer cells via autophagy, apoptosis, cell cycle arrest and interference of cellular signal transduction (Gan et al., 2015; Milutinović et al., 2015).

Bioactive peptides derived from marine sources such as tuna (Hung et al., 2014) and oyster (Umayaparvathi et al., 2014), elucidated anti-proliferative effects on cancer cells.
However, the exact mechanisms of the anti-proliferative effects of bioactive peptides have not been investigated in depth.

1.7 Aim and objectives

The main aim of this project is to purify and identify peptides with bioactive properties from salmon (*Salmo salar*) skin gelatin. The objectives below were considered necessary to achieve the aim:

- To extract gelatin from salmon skin
- To prepare protein hydrolysates using alcalase, pepsin and collagenase
- To purify a bioactive peptide fraction with the highest antioxidant and ACE inhibition activity using ultrafiltration and gel filtration
- To investigate the antioxidant mechanisms of the purified fraction
- To investigate the ACE inhibition mechanisms of the purified fraction
- To investigate the anti-proliferation effect of the purified fraction on human hepatocellular carcinoma
- To investigate the effect of the purified fraction on the antioxidant enzymes of human hepatocellular carcinoma
- To investigate the effect as well as the mechanisms of anti-proliferative activity of the purified fraction on human colorectal adenocarcinoma
- To analyse the amino acid composition of the isolated fraction
Chapter 2
2 EXTRACTION AND PURIFICATION OF ANTIHYPERTENSIVE BIOACTIVE PEPTIDES FROM SALMON SKIN GELATIN

2.1 Introduction

High blood pressure or hypertension is a condition of the persistent increase in blood pressure. Hypertension is diagnosed when both the systolic and diastolic blood pressure consistently exceeds 140 mmHg and 90 mmHg respectively. According to Public Health England, more than 1 in 4 adults had hypertension in 2014 (Public Health England, 2014). Although normally asymptomatic, hypertension can lead to significant impact on health. It accounts for 45% of deaths from heart disease (WHO, 2011).

Several mechanisms are involved in the regulation of blood pressure in the human body. These include the kinin-nitric oxide system (KNOS), neutral endopeptidase system (NEPS), the renin-chymase system (RCS) as well as the renin-angiotensin-aldosterone system (RAAS). RAAS is considered as the major regulator of the long term control of arterial pressure (Carey et al., 2000). As RAAS maintains blood pressure via sodium absorption, overactivity of RAAS can lead to the increased vasoconstriction thus the occurrence of hypertension. Elevation of blood pressure is achieved when angiotensin-I is cleaved to angiotensin-II which is a potent vasoconstrictor. The main enzyme responsible for the production of angiotensin-II is angiotensin converting enzyme (ACE). ACE works by hydrolysing the N-terminal dipeptide His-Leu of angiotensin-I (decapeptide) to produce angiotensin-II (octapeptide) (Crowley et al., 2006).

ACE is a dipeptidyl carboxypeptidase which mainly consists of \( \alpha \)-helices. A zinc ion and 2 chloride ions are also incorporated in ACE. Zinc ion plays an important role in the
catalytic activity of ACE (Cushman and Andotti, 1999) whereas chloride ions might interact with both the substrate and enzyme. The active site of ACE is divided into 2 subdomains by a deep and narrow channel. A ‘lid’ from α-helices is located in the N-terminal of the active site which restricts access to large polypeptides that only permits small peptides as substrates for the active site (Natesh et al., 2004).

Angiotensin receptors are divided into angiotensin type 1 or 2 receptors (AT$_1$ or AT$_2$) which have different functions. AT$_1$ is expressed ubiquitously in the cardiovascular system whereas AT$_2$ is mostly found in the developing foetus but its expression in the cardiovascular system is very low (Lemarié and Schiffrin, 2010). Binding of angiotensin-II to angiotensin type I receptor (AT$_1$) would lead to a cascade of events which result in vascular smooth muscle contraction and the release of adrenaline. This induces artery constriction and the increase of heart rate. Angiotensin-II also stimulates the release of aldosterone from the adrenal cortex, which leads to the retention of sodium in the distal nephron. In the kidney, activation of AT$_1$ would lead to vasoconstriction and antinatriuresis (Klingbeil et al., 2000).

Apart from being responsible for the production of the potent vasoconstrictor angiotensin-II, ACE degrades bradykinin which acts as a vasodilator (Dendorfer et al., 2001). Thus, many hypertensive drugs are designed to act as ACE inhibitors. Although synthetic ACE inhibitors exhibit potent hypotensive activity, they can lead to many adverse effects including dry cough, skin rash as well as headache (Chu et al., 2014).

As mentioned above, ACE inhibitors currently used to treat hypertension have significant adverse effects on health. Recent studies have shown the production of food derived ACE inhibitors. These ACE inhibitor peptides were successfully produced from different
sources especially marine sources such as sea bream bones and scales (Akagündüz et al., 2014), cuttlefish wastewater (Amado et al., 2014) and snakehead fish (Ghassem et al., 2013). The antihypertensive activity of the peptides were also confirmed in in vivo where oral administration of the bioactive peptides successfully decreased blood pressure in spontaneously hypertensive rats (Jung et al., 2006; Wang et al., 2008).

The aims of this study were to isolate and characterise peptides with ACE inhibition activity from salmon skin gelatin. Gelatin from salmon skin was hydrolysed with different enzymes to compare their ACE inhibition activity. The best hydrolysate was further purified and compared in terms of their ACE inhibition activity. The ACE inhibition mechanisms and amino acid composition of the isolated peptide were also investigated in this study.
## 2.2 Method

### 2.2.1 Materials and reagents

The following materials and reagents were purchased from the different suppliers:

<table>
<thead>
<tr>
<th>Materials</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmon</td>
<td>M&amp;J Seafood (Aylesbury, UK)</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate (NaHCO$_3$)</td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td></td>
</tr>
<tr>
<td>Whatman paper No. 4</td>
<td></td>
</tr>
<tr>
<td>Alcalase, ≥2.4 U/g</td>
<td></td>
</tr>
<tr>
<td>Collagenase, ≥125 CDU/mg solid</td>
<td>Sigma Aldrich (Dorset, UK)</td>
</tr>
<tr>
<td>Pepsin, ≥250 units/mg protein</td>
<td></td>
</tr>
<tr>
<td>Hydrochloric acid (HCl), ACS reagent</td>
<td></td>
</tr>
<tr>
<td>Sephadex G-25</td>
<td></td>
</tr>
<tr>
<td>Boric acid</td>
<td></td>
</tr>
<tr>
<td>Sodium chloride</td>
<td></td>
</tr>
<tr>
<td>Angiotensin converting enzyme (ACE), ≥2.0 units/mg protein</td>
<td></td>
</tr>
<tr>
<td>Hippuryl-Histidyl-Leucine (HHL)</td>
<td></td>
</tr>
<tr>
<td>Zinc chloride (ZnCl$_2$)</td>
<td></td>
</tr>
<tr>
<td>Triethylamine (TEA), HPLC grade</td>
<td></td>
</tr>
<tr>
<td>Phenylisothiocyanate (PITC), HPLC grade</td>
<td></td>
</tr>
<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>Fisher Scientific (Leicester, UK)</td>
</tr>
<tr>
<td>Sulphuric acid</td>
<td></td>
</tr>
<tr>
<td>Materials</td>
<td>Suppliers</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Acetonitrile (ACN), HPLC grade</td>
<td>Fisher Scientific (Leicester, UK)</td>
</tr>
<tr>
<td>Trifluoroacetic acid (TFA), HPLC grade</td>
<td>Millipore (Feltham, UK)</td>
</tr>
<tr>
<td>0.22 µm filter</td>
<td>Vivaspin (Epsom, UK)</td>
</tr>
<tr>
<td>Ultrafiltration membrane cartridges</td>
<td></td>
</tr>
</tbody>
</table>

### 2.2.2 Gelatin extraction from salmon skin gelatin

Gelatin was extracted from salmon skin using the method of Badii and Howell (2006). Salmon skin was descaled, cleaned and cut into small pieces (3 cm x 3 cm). The skin was frozen at -80 °C and then freeze dried. Freeze dried salmon skin (200 g) was defatted using 1000 mL NaHCO₃ (0.125 %) with 200 g of ice. The mixture was stirred at 4 °C for 30 min and centrifuged at 4500 x g for 5 min. The supernatant was discarded and the procedure with NaHCO₃ was repeated until no fat was visible in the supernatant. The defatted fish skin was treated with 1000 mL 0.2 % NaOH for 15 h at 4 °C. The NaOH solution was discarded and the skin was rinsed with water. Then, the fish skin was treated with 0.2 % sulphuric acid (1000 mL) at 4 °C. After 8 h, the skin was again removed and rinsed with water. Next, the skin was treated with 1000 mL 0.7 % citric acid for another 12 h at 4 °C, then rinsed thoroughly with water. Gelatin was extracted from the treated skin with Milli-Q water in 45 °C for 12 h. The skin was discarded and solution was filtered using a Whatman paper No. 4 in a Büchner funnel. The solution was frozen at -80 °C and freeze dried to obtain the gelatin.
2.2.3 Measurement of angiotensin converting enzyme (ACE) inhibition activity

The ACE inhibition activity of samples was determined using the method from Wu et al. (2002) with slight modifications. All samples and reagents in this experiment were dissolved in 0.1 M borate buffer with 0.3 M sodium chloride (pH 8.3). Hippuryl-His-Leu (HHL) (2mM) was used a substrate for ACE. Samples (5 mg/mL) or captopril (0.5 mg/mL) were added to 50 µL of HHL and incubated at 37 °C for 10 min. The reaction was then stopped by the addition of 100 µL 1 M hydrochloric acid. Buffer was used as the control instead of the sample.

Samples were analyzed in the HPLC (Ultimate 3000, Dionex) and eluted using two solvent systems: (A) 0.05 % trifluoroacetic acid (TFA) in water and (B) 0.05 % TFA in acetonitrile (ACN) at 1 mL/min. The gradient step for the elution used was 5- 60 % solvent A in 0-10 min, followed by a constant 60 % for 2 min, then returned to 5 % in 1 min. This was followed by isocratic elution for 4 min. Hippuric acid (HA) was detected at 228 nm using a Gemini-NX C18 column (Phenomenex). Data was analysed using the Chromeleon Software. The ACE inhibition activity was calculated using the formulae:

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

Where \( C \) = Area under the peak of control

\( S \) = Area under the peak of samples/ positive control (captopril)
2.2.4 Preparation and purification of bioactive peptides with ACE inhibition activity

2.2.4.1 Preparation of hydrolysates from salmon gelatin

The gelatin obtained was hydrolysed with three different enzymes. Gelatin solutions (1:50 w/v) were made from the gelatin prepared in 2.2.2. The hydrolysis conditions, with modifications, for alcalase as well as collagenase and pepsin were as described by See et al. (2011) and Ranathunga et al. (2006) respectively. The temperature and pH (1 M HCl or 1 M NaOH) were adjusted according to the enzymes used. The optimum conditions for the different enzymes used for the hydrolysis are shown in the table below:

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Optimum conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
</tr>
<tr>
<td>Pepsin</td>
<td>2.0</td>
</tr>
<tr>
<td>Collagenase</td>
<td>7.5</td>
</tr>
<tr>
<td>Alcalase</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Table 2.1 Optimum pH and temperature used for pepsin, collagenase and alcalase to hydrolyse salmon skin gelatin.

Enzymes (1 %) were added to the gelatin solutions and were incubated for 8 h whilst maintaining their appropriate temperature and pH. After the completion of digestion, the mixture was put into boiling water for 15 min to inactivate the enzymes, thus terminating hydrolysis of the gelatin. The hydrolysed protein was centrifuged (5000 x g) and the supernatant collected, lyophilised and stored at - 80°C. ACE inhibition activity of the hydrolysates was compared using the method in 2.2.3 the hydrolysate with the highest ACE inhibition activity was chosen for further studies.
2.2.4.2 Ultrafiltration of salmon skin hydrolysate

Freeze dried alcalase hydrolysates of salmon skin were dissolved in Milli-Q water (1: 10 w/v) and vortexed to homogenise the solution. The solution was sequentially fractionated into different fractions using cartridges with different molecular weight (MW) cut-offs of 5 kDa and 2 kDa. After passage through the 5 kDa membrane cartridges, aliquots were collected and further fractionated with the 2 kDa membrane cartridges. Fractions collected were labelled according to their MW range: Ultrafiltration fraction >5 kDa (UF-1), ultrafiltration fraction 2-5 kDa (UF-2) and ultrafiltration fraction <2 kDa (UF-3). The fractions were lyophilised and stored at –80 °C. The ACE inhibition activity amongst the ultrafiltration fractions were compared as in 2.2.3 and the best fraction was chosen for further purification via gel filtration.

2.2.4.3 Gel filtration chromatography

UF-3 was further purified by gel filtration chromatography using HPLC with a gel filtration column (2.5 cm x 90.0 cm). 250 mg samples were dissolved in 5 mL 50 mM sodium phosphate buffer (pH 7) and filtered through a 0.22 µm filter. The gel filtration column was packed with Sephadex G-25 gel and the samples were injected onto the column. Samples were then run with the 50 mM sodium phosphate buffer for 10 h at a constant flow rate of 1 mL/min. A total of 121 fractions were collected and sufficient samples were pooled from 12 runs. The absorbance at 228 nm was determined and only fractions with a value of > 1 were collected to determine their ACE inhibition activity (2.2.3). The fraction with the highest activity was chosen to determine its ACE inhibition mechanisms.
2.2.5 ACE inhibition mechanisms

2.2.5.1 Enzyme kinetics

Different concentrations of HHL substrate (0, 2, 3, 4 and 6 mM) and peptide (0, 2.5 and 5.0 mg/mL) were used in this experiment. HA produced from the different combinations of the HHL and peptide concentrations at reaction times (0-30 min) were determined using the method in 2.2.3. A Michaelis-Menten graph was first plotted to determine the maximum velocity ($V_{\text{max}}$) of the reaction and the concentration of substrate leading to half of maximum velocity ($K_m$). The Lineweaver Burk plot was also plotted to determine the enzyme kinetics.

2.2.5.2 Differential scanning calorimetry (DSC) of GF28 and zinc chloride (ZnCl$_2$)

Micro DSCVII (Setaram, France) was used to determine the thermodynamics of 5 mg/mL GF28, 5 mM ZnCl$_2$ and GF28-ZnCl$_2$. The method was based on Badii and Howell (2002) where an equal amount of sample (750 mg) and reference (Milli-Q water) was heated from 8-80 °C (rate of 0.5 °C/min) then cooled back to 8 °C. The temperature when the peptide is denatured halfway is referred as transition temperature ($T_m$). $T_m$ was measured using the tip of the peak. The enthalpy change was also measured by integrating the area under the peaks using the software provided (SETSOFT 2000).
2.2.6 Amino acid analysis of GF28

2.2.6.1 Sample preparation

GF28 (25 mg) was dissolved in 5 mL 6 N HCl in dark screw-cap vials. The vials were purged with nitrogen and stored at 110 °C for 24 h to completely hydrolyse the peptide.

2.2.6.2 Derivatisation of amino acids with phenylisothiocyanate (PITC)

Derivatisation of the amino acids of GF28 was according to Badii and Howell (2001). 1 mL of the sample solution from 2.2.6.1 was centrifuged and 20 µL was transferred to glass microtubes. Amino acid standard (20 µL) was also added to another glass microtube. The samples and standard were mixed with 20 µL drying reagent and vacuum dried for 15 min. Derivatisation reagent (10 µL) was added to the microtubes, sealed and left at room temperature for 20 min. The mixtures were further vortexed and vacuum dried. After 10 min, 20 µL of methanol was added and completely dried under vacuum. The residue in the glass microtubes were reconstituted with eluent A (0.05 % TFA in water) and analysed in the HPLC.

Drying reagent: Methanol: Water: Triethylamine (TEA) (2:2:1)

2.2.6.3 Amino acid analysis using HPLC

The prepared and derivatised samples/ control were analysed using HPLC (Ultimate 3000). Amino acids were separated using a C18 reverse-phase column (Phenomenex). Mobile phases of the analysis were: Eluent A: 0.22 M sodium acetate buffer with 0.05 % TEA, pH 6.2 and Eluent B: ACN: water (60:40). The gradient profile used was: 0-10 min (A: 100 %,
B: 0 %), 10-10.5 min (A: 54 %, B: 46 %), 10.5-12.5 min (A: 0 %, B: 100 %), 12.5-20.5 min (A: 100 %, B: 0 %). Amino acids were detected at 254 nm using integrated software (Chromeleon).

2.2.7 Statistical analysis

All experiments were done in triplicate (unless stated otherwise) and in three different experiments. Results were analysed using GraphPad Prism version 6.0. One way analysis of variance (ANOVA) was used to compare the means of the data followed by the Tukey’s post-hoc test unless stated otherwise. The details of the statistical tests were provided in the figure legends. A statistical difference was observed when $p \leq 0.05$. Data were presented as mean ± standard error of mean (SEM).
2.3 Results

2.3.1 Purification of salmon skin gelatin peptides with ACE inhibition activity

2.3.1.1 ACE inhibition activity of pepsin, collagenase and alcalase hydrolysates from salmon skin gelatin

Gelatin extracted from salmon skin was hydrolysed with three different proteases: pepsin, collagenase and alcalase. Figure 2.1 shows the ACE inhibition activity of the hydrolysates compared to captopril. All three hydrolysates showed significantly lower ACE inhibition activity compared to captopril (68.1 %). Alcalase hydrolysed salmon skin gelatin exhibited highest ACE inhibition activity (59.0 %) followed by collagenase hydrolysate (53.7 %). Pepsin hydrolysate showed lowest ACE inhibition activity of 43.6 %. Thus, alcalase hydrolysed salmon skin gelatin was chosen for further purification by ultrafiltration.

![Figure 2.1](image)

**Figure 2.1** ACE inhibition activity of 5 mg/mL salmon skin gelatin digested by pepsin, collagenase and alcalase. Captopril (0.5 mg/mL) was used as a positive control. Means were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference ($p \leq 0.05$) to the control was represented by c. Data were represented as mean ± SEM ($n = 9$).
2.3.1.2 ACE inhibition activity of ultrafiltration fractions from salmon skin alcalase hydrolysate

The ACE inhibition activity of ultrafiltration fractions of alcalase hydrolysates compared to captopril were presented in Figure 2.2. An increase in ACE inhibition activity was shown with a decrease in molecular weight of the alcalase hydrolysate of salmon skin gelatin. The ACE inhibition activity of ultrafiltration hydrolysates of UF-1 and UF-2 were 52.4 % and 59.5 % respectively. Both showed significantly lower ACE inhibition activity than captopril (71.1 %). Only UF-3 (71.1 %) had ACE inhibition activity that was similar to captopril. UF-3 was then chosen for further purification using gel filtration via HPLC.

![Figure 2.2](image)

**Figure 2.2** ACE inhibition activity of ultrafiltration fractions of alcalase treated hydrolysate. Captopril (0.5 mg/mL) was used as a positive control. Means were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference ($p \leq 0.05$) to the control was represented by c. Data were represented as mean ± SEM (n = 9).
2.3.1.3 ACE inhibition activity of gel filtration fractions

Gel filtration fractions were derived using Sephadex G25, collected and read using a spectrophotometer at 218 nm. Only GF-23 to GF-32 showed an absorbance higher than 1.0 which indicated a high amount of peptide. These were chosen to investigate their ACE inhibition activity and the results were shown in Figure 2.3. Captopril exhibited ACE inhibition activity of 82.5 % but GF23 to GF32 showed significantly lower ACE inhibition activity than captopril. ACE inhibition activity increased from GF23 (16.3 %) to GF28 (46.5 %), then subsequently decreased to 13.1 % in GF-32. GF28 exhibited significantly higher ACE inhibition activity than other fractions collected. Thus, GF28 was chosen as the sample used to investigate the ACE mechanisms.

![Figure 2.3 ACE inhibition activity of gel filtration fractions of alcalase hydrolysate against 0.5 mg/mL captopril as positive control. Means were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference (p ≤ 0.05) to the control was represented by c. Data were represented as mean ± SEM (n = 9).](image-url)
2.3.2 ACE inhibition mechanisms of GF28

2.3.2.1 Enzyme kinetics of ACE with GF28

Lineweaver Burk plot using 1/initial velocity (1/V<sub>0</sub>) against 1/Substrate concentration (1/[Substrate]) were plotted in Figure 2.4. According to the enzyme kinetics in Table 2.2, the maximum velocity (V<sub>max</sub>) of the samples: control, 2.5 mg/mL GF28 and 5.0 mg/mL GF28 were similar (10.5 mM/min). On the other hand, the concentration of substrate leading to half of maximum velocity (K<sub>m</sub>) increased with the concentration of GF28 used. K<sub>m</sub> values for control, 2.5 mg/mL GF28 and 5.0 mg/mL GF28 were 4.92, 6.61 and 9.64 mM respectively. The characteristic of similar V<sub>max</sub> values but an increase in K<sub>m</sub> indicated competitive inhibition of GF28 towards ACE.

<table>
<thead>
<tr>
<th></th>
<th>Control (No inhibitor)</th>
<th>GF28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2.5 mg/mL</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt; (mM/min)</td>
<td>10.46 ± 0.16</td>
<td>10.48 ± 0.21</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt; (mM)</td>
<td>4.92 ± 0.11</td>
<td>6.61 ± 0.12</td>
</tr>
</tbody>
</table>

Table 2.2 V<sub>max</sub> and K<sub>m</sub> values of control (no inhibitor), 2.5 and 5.0 mg/mL of GF28. Data were represented as mean ± SEM (n = 9).

Figure 2.4 Lineweaver Burk plot of 0, 2.5 and 5.0 mg/mL GF28. Substrate (HHL) concentrations used were 2-6 mM. Data were represented as mean (n = 9).
2.3.2.2 Thermodynamics of GF28 in the presence of Zn$^{2+}$

In Figure 2.5, zinc chloride alone showed a straight line up to 80°C in DSC where no changes in the structure occurred. Apart from that, thermodynamics of GF28 alone exhibited 4 peaks with different $T_m$ ($a = 25.1°C$, $b = 35.3°C$, $c = 46.3°C$, $d = 50.0°C$) with an increase of temperature (Table 2.3) Different peaks also exhibited different enthalpy changes indicating that 1.59 J/g was needed at $T_m$ ($a = 25.1 °C$) to denature the peptide. At $d$ (50.0°C), the enthalpy change was 0.19 J/g where GF28 was completely denatured after this reaction. The thermodynamics of GF28 with ZnCl$_2$ exhibited a different slope compared to ZnCl$_2$ and GF28 alone.

![Figure 2.5](image)

**Figure 2.5** Thermodynamics of 5 mM ZnCl$_2$ and GF28 alone as well as GF28 and ZnCl$_2$ up to 90 °C using differential scanning calorimetry. Peaks detected were represented as a, b, c and d respectively. Data were represented as mean (n = 9).

<table>
<thead>
<tr>
<th>Peak</th>
<th>Transition temperature, $T_m$ (°C)</th>
<th>Enthalphy change (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>25.07 ± 0.14</td>
<td>1.59 ± 0.02</td>
</tr>
<tr>
<td>b</td>
<td>36.34 ± 0.26</td>
<td>0.26 ± 0.31</td>
</tr>
<tr>
<td>c</td>
<td>46.34 ± 0.04</td>
<td>0.14 ± 0.15</td>
</tr>
<tr>
<td>d</td>
<td>49.95 ± 0.28</td>
<td>0.19 ± 0.20</td>
</tr>
</tbody>
</table>

**Table 2.3** DSC results for the transition temperature and enthalpy change exhibited in the denaturation of GF28. Peaks were represented by a, b, c and d respectively. Data were represented as mean ± SEM (n = 9).
2.3.3 Amino acid analysis

Amino acid analysis of GF28 was represented in Table 2.4. Most amino acid can be found in GF28 where glycine was the most abundant amino acid (42.2 %). This was followed by alanine (12.9 %) and other amino acids were found to be less than 10 % in GF28. Apart from that, the predominant group of amino acids in GF28 were hydrophobic amino acids (28.3 %) (expressed as the sum of Ala, Val, Met, Isoleu, Leu, Phe, Pro, Tyr). Only 9.4 % of GF28 consisted of aromatic amino acids.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>% amino acid in GF28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>1.3 ± 0.04</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4.4 ± 0.01</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>3.1 ± 0.01</td>
</tr>
<tr>
<td>Serine</td>
<td>2.5 ± 0.002</td>
</tr>
<tr>
<td>Glycine</td>
<td>42.2 ± 0.05</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.1 ± 0.02</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.1 ± 0.01</td>
</tr>
<tr>
<td>Threonine</td>
<td>8.9 ± 0.008</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.9 ± 0.04</td>
</tr>
<tr>
<td>Proline</td>
<td>2.3 ± 0.11</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.9 ± 0.03</td>
</tr>
<tr>
<td>Valine</td>
<td>2.0 ± 0.01</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.6 ± 0.06</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.9 ± 0.07</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.1 ± 0.01</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.5 ± 0.02</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.7 ± 0.02</td>
</tr>
<tr>
<td>Amino Acid Categories</td>
<td>Percentage (± SEM)</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>4.7 ± 0.01</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.8 ± 0.09</td>
</tr>
<tr>
<td>Aromatic amino acids</td>
<td>9.4 ± 0.06</td>
</tr>
<tr>
<td>Hydrophobic amino acids</td>
<td>28.3 ± 0.07</td>
</tr>
</tbody>
</table>

**Table 2.4** Amino acid composition (%) of GF28. Aromatic amino acids are expressed as the sum of Phe, His, Trp and Tyr per 100 amino acid residues and hydrophobic amino acids are the sum of Ala, Val, Met, Isoleu, Leu, Phe, Pro and Tyr per 100 amino acid residues. Data were represented as mean ± SEM (n = 9).
2.4 Discussion

2.4.1 Gelatin extraction and purification of bioactive peptides with ACE inhibitory activity from salmon skin gelatin

In the past, bioactive peptides exhibiting ACE inhibition activity were isolated from different sources such as milk or muscle protein (FitzGerald and Meisel, 2000; Ryan et al., 2011). However, the high amount of fish filleting by-products generated such as skin is also a source of valuable protein and could be used to obtain bioactive peptides.

In this study, ACE inhibitory peptides were successfully isolated from salmon skin gelatin and further digested with three different proteases: pepsin, collagenase and alcalase. The ACE inhibition activity of the hydrolysates was investigated using the HPLC method. The alcalase hydrolysate (59.0 %) exhibited the highest ACE inhibition amongst the hydrolysates. However, the ACE inhibition activity of alcalase hydrolysate was still significantly lower than captopril (68.1 %). The difference in ACE inhibition activity of the hydrolysates may be due to the different enzyme cleavage specificity. Collagenase has a strong affinity to cleave at the peptide bond between Gly and a hydrophobic amino acid (Seltzer et al., 1990) whereas pepsin prefers to cleave after bulky amino acids such as leucine and phenylalanine (Palashoff, 2008). Alcalase exists as a crude enzyme and contains several different proteases apart from its main component subtilin Carlsberg (Doucet et al., 2003). The different proteases in alcalase also have different peptide cleavage sites which could lead to a higher degree of hydrolysis compared to pepsin and collagenase. Thus, more bioactive peptides that possessed ACE inhibition activity can be obtained when digested with alcalase.
The alcalase hydrolysate of salmon skin gelatin was ultrafiltered and separated into 3 different MW range. As shown in Figure 2.2, the ACE inhibition activity was found to be inversely proportional to the MW of the fractions. The ACE inhibition activity increases from 52.4 % in UF-1 (highest MW fraction) to 71.1 % in UF-3 (lowest MW fraction). Smaller MW peptides may be an advantage in the inhibition of ACE as only small peptides are accessible to the active site, thus the inhibition of ACE (Natesh et al., 2004). UF-3 was chosen to be further purified by gel filtration.

Amongst the 121 fractions collected, only gel filtration fraction number (GF) 23 to GF32 exhibited an absorption higher than 1.0 at 228 nm. So, the ACE inhibition activity of GF23 to GF32 was investigated. Amongst the fractions, the ACE inhibition activity of GF28 was the highest (46.5 %) but significantly lower than captopril (82.5 %). However, a lower ACE inhibition activity of the gel filtration fraction was observed compared to ultrafiltration fraction (UF-3). Most studies (Zhuang et al., 2012; Zou et al., 2013) found an increasing ACE inhibition activity with purification but results found in this study were similar to the one observed in Campos et al. (2013). The ACE inhibition mechanisms of GF28 were investigated using enzyme kinetics and thermodynamics studies of the peptide.

2.4.2 ACE inhibition mechanisms of GF28

According to the enzyme kinetics, data provided in the Michaelis-Menten graph is summarised in Table 2.2. $V_{\text{max}}$ was found to be constant at 10.5 mM/min and a gradual increase in $K_m$ when the concentration of GF28 was increased. As an inhibitor, GF28 had no effect on the $V_{\text{max}}$ but increases $K_m$ suggests that GF28 acted as a competitive inhibitor of ACE (Berg et al., 2002). The intercepts on the $1/V_0$ axis of the Line-Weaver Burk plot (Figure 2.4) also showed that GF28 is a competitive inhibitor which competes with the
substrate (HHL) at the active site of ACE. This inhibitory mechanism of GF28 is similar to the antihypertensive drug (captopril) used in this study (Chen et al., 2013). Most studies showed peptides from fish sources that exhibited ACE inhibition activity are mainly via mixed type (Mäkinen et al., 2012) or non-competitive inhibition (Qian et al., 2007; Wang et al., 2008; Lee et al., 2014). Only a few peptides from marine sources were shown to exhibit competitive inhibition of ACE such as from fermented oyster sauce (Je et al., 2005).

ACE is a metalloprotein exopeptidase. A zinc ion can be found in the active site of ACE which plays a role in the hydrolysis action of the enzyme. Thus, the inhibition of the zinc ions may lead to the inhibition of ACE activity (Cushman and Andotti, 1999). The ability of GF28 to bind to zinc ions was investigated using DSC. In Figure 2.5, increase in temperature did not show an effect on ZnCl₂ and 4 peaks were detected in the thermodynamics of GF28 alone. The 4 peaks may indicate 4 different peptides with different degradation temperature shown in Table 2.3. The Tₘ of the last peak (d), 49.95 °C, suggested that GF28 was half degraded at this temperature. This characteristic of the four different peaks were missing in the thermodynamics of GF28 with ZnCl₂ mixture. Apart from that, the graph was also different to the graph of ZnCl₂ alone. This indicated that GF28 was able to bind to zinc ions in the active site of ACE which lead to the inhibition of ACE.

2.4.3 Amino acid analysis

Amino acid analysis results in Table 2.4 showed that GF28 is composed mainly of glycine (42.2 %) and the predominant group of amino acid is hydrophobic amino acid (28.3 %). Three subsites, S1 (antepenultimate), S1’ (penultimate) and S2’ (ultimate) are located in
the two active sites of ACE. Apart from that, the subsites have a preference for hydrophobic tri-peptide residues at the C terminal of the ACE inhibitory peptides (Pripp et al., 2005). There was also a preference for bulky aromatic amino acids such as Phe, His, Tyr and Trp at the S’ position, which may increase the binding of the peptides to ACE. Aromatic amino acids, which account for 9.4 %, in GF28 may block access of the substrate to the active site thus increasing the ACE inhibition activity of the peptide (Kobayashi et al., 2008).
2.5 Conclusion

Gelatin was successfully extracted from salmon skin and hydrolysed using different proteases. Alcalase hydrolysate was found to possess the highest ACE inhibition activity compared to collagenase and pepsin. A higher degree of hydrolysis was achieved by using alcalase as mentioned in the discussion. Alcalase hydrolysate was further purified using ultrafiltration and gel filtration chromatography. The ACE inhibition activity of the gel filtration fraction (GF28: 46.5 %) was lower than the ultrafiltration fraction (UF-3: 71.1 %). This may be due to the exclusion of large aromatic amino acids responsible for the ACE inhibition activity during gel filtration. Thus, the amount of aromatic amino acids may be lower in GF28 than UF-3.

The ACE inhibition mechanisms were elucidated using GF28. Enzyme kinetics showed competitive inhibition of ACE. Thermodynamics of GF28 in the presence of ZnCl₂ also showed that GF28 was able to bind to Zn²⁺. This further confirmed the competitive inhibition of ACE as Zn²⁺ acts as an important factor in the active site of ACE. The presence of high amount of hydrophobic amino acids (28.3 %) as well as aromatic amino acids (9.4 %) in GF28 may also be responsible for the ACE inhibition activity of the peptide. Although these findings indicate the potential for producing peptides with anti-hypertensive abilities, in vivo and clinical studies are needed before they can be used for nutraceutical applications and functional foods.
Chapter 3
3 EXTRACTION AND PURIFICATION OF BIOACTIVE PEPTIDES WITH ANTIOXIDANT ACTIVITY FROM SALMON SKIN GELATIN

3.1 Introduction

Lipid peroxidation is the oxidative degradation of lipids which can lead to many undesirable effects. In food products, lipid degradation causes undesirable effects in the colour, texture, flavour and smell of food which also affects the quality and shelf life. Evidence has shown that *in vivo* lipid peroxidation is associated with many diseases including neurological disorders (Shichiri, 2014), atherosclerosis (Chisolm and Steinberg, 2000), type 2 diabetes (Santoro *et al.*, 2014) and cancer (Thanan *et al.*, 2015; Zhong and Yin, 2015).

Unsaturated fatty acids, especially polyunsaturated fatty acids (PUFA), are more susceptible to lipid peroxidation than saturated fatty acids as lipid peroxidation increases with the amount of double bonds present in the lipid. Antioxidants play an important role in food products, where they help to maintain the sensory and nutritional quality of the food as well as the shelf life. Synthetic antioxidants are normally phenolic compounds which include 2-tert-butyl-4-hydroxyanisole (BHA) and 2,6-di-tert-butyl-hydroxytoluene (BHT). These synthetic phenolic antioxidants are cheap and very effective in protecting lipids against peroxidation (Sarin *et al.*, 2010).

The European Food Safety Authority concluded that there were no concerns in genotoxicity regarding the use of BHA. The long-term toxicity and carcinogenicity in the fore-stomach of rats were also not relevant to human risk assessment (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2011). However, high doses of
phenolic synthetic antioxidants showed endocrine disruptive effects (Pop et al., 2013). Apart from that, there is a consumer led demand in the reduction of synthetic food additives including synthetic antioxidants as well as the preference for natural food additives. In different mixtures of synthetic antioxidants, the interaction between antioxidants at high temperatures could lead to a negative synergism (Allam and Mohamed, 2002).

Ascorbic acid and tocopherols are the most common natural antioxidants used in food products. Plant extracts exhibited potent antioxidant activity as the activity is strongly associated with the amount of phenolic compounds present (Velioglu et al., 1998). However, the use of plant extracts, particularly extracts from herbs and spices, should be carefully evaluated as they can influence the sensory acceptability of the food product (Yanishlieva et al., 2006).

Bioactive peptides exhibiting antioxidant activities were successfully isolated from marine sources such as croceine croaker (Chi et al., 2015), cod (Girgih et al., 2015), squid (Nakchum and Kim, 2015) and half-fin anchovy (Song et al., 2015). Depending on the amino acid composition and sequence, the bioactive peptides isolated showed antioxidant activity via different antioxidant mechanisms. The main antioxidant mechanisms were metal ion chelation, reducing power activity and radical scavenging activity.

In this study, gelatin was isolated from salmon skin and hydrolysed with different proteases acting at different cleavage sites. The hydrolysate exhibiting the highest peroxide formation inhibition activity was further purified via ultrafiltration and gel filtration chromatography. The fraction with the most potent antioxidant activity was determined using the ferric thiocyanate (FTC) method. Then, the different antioxidant mechanisms of
the fraction was investigated using the reducing power assay, ABTS and DPPH scavenging activity assays as well as the copper and ferrous ion chelating activity assays.
3.2 Methods

3.2.1 Materials and reagents

The following materials and reagents were purchased from the different suppliers:

<table>
<thead>
<tr>
<th>Materials</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbate, ≥99.0 %</td>
<td></td>
</tr>
<tr>
<td>Trolox, 97 %</td>
<td></td>
</tr>
<tr>
<td>Linoleic acid, ≥99.0 %</td>
<td></td>
</tr>
<tr>
<td>Ferrous chloride (FeCl(_2)), 98 %</td>
<td></td>
</tr>
<tr>
<td>Ferric chloride (FeCl(_3)), reagent grade</td>
<td></td>
</tr>
<tr>
<td>Potassium ferricyanide</td>
<td></td>
</tr>
<tr>
<td>Trichloroacetic acid (TCA), ACS reagent</td>
<td>Sigma Aldrich (Dorset, UK)</td>
</tr>
<tr>
<td>Ammonium thiocyanate, ACS reagent</td>
<td></td>
</tr>
<tr>
<td>1,1-diphenyl-2-picrylhydrazyl (DPPH)</td>
<td></td>
</tr>
<tr>
<td>2-tert-butyl-4-hydroxyanisole (BHA), ≥98.5 %</td>
<td></td>
</tr>
<tr>
<td>2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), HPLC grade</td>
<td></td>
</tr>
<tr>
<td>Potassium persulfate, ACS reagent</td>
<td></td>
</tr>
<tr>
<td>Ferrozine</td>
<td></td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td></td>
</tr>
<tr>
<td>Copper (II) sulphate pentahydrate, ACS reagent</td>
<td></td>
</tr>
<tr>
<td>Pyrocatechol violet</td>
<td></td>
</tr>
<tr>
<td>Sodium acetate, ACS reagent</td>
<td></td>
</tr>
<tr>
<td>Absolute ethanol, HPLC grade</td>
<td>Fisher Scientific (Leicester, UK)</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td></td>
</tr>
</tbody>
</table>
3.2.2 Isolation and purification of peptides with antioxidant activity from salmon skin

Gelatin was extracted from salmon skin using the method in 2.2.2. GF28 was further isolated from the alcalase hydrolysate according to methods in 2.2.4. The peroxide formation inhibition activity was compared amongst the different hydrolysates as well as the ultrafiltration and gel filtration fractions using the ferric thiocyanate (FTC) method. The FTC method is described below (Section 3.2.3).

3.2.3 Ferric thiocyanate (FTC) method

The peroxide formation inhibition activity of samples was evaluated using the FTC method. The method from Kikuzaki and Nakatani (1993) with slight modifications was used to prepare a linoleic acid model system. Samples (20 mg) or 0.01 % (w/v) positive controls (ascorbate and trolox) were mixed with 10 mL absolute ethanol, 10 mL 1 M (w/v) phosphate buffer (pH 7.0), 4.78 mL milli-Q water and 0.12 mL linoleic acid. The mixtures were vortexed and kept at 40 °C in the dark. Samples were taken from the emulsion systems every 24 h to evaluate the peroxide formation inhibition activity of samples/control.

The FTC method is based on the production of the peroxide which is the product of lipid peroxidation. Upon reaction with ferrous chloride, peroxide forms ferric chloride which is a red coloured compound. At 24 h intervals, 100 µL samples were added to a mixture of 4.7 mL 75 % (v/v) ethanol and 100 µL 30 % (w/v) ammonium thiocyanate. Absorbance was read at 500 nm after the addition of 20 mM (w/v) ferrous chloride (FeCl₂) to the mixture for 3 min. The peroxide formation inhibition activity was calculated using:
Peroxide formation inhibition (%) = \[\frac{(C-S)}{C} \times 100\]

Where C is defined as the absorbance of control where milli-Q water is used instead of sample

S is defined as the absorbance of sample or positive controls (ascorbate and trolox)

### 3.2.4 Antioxidant mechanisms of GF28

#### 3.2.4.1 Reducing power assay

The ability of GF28 to act as a reducing agent by donating an electron or H\(^+\) was investigated using the reducing power assay by Yildirim et al. (2001) with slight modifications. This method was based on the reduction of ferric thiocyanate via reducing agents. The ferrocyanides reacted with ferric chloride to form a greenish blue compound known as the Perls’ Prussian Blue complex detected at 700 nm. Depending on the extent of reduction, the yellow colour of the reaction mixture changes to different shades of greenish blue mixtures depending on the amount of Perls’ Prussian Blue complex present.

GF28 (1 mL) was mixed with 2.5 mL 0.2 M (w/v) phosphate buffer (pH 6.6) and 2.5 mL 1% (w/v) potassium ferricyanide. The concentration of GF28 used in this experiment was 0.5-10 mg/mL. The mixture was incubated at 50 °C for 30 min. Followed by the addition of 2.5 mL 10% (w/v) trichloroacetic acid, the mixture was centrifuged at 1,500 x g for 5 min. In a 96 well plate, 125 µL of the supernatant was added to 125 µL milli-Q water and 25 µL (w/v) ferric chloride. The absorbance of the solution was read at 700 nm after 10 min. The reducing power of GF28 was expressed as the reducing power activity normalised to the control. The reducing power activity was calculated as follows:
Reducing power assay activity (%) = \[(S – C)/C\] x 100

Where S is defined as the absorbance of samples or positive control (0.01 % BHA)

C is defined as the absorbance of control using 1 mL of milli-Q water instead of samples

3.2.4.2 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical is a stable water soluble radical which is characterised by its violet colour. When reacted with a hydrogen or electron donating agent, DPPH forms diphenyl-picrylhydrazine (DPPH-H). Thus, the violet colour fades to a yellow colour when DPPH-H is formed.

The method from Bersuder et al. (1998) with slight modifications was used to evaluate the DPPH scavenging activity of GF28. DPPH was prepared at a concentration of 0.02 % in ethanol (w/v). In a 96 well plate, 50 µL GF28 (0.5-10.0 mg/mL) was mixed with 50 µL absolute ethanol and 12.5 µL of DPPH previously prepared. The mixture was shaken and kept at room temperature in the dark. After 1 h, the mixture was measured at 517 nm.

BHA (0.01 % w/v) was used as a positive control in this experiment. The DPPH radical scavenging activity of GF28 was calculated as follows:

DPPH radical scavenging activity (%) = \([(C – S)/C\] x 100

Where C is the absorbance of the control using 100 µL of milli-Q water instead of sample

S is the absorbance of samples or positive control (0.01 % BHA)
3.2.4.3 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging ability of GF28

The ABTS radical scavenging method of GF28 was based on Re et al. (1999). ABTS (7 mM) was prepared with 2.45 mM potassium persulfate. The ABTS solution was kept in the dark and allowed to stand in room temperature for 12 h. Before use, ABTS solution was diluted with phosphate buffer (pH 7.4) until the absorbance reached 0.70 ± 0.02. GF28 dissolved in ethanol (10 µL) was added to 1 mL prepared ABTS solution. The decolourisation of ABTS radicals in the presence of reducing agent was read at 734 nm after 5 min. The following formula was used to calculate the ABTS radical scavenging ability of GF28:

\[
\text{ABTS radical scavenging activity (\%) } = \left( \frac{C - S}{C} \right) \times 100
\]

Where C is the absorbance of control using ethanol instead of samples

S is the absorbance of sample or positive control (0.01 % BHA)

3.2.4.4 Ferrous chelating activity assay

Ferrous ions form a water soluble magenta complex when reacted with ferrozine. In the presence of chelating agents, the formation of this Fe$^{2+}$-ferrozine magenta complex is disrupted leading to the reduction of the intensity of magenta colour. Thus, the lower absorbance of mixtures indicates a higher chelating activity.

The activity of GF28 to chelate Fe$^{2+}$ was measured according to Dinis et al. (1994) with slight modifications. In a 96 well plate, 50 µL GF28 (0.5-10 mg/mL) were mixed with 160 µL milli-Q water and 5 µL 2 mM (w/v) ferrous chloride. After 1 min, 10 µL of 5 mM (w/v)
ferrozine was added. The magenta complex formed was measured at 563 nm after an incubation period of 10 min. 0.01 % (w/v) ethylenediaminetetraacetic acid (EDTA) was used as a positive control. The ferrous chelating activity of GF28 was calculated as follows:

$$\text{Fe}^{2+} \text{ chelating activity (}) \%\text{ = } \frac{(C - S)}{C} \times 100$$

Where C is the absorbance of control using milli-Q water instead of samples

S is the absorbance of samples or EDTA as positive control

3.2.4.5 Copper ion chelating activity assay

The colorimetric method from Megías et al. (2009) was modified to determine the copper ion chelating activity of GF28. All reagents or samples were prepared in 50 mM sodium acetate buffer (w/v) pH 6. GF28 (20 µL) or 0.01 % w/v EDTA (20 µL) was added to 10 µL 1mM pyrocatechol violet (w/v) in a 96-well plate. Then, 2mM w/v copper (II) sulphate pentahydrate (10 µL) was added to the mixture and the absorbance read at 632 nm.

The following formula was used to calculate the Cu$^{2+}$ chelating activity:

$$\text{Cu}^{2+} \text{ chelating activity (}) \%\text{ = } \frac{(C - S)}{C} \times 100$$

Where C is the absorbance of control using Milli-Q water instead of samples

S is the absorbance of samples or positive control (0.01 % EDTA)
3.2.5 Statistical analysis

All experiments were done in triplicate (unless stated otherwise) and in three different experiments. Results were analysed using GraphPad Prism version 6.0. ANOVA was used to compare the means of the data followed by the Tukey’s post-hoc test unless stated otherwise. The details of the statistical tests were provided in the figure legends. A statistical difference was observed when $p \leq 0.05$. Data were presented as mean ± SEM.
3.3 Results

3.3.1 Purification of salmon skin gelatin peptides with peroxide formation inhibition activity

3.3.1.1 Antioxidant activity of salmon skin gelatin digested by pepsin, collagenase and alcalase

Peroxide formation inhibition of pepsin, collagenase and alcalase digested salmon skin gelatin was compared in Figure 3.1. Both positive controls, ascorbate and trolox, had a peroxide inhibition activity of 56.8 % and 66.1 % respectively. Salmon skin gelatin digested by pepsin exhibited the lowest peroxide formation inhibition activity (45.1 %) which was significantly lower compared to the positive controls. This was followed by the collagenase hydrolysate (53.8 %) where its antioxidant activity is comparable to ascorbate. Alcalase hydrolysate (61.6 %) showed the highest peroxide formation inhibition activity. Only the antioxidant activity of the alcalase hydrolysate was not significantly different to both ascorbate and trolox.

Figure 3.1 Peroxide formation inhibition of salmon skin gelatin digested using pepsin, collagenase and alcalase. Ascorbate and trolox were used as positive controls. Means were compared using ANOVA followed by the Tukey’s post hoc test. Significant differences (p ≤ 0.05) to ascorbate and trolox were represented by a and b respectively. Data were expressed as mean ± SEM (n = 9).
3.3.1.2 Peroxide formation inhibition activity of ultrafiltration fractions

As the alcalase hydrolysate exhibited the highest peroxide formation inhibition activity, the peptide was further purified using different MWCO cartridges. The peroxide formation inhibition activity of the ultrafiltration fractions are presented in Figure 3.2. The antioxidant activity of UF-1 (62.2 %), the fraction with the highest MW peptides, was non-significantly different to ascorbate (57.4 %) and trolox (66.1 %). UF-2 and UF-3 exhibited peroxide inhibition formation activity of 65.5 % and 66.1 % respectively. Both fractions had similar antioxidant activity to trolox but significantly higher activity than ascorbate. However, there were no significant differences in the peroxide formation inhibition activity amongst the ultrafiltration fractions.

![Figure 3.2](image.png)

**Figure 3.2** Peroxide formation inhibition of ultrafiltration fractions of alcalase digested salmon skin gelatin. Ascorbate and trolox were used as positive controls. Means were compared using ANOVA followed by the Tukey’s post hoc test. Significant differences (p ≤ 0.05) to ascorbate were represented by a. Data were expressed as mean ± SEM (n = 9).
3.3.1.3 Antioxidant activity of gel filtration fractions

UF-3 with the highest antioxidant activity was pooled from the ultrafiltration process and separated using gel filtration chromatography with a Sephadex G-25 gel to obtain peptides with higher purity. All gel filtration fractions collected were read at 228 nm. A higher absorbance indicated a higher amount of peptides in the fraction. Thus, the absorbance of 1.0 was used as a cut-off point for the selection of fractions. Only GF28 to GF32 were selected and the antioxidant activity was compared using the ferric thiocyanate method as shown in Figure 3.3.

Ascorbate and trolox were used as positive controls for this experiment. GF23 to GF32 exhibited similar antioxidant activity to one another where GF32 (61.1 \%) showed the lowest activity. Apart from GF 32, GF23 to GF31 exhibited significantly higher antioxidant activity compared to ascorbate (56. \%). GF 32 also showed significantly lower antioxidant activity than trolox (67.3 \%). Fractions with highest peroxide formation inhibition activity were GF26 (67.3 \%), GF27 (68.8 \%) and GF28 (67.1 \%).

Although the peroxide formation inhibition activity of GF23 to GF32 were similar, only GF28 showed significantly higher ACE inhibition activity in Chapter 1. Thus, GF28 was chosen for further studies in the elucidation of its antioxidant mechanisms.
Figure 3.3 Peroxide formation inhibition of UF-3 gel filtration fractions. Ascorbate and trolox were used as positive controls. Means were compared using ANOVA followed by the Tukey’s post hoc test. Significant differences ($p \leq 0.05$) to ascorbate and trolox were represented by a and b respectively. Data were expressed as mean ± SEM (n = 9).
3.3.2 Antioxidant mechanisms of GF28

3.3.2.1 Reducing power assay of GF28

The reducing power assay of 0.5 to 20.0 mg/mL GF28 was investigated in Figure 3.4. This assay is based on the detection of the Perls’ Prussian Blue complex at 700 nm. In the presence of reducing agents, ferric thiocyanate is reduced to the ferrous form. The addition of FeCl$_3$ forms the ferrous-ferric complex known as the Perls’ Prussian Blue complex.

BHA was used as a positive control which acts as an efficient reducing agent. Thus, the reducing power assay activity was shown to be 69.0 %. A gradual increase in reducing power assay activity was observed (0.9 % to 9.8 %) in 0.5 to 10.0 mg/mL GF28. However, all concentrations were shown to have a significantly lower reducing power activity than BHA. Reducing power assay activity of 10.0 mg/mL GF28 (9.8 %) was significantly higher than the other concentrations used apart from 7.5 mg/mL GF28 (8.2 %).

![Figure 3.4 Reducing power assay of 0.5 to 10.0 mg/mL GF28. The formation of Perls’ Prussian Blue complex was detected at 700 nm. A significant difference ($p < 0.05$) to the positive control, BHA, was represented by a. Means were compared using ANOVA followed by the Tukey’s post hoc test. Data correspond to the mean ± SEM ($n = 9$).](image)
3.3.2.2 DPPH radical scavenging activity

Figure 3.5 showed the DPPH radical scavenging activity of 0.5 to 10.0 mg/mL GF28 compared to BHA as a positive control. GF28 showed very low DPPH radical scavenging activity where the highest concentration used, 10.0 mg/mL GF28, only showed 3.5 % activity.

Figure 3.5 DPPH scavenging activity of 0.5 to 10.0 mg/mL GF28. Activity was measured at 517 nm and compared to 0.02 % BHA as a natural antioxidant. Means were compared using ANOVA followed by the Tukey’s post hoc test. Data correspond to the mean ± SEM (n = 9). A significant difference ($p < 0.05$) to the positive control, BHA, was represented by a.
3.3.2.3 ABTS assay

In this assay, ABTS is generated and the decolourisation of this blue green molecule is detected at 734 nm. The ABTS radical scavenging activity of GF28 compared to BHA as a positive control is shown in Figure 3.6. ABTS radical scavenging activity exhibited by GF28 was significantly lower than BHA (75.5 %). There was only a slight increase in ABTS radical scavenging activity when the concentration of GF28 increased. The highest ABTS radical scavenging activity exhibited in GF28 (10.0 mg/mL) was 4.5 %.

![Figure 3.6 ABTS radical scavenging activity of 0.5 to 10.0 mg/mL GF28. Activity was measured at 734 nm compared to 0.02 % BHA as a natural antioxidant. Means were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference (p < 0.05) to the positive control, BHA, was represented by a. Data correspond to the mean ± SEM (n = 9).](image)
3.3.2.4 Fe\textsuperscript{2+} chelating activity assay

In Figure 3.7, the chelation activity of GF28 increased in a dose dependent manner with the concentration used. EDTA is an effective metal chelator, the ferrous chelating activity was 84.1 %. Ferrous chelating activity of 2.5 mg/mL GF28 (68.7 %) was not significantly different to EDTA (84.1 %). GF28 reached maximum ferrous chelating activity at 5.0 mg/mL (87.0 %). Further increase in GF28 concentration to 7.5 and 10.0 mg/mL did not significantly increase the ferrous chelation activity (90.0 % and 90.0 % respectively). IC\textsubscript{50} of GF28 was determined to be 1.0 mg/mL.

Figure 3.7 Ferrous chelating activity of 0.5 to 10.0 mg/mL GF28 compared to 0.02 % EDTA as a positive control. Means were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference (\(p \leq 0.05\)) to EDTA was represented by a. Data correspond to the mean ± SEM (n = 9).
3.3.2.5 Cu\textsuperscript{2+} chelating activity assay

Similar to the ferrous chelating activity assay, the copper chelating activity assay is shown to be proportional to the concentration of GF28 used (Figure 3.8). The copper chelating activity of GF28 increased from 1.6 \% to 58.1 \% when concentrations used increased from 0.5 to 10.0 mg/mL. However, the highest concentration of GF28 used, 10.0 mg/mL, had significantly lower copper chelating activity than EDTA (58.1 \% and 86.3 \% respectively). IC\textsubscript{50} of GF28 was also shown as 7.6 mg/mL. The activity of 10.0 mg/mL GF28 was not significantly higher than 7.5 mg/mL GF28 (50.3 \%).

![Copper chelating activity of 0.5 to 10.0 mg/mL GF28 compared to 0.02 % EDTA as a positive control. Means were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference (p \leq 0.05) to EDTA was represented by a. Data correspond to the mean ± SEM (n = 9).](image)

Figure 3.8
3.4 Discussion

3.4.1 Purification of salmon skin gelatin with antioxidant activity

The antioxidant activity of salmon skin gelatin hydrolysed by different enzymes as well as the further purified protein fractions were evaluated in their peroxide formation inhibition activity using the FTC method. Ascorbate and trolox were used as positive controls in the experiments as both are commercially used antioxidants in food products. Ascorbate acts as a potent electron donor and trolox is the water soluble analogue of Vitamin E.

Gelatin was extracted from salmon skin and digested using pepsin, collagenase and alcalase and their antioxidant activity was compared in Figure 3.1. Pepsin digested hydrolysate (45.1 %) exhibited the lowest antioxidant activity. The alcalase hydrolysate exhibited the highest peroxide formation inhibition (61.6 %) which was not significantly different to the activity of ascorbate and trolox (56.8 % and 66.1 % respectively). As discussed in 2.4.1, the higher activity of the alcalase hydrolysates may be due to the enzyme alcalase exists as a crude enzyme of several proteases which have different cleavage sites. This can lead to a greater degree of hydrolysis and the production of more bioactive peptides.

Alcalase hydrolysate was subjected to further purification using ultrafiltration cartridges with different MWCO. Antioxidant activity of UF-2 (65.5 %) and UF-3 (66.1 %) were similar. Apart from that, the peroxide formation inhibition activities of UF-2 and UF-3 were also significantly higher than ascorbate (57.4 %). However, compared to the alcalase hydrolysate (61.6 %), there was only a slight increase in antioxidant activity of ultrafiltration fractions. UF-3 was further purified by gel filtration column using Sephadex
G25 gel. In Figure 3.3, GF23 to GF32 exhibited peroxide formation inhibition activity that was not significantly different to one another. This is in contrary to some studies that the antioxidant activity of peptides significantly increased with the reduction of MW such as soy protein fractions (Moure et al., 2006) and cod (Farvin et al., 2014). However, GF28 was chosen for further studies as only this fraction showed significantly higher ACE inhibition activity (Chapter 2) than the others.

3.4.2 Antioxidant mechanisms of GF28

GF28 exhibited low reducing power activity (Figure 3.4) where 10 mg/mL GF28 showed 9.8 % reducing power activity when normalised to control. Both DPPH and ABTS radical scavenging activity assay measure the ability of GF28 peptide to act as an electron donor or hydrogen donor. DPPH exists as a lipid soluble radical whereas ABTS as a water soluble radical. GF28 also showed minimal DPPH and ABTS radical scavenging abilities (Figure 3.5 and Figure 3.6 respectively). GF28 (10 mg/mL) showed DPPH and ABTS radical scavenging activity of 3.5 % and 4.5 % respectively. This indicated that GF28 was not efficient as an antioxidant that scavenges free radicals either in a hydrophobic or hydrophilic environment.

Transition metal ions react with lipid hydroperoxides to form alkoxy radicals (Repetto et al., 2010). Alkoxy radicals are highly reactive and can propagate lipid peroxidation. Thus, the inhibition of transition metal ions could decrease lipid peroxidation. GF28 exhibited high chelation activity of iron and copper transition ions (Figure 3.7 and Figure 3.8). The GF28 for the IC$_{50}$ of Fe$^{2+}$ and Cu$^{2+}$ were 1.0 and 7.6 mg/mL respectively.

It was observed that the chelation activity was the main antioxidant mechanism of GF28. GF28 also showed low reducing power activity as well as non-significant DPPH and
ABTS radical scavenging abilities. Most studies revealed that marine sourced peptides such as patin (Najafian and Babji et al., 2015) and croceine croaker (Chi et al., 2015) exhibited high DPPH and ABTS radical scavenging ability which may be attributed to the high amount of hydrophobic and aromatic amino acids of the peptides. This was contrary to the findings of this study and suggests that the amount of hydrophobic and aromatic amino acids is less important than the sequence of the peptide in its antioxidant mechanisms. The amount of Cys, His, Asp and Glu in GF28 (8.1 %) may play a role in the chelation activity of the peptide as the amino acids above are shown to be responsible for the metal chelating activity of peptides in Guo et al. (2015). Histidine also contributes to the metal chelating activity of peptides where the imidazole ring was shown to form copper chelates to retard copper mediated lipid oxidation (Torres-Fuentes et al., 2014). Histidine only accounted for 2.1 % of GF28. Pownall et al. (2010) also showed that peptides that exerted the highest metal chelation activity had low amounts of histidine; this study also showed that the metal chelation activities were associated with hydrophobic amino acids which can be found in high amounts in GF28 (28.3 %).
3.5 Conclusion

Gelatin was successfully extracted from salmon skin and digested with pepsin, collagenase and alcalase. The alcalase hydrolysate exhibited the highest peroxide formation inhibition and was chosen for further purification using ultrafiltration and gel filtration chromatography. The purified fractions were compared in terms of their antioxidant activity by measuring the peroxide formation inhibition using FTC. It was shown that a decrease in MW of the peptide did not significantly increase the peroxide formation inhibition activity. The antioxidant activities of the ultrafiltration as well as gel filtration fractions were not significantly different to the alcalase hydrolysate. However, GF28 was still chosen for further studies as it exhibited the highest ACE inhibition amongst the fractions.

The main antioxidant mechanism of GF28 was chelation of transition metal ions such as Fe$^{2+}$ and Cu$^{2+}$. However, GF28 showed low reducing power activity. The ABTS and DPPH radical scavenging activities were poor in GF28. This indicated that GF28 have low scavenging activity of both water and lipid soluble radicals. The amino acid analysis of GF28 (Section 2.3.3) indicated that the amount of hydrophobic amino acids (28.3 %) and aromatic amino acids (9.4 %) may contribute to the antioxidant mechanisms of the peptide. However, the sequence of the amino acids may also be essential to the antioxidant mechanisms of bioactive peptides.
Chapter 4
4 EFFECT OF GF28, A SALMON SKIN GELATIN PEPTIDE, ON THE ENDOGENOUS ANTIOXIDANT DEFENCE SYSTEM IN HEPG2 CELLS

4.1 Introduction

According to the NHS (2014), cancer is defined as the condition where the cells in a certain part of the body, divide and grow uncontrollably. In the UK, liver cancer accounts for 1% of all cancer and 3% of deaths due to cancer (Cancer Research UK, 2015a). Liver cell cancers account for approximately half of the liver cancers followed by intrahepatic bile duct carcinomas and unspecified types (West et al., 2006). The majority of liver cell cancers are hepatocellular carcinoma.

Reactive oxygen species (ROS) are produced resulting from normal cellular metabolism and xenobiotic detoxification. Although low to moderate amounts of ROS are essential, an excess of ROS (defined as oxidative stress) can lead to DNA, lipid and protein damage in cells. Oxidative stress is linked to the development and progression of ageing, cancer, diabetes and cardiovascular diseases (Valko et al., 2007). Oxidative stress is considered to be highly involved in the pathogenesis of cancer (Valko et al., 2006; Sosa et al., 2013). Thus, the homeostasis between the production and elimination of ROS is essential for the normal function of cells.

In response to oxidative stress, the endogenous antioxidant defence system of the body is involved in the removal and elimination of ROS in the body. The liver is the second largest organ in the body; one of the main functions of the liver is the detoxification of alcohol and toxins in the body.
As the liver is the main detoxification organ of the body, the endogenous antioxidant defence systems such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase are crucial. Lower levels of GPx and GSH were found in liver cancer patients (Arslan et al., 2014). SOD expression was also shown to increase in cancerous cell lines (Liu et al., 2012; Chen et al., 2014).

Recent studies of bioactive peptides showed growth inhibition effects on cancer cell lines. Many marine sourced bioactive peptides exerted anti-proliferative effects on different types of cancer cell lines such as half-fin anchovy on prostate cancer cells (Song et al., 2014), tuna on breast cancer cell cells (Hung et al., 2014) and loach on colon cancer cells (You et al., 2011). However, only one study regarding the effect of bioactive peptides (from rice bran) on liver cancer cells was found (Kannan et al., 2010).

Thus, GF28 was isolated and purified from salmon skin gelatin and its effect on HepG2 cells, a human hepatocellular carcinoma cell line, was investigated. Using the MTT assay, the anti-proliferative effect and IC$_{50}$ of GF28 was determined. In addition, the effect of GF28 on the different endogenous antioxidant defence systems of HepG2 cells was investigated. These included SOD, catalase, GSH, GPx and GR.
### 4.2 Methods

#### 4.2.1 Materials and reagents

The following materials and reagents were purchased from the different suppliers:

<table>
<thead>
<tr>
<th>Materials</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2 cell line</td>
<td>Sigma-Aldrich (Dorset, UK)</td>
</tr>
<tr>
<td>Thiazolyl blue tetrazolium bromide</td>
<td></td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td></td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle Medium (Gibco)</td>
<td></td>
</tr>
<tr>
<td>Foetal Bovine Serum (Gibco)</td>
<td>Fisher Scientific (Leicester, UK)</td>
</tr>
<tr>
<td>Penicillin-streptomycin (Gibco)</td>
<td></td>
</tr>
<tr>
<td>L-Glutamine 200 mM (Gibco)</td>
<td></td>
</tr>
<tr>
<td>1x Trypsin (Gibco)</td>
<td></td>
</tr>
<tr>
<td>Phosphate buffer saline pH 7 (Gibco)</td>
<td></td>
</tr>
<tr>
<td>Non-essential amino acid (Gibco)</td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase assay kit</td>
<td>Cambridge Bioscience Ltd (Cambridge, UK)</td>
</tr>
<tr>
<td>Glutathione reductase assay kit</td>
<td></td>
</tr>
<tr>
<td>OxiSelect Total Glutathione Assay Kit</td>
<td></td>
</tr>
<tr>
<td>Amplex Red Catalase Assay Kit</td>
<td>Abcam (Cambridge, UK)</td>
</tr>
<tr>
<td>Superoxide Dismutase Activity Assay Kit (colorimetric)</td>
<td></td>
</tr>
</tbody>
</table>
4.2.2 Extraction and isolation of GF28

Gelatin was extracted from salmon skin using the method in 2.2.2. GF28 was then further isolated from the alcalase hydrolysate according to methods in 2.2.4.

4.2.3 Cell culture

HepG2 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10 % foetal bovine serum, 1 % penicillin-streptomycin, 1 % non-essential amino acid and 1 % L-glutamine. Cells were sub-cultured when cells were 80 % confluent using 1 mL (25 cm² flasks) or 2 mL (75 cm² flasks) 0.01 % trypsin after washing with 5 mL phosphate buffer saline pH 7. Seeded cells were incubated at 37 °C in a 5 % CO₂ humidified incubator. HepG2 cells were obtained from Sigma-Aldrich at Passage 48 (P 48). HepG2 P48 to P60 were used in the experiments below.

4.2.4 Effect of GF28 on the cell viability of HepG2 cells (MTT assay)

The MTT assay was used to measure the number of viable cells using the tetrazolium dye thiazolyl blue tetrazolium bromide (MTT). Oxidoreductase enzymes found in viable cells will reduce the MTT dye to a purple insoluble formazan. Thus, the higher intensity of the purple colour reflects a higher number of viable cells present.

For this assay, cells were cultured in 96-well tissue culture plates at $1 \times 10^4$ cells/ 100 µL for each well. After 24 h when cells were 50 % confluent, 100 µL of a concentration range of GF28 (0.05-1.00 mg/mL) was used to treat the cells for another 24 h. Then, 20 µL MTT dye prepared at 5 mg/mL in phosphate buffer saline (PBS) was added to each individual wells and incubated in 37 °C and 5 % CO₂ humidified incubator.
After a 2 h incubation period, the culture medium and MTT dye mixture was aspirated and 100 µL dimethyl sulfoxide (DMSO) was further added to the wells to solubilise the formazan crystals. The 96-well tissue culture plate was shaken for 3 min at room temperature and the intensity of the purple colour was measured at 570 nm using a plate reader (FLUOstar Omega, BMG Labtech).

Cells treated with just media were used as control. Each sample was repeated 6 times and the experiment was also repeated on three different separate occasions to ensure reproducibility. Cell viability (%) was calculated using the following formulae:

\[
\text{Cell viability} \left(\%\right) = 100 - \left[\left(\frac{C - S}{C}\right) \times 100\right]
\]

Where C is defined as the absorbance of control (treated with media only) at 570 nm

\( S \) is defined as the absorbance of samples (treated with GF28) at 570 nm

4.2.5 Effect of GF28 on HepG2 cell morphology

HepG2 cells were seeded at 1 x 10^6 cells/ 5 mL in 25 cm² cell culture flasks and incubated for 24 h. When cells were 50 % confluent, different concentrations (0.100, 0.150 and 0.175 mg/mL) of GF28 were added to individual flasks and incubated for another 24 h in a humidified incubator (37 °C, 5 % CO₂). Changes in cell morphology were observed under a phase contrast microscope fitted with a camera (Nikon Eclipse TS100) at a magnification of 10 x.
4.2.6 Effect of GF28 on the endogenous antioxidant defence system

4.2.6.1 Collection of cell lysate

HepG2 cells were seeded at 1 x 10^6 cells/5 mL in 25 cm^2 cell culture flasks for 24 h. When cells were 50 % confluent, different concentrations (0.100, 0.150 and 0.175 mg/mL) of GF28 were added to individual flasks and incubated for another 24 h in a humidified incubator (37 °C, 5 % CO₂). The media solution of the flasks were aspirated and washed with 3 mL phosphate buffer solution. Cold PBS (1 mL) was added and the cells were collected using a rubber policeman. The solution was collected in an eppendorf and centrifuged for 10,000 x g for 3 min. Then, the supernatant containing the cell lysate was collected and stored at -80 °C for further use. Cell lysates from flasks treated with media alone were also used as control.

4.2.6.2 Superoxide dismutase activity assay

The superoxide dismutase (SOD) activity of HepG2 cell lysate was determined according to the manufacturer’s instructions using the Superoxide Dismutase Activity Assay Kit (Colorimetric). This assay was based on the reduction of a superoxide anion (O₂⁻); the reagent WST-1 produces a water-soluble formazan upon O₂⁻ reduction. The absorbance of the water-soluble formazan was read at 450 nm. However, the rate of reduction of O₂⁻ using WST-1 was inhibited by SOD. Thus, higher absorbance indicated lower SOD activity. Cell lysates collected in 4.2.6.1 were used in this experiment. All samples were run in triplicate and the experiment was also repeated on three separate occasions.
4.2.6.3 Catalase assay

The effect of GF28 on the catalase activity in HepG2 cells was further investigated. The experiment was conducted according to the manufacturer’s instructions. Catalase reacts with $\text{H}_2\text{O}_2$ to produce water and oxygen. This assay is based on the production of resorufin. In the presence of horse radish peroxidase, the Amplex Red reagent reacts with excess $\text{H}_2\text{O}_2$ to produce resorufin. Resorufin is detected at 560 nm in a plate reader. Thus, a higher amount of resorufin produced reflected lower catalase activity. Cell lysates collected in 4.2.6.1 were used in this experiment. All samples were run in triplicate and the experiment was also repeated in three separate occasions.

4.2.6.4 Total glutathione assay

Total glutathione was quantified according to the instructions in the OxiSelect Total Glutathione Assay Kit. Glutathione reductase (GR) was used to reduce oxidised glutathione (GSSG) to the reduced form (GSH). Then, the thiol found in GSH reacts with the chromogen to produce a coloured compound with an absorbance at 405 nm. Thus, higher amounts of GSH and GSSG lead to a higher absorbance at 405 nm. Cell lysates collected in 4.2.6.1 were used in this experiment. All samples were run in triplicate and the experiment was also repeated on three separate occasions.
4.2.6.5 Glutathione peroxidase assay

The glutathione peroxidase (GPx) activity of cell lysates prepared in 4.2.6.1 was determined in accordance to the kit’s instructions. In the presence of hydroperoxides, glutathione peroxidase (GPx) oxidises GSH to GSSG. Then by oxidising NADPH to NADP⁺, glutathione reductase (GR) recycles GSSG into the reduced form. The kit was based on the reduction of absorbance at 340 nm when NADPH was oxidised to NADP⁺. The reduction of absorbance was proportional to the GPx activity. All samples were run in triplicate and the experiment was further repeated on three separate occasions.

4.2.6.6 Glutathione reductase assay

Similar to the principle in 4.2.6.5, the GR activity of cell lysates from 4.2.6.1 was based on the reduction of absorbance at 340 nm when NADPH oxidised to NADP⁺. The reduction in absorbance was proportional to the GR activity. All samples were run in triplicate and the experiment was repeated on three separate occasions.

4.2.7 Statistical analysis

All experiments were done in triplicate (unless stated otherwise) and on three different occasions. Results were analysed using GraphPad Prism version 6.0. ANOVA was used to compare the means of the data followed by the Tukey’s post-hoc test unless stated otherwise. The details of the statistical tests were provided in the figure legends. A statistical difference was observed when $p \leq 0.05$. Data were presented as mean ± SEM.
4.3 Results

4.3.1 Cell viability assay of HepG2

The effect of GF28 on the cell viability of HepG2 cells was investigated using the MTT assay. HepG2 cells were treated with 0.05-1.0 mg/mL GF28 for 24 h. Figure 4.1 showed that GF28 exhibited anti-proliferative effects on HepG2. The cell viability of HepG2 was also inversely correlated to the concentration of GF28. Marked reduction in the cell viability was observed in HepG2 treated with 0.125 mg/mL GF28 (67.9%). Cell viability was also shown to significantly decrease with increased GF28 starting from 0.125-1.000 mg/mL compared to untreated cells. IC$_{50}$ of GF28 on HepG2 cells was determined to be 0.154 mg/mL. Based on the results obtained, the concentrations of GF28 used for further studies were determined to be 0.100, 0.150 and 0.175 mg/mL respectively.

Figure 4.1 Cell viability of HepG2 cells treated with GF28 for 24 h. HepG2 cells were cultured and incubated with GF28 over a concentration range of 0.05-1.00 mg/mL respectively. Means were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference ($p \leq 0.05$) to the cell viability of untreated cells was represented by a. Data correspond to the mean ± SEM ($n = 18$).
4.3.2 Effect of GF28 on the morphology of HepG2 cells

Figure 4.2 shows the cell morphology of HepG2 cells treated with 0.100, 0.150 and 0.175 mg/mL GF28 respectively. The morphology of the GF28-treated cells were compared to the untreated HepG2 cells using light microscopy at 10 x magnification. The morphology of cells treated with 0.100 mg/mL showed no significant difference to the untreated cells. On the other hand, it can be observed that increase in GF28 concentration increased HepG2 cell death under the microscope. 0.150 mg/mL GF28-treated cells showed small amount of dead cells whilst 0.175 mg/mL GF28-treated cells showed greater amount of unhealthy or dead cells than the smaller GF28 concentrations.

Figure 4.2 Morphology of HepG2 cell line in (A) untreated cells, (B) cells treated with 0.100 mg/mL GF28, (C) cells treated with 0.150 mg/mL GF28 and (D) cells treated with 0.175 mg/mL GF28. HepG2 cells were incubated with the treatments above for 24 h and observed under light microscope. Images captured were at 10 x magnification.
4.3.3 Effect of GF28 on endogenous antioxidant defence system

4.3.3.1 Superoxide dismutase (SOD) activity

Superoxide dismutase activity of untreated cells as control as well as cells treated with a concentration range of GF28 (0.100, 0.150 and 0.175 mg/mL) was shown in Figure 4.3. SOD activity of 0.100 mg/mL GF28-treated cells was similar to the control (70.2 % vs 73.5 %). 0.150 and 0.175 mg/mL GF28-treated cells (60.4 % and 46.2 % respectively) showed significantly lower SOD activities than the control. Thus, it can be observed that an increase in GF28 concentration reduces the SOD activity in HepG2 cells.

![Figure 4.3 Superoxide dismutase activity of HepG2 cells treated with GF28 for 24 h. HepG2 cells were cultured and incubated with GF28 of 0.100, 0.150 and 0.175 mg/mL respectively. HepG2 cells incubated with media alone were used as control. Means were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference (p ≤ 0.05) to the SOD activity of the control was represented by a. Data correspond to the mean ± SEM (n = 9).]
4.3.3.2 Effect of GF28 on the activity of catalase in HepG2 cells

The activity of catalase in HepG2 cells subjected to different treatments of GF28 was presented in Figure 4.4. The catalase activity in untreated cells (control) shown was 41.8 mU. There was no significant change in catalase activity when cells were treated with 0.100 mg/mL GF28 (47.9 mU). A significant increase of the catalase activity compared to the control was also observed in 0.150 mg/mL (70.5 mU) and 0.175 mg/mL (77.0 mU) GF28-treated cells. However, there was no significant difference in the activity of catalase between the two concentrations of GF28-treated cells (0.150 mg/mL and 0.175 mg/mL).

Figure 4.4 The catalase activity in HepG2 cells treated with GF28 for 24 h. HepG2 cells were cultured and incubated with GF28 of 0.100, 0.150 and 0.175 mg/mL respectively. HepG2 cells incubated with media alone were used as control. Means were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference (p ≤ 0.05) to the activity of catalase in the control was represented by a. Data correspond to the mean ± SEM (n = 9).
4.3.3.3 Effect of GF28 on the total amount of glutathione

The total amount of glutathione in untreated cells was 5.4 µM (Figure 4.5). A significant reduction of the amount of total glutathione was observed in cells treated with GF28. The total glutathione amount in 0.100 mg/mL GF28-treated cells was 3.3 µM and followed by 0.150 mg/mL (1.9 µM). Cells treated with 0.175 mg/mL GF28 showed the lowest amount of total glutathione (1.0 µM). Thus, it was shown that the amount of total glutathione was inversely correlated to the GF28 concentration used to treat HepG2 cells.

Figure 4.5 The amount of total glutathione in HepG2 cells treated with GF28 for 24 h. HepG2 cells were cultured and incubated with GF28 of 0.100, 0.150 and 0.175 mg/mL respectively. HepG2 cells incubated with media alone were used as control. Means were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference ($p \leq 0.05$) to the amount total glutathione in the control was represented by a. Data correspond to the mean ± SEM (n = 9).
4.3.3.4 Effect of GF28 on glutathione peroxidase (GPx) activity of HepG2 cells

The glutathione peroxidase (GPx) activity of HepG2 cells decreased when the concentration of GF28 used increased (Figure 4.6). GPx activity of untreated cells were normalised to 50 %. GPx activity of HepG2 cells treated with the range of GF28 (0.100, 0.150 and 0.175 mg/mL) were significantly lower than the control. On the other hand, the GPx activity of 0.175 mg/mL GF28-treated cells showed approximately half the activity of untreated cells (27.4 % vs 50.0 %).

Figure 4.6 Glutathione peroxidase activity normalised to the control in HepG2 cells treated with GF28 for 24 h. HepG2 cells were cultured and incubated with GF28 of 0.100, 0.150 and 0.175 mg/mL respectively. HepG2 cells incubated with media alone were used as control. Means were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference ($p \leq 0.05$) to the glutathione peroxidase activity in the control was represented by a. Data correspond to the mean ± SEM ($n = 9$).
4.3.3.5 Effect of GF28 on glutathione reductase (GR) activity of HepG2 cells

Figure 4.7 presented the glutathione reductase (GR) activity of HepG2 cells treated with a range of GF28 concentrations (0.100, 0.150 and 0.175 mg/mL). GR activity of untreated cells (control) was normalised to 50%. It was shown that GF28 did not affect the GR activity in HepG2 cells. All GF28-treated cells showed similar GR activity to the control.

![Figure 4.7 Glutathione reductase activity normalised to the control in HepG2 cells treated with GF28 for 24 h. HepG2 cells were cultured and incubated with GF28 of 0.100, 0.150 and 0.175 mg/mL respectively. HepG2 cells incubated with media alone were used as control. Means were compared using ANOVA followed by the Tukey’s post hoc test. Data correspond to the mean ± SEM (n = 9).](image-url)
4.4 Discussion

4.4.1 Effect of GF28 on cell viability assay and morphological changes in HepG2 cell line

HepG2 cells were incubated with a concentration range of GF28 for 24 h and the cell viability was determined using the MTT assay. GF28 exhibited anti-proliferative effects on HepG2 cells where cell death increased with increasing concentration of GF28. The effect of GF28 on HepG2 cells was observed under a light microscope. A visible amount of dead cells was also observed for 0.175 mg/mL GF28-treated cells when compared to 0.150 mg/mL.

4.4.2 Effect of GF28 on the endogenous antioxidant defence system

Superoxide dismutase plays an essential role in the antioxidant defence system whereby SOD converts superoxide radicals (\(O_2^-\)) to hydrogen peroxide (\(H_2O_2\)). It was found that SOD expression in HepG2 cells was twice the amount when compared to normal cells (Hanif et al., 2005). Clavijo-Cornejo et al. (2014) found that the transiently impaired SOD can be rapidly recovered in HepG2 cells. This may pose as a survival advantage in cancer cells and SOD can be considered as a target for liver cancer. The inhibition of SOD in cancerous cells may induce the accumulation of \(O_2^-\) and lead to reactive oxygen species (ROS) induced cellular damage and apoptosis (Huang et al., 2000). In Figure 4.3, GF28 reduced SOD activity in HepG2 cell line. An increase in GF28 concentration also led to the decrease in SOD activity. SOD activity in 0.175 mg/mL treated cells was approximately half the activity in control.
Characterised by the different redox active metals in their catalytic sites, SOD also exists as different isotypes. The cystolic and mitochondrial SOD are major enzymes in the antioxidant defence system. Cystolic SOD contains copper/zinc whereas mitochondrial SOD contains manganese. In Chapter 3, GF28 was shown to be a very effective metal chelator. GF28 would be able to bind to the metal ion in the catalytic site of SOD. This would then able to reduce the SOD activity.

Catalase catalyses the conversion of \( \text{H}_2\text{O}_2 \) produced by SOD to water and oxygen. It was expected that the reduction in SOD activity would reduce \( \text{H}_2\text{O}_2 \) and thus the catalase activity. However, the opposite was observed in Figure 4.4 where the catalase activity increased when SOD activity decreased with increasing GF28 concentration. Apart from SOD, other enzymatic systems in different cellular compartments including sulfhydryl oxidase in the endoplasmic reticulum (ER) as well as amino acid oxidases and lipid oxygenase in the cytosol can generate \( \text{H}_2\text{O}_2 \) (Giorgio et al., 2007). A possible explanation for the results may be the inhibition of SOD by GF28 that can lead to the accumulation of ROS which induced ER stress (Qu et al., 2013). ER stress further activates the unfolded protein response (UPR) which also leads to the increase of protein folding capacity in the ER (Wang and Kaufman, 2014). In the ER, sulfhydryl oxidases are responsible for oxidising sulfhydryl groups to form disulphide bonds in the folding of proteins. The formation of disulphide bonds via sulfhydryl oxidases produces \( \text{H}_2\text{O}_2 \) (Faccio et al., 2011). Thus, the increased ER stress induced by GF28 causes UPR which increases \( \text{H}_2\text{O}_2 \) production. The increased amount of \( \text{H}_2\text{O}_2 \) produced may lead to the increase of catalase activity seen in Figure 4.4.

Glutathione (GSH) is a tripeptide responsible for preventing the ROS damage of cellular components. The thiol group of the cysteine in GSH acts as a reducing agent which
inactivates ROS and produces oxidised glutathione (GSSG). The amount of total glutathione in HepG2 cells are inversely correlated with GF28 concentration (Figure 4.5). As glutathione is an essential component in the endogenous antioxidant defence system, the enzymes involved in glutathione metabolism are important. GPx are selenoproteins responsible for the conversion of GSH to GSSG whilst reducing lipid hydroperoxides to alcohols or H$_2$O$_2$ to water and oxygen (Görlach, 2014). On the other hand, GR is an important enzyme that catalyses the conversion of GSSG to GSH to regenerate the antioxidant activity of GSH. GPx activity of GF28-treated HepG2 cells was significantly lower than the control (Figure 4.6) whereas GF28 had no effect on GR activity (Figure 4.7).
4.5 Conclusion

GF28 is a peptide derived from salmon (*Salmo salar*) skin gelatin and exhibited anti-proliferative effects on HepG2 cells. MTT results showed significantly lower cell viability in 0.125 mg/mL GF28-treated HepG2 cells. IC$_{50}$ of GF28 was also determined to be 0.154 mg/mL. Under the light microscope, similar results can be observed where increased HepG2 cell death was observed with increasing GF28 concentration.

The effect of GF28 on the endogenous antioxidant system of HepG2 cells was also investigated. SOD activity was significantly decreased in 0.150 mg/mL GF28-treated cells. As GF28 was shown to exhibit effective metal chelation activity in the previous chapter, GF28 may be able to inhibit the redox active metals in the catalytic site of SOD and thus the SOD activity. As SOD was also found to be higher in cancerous cells, the inhibition of SOD activity of GF28 could be considered as a target for liver cancer treatment.

However, the activity of catalase increased in spite of the decreased H$_2$O$_2$ due to a reduction in SOD activity with GF28. This may be due to GF28 that is able to induce UPR via ER stress and the increased protein folding in the ER with sulfhydryl oxidases would increase H$_2$O$_2$ production. Thus, sulfhydryl oxidase in the increased protein folding posed as an alternative source of H$_2$O$_2$ apart from SOD causing the increase in catalase activity.

GF28 also affected the endogenous antioxidant system by decreasing the amount of total glutathione and GPx activity. Apart from that, the GR activity was unchanged in GF28-treated HepG2 cells.

In conclusion, GF28 was able to inhibit proliferation of hepatocellular carcinoma. The anti-proliferation activity of GF28 may be attributed to the effective inhibition of SOD
which was speculated to be an effective target for liver cancer treatment. The decrease of GSH and GPx activities by GF28 may also contribute to its anti-proliferative activity in HepG2 cells. However, more *in vitro* as well as *in vivo* research is needed.
Chapter 5
5 ANTI-PROLIFERATIVE EFFECT AND MECHANISMS OF GF28 ON HUMAN COLORECTAL ADENOCARCINOMA CELLS

5.1 Introduction

Colorectal cancer is the fourth most common cancer that occurs in the UK and accounts for 13% of all cancer cases (Cancer Research UK, 2011). Colorectal cancer is also known as bowel cancer which includes colon cancer and rectal cancer depending on the site of the cancer. More than 95% of colorectal cancer is comprised of adenocarcinoma (Cancer Research UK, 2013). Colorectal adenocarcinoma is the cancer that occurs at the gland cells that line the walls of the bowel.

The causes and progression of colorectal cancer can be attributed to a wide range of risk factors. Studies show that dietary patterns such as excessive alcohol, red and processed meat consumption increased colorectal cancer risk whereas the risk decreases with a high consumption of fibre as well as fruits and vegetables (Vargas and Thompson, 2012). Physical activity was also found to decrease the risk for colorectal cancer (Wolin et al., 2009).

Bioactive compounds are essential (e.g. proteins) and non-essential (e.g. polyphenols) components derived from food constituents that have an effect on human health (Biesalski et al., 2009). Studies have shown that bioactive compounds are able to exert anti-proliferative effects in different types of cancer cells via autophagy, apoptosis, cell cycle arrest and interference of cellular signal transduction (Xia et al., 2011; Gan et al., 2015; Milutinović et al., 2015).
Bioactive peptides derived from various marine sources such as oyster (Umayaparvathi et al., 2014), tuna (Hung et al., 2014) as well as Japanese threadfin bream and two-wing flying fish (Naqash and Nazeer, 2010) were also found to have anti-proliferative activities in cancer cells. Although marine sourced bioactive peptides exhibited anti-proliferative effects on cancer cells, the exact mechanisms of the peptides on cancer cells were not reported.

Thus, this study uses GF28, a bioactive peptide derived from salmon skin gelatin and further purified using ultrafiltration as well as gel filtration. GF28 was used to treat Caco-2 cells and morphological changes were also observed using a phase contrast microscope. The anti-proliferative effects of GF28-treated Caco-2 cells were further elucidated in cellular and mitochondria reactive oxygen species (ROS) production as well as other cell death mechanisms.
5.2 Methods

5.2.1 Materials and reagents

The following materials and reagents were purchased from the different suppliers:

<table>
<thead>
<tr>
<th>Materials</th>
<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td>Caco-2 cell line</td>
<td></td>
</tr>
<tr>
<td>RNase</td>
<td></td>
</tr>
<tr>
<td>Propidium iodide (PI)</td>
<td>Sigma-Aldrich (Dorset, UK)</td>
</tr>
<tr>
<td>RIPA buffer</td>
<td></td>
</tr>
<tr>
<td>Phenylmethanesulfonylfluoride (PMSF)</td>
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</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO), HPLC grade</td>
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<tr>
<td>NuPage Sample Reducing Agent</td>
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<tr>
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</tr>
<tr>
<td>Novex Sharp Pre-stained Protein Standard</td>
<td>Life Technologies (Paisley, UK)</td>
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<td>NuPage Transfer Buffer</td>
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<tr>
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</tr>
<tr>
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<tr>
<td>WesternBreeze Wash solution</td>
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<tr>
<td>Hank’s Balanced Salt Solution (HBSS) (Gibco)</td>
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<tr>
<td>CM-H₂DCFDA (Molecular Probes)</td>
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<td>MitoSOX (Molecular Probes)</td>
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<tr>
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<td>Caspase-Glo 9 Assay</td>
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<tr>
<td>β-Actin Mouse mAB</td>
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</table>
5.2.2 Extraction and isolation of GF28

Gelatin was extracted from salmon skin using the method described in 2.2.2. GF28 was then further isolated from the alcalase hydrolysate according to methods in 2.2.4.

5.2.3 Cell culture

Caco-2 cells (P 32) were grown in similar conditions as described in 4.2.3. P32 to P55 was used in all experiments below.

5.2.4 Effect of GF28 on the cell viability of Caco-2 cells (MTT assay)

The MTT assay to observe the effect of GF28 (0.05 – 1.00 mg/mL) on Caco-2 cells was performed according to 4.2.4.

5.2.5 Effect of GF28 on Caco-2 cell morphology

Cell morphology of 0.10 and 0.15 mg/mL GF28-treated Caco-2 cells were observed under a phase contrast microscope according to 4.2.5.

5.2.6 Effect of GF28 on intracellular reactive oxygen species (ROS) production in Caco-2 cells

The chloromethyl derivative of 2’, 7’-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) dye was used in the assessment of intracellular ROS levels using flow cytometry. CM-H₂DCFDA is a cell membrane permeable probe. Once inside the cell, the probe will undergo cleavage at the acetate group by intracellular esterases. Further
oxidation of the probe by ROS converts the probe into a highly fluorescent compound 2’, 7’-dichloroflourescein (DCF).

Caco-2 cells were seeded at 1 x 10^6 cells/ 5 mL in 25 cm² flasks. At 50 % confluency, cells were treated with 0.10 and 0.15 mg/mL GF28 or media alone, respectively, for 24 h. The media was poured into 15 mL centrifuge tubes and washed with 2 mL PBS. Cells were harvested using 2 mL trypsin. The trypsinised cells were resuspended using their media collected previously and centrifuged at 500 x g for 3 min. The media was then aspirated leaving the cell pellet at the bottom of the centrifuge tube. PBS (0.5 mL) and 5 µM CM-H₂DCFDA (5 µL) prepared in DMSO was added to the cell pellet and incubated for 30 min at 37 °C in 5 % CO₂. After the incubation period, cells were kept in the dark and on ice. The cellular ROS levels of 10,000 events for each samples were measured using a BDFACS Canto flow cytometer (BD Biosciences, USA) using an excitation and emission wavelength of 495 and 520 nm respectively.

5.2.7 Mitochondrial reactive oxygen species (ROS) production of GF28-treated cells

The amount of mitochondrial ROS in Caco-2 cells was determined using MitoSOX. Similar to CM-H₂DCFDA, MitoSOX is also cell-permeable and specifically targets the mitochondria. Once in the mitochondria, MitoSOX is oxidised by superoxide and turns to a red fluorescent probe which the fluorescent intensity can be detected using flow cytometry.

The method for this assay was similar to CM-H₂DCFDA (5.2.6) where 5 µM MitoSOX (5 µL) was added with 0.5 mL cold (Hank’s balanced salt solution) HBSS instead of PBS and incubated for 10 min in a 37 °C, 5 % CO₂ humidified incubator. After the incubation period, cells were kept in the dark and on ice. The mitochondrial ROS levels for 10,000
events in each sample were measured using a BD FACS Canto flow cytometer (BDBiosciences, USA) with an excitation and emission wavelength of 510 and 580 nm respectively.

5.2.8 Cell cycle assessment of GF28-treated cells

The effect of GF28 on the cell cycle of Caco-2 cells was investigated using flow cytometry. 1 x 10⁶ cells/ 5 mL were seeded in 25cm² flasks for 24 h until 50 % confluent. Then, 0.10 and 0.15 mg/mL GF28 as well as media alone were used to treat the cells respectively for a further 24 h. Media was collected in 15 mL centrifuge tubes and poured back to the corresponding flasks after the flasks were washed with 2 mL PBS and trypsinised with 0.01% trypsin (2 mL). The detached cells in the media were collected in centrifuge tubes and pelleted at 500 x g for 3 min in a centrifuge (Labofuge 400R, Heraeus Instruments). Media was aspirated and 200 µL of cold PBS and 1 mL of 70 % cold ethanol in PBS were added to the cells followed by vortexing. Cells were fixed for 12 h at 4 °C. Then, the tubes were centrifuged at 500 x g for 3 min and the fixing solution was discarded. PBS (1 mL) was added to wash the cells and 10 µL of RNase was added. The solution was incubated for 30 min at 37 °C and 10 µL of propidium iodide (PI) was added to stain the cells. Cells were kept on ice and in the dark until analysis using a BD FACS Canto flow cytometer (BDBiosciences, USA). 10,000 events were recorded for each sample at 530 nm and 620 nm as excitation and emission wavelengths respectively.
5.2.9 Annexin V and propidium iodide (PI) staining of GF28-treated Caco-2 cells

Anti-proliferative effect pathway of GF28 was investigated by probing treated cells with Annexin V (dyes apoptotic cells) and PI (dyes necrotic cells). Cells were seeded at 1 x 10^6/5 mL in 25cm² flasks for 24 h. Once 50% confluent, the cells were treated with 0.10 and 0.15 mg/mL GF28 or control and incubated a further 24 h. Media was collected in 15 mL centrifuge tubes and added back to the respective flasks after the flasks were washed with 2 mL PBS and trypsinised with 2 mL 0.01% trypsin. Cells were pelleted using centrifugation at 500 x g for 3 min and the media was aspirated. Assay buffer (0.5 mL), Annexin V (5 µL) and PI (5 µL) were added in sequence and kept in ice until further analysis. A sample treated with just media was used for gating. Events (10,000) were recorded for each sample where 520 nm was used to detect Annexin V labelling and 625 nm was used to detect PI labelling in samples.

5.2.10 Caspase 3/7, 8 and 9 assays

The caspase 3/7, 8 and 9 assays were done according to the manufacturer’s instructions. Briefly, cells were seeded at 1 x 10^4 cells/100 µL in white 96 well plates. After reaching 50% confluency, cells were treated with GF28 (0.10 and 0.15 mg/mL) or media alone as control for 24 h. To prepare the reaction reagent, 10 mL of buffer provided was added to the substrate. Then, 100 µL of the reaction reagent was added to each well and incubated at room temperature for 30 min. The luminescence of each well was read at an excitation and emission wavelength of 490 nm and 510-570 nm respectively. For caspase 8 and 9 assays, an additional 30 µL of MG132 inhibitor was added to the reaction reagent.
5.2.11 Western blotting

5.2.11.1 Cell treatment and sample collection

1 x 10⁶ cells/ 5 mL were seeded in T25 flasks. Once 50% confluent, cells were treated with GF28 (0.10 and 0.15 mg/mL) or media alone as control for 24 h. Cells adhered to the flasks were scrapped using rubber-policemen and poured into 15 mL centrifuge tubes. The tubes were centrifuged at 500 x g for 3 min at 4 °C and the media was aspirated. Cold PBS was added to wash the cells and the cells were again centrifuged and PBS aspirated. Lysis solution with 1mM phenylmethanesulfonylfluoride (PMSF) (300 µL) was added to each tube. Cells were immediately sonicated and put on ice for 30 min. The tubes were centrifuged at 500 x g for 3 min and the supernatant was aliquoted and stored at - 80°C until further use.

5.2.11.2 Sample preparation, loading and electrophoresis

Samples (15 µg) were added to 2 µL of reducing agent and 4 µL sample buffer. The total volume of samples were made up to 15 µL using milliQ water. Samples were heated at 70 °C for 10 min and chilled on ice prior to use.

A gel was removed from its plastic packaging and the white strip and comb was also removed. Running buffer was prepared by adding 50 mL running buffer to 950 mL milliQ water. The running buffer was used to rinse the wells of the gel to remove any air bubbles. The gel was placed and secured in a chamber. Running buffer was then poured into the chamber covering the wells. Samples (15 µL) and protein ladder was added to each well respectively. Electrophoresis was run at 120 V for 2 h.
5.2.11.3 Gel transfer to membrane

Before the transfer, transfer buffer was prepared by adding 50 mL transfer buffer to 100 mL methanol and 850 mL milliQ water. Sponge pads, filter papers as well as the transfer membrane were soaked in the transfer buffer for at least 5 min. Then, the gel was removed from the chamber. The plastic casing was cracked open and the gel was rinsed with the transfer buffer thoroughly. A transfer sandwich was assembled using the sequence as follow: sponge pad, sponge pad, sponge pad, filter paper, transfer membrane, gel, filter paper, sponge pad, sponge pad. The transfer sandwich was placed into a transfer cartridge with the gel side of the sandwich facing the cathode (-) electrode. Transfer buffer was then used to fill the chamber and run at 30 V for 2.5 h.

5.2.11.4 Blocking

The transfer membrane was removed from the chamber and washed with milliQ water for 5 min, three times. The membrane was then rolled into a 50 mL centrifuge tube with the blocking solution (2 mL diluent A + 3 mL diluents B + 5 % skimmed milk powder) and placed in a roller for 1 h at room temperature.

5.2.11.5 Primary antibody probing

The membrane was again removed from the centrifuge tube and rinsed with milliQ water for 5 min, three times. The membrane was incubated with the primary antibody solution (2 mL diluent A + 1 mL diluents B + 7 mL milliQ water + 5 % skimmed milk powder + 1:1000 primary antibody) at 4 °C overnight. Mouse monoclonal antibodies were used for poly ADP ribose polymerase (PARP) and β-actin probing whereas rabbit monoclonal antibody was used for LaminA/C probing.
5.2.11.6 Washing and secondary antibody probing

The membrane was washed with 20 mL of wash solution (10 mL antibody wash solution + 150 mL milliQ water) for 5 min. Washing of membrane was repeated three times. Then, the membrane was incubated with the secondary antibody solution (2 mL diluent A + 1 ml diluents B + 7 mL milliQ water + 1: 15,000 secondary antibody solution) for 30 min at room temperature. The appropriate anti-mouse and anti-rabbit secondary antibodies were used according to the type of antibodies in the primary antibody probing. During the incubation period, the membrane was kept in the dark to prevent light exposure.

5.2.11.7 Membrane imaging and band density analysis

All membranes were scanned using the Odyssey CLx (LI-COR, USA) with the imaging software (Image Studio ver 3.1). A scan resolution of 169 µm was used. Band density analysis was performed using Image Studio.

5.2.12 Statistical analysis

All experiments were done in triplicate (unless stated otherwise) and on three different occasions. Results were analysed using GraphPad Prism version 6.0. One way analysis of variance (ANOVA) was used to compare the means of the data followed by the Tukey’s post-hoc test unless stated otherwise. The details of the statistical tests were provided in the figure legends. A statistical difference was observed when $p \leq 0.05$. Data were presented as mean ± standard error of mean (SEM).
5.3 Results

5.3.1 Cell viability of Caco-2 cells treated with GF28

The effect of GF28 was investigated in human colorectal adenocarcinoma cells, Caco-2 cells. In Figure 5.1, 0.05-1.00 mg/mL GF28 was used to treat the Caco-2 cells for 24 h. GF28 showed anti-proliferative effects in Caco-2 cells where cell viability was shown to decrease with increasing concentrations of GF28. The number of viable cells was significantly lower than the untreated cells when treated with 0.15-1.00 mg/mL GF28. Cells treated with lower than 0.15 mg/mL GF28 had no significant differences in cell viability compared to the control. The number of viable cells treated with 0.15 mg/mL was 64.9 %. Cell viability was 32.0 % in 1.0 mg/mL GF28-treated cells.

To further elucidate the anti-proliferative effect of GF28 on Caco-2 cells, GF28 concentrations used were 0.10 and 0.15 mg/mL respectively.

Figure 5.1 Cell viability of Caco-2 cells treated with GF28 for 24 h. Caco-2 cells were cultured and incubated with GF28 over a concentration range of 0.05-1.00 mg/mL respectively. Means were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference ($p \leq 0.05$) to the cell viability of untreated cells was represented by a. Data correspond to the mean ± SEM ($n = 18$).
5.3.2 Morphological changes of Caco-2 cells treated with GF28

After a 24 h incubation period, untreated and treated (0.10 and 0.15 mg/mL GF28) Caco-2 cells were observed under a phase contrast microscope at 10 x magnification (Figure 5.2). Treated cells (0.10 mg/mL GF28) showed slight differences in the morphology to the untreated cells, whereas 0.15 mg/mL GF28-treated cells were visibly different from the untreated cells. In Figure 5.2C, the cell membrane and cellular contents were not clearly visible in cells treated with 0.15 mg/mL GF28. Apart from that, a higher amount of dead cells were observed in 0.15 mg/mL treated cells compared to the untreated cells.

Figure 5.2 Morphology of Caco-2 cell line in (A) untreated cells, (B) cells treated with 0.10 mg/mL GF28, and (C) cells treated with 0.15 mg/mL GF28. Caco-2 cells were incubated with the treatments above for 24 h and observed under light microscope. Images captured were at 10 x magnification.
5.3.3 Intracellular and mitochondrial reactive oxygen species (ROS) by GF28-treated Caco-2 cells

Figure 5.3 showed the ROS levels produced when Caco-2 cells were treated with 0.10 and 0.15 mg/mL GF28 respectively compared to the control. Intracellular and mitochondrial ROS production (Figure 5.3A and Figure 5.3B respectively) in GF28-treated Caco-2 cells was lower compared to the control. Intracellular and mitochondrial ROS production decreased when concentrations of GF28 used increased. In Figure 5.3A, intracellular ROS production was 23.6 % (0.10 mg/mL) and 15.3 % (0.15 mg/mL) compared to 32.9 % for the control. Mitochondrial ROS production in the control was 25.2 % compared to 14.3 % and 7.8 % in 0.10 and 0.15 mg/mL GF28-treated cells (Figure 5.3B). In both intracellular and mitochondrial ROS production, 0.15 mg/mL GF28-treated Caco-2 cells were significantly lower than the control.

![Figure 5.3](image.png)

**Figure 5.3** (A) Intracellular ROS and (B) mitochondrial ROS production of Caco-2 cells treated with GF28 for 24 h. Caco-2 cells were cultured and incubated with GF28 of 0.10 and 0.15 mg/mL respectively. Caco-2 cells incubated with media alone were used as control. Means were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference ($p \leq 0.05$) to the ROS production of the control was represented by a. Data correspond to the mean ± SEM (n = 9).
5.3.4 Cell cycle assessment of GF28-treated Caco-2 cells

As GF28 showed anti-proliferative effects on Caco-2 cells, the cell cycle arrest of GF28-treated cells was investigated (Figure 5.4). In this assay, cells were treated with 0.10 and 0.15 mg/mL GF28 respectively for 24 h. Propidium iodide (PI) was then used to stain DNA where the intensity of the PI signal is proportional to the amount of DNA present.

Prior to mitosis, the different phases of the cell cycle are as follows: G1 phase (Growth phase 1), S phase (DNA synthesis phase) and G2 phase (Growth phase 2). The cell population in the different cell cycle phases of the control were 69.9 % (G1), 10.5 % (S) and 19.5 % (G2). Cells treated with 0.10 mg/mL GF28 exhibited similar cell cycle patterns to the control (69.1 %, 9.2 % and 21.8 % for G1, S and G2 phases respectively). However, when Caco-2 cells were treated with 0.15 mg/mL GF28, G1 phase significantly increased (79.4 % vs 69.9 %) and G2 phase significantly decreased (12.8 % vs 19.5 %) compared to the control.

![Figure 5.4 Effect of GF28 on cell cycle in Caco-2 cells using flow cytometry. Caco-2 cells were cultured and incubated with GF28 of 0.10 and 0.15 mg/mL respectively for 24 h. Caco-2 cells incubated with media alone were used as control. Then, cells were fixed using 70 % ethanol and stained with PI. 10,000 cells were counted for each sample. Phases of cell cycle of Caco-2 cells were classified into G1 (Growth phase 1), S (DNA synthesis phase) and G2 (Growth phase 2). Means of the different phases between the different samples were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference ($p \leq 0.05$) to the control was represented by a. Data correspond to the mean ± SEM ($n = 9$).]
5.3.5 Annexin V and propidium iodide (PI) staining of GF28-treated Caco-2 cells

The effect of GF28 on phosphatidylserine (PS) externalisation in Caco-2 cells were also investigated (Figure 5.5). Essentially, Annexin V conjugates and stains PS which indicates cell death via apoptosis whereas PI stains DNA which indicates cell death via necrosis.

Flow cytometry charts of the control as well as treated Caco-2 cells (0.10 and 0.15 mg/mL GF28 respectively) are shown in Figure 5.5A. Each graph consist of four quadrants which represent: healthy (H), apoptotic (A), necrotic (N) as well as necrotic and/or late apoptotic (NA) cells. Compared to the control, it can be observed from the flow cytometry charts that when the concentration of GF28 increased there was a gradual increase in the cell population in A and NA quadrants as well as a decrease in the H quadrant.

The charts were then further represented using bar graphs in Figure 5.5B. The cell population of the untreated cells (control) in the different quadrants are 90.7 % (H), 0.8 % (N), 1.1 % (A) and 7.4 % (NA). In general, apart from the cell population in the N quadrant the cell population in H, A and NA quadrants in GF28-treated cells were significantly different to the control. The number of healthy cells significantly decreased when cells were treated with 0.10 and 0.15 mg/mL GF28 (70.8 % and 43.3 % respectively) when compared to 90.7 % control. Apart from that, the cell population of 0.10 and 0.15 mg/mL GF28-treated Caco-2 cells in A (6.5 % and 29.2 % respectively) and NA (21.5 % and 24.0 % respectively) was significantly higher than the control.
Figure 5.5 Effect of GF28 on cell death pathway of Caco-2 cells using flow cytometry. Caco-2 cells were cultured and incubated with GF28 of 0.10 and 0.15 mg/mL respectively for 24 h. Caco-2 cells incubated with media alone were used as control. (A) Flow cytometry charts of cells treated with (i) control (ii) 0.10 mg/mL GF28 and (iii) 0.15 mg/mL GF28. Cells were then stained with Annexin-V and PI. Then, 10,000 cells were counted for each sample where the cell population was categorised into four quadrants: Healthy (H), Apoptotic (A), Necrotic (N) as well as Necrotic and/or apoptotic (NA). (B) Graphical representation of the flow cytometry charts. Means of the different quadrants between the different samples were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference ($p \leq 0.05$) to the control was represented by a. Data correspond to the mean ± SEM ($n = 9$).
5.3.6 Intrinsic pathway of apoptosis

5.3.6.1 Expression of caspase-9

As staining of Caco-2 cells treated with GF28 showed cell death via the apoptosis. The different pathways of apoptosis were investigated. Caspase-9 is involved in the intrinsic signalling pathway of apoptosis. **Figure 5.6** illustrated the effect of GF28 on the caspase-9 expression of Caco-2 cells. The luminescence unit of the control was set at 50 RLU. Caspase 9 expression of 0.10 mg/mL GF28-treated cells (59.3 RLU) was not significantly different to the control. However, the caspase-9 expression was shown to significantly increase in 0.15 mg/mL GF28-treated cells (116.3 RLU) compared to the control.

![Figure 5.6](image)

**Figure 5.6** Effect of GF28 on caspase-9 expression using luminescence assay. Caco-2 cells were cultured and incubated with GF28 of 0.10 and 0.15 mg/mL respectively for 24 h. Caco-2 cells incubated with media alone were used as control. Excitation wavelength used was 490 nm and the emission wavelength was 510 nm. The luminescence of each sample was expressed as the relative luminescence units (RLU) normalised to the control (50 RLU). Mean RLU of the different samples were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference ($p \leq 0.05$) to the control was represented by a. Data correspond to the mean ± SEM ($n = 9$).
5.3.6.2 Effect of GF28 on the activation of Lamin A and Lamin C in Caco-2 cells

As GF28 showed anti-proliferative effects on Caco-2 cells via caspase-9 activation, the effects of GF28 on cleaved Lamin A and C were further investigated in Figure 5.7. The expression of cleaved Lamin A and C in Caco-2 cells treated with GF28 were compared to untreated cells (control) using western blot analysis. Figure 5.7A showed the western blot membrane of the expression of cleaved Lamin A and C using β-actin as the loading control. The activation of Lamin A and C can be observed from the amount of Lamin A and C cleaved. Darker bands for cleaved Lamin A (41 kDa) and cleaved Lamin C (28 kDa) were observed in 0.15 mg/mL GF28-treated Caco-2 cells compared to the control.

In Figure 5.7B, the activation of both Lamin A and Lamin C was standardised to 1. Lamin A activation of 0.10 and 0.15 mg/mL GF28-treated Caco-2 cells were 2.9 and 9.7 fold respectively when compared to the control. In addition, Lamin C activation for GF28-treated cells were 2.5 and 9.6 fold (0.10 and 0.15 mg/mL GF28 respectively) to the expression of control. In conclusion, compared to the control, the Lamin A and C activation significantly increased in 0.15 mg/mL GF28-treated Caco-2 cells whereas the 0.10 mg/mL GF28-treated cells showed no difference in its Lamin A and C activation.
Figure 5.7 Effect of GF28 on Lamin A and C activation using western blot. Caco-2 cells were cultured and incubated with GF28 of 0.10 and 0.15 mg/mL respectively for 24 h. Caco-2 cells incubated with media alone were used as control. Cell lysate was then collected using RIPA buffer with a protease inhibitor cocktail and PMSF. (A) Western blot membrane showing Lamin A (74 kDa), Lamin C (63 kDa), cleaved Lamin A (41 kDa) and cleaved Lamin C (28 kDa) for each sample. β-actin (45 kDa) was used as a loading control. (B) Graphical representation of (i) Lamin A and (ii) Lamin C activation by the band density of cleaved Lamin A and cleaved Lamin C respectively. The band density of each sample was expressed as activation of Lamin A or C of sample normalised to the control which was set at 1. Expression of the different samples was compared using ANOVA followed by the Tukey’s post hoc test. A significant difference ($p \leq 0.05$) to the control was represented by $\text{a}$. Data correspond to the mean ± SEM ($n = 9$).
5.3.7 Extrinsic pathway of apoptosis

5.3.7.1 Expression of caspase-8 and caspase-3/7 in GF28-treated cells compared to untreated cells

Although apoptosis can be regulated by the intrinsic pathway, apoptosis can also be activated via the extrinsic pathway. Caspase-8 and caspase-3/7 are located at the downstream signalling of the extrinsic pathway.

Caspase-8 and caspase-3/7 expression are expressed as RLU in Caco-2 cells treated with GF28 compared to untreated cells (control) in Figure 5.8. Caspase-8 expression in GF28-treated cells (78.0 RLU and 112.5 RLU for 0.10 and 0.15 mg/mL respectively) were significantly higher compared to the control (50 RLU). For caspase-3/7 expression, the RLU of 0.10 and 0.15 mg/mL GF28 (78.6 RLU and 122.6 RLU respectively) treated cells were also significantly higher compared to the control (50 RLU). Thus, indicating that the anti-proliferative effect of GF28 in Caco-2 was also due to the activation of caspase-8 as well as caspase-3/7 which are responsible for the extrinsic pathway of apoptosis.

Figure 5.8 Effect of GF28 on (A) caspase-8 and (B) caspase-3/7 expression using luminescence assay. Caco-2 cells were cultured and incubated with GF28 of 0.10 and 0.15 mg/mL respectively for 24 h. Caco-2 cells incubated with media alone were used as control. Excitation wavelength used was 490 nm and the emission wavelength was 510 nm. The luminescence of each sample was expressed as the relative luminescence units (RLU) normalised to the control (50 RLU). Mean RLU of the different samples were compared using ANOVA followed by the Tukey’s post hoc test. A significant difference ($p \leq 0.05$) to the control was represented by $a$. Data correspond to the mean ± SEM ($n = 9$).
5.3.7.2 Effect of GF28 on PARP activation

The activation of caspase-3/7 in GF28-treated Caco-2 cells indicated that the extrinsic pathway as one of its anti-proliferative apoptotic pathway. Effect of GF28 on the activation of PARP was further investigated in Figure 5.9. Using β-actin as a loading control, the western blot membrane (Figure 5.9A) showed darker bands of cleaved PARP (89 kDa) in GF28-treated Caco-2 cells compared to the control. PARP activation is indicated by PARP cleavage, thus 0.15 mg/mL showed high PARP activation by its darker band in the cleaved PARP.

PARP activation is further represented in a bar graph (Figure 5.9B). PARP activation normalised to the control of 0.10 mg/mL and 0.15 mg/mL (3.2 fold and 5.2 fold respectively) were significantly higher than the control (normalised to 1).

![Figure 5.9](image)

**Figure 5.9** Effect of GF28 on PARP activation using western blot. Caco-2 cells were cultured and incubated with GF28 of 0.10 and 0.15 mg/mL respectively for 24 h. Caco-2 cells incubated with media alone were used as control. Cell lysate was then collected using RIPA buffer with a protease inhibitor cocktail and PMSF. (A) Western blot membrane PARP (116 kDa) and cleaved PARP (89 kDa) for each sample. β-actin (45 kDa) was used as a loading control. (B) Graphical representation of PARP activation by the band density of cleaved PARP. The band density of each sample was expressed as activation of sample normalised to the control which was set at 1. Activation of PARP in different samples was compared using ANOVA followed by the Tukey’s post hoc test. A significant difference \( p \leq 0.05 \) to the control was represented by a. Data correspond to the mean ± SEM \( (n = 9) \).
5.4 Discussion

The cell viability results (Figure 5.1) and morphological observations (Figure 5.2) indicated that GF28 had anti-proliferative effects on human colorectal adenocarcinoma cells. Caco-2 cells treated with GF28 concentration of 0.15 mg/mL were able to show significant cell death after 24 h compared to the untreated cells as control. IC$_{50}$ of GF28 was also shown to be 0.16 mg/mL from the MTT results. Under the phase contrast microscope, 0.10 mg/mL GF28-treated cells had similar morphology whereas 0.15 mg/mL GF28-treated Caco-2 cells exhibited significant morphological changes compared to the control. Therefore, the GF28 concentration used in this study to further elucidate the cell death mechanism in Caco-2 cells was 0.10 and 0.15 mg/mL.

Apart from causing damage to proteins, lipids and DNA, excess ROS can also act as mediators towards cell death (Marchi et al., 2012). Mounting evidence of mitochondrial ROS in particular, can lead to the release of pro-apoptotic proteins which can trigger programmed cell death (Thornton and Hagberg, 2015; Fleury et al., 2002). Thus, GF28 can cause mitochondrial dysfunction which lead to excess ROS production and cell death.

Although GF28 showed anti-proliferative effects according to the MTT and morphological observations, intracellular and mitochondrial ROS decreased in GF28-treated cells compared to control (Figure 5.3). The observed results may be due to GF28 acting as an efficient antioxidant as described in Chapter 3.

GF28-treated Caco-2 cells also showed cell cycle arrest at the G1 phase (Growth phase 1). The accumulation of cell population in the G1 phase suggested that cells are blocked in the transition of G1 to S phase. During the G1 phase, DNA replication and the transition to S
phase is initiated via the activity of growth dependant cyclin dependant kinase (CDK) (Bertoli et al., 2013). This suggests that GF28 may be able to alter CDK activity which could prevent Caco-2 cells progressing to the S phase, leading to the prevention of Caco-2 cell growth and proliferation.

As cell death mechanisms can be divided into apoptosis, necrosis and necroptosis, the anti-proliferative mechanism of GF28 in Caco-2 cells was further investigated. Annexin V and PI were used to identify the cell death pathway of the Caco-2 cells treated with GF28 for 24 h. Annexin V conjugates and stains apoptotic cells whereas PI stains necrotic cells. It was shown that increasing GF28 concentration increased the number of apoptotic as well as late apoptotic and/or necrotic cells. The cell population classified as apoptotic cells were 6.5 % and 29.2 % (0.10 and 0.15 mg/mL respectively) compared to 1.1 % in the control. Thus, GF28 was shown to cause cell death in Caco-2 cells via apoptosis.

The different apoptosis pathway of Caco-2 cells treated with GF28 was further investigated in this study. There are two general apoptosis signalling pathway: the intrinsic pathway is activated by cellular stress and involves the mitochondria signalling cascade and caspase-9 downstream; the extrinsic pathway involves the activation of death receptor ligands and caspase-8 downstream (Fulda and Debatin, 2006). Both intrinsic and extrinsic pathways will cleave caspase-3/7 which would further induce apoptosis (Chipuk and Green, 2006).

GF28 showed induction of apoptosis via the intrinsic pathway by caspase-9 activation. Caco-2 cells treated with 0.15 mg/mL GF28 showed 116.3 RLU compared to 50.0 RLU in the control. The downstream activation of the intrinsic pathway was also further confirmed via the activation of Lamin A and C. Both Lamin A and C are cleaved by caspase-6 which
is the downstream of caspase-9. GF28-treated cells showed significantly higher Lamin A and C cleavage compared to the control. Cleavage of Lamin A and C shown in Figure 5.7 also indicated the activation of both Lamin A and C. This is responsible for the shrinkage of cell and membrane blebbing during apoptosis.

Activation of caspase-8 is also one of the key signalling mediators in the extrinsic pathway of apoptosis. As illustrated in Figure 5.8, GF28 was also able to induce higher expression of caspase-8 in Caco-2 cells. Apart from that, GF28 also showed activation of caspase-3/7 which is downstream of both the extrinsic and intrinsic pathway.

The activation of caspase-3/7 was confirmed via western blotting of PARP (Figure 5.9). In the attempt of cellular repair, activation of PARP causes ATP depletion. However, excess ATP depletion can also trigger cell lysis and cell death via necrosis (Los et al., 2002; Fan and Jong, 2014). Apart from that, upon activation of PARP, poly(ADP-ribose) (PAR) will translocate from the cytosol to the outer membrane of mitochondria (Yu et al., 2006). This would lead to the release of apoptosis inducing factor (AIF) which can cause pro-apoptotic activities of DNA fragmentation and chromatin condensation (McGill et al., 2012; Doti et al., 2014).
5.5 Conclusion

GF28, a peptide derived from salmon skin gelatin, exhibited anti-proliferative effects in human colorectal adenocarcinoma cells (Caco-2). MTT results exhibited IC$_{50}$ of 0.16 mg/mL for GF28. Caco-2 cells treated with 0.15 mg/mL GF28 also showed significant morphological changes when compared to the control. Intracellular and mitochondrial ROS was also significantly lower in GF28-treated cells.

Cell cycle studies in Caco-2 cells also showed that cells treated with GF28 induced cell cycle arrest at the G1 phase, preventing the cells to continue to the S phase for DNA replication. Apart from that, it was shown that GF28-treated cells undergo apoptosis instead of necrosis as the cell death mechanism.

Apoptosis can be divided into the intrinsic and extrinsic pathway. GF28 was also able to induce caspase-9 cleavage and activation of Lamin A and C which are involved in the extrinsic apoptosis pathway. Besides that, Caco-2 treated with GF28 also showed significantly higher activation of caspase-8 which is a key mediator in the intrinsic apoptotic pathway. Results also showed the activation of caspase-3/7 and PARP which are the downstream of both the extrinsic and intrinsic pathway.

In conclusion, GF28 showed anti-proliferative effects on Caco-2 cells; the cells underwent programmed cell death, apoptosis. The apoptosis pathway induced by GF28 was also elucidated in this study. GF28 showed promising results in terms of the anti-proliferative mechanism which involved apoptosis in colorectal carcinoma cells. However, more studies
are still needed to further investigate the exact anti-proliferative pathways induced by GF28.
Chapter 6
6 General discussion and conclusion

6.1 General discussion

6.1.1 Isolation and purification of peptides from salmon skin gelatin

The increasing demand for products derived from natural sources over the past decade can be attributed to the increased awareness in the potential health benefits of these products. Bioactive peptides in particular, are given much attention as they can be easily derived from natural sources and many studies showed that bioactive peptides exhibited a wide range of health benefits.

In this study, gelatin was extracted from salmon skin using an acidic extraction method. Pepsin, alcalase and collagenase were used to hydrolyse the extracted gelatin. The most potent hydrolysate was further purified by ultrafiltration and gel filtration. To obtain a peptide fraction with the highest activity, the antioxidant and ACE inhibition activity amongst the hydrolysates as well as the ultrafiltered and gel filtered purified fractions were compared.

Antioxidant and ACE inhibition activity (61.6 % and 59.0 %, respectively) of the alcalase hydrolysate were higher compared to the pepsin and collagenase hydrolysates. As mentioned above, the alcalase hydrolysate was further purified. The antioxidant activity did not significantly differ amongst the purified fractions whereas GF28 showed the highest ACE inhibition activity. Thus, GF28 from the gel filtration fraction was chosen for further investigation in this study.
6.1.2 ACE inhibition mechanisms of GF28

The ACE inhibition mechanisms of GF28 were investigated with enzyme kinetics. A Lineweaver Burk plot illustrated similar $V_{\text{max}}$ values (10.5 mM/min) but different $K_{\text{m}}$ values; this indicated that GF28 acts as a competitive inhibitor on ACE.

A zinc ion can be found in the active site of ACE and the chelation of the metal ion can lead to ACE inhibition effects. Thermodynamics results showed GF28 was able to bind to zinc ions. This result further confirmed the enzyme kinetic findings which showed competitive inhibition of ACE by GF28.

6.1.3 Antioxidant mechanisms of GF28

The reducing power, radical scavenging ability and metal ion chelation ability of GF28 were investigated to determine its antioxidant mechanism. There was only a slight increase in the reducing power activity with increasing concentration of GF28. The maximum reducing power activity exhibited by 10 mg/mL GF28 was 9.8 %. DPPH and ABTS radical scavenging assays measure the ability of GF28 to act as an electron donor in both hydrophobic and hydrophilic environment. GF28 showed poor DPPH and ABTS scavenging activity (3.5 % and 4.5 % respectively). However, the transition metal ions chelation activity of GF28 was high. GF28 was able to effectively chelate iron and copper transition ions ($IC_{50}$: 1.0 and 7.6 mg/mL respectively).

Thus, the high antioxidant activity observed in GF28 may be mainly attributed to the high transition metal ion chelating activity.
6.1.4 Amino acid composition of GF28

Amino acid results showed that GF28 was mainly comprised of glycine (42.2%) and hydrophobic amino acid (28.3 %) was the predominant amino acid group. The presence of hydrophobic as well as bulky aromatic amino acids (9.2 %) play a role in ACE inhibition whereby aromatic amino acids can bind to ACE, blocking its active site. The amount of hydrophobic amino acid may also be responsible for the antioxidant activity of GF28. The high transition metal ion chelating activity of GF28 may be associated with the hydrophobic amino acid.

6.1.5 Effect of GF28 in hepatocellular carcinoma cells

HepG2 cells were used as a model of hepatocellular carcinoma cells to investigate the effect of GF28. MTT results showed that GF28 exhibited anti-proliferative effects in HepG2 cells; the IC$_{50}$ of GF28 was determined as 0.154 mg/mL. This was confirmed in GF28-treated HepG2 cells observed under the phase contrast microscope. The increase in GF28 concentration in HepG2 cells led to an increase in cell death. As liver is the main detoxifying organ in the body and GF28 showed anti-proliferation effects on HepG2 cells, the effect of GF28 in the endogenous antioxidant system was investigated.

A higher amount of SOD is expressed in cancer cells compared to normal cells. Apart from that, the rapid recovery of SOD in injured cancer cells may pose as a survival mechanism for cancer cells. Thus SOD may act as a target for hepatocellular carcinoma cells. An increase in GF28 concentration led to a significant decrease in SOD activity. The SOD activity in 0.175 mg/mL GF28-treated cells was significantly lower than untreated cells (46.2 % vs 73.5 %). SOD can be divided into several isoforms according to the
different redox active metals at the catalytic site. The significant decrease in SOD activity may be due to the high metal ion chelation activity of GF28.

Due to the decrease of SOD in GF28-treated cells, the amount of H$_2$O$_2$ produced will decrease. Thus the decrease in catalase activity is expected in GF28-treated cells compared to untreated cells as catalase catalyses the conversion of H$_2$O$_2$ to oxygen and water. However, GF28-treated HepG2 cells showed an increase in catalase activity. GF28 may induce ER stress which can lead to UPR activation, acting as another source of H$_2$O$_2$.

GF28 also affected the endogenous antioxidant system by decreasing the amount of total glutathione and GPx activity. In addition, the GR activity was unchanged in GF28-treated HepG2 cells.

The above effects of GF28 on the endogenous antioxidant defence system may play a role in the development of liver cancer treatment.

**6.1.6 Effect of GF28 on colorectal adenocarcinoma cells**

Colorectal cancer is the fourth most common cancer in the UK. Caco-2 cells were used in this study to illustrate the effect of GF28 on colorectal adenocarcinoma cells. GF28 showed toxicity effects on Caco-2 cells causing cell death. IC$_{50}$ was determined to be 0.16 mg/mL. Morphological changes of GF28-treated Caco-2 cells also confirmed the anti-proliferative effects of GF28.

In general, injured cells can lead to increased ROS production. However, intracellular and mitochondria ROS production in 0.15mg/mL GF28-treated Caco-2 cells were significantly lower than untreated cells. The lower ROS produced may be due to the antioxidant activity
of GF28. Further cell cycle studies of GF28-treated Caco-2 showed cell cycle arrest at the G1 phase. The accumulation of cell population in the G1 phase suggested that cells are blocked in the transition of G1 to S phase.

Cell death can be divided into apoptosis, necrosis and necroptosis. As GF28 induced cell death in Caco-2 cells, flow cytometry using Annexin V and PI was used to stain dying cells to determine the cell death pathway. It was shown that increasing GF28 concentration increased the number of apoptotic as well as late apoptotic and/or necrotic cells.

The apoptotic pathway of GF28-treated Caco-2 cells was further investigated. The extrinsic as well as intrinsic apoptotic pathways were activated in GF28-treated Caco-2 cells. GF28 was found to activate the intrinsic apoptotic pathway via the activation of caspase-9 as well as the downstream signaling of Lamin A and Lamin C which are responsible for cell shrinkage and membrane blebbing. The extrinsic apoptotic pathway was also activated by GF28. Caspase-8 and its downstream signaling of caspase-3/7 were activated. This was confirmed via western blotting of PARP that is responsible for DNA fragmentation and chromatin condensation during apoptosis.
6.2 General conclusions

- Alcalase hydrolysed salmon skin gelatin hydrolysate had higher antioxidant and ACE inhibition activity than pepsin and collagenase hydrolysates.
- The most potent fraction of the alcalase hydrolysate purified by ultrafiltration and gel filtration was GF28.
- Enzyme kinetics showed that GF28 acts as a competitive inhibitor of ACE. This was further confirmed by the thermodynamic study which showed that GF28 was able to bind zinc. Zinc ions are located in the active site of ACE, which play a role in its activity.
- GF28 showed potent transition metal ion chelating activity and low reducing power activity. Radical scavenging activity was also poor in GF28.
- Amino acid composition of GF28 indicated the high amount of hydrophobic amino acids (28.3 %) may play a role in its antioxidant and ACE inhibition activity.
- GF28 exhibited anti-proliferative effects in both hepatocellular carcinoma (HepG2) and colorectal adenocarcinoma (Caco-2) cell lines.
- The effects of GF28 on the endogenous antioxidant defence system may play a role in the cytotoxicity effects on HepG2 cells.
- GF28 decreased SOD and GPx activities as well as the amount of total glutathione whereas it increased catalase activity. However, GF28 did not affect GR in HepG2 cells.
- Although GF28 induced cell death in Caco-2 cells, intracellular and mitochondrial ROS decreased; this may be due to the antioxidant activity of GF28.
- Caco-2 cells treated with GF28 showed cell cycle arrest at the G1 phase. Caco-2 cells were prevented from progressing to the S phase in which DNA replication takes place.
- Caco-2 cells conjugate with Annexin V and stained with PI showed GF28 induced cell death via apoptosis.
- Intrinsic apoptotic pathway was activated in GF28-treated Caco-2 cells. Caspase-9 as well as Lamin A and Lamin C, responsible for membrane blebbing and cell shrinkage, were significantly higher in GF28-treated Caco-2 cells than untreated cells.
- GF28 also led to apoptosis via the extrinsic pathway. Caspase-8, caspase-3/7 and PARP cleavage were observed, indicating apoptotic cell death via death receptor ligand activation.

6.3 Future work

In this study, several bioactive properties were elucidated using a bioactive peptide, GF28, produced from salmon skin gelatin. This included ACE inhibition activity and antioxidant properties, as well as anti-proliferative effects on hepatocellular and colorectal adenocarcinoma cell lines. However, more research can be done in each of these areas e.g. elucidation of structure-function relationship and absorption of the peptide using a Caco-2 cell line monolayer model.

The ACE inhibition activity study can be further investigated in animal studies. Spontaneously hypertensive rats are commonly used as hypertensive models. In addition, the effect of GF28 on other blood pressure regulation system or mediators such as calcium ions can also be investigated using cell lines.
Compared to most antioxidants used in food products which act as hydrogen donors, GF28 is an effective metal chelator. For future studies, GF28 may be incorporated into food products with other hydrogen donating antioxidants such as ascorbate. This combination of antioxidants with different antioxidant mechanisms may show synergistic effects that retard lipid oxidation more effectively.

As GF28 also showed promising anti-proliferative results in liver and colorectal cancer, more evidence is needed to further develop GF28 as a cancer therapy. Microarray analysis is a useful technique to further elucidate the effect of GF28 on gene expression. Other cell death pathways such as autophagy and necroptosis can also be investigated. Cell death pathways induced by GF28 can also be compared to known anti-cancer drugs.

As elucidated in this study, bioactive peptides have many health benefits. Different bioactive peptides can be derived from different sources using different enzymes. This can lead to bioactive peptides with different lengths and amino acid sequence, thus different bioactive properties as well as mechanisms. There is a huge potential in the field of bioactive peptides but more detailed studies have to be undertaken.
References


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