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**Characterisation of storage proteins and their digesta from
oat bran, wheat bran and barley, and identification of
bioactive peptides derived from oat bran**

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy

at
Lincoln University

by
Xi Gong

Lincoln University

2023

I Humbly Dedicate This Piece of Work to My Be Loved Parents

Who Never Lose Their Faith in Me

Not all those who wander are lost. – J. J. R. Tolkien

Declaration

Parts of this thesis are submitted or accepted for publication and/or presented in advance of submission of the thesis:

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Abstract of a thesis submitted in partial fulfilment of the requirements for
the Degree of Doctor of Philosophy

Characterisation of storage proteins and their digesta from oat bran, wheat
bran and barley, and identification of bioactive peptides derived from oat bran

by
Xi Gong

Aims: The aims of this thesis were to characterise storage proteins extracted from oat bran, wheat bran and barley and the digesta produced from them in an *in vitro* digestion model, followed by investigating the antioxidant and angiotensin-converting enzyme (ACE) inhibitory activity of the digesta, and finally to identify novel peptides with antioxidant and ACE inhibitory activity in oat bran intestinal digesta.

Method: Storage protein concentrates of oat bran, wheat bran and barley were obtained by alkaline extraction and isoelectric precipitation. *In vitro* digestion model was adapted to generate their digesta protein where their protein/peptide profiles were analysed using sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The digestibility, degree of hydrolysis and the release of the free amino acids (FAAs) were determined on these digesta. Antioxidant assays including 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), total phenolic content (TPC), ferric ion reducing antioxidant power (FRAP) assay and oxygen radical absorbance capacity (ORAC) assay were conducted to evaluate the overall antioxidant activity of the crude protein and digesta. The samples were also assessed for ACE inhibitory activity. The storage proteins in oat bran were selected for further analysis by two-dimensional electrophoresis (2-D electrophoresis). The small-intestinal digesta of oat bran protein with the molecular weight (MW) of < 3 kDa was purified by anion-exchange chromatography and the selected fractions were subjected to mass spectrometry (MS) to determine the peptide sequences.

Findings: The major storage protein in oat bran protein concentrate were globulin, albumin and avenins, while prolamin, gliadin and albumin predominated in wheat bran protein concentrate, and hordein, albumin and globulin in the barley protein concentrate. These proteins were mostly

degraded into polypeptides and smaller protein fractions by the end of intestinal digestion. This process resulted in significantly higher overall antioxidant activity and ACE inhibitory power in digesta. Oat gastric and intestinal digesta showed the highest ABTS scavenging activity of 2 Trolox equivalent mM/g sample. Oat digesta also showed a higher TPC value (200 gallic acid equivalent mg/g sample) compared to wheat bran and barley digesta (150 gallic acid equivalent mg/g sample and 40 gallic acid equivalent mg/g sample). The ACE inhibitory power of all three intestinal digesta was greater than 80%. Because oat digesta showed the highest overall antioxidant and ACE inhibitory activity, oat intestinal digesta was selected for separation by anion exchange chromatography. In all elutes, the highest ABTS scavenging activity reached to 275 Trolox equivalent $\mu\text{M/g}$ sample and the highest ORAC scavenging activity was 170 Trolox equivalent $\mu\text{M/g}$ sample. Four antioxidant anionic fractions and two ACE inhibitory fractions along with the digesta < 3 kDa were subjected to reverse-phase high performance liquid chromatography-tandem mass spectrometry (R-HPLC-MS/MS). Three peptides (FEPL, LSEAL and SQAGITEY) were identified as potential antioxidants while LQAFEPL, ALPVDV and NDIL were potential ACE inhibitory peptides. The peptide LIVPQ derived from digesta < 3 kDa showed the potential to possess both antioxidant and ACE inhibitory activity.

Conclusion: These investigations have shown that storage proteins derived from oat bran, wheat bran and wholegrain barley are ideal sources to produce bioactive digesta after digestion. The peptides identified in oat bran intestinal digesta displays bioactive nature as an intermediate ACE inhibitor, with the potential of future applications in improving human health.

Keywords: Bran protein, protein extraction, *in vitro* digestion, digestibility, free amino acid profile, anion exchange chromatography, 2-D electrophoresis, oat bran digesta, antioxidant oat peptide, ACE inhibitory oat peptide

Abbreviation

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid

ACE: angiotensin-converting enzyme

BPC: barley protein concentrate

DPPH: 2,2-diphenyl-1-picrylhydrazyl

FPLC: Fast Protein Liquid Chromatography

FRAP: ferric reducing antioxidant power

GI digestion/GI tract: gastrointestinal digestion/gastrointestinal tract

IC₅₀ : half maximal inhibitory concentration

MS: mass spectrometry

MW: molecular weight

OBPC: oat bran protein concentrate

ORAC: oxygen radical absorbance capacity

pI: isoelectric point

RBPHs: rice bran protein hydrolysates

R-HPLC-MS/MS: reverse-phase high performance liquid chromatography-tandem mass spectrometry

SDS-PAGE: sodium dodecyl-sulfate polyacrylamide gel electrophoresis

TPC: total phenol content

WBPC: wheat bran protein concentrate

2-D electrophoresis/2-D gel: two-dimensional electrophoresis/two-dimensional gel

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As with any PhD project that comes to an end, I would like to mark the end of this amazing journey with a short piece. No one who achieves success do so without acknowledging the help of others and this one is no exception. In the last four years of commitment and endeavour, I received endless love and support from so many people and without whom I would not have succeed.

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Chapter 1

Literature Review

1.1 Introduction

Wheat, oats and barley have long played a crucial role in human diet as the staple cereal in the western world. Wheat, barley and oats have been top cereals produced worldwide and they served different purposes in terms of food processing: wheat has been used for bakery for centuries; oats have been getting popular in the last decades for being a healthy breakfast; barley was the main gradient for making beer. In 2021, the production of these three cereal crops were over 770 million tonnes, 23 million tonnes and 150 million tonnes (FAO, 2023) making a magnificent contribution to the market and the economy growth. Today, with the growing attention in the area of the waste recovery, cereal industries produce millfeed, the by-products (as bran, shorts, middlings) of the milling of cereal flours, as a commercial product for animal feeds and functional ingredients in food innovation area.

Many studies in recent years have focused on the functional and nutritional properties of proteins in the cereal, in particular the bran- the hard/outer layers of cereal grain (Alu'datt et al., 2012; Balandrán-Quintana et al., 2015; Van den Broeck et al., 2016) and the bioactive attributes of peptides derived from the hydrolysis of these proteins (Shobako et al., 2018; Walters et al., 2020; Yang et al., 2020). Bran has been known to contain the variety of different nutritional components including proteins, starch, lipid, dietary and bioactive compounds such as phenolic components (Fărcaș et al., 2022; Laddomada et al., 2015; Mao et al., 2022; Sidhu et al., 2007). Wheat bran contains storage protein range between 15.2% - 16.9% (Chalamacharla et al., 2018) and can be separated into major protein groups; the non-prolamin (albumin, globulin), the prolamin (gliadin) and the glutenin groups (Malik, 2009). In wholegrain wheat, gluten is the mixture of glutenin and gliadin which gives the unique elastic nature to the bread structure. Oat bran constitutes 15% - 18% protein (Duță et al., 2018) with similar storage protein groups but a

unique prolamin named avenin. Barley contains 7% - 25% protein with four major storage protein groups: albumin, globulin, prolamin and glutelin in which hordein, a unique prolamin in barley, found only in the endosperm comprising 30% - 50% of total protein while much less protein is distributed in the husk and bran (Arendt & Zannini, 2013).

In this chapter, comprehensive yet extensive literature reviews are focused in two key area relevant to the current investigation; the dominant storage proteins in wheat, barley and oat, and the proteomic studies that have been used to detect specific bioactive peptides derived from these storage proteins. The guideline of PRISMA was also utilised to create the literature review to record and investigate the studies exploring the antioxidant and angiotensin-converting enzyme (ACE) inhibitory peptides originated from oat as well as wheat, barley and rice.

1.2 Structure of Cereal Grain

Grains, including oats, wheat and barley, consist of three main parts: the bran, endosperm, and embryo (Figure 1.2.1 showing the anatomical structure). Grains have a tough outer layer of protection known as hull or husk with high content of cellulose, hemicellulose, lignin, arabinoxylan and other carbohydrate polymers. Bran is located in between the husk and endosperm with multiple layers serving as the coat of the seed kernel. The seed kernel usually refers to endosperm and embryo which are required for seed to grow. As shown in Figure 1.2.1, endosperm contains high protein and starch content acting as the nutrient reservoir of grains while germs are rich in lipid, proteins and vitamins and is also the core of seeds where the germination occurs. Bran, on the other hand, contains high storage protein content and is edible but could be removed and wasted during flour milling. Bran protein distribute mostly in aleurone layer of the bran followed by testa and pericarp, respectively (Balandrán-Quintana et al., 2015). Wheat bran protein content ranges between 15.2% - 16.9% (Chalamacharla et al., 2018) while oat bran contains 15% - 18% protein (Duță et al., 2018). No study has been found indicating the presence of any different type of storage protein in the bran compared to the storage protein found in kernel.

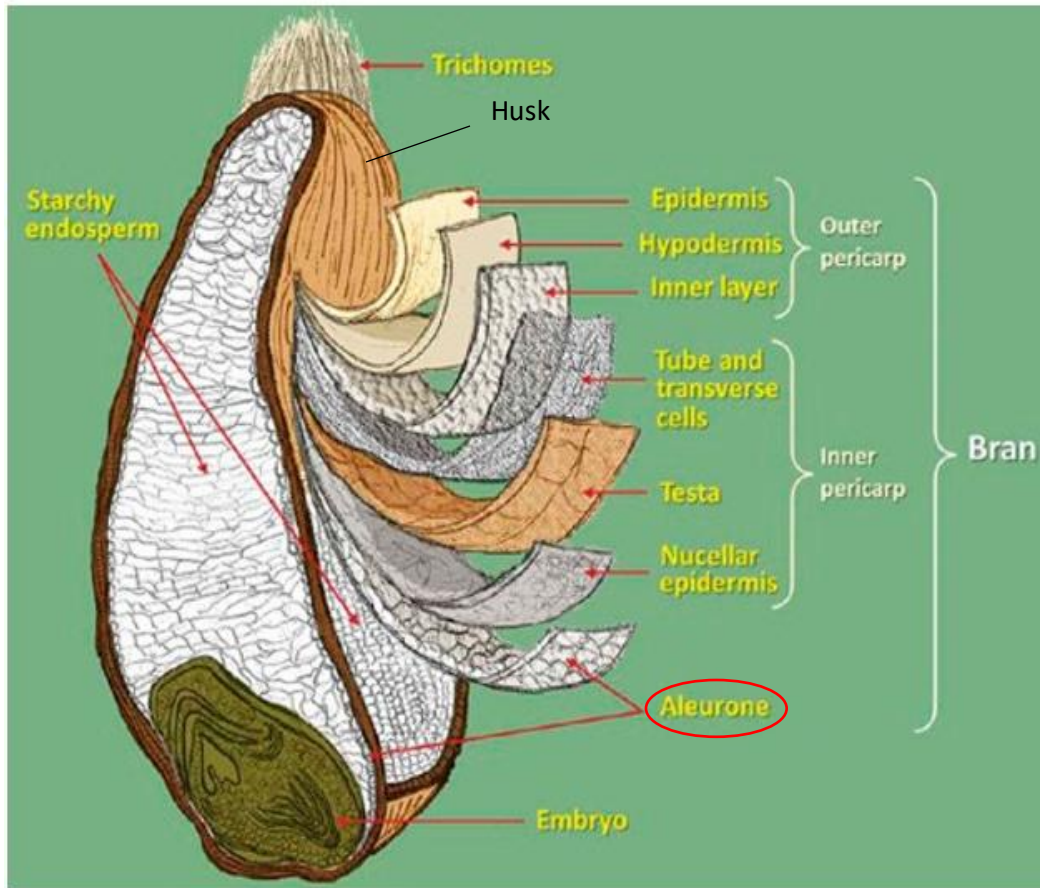


Figure 1.2.1 The detailed structure of the cereal grain (Balandrán-Quintana et al., 2015).

The red cycle emphasizes aleurone layer where the storage protein of bran is reserved.

1.3 Major Storage Proteins in Cereals

Historically, Osborne (1908) concluded storage proteins in plants were comprised of albumin (water-soluble), globulin (salt-soluble), prolamin (aqueous alcohol-soluble) and glutelin (residue protein; extracted by alkali solution) (Malik, 2009).

Albumins and globulins are non-prolamin proteins contributing 15% to 20% of total proteins in wheat flours (Malik, 2009). The molecular weight (MW) of albumins and globulins are mostly below 25 kilo dalton (kDa) whereas other unidentified proteins in the wheat flour have a MW from 60 to 70 kDa (Malik, 2009). Triticin was a minor group of storage protein found in wheats and was a polymeric storage protein found in seeds. Albumins and globulins are believed to have better nutritional composition compared to other storage proteins presented in wheat due to their high lysine and methionine contents which are considered absent in other storage proteins. Alpha-amylase/trypsin, serpins and purothionins are predominant albumins and globulins in wheat flour and their connection to dough-forming properties of wheat flours in breadmaking remain a mystery (Goesaert et al., 2005). In rice flour, it was well illustrated that decomposition by protease produced albumin with a MW of 14-16 kDa albumin and 27.5 kDa α -Globulin which were believed to have allergenic reactions to IgE from patients (Ito et al., 2005). As for proteins in wholegrain oats, albumin accounts for 1-12% of total protein contents and the 2-S albumin are predominant within, with MW vary from 14 to 17 kDa to 36 to 47 kDa (Klose & Arendt, 2012). The term, 2-S albumin, is defined based on the sedimentation coefficient of the protein which describes the rate of movement of the protein during centrifugation (Moreno & Clemente, 2008). Avenins are oat prolamins with a low MW of 30 kDa (Rasane et al., 2015).

The non-sticky rubbery remains after washing wheat with water are called gluten. Gluten consists of 80–85% protein, 5% lipids and minor contents of starch (Malik, 2009). Gluten plays a crucial role in dough making while forming a gluten-starch network locking CO₂ bubbles in dough during fermentation providing spongy structure. Gluten is only found in grain endosperm while forming a long matrix

around starch granules. Gluten protein accounts for 80% to 85% of total wheat proteins belonging to the prolamin class (Shewry & Halford, 2002). Two major groups of gluten are distinguished: monomeric gliadins and polymeric glutenins. α -, γ - and ω -gliadins make up to monomeric gliadins while polymeric glutenins contain low molecular weight subunits (LMW) and high molecular weight subunits (HMW). Gliadins are abundant in wheat and barley but absent in oats and rice. A recent study conducted by Kajendran et al. (2019) indicated that gliadin was found in transgenic rice Bg 250 with a molecular weight of 27 kDa while the confirmation that α -helices and β -strands were found. As for glutenin, HMW glutenin has a molecular weight between 80-130 kDa on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) while LMW glutenin has a molecular weight of 58 kDa, 42-51 kDa and 30-40 kDa belonging to different subgroups (Malik, 2009). The comprehensive data including the important elements of the sequence length, definition, source, reference, features and the original sequence of cereal storage proteins are available on UniProt databases (<https://www.uniprot.org/>).

1.3.1 Globulins and Albumins

In wheat, globulin and albumins (the non-prolamin group) are the minor storage proteins (5% - 10%) compared to prolamins and glutenin (MARCONE & YADA, 1994), yet for their bioactive properties has been well studied (Liaoa et al., 2023). Wheat globulin/albumins shows a board range of molecular size between 6 – 100 kDa (DuPont et al., 2008). The major varieties were located at 37 kDa and 55 kDa which was considered to as 8S globulin while intense bands were also shown at 22 kDa and 40 kDa corresponding to 11S globulins. Merlino et al. (2009) utilised Two-dimensional (2-D) electrophoresis revealing 2,250 spots related to wheat globulins and albumins. The major spots were located at MW between 13 to 100 kDa and at the isoelectric point (pI) between 4 to 7.

In oat, globulin is the predominant protein comprising 50% - 80% of the whole protein in oats and is soluble in buffered salt solution (Klose & Arendt, 2012). The rich globulin in oats contributes to the balanced amino acid profile (Kaukovirta-Norja

et al., 2004) due to the comparatively high lysine content in oat globulin (2.6%), slightly higher than rice globulin (2.56%) and much higher than wheat protein (Peterson, 1978; Vasal, 2004). Oat globulin has a wide range of pI between 5.0 – 9.0 with an optimal resolution at about pH 8.0 and a MW mainly between 22 kDa – 57 kDa with some minor peaks at 6 kDa and 150 kDa resolved by column chromatography (Galova et al., 2019; Ma & Harwalkar, 1984). Oat globulin structure was first characterised by Peterson (1978) as containing α -subunits (21.7 kDa) and β -subunits (31.7 kDa). The α -subunits had a significantly higher content of aspartic acid/asparagine and arginine and a lower content of glutamic acid/glutamine. Burgess et al. (1983) separated oat globulins into 3S (MW: < 20 kDa), 7S (MW: 55 kDa) and 12S (MW: 54 kDa) globulin fractions. The 3S and 7S globulin structurally resembled vicilin proteins in legumes. The 12S globulin had a similar structure to the legume S11 globulin with two subunits form a dimer stabilised by disulfide bonds. Oat globulin shows a low degree of homogeneity to legume storage proteins but high homogeneity to other cereal globulins (Klose & Arendt, 2012). Shotwell et al. (1988) compared oat globulin to the globulin from other plant sources and found that the amino acid sequence deduced from oat globulin cDNA clone had 31% sequence identity with soybean glycinin, 38% with legume and 70% with rice globulin.

1.3.2 Prolamin

Avenin (the oat prolamin) comprises 4 - 15% of the total storage protein and is the second most abundant storage protein in oats (Klose & Arendt, 2012). Like other prolamins in cereals, avenin is soluble in ethanol, which makes aqueous ethanol extraction a common method to prepare avenin for sample characterisation (Gimenez et al., 2017; Nałęcz et al., 2009; Schalk et al., 2017). With the MW at 20 kDa – 30 kDa and the pI value between the pH of 5 – 9, avenin is a gluten-like prolamin that may trigger celiac disease, a chronic immune mediated disorder, which affects 1.4% of the global population (Gimenez et al., 2017; Tanner et al., 2019). Avenin is rich in proline and glutamic acid but has a low basic amino acid content (Klose & Arendt, 2012). The schematic structure showed conserved regions A, B and

C which are also present in the prolamins of the *Triticeae*, oats, and rice, and the zeins of maize, and are homologous to each other with conserved cysteine residues (Shewry et al., 1995). Shotwell et al. (1988) isolated both globulin and avenin genomic clones from an oat genomic sequence library followed by hybridisation and sequence analysis. The authors found the same amount of globulin and avenin mRNA during the development of the oat endosperm, yet the content of globulin was over five times higher than avenin in the mature grains, which indicated that the regulation of storage protein synthesis was affected not only by transcription, but that the hairpin loop structure of the coding sequence of the avenin mRNA might reduce the rate of the translation. Plus, this study also indicated that the avenin genomic clones encoded a polypeptide with 209 amino acids with a signal peptide of 19 amino acids and was rich in glutamine and proline with lysine or tryptophan absent. By using expressed sequence tag analysis, Anderson (2014) indicated that the avenin polypeptides structure included six regions in which regions II and IV were rich in glutamine and proline, and the content of the glutamine plus proline in avenin ranged from 39.4% - 47.6%, much higher than that in oat globulin (16.7% - 19.1%).

Hordeins are storage proteins found only in barley. With high proline and glutamine contents, it is classified into the family of prolamin. The extraction of hordeins is simply extracting by 70% ethanol or 55% isopropanol. By using gel electrophoresis, hordein was resolved into three groups of peptides with MW of 10 kDa, 15 kDa and 60 kDa respectively (Baxter, 1981). Chanput et al. (2009) utilised pepsin and trypsin to hydrolyse hordein and indicated the significant increase of antioxidant activity of hordein hydrolysate; nearly activity of all purified fractions increased by over 25% while one fraction showed double antioxidant activity. B1-hordein, B3-hordein, C-hordein and gamma-hordein were predicted as the origin of antioxidant peptides by using AnOxPrep (Dinkçi et al., 2023).

Gliadin is the prolamin found only in wheat. It is commonly classified as α -, β -, γ - and ω - isotypes based on the electrophoretic mobility in 2-D electrophoresis (Jackson et al., 1983). In general, α - gliadins comprise 33% - 51% of total proteins of different

wheat species followed by γ -gliadins (24% - 36%) and ω -gliadins (5%-19%) (Wieser et al., 2023). The extraction of gliadin showed protein bands on SDS-PAGE from 10 kDa to 150 kDa with intense bands located between 25 kDa to 75 kDa (Chen et al., 2011). Malik (2009) indicated that the MW of α -, β -, γ - and ω - gliadin was ranged between 30 kDa to 80 kDa and were rich in proline and glutamine content. The structure of the isotypes of gliadins was well established by Urade et al. (2018). Alpha-gliadins (30 kDa - 34 kDa) comprise of a signal peptide, an N-terminal region, a repetitive domain, and the C-terminal region featured by a cysteine-rich region (CI), a glutamine-rich region (CII) and the region distinguished by two cysteine residues (CIII). The structure of γ - gliadin (26 kDa to 36 kDa) is similar to α -gliadin except for that α -gliadin contains two stretches of polyglutamine in the C-terminal of the repetitive domain and in CII while ω - gliadin shows simpler structure with a long repetitive domain (238 residues) and a C-terminal region containing no cysteine. Gliadin has been known to cause celiac disease (De Re et al., 2013; Maiuri et al., 2019; Salentijn et al., 2012) by stimulating T-cells by presenting epitopes from α - and γ - gliadins (Salentijn et al., 2012).

1.3.3 Glutelins

Glutelin is a part of wheat gluten which showed a similar amino acids composition to wheat gliadin with high glutamine and proline content but less charged amino acids (Malik, 2009). Gluten is the major storage protein (85-90%) found in wheat kernel and make up about 30 – 50% of total protein in wheat grains. Gluten is also found in other cereals such as rye, spelt and barley (Biesiekierski, 2017) and is mostly made of gliadin and glutelin (Flambeau et al., 2017). Glutelin is classified into high-molecular-weight glutelin subunits (HWM-GS) and low-molecular-weight glutelin subunits (LWM-GS). HWM-GS have MW ranged from 65 kDa to 120 kDa on SDS-PAGE and is encoded by *Glu-A1*, *Glu-B1* and *Glu-D1* loci on the long arms of homoeologous group-1 chromosomes (Dong et al., 2009; Liu et al., 2010). The primary structure of HWM-GS consists of a non-repetitive N-terminal domain with 81-104 residues, a C-terminal with 42 residues and a central domain comprising 481- 872 residues while the central domain is featured by x-type subunits (the repeat units include

tripeptides (GQQ), hexapeptides (PGQGQQ), and nonapeptides (GYPTSPQQ)) and γ -type subunits (the repeat units are the hexapeptides (PGQGQQ) and nonapeptides (GYPTSLQQ)) (Li et al., 2020). X-type and γ -type subunits contains 4 and 7 cysteine in their structure (Li et al., 2020), respectively, which are important as cysteine presents in intrachain di-sulfide bonds (Shewry et al., 2003; L. Wang et al., 2021) and enables the formation of gluten aggregates contributing to the elasticity of dough making (Li et al., 2020; Zhang et al., 2018). HMW-GS can be isolated by reverse-phase high-performance liquid chromatography (R-HPLC) and sequenced by tandem mass spectrometry (MS/MS) (Dong et al., 2009). LWM-GS are similar in size and structure to the γ -gliadin (Shewry & Tatham, 1990). Ikeda et al. (2002) cloned and isolated the gene of LMW-GS and deduced the LMW-GS fell into two types: the one with a N-terminal contains the isoleucine as the first amino acid and the other one with methionine as the first amino acids. Verbruggen et al. (2001) indicated that the addition of HMW-GS to the controlled glutelin network increased the dough strength and extension properties while the addition of LMW-GS also showed increased dough strength but the induced effect it not as significant.

1.4 Protein Extraction for Analysis

Protein extraction is the first step before characterising proteins in cereals, with the intention to isolate one, a few or the whole protein contents from the complexity. Alkaline extraction followed by iso-electric precipitation has been accepted as a accepted approach to extract whole protein content from cereal grains in order to characterise the bioactive values of derived peptides (Alu'datt et al., 2012; Walters et al., 2020; Wang et al., 2015; Yadav et al., 2011). The yield of oat protein extraction by this method, with the increase of pH (towards alkaline condition), could reach to 75.5% (Kriger et al., 2018). This method was developed based on the theory that alkaline solution degrades cellulose and destroys the fibril network to release the protein fractions (Knill & Kennedy, 2003). Another method used for extracting whole protein content from oats is to use an extraction buffer containing urea, a reducing agent (Dithiothreitol (DTT) or 2-mercaptoethanol) and a detergent (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS)) (Chen et al.,

2016; Galova et al., 2019; Nałęcz et al., 2009). DuPont et al. (2008) used a detergent, sodium dodecyl sulfate (SDS), to assist wheat protein extraction and the yield of gliadin recovery reach to 95%. The alkali extraction and isoelectric precipitation method usually prepares the cereal proteins to be characterised and identified via 2-D electrophoresis technique. The reducing agents and detergents in the extraction buffer is necessary to solubilise proteins and gives high resolution to the 2-D electrophoresis analysis.

The methods to separate individual storage protein from whole storage protein extracts have been modified based on Osborne's method established in 1907 (Snyder, 1907). According to the solubility of different storage proteins in different chemical solutions, albumin and globulin fractions, prolamin (avenins) and glutenins can be extracted by distilled water, saline solution, 70% ethanol and 55% 2-propanol, respectively; this method was used to prepare cereal protein isolate for the bioactivity determination and for characterising protein and peptides (Capouchova et al., 2004; Comino et al., 2016; Drakos et al., 2017; Nałęcz et al., 2009; Zhang et al., 2014; Zhang et al., 2019).

1.5 *In vitro* Digestion

Proteases are used to hydrolyse the storage proteins of cereals and produce functional bioactive peptides. Different food-grade proteases have been used in order to obtain peptides with different functions (see tables in section 1.8). The simulated *in vitro* digestion model has been developed as a cost-effective alternative to the expensive, challenging, and time-consuming *in vivo* studies of digestion. Though the system only partially mimics the complexity of the gastrointestinal (GI) system, it has become a standard method to determine the digestibility (%), Degree of Hydrolysis (DH%), change of the structure and release of bioactive peptides and essential amino acids from a range of foods. Recently, INFOGEST *in vitro* digestion model has been widely adopted as a standardised method to impedes the results

capable for intercomparison (Brodkorb et al., 2019). However, in the studies investigating the degradation of protein in cereals, *in vitro* digestion was applied in different conditions where the ratio of protein and enzyme was set to 100:1 (w/w) which led to significantly lower enzyme activity in the digesta slurry, however sufficient to obtain complete degradation of protein at the end stage of digestion (Uraipong and Zhao, 2018; Phongthai et al., 2018).

1.6 Bottom-up and Top-down Methods in Cereal Protein Proteomics

Two classic approaches in combination with Mass Spectrometry (MS) for identifying and characterising cereal proteins are termed as the “bottom-up” and “top-down” methods (shown in Figure 1.6.1). Theoretically, bottom-up proteomics requires the hydrolysis of the protein into peptides before the sample is subjected to MS (Alves et al., 2019). In contrast, with the top-down method the isolated protein is directly injected into the MS without enzyme cleavage to assess the complete protein sequences. The major differences between the bottom-up and top-down methods are illustrated in Figure 1.6.1. Comparatively, the top-down method provides useful data of the full protein or large peptide sequences and is capable to localise multiple post-translational modifications (PTMs). However, the top-down method is expensive and time-consuming requiring the assistance of proteomic software such as ProSightPC and PIITA to find protein in the databases with the highest Protein-Spectra-Match (PrSM) score to determine the significant differences and thereby requires the complimentary data provided by the bottom-up method (Catherman et al., 2014; Cunsolo et al., 2004; Liu et al., 2012). The top-down method has been widely used to identify the isotypes of allergic proteins in cereals (Cho et al., 2018; Comino et al., 2016; Cunsolo et al., 2004; Egorov et al., 1994; Uvackova et al., 2013). For example, Ahola et al. (2020) extracted avenin from oat flour by the peak retention time of R-HPLC and subjected the fractions to tandem mass spectrometry; Fractions contained proteins which were identified as avenins including avenin LOL6J0 (32.8 kDa, featuring the tryptic peptides QAICQVTR, QLAQIPEQLR and

QFLVQQCSPVAAVPFLR) and proteins that could possibly be avenin Q09114 (21.0 kDa), LOL418 (29.1 kDa), LOL5G8 (25.7 kDa), LOL841 (22.9 kDa), LOL5H5 (24.5 kDa), LOL6K5 (23.1 kDa), LOL8B6 (22.9 kDa), or F4MJY1 (25.6 kDa), identifiable by the two tryptic peptides QFLVQQCSPVAVVPFLR and QLAQIPEQLR. Trypsin or chymotrypsin digestion prior to MS analysis have always been a gold standard in bottom-up methods due to the broad cleavage ability which produces peptides with average MW of 0.7 kDa - 1.5 kDa, a suitable range for MS (Laskay et al., 2013). The bottom-up proteomic methods have been adopted in many recent studies which have characterised and identified the bioactive peptides. In these studies, different proteases was used to identify novel bioactive peptides (Liu et al., 2021; Sánchez-Velázquez et al., 2021; Tok et al., 2021; Vanvi & Tsopmo, 2016; Zaky et al., 2020).

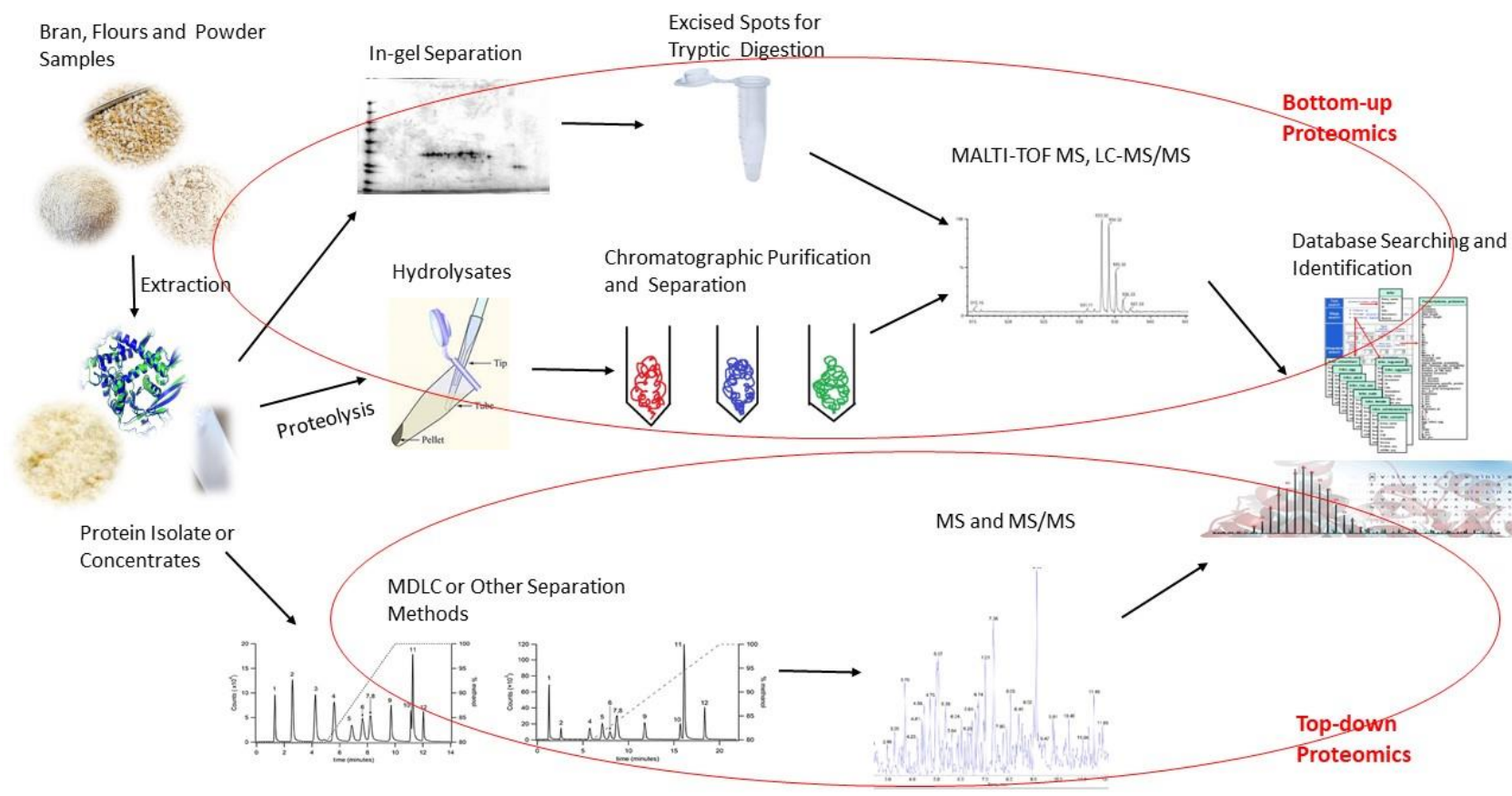


Figure 1.6.1 The bottom-up and top-down proteomics workflow in characterising oat proteins and derived peptides.

1.6.1 In-gel Techniques

The in-gel techniques, by principle, refers to the approaches to visualise and characterise proteins which migrate through the pores of the polyacrylamide matrix with the application of the electric field. The utilisation of gel to characterise cereal proteins started in 1964 when SDS was found to enhance the electrophoretic resolution (Ornstein, 1964; Pederson, 2008), SDS-PAGE has become one of the most important proteomic tools in analysing proteins in wheat and barley seeds (Bietz & Wall, 1973; Fullington et al., 1980; Shewry et al., 1978). Characterisation of oat storage proteins by using SDS-PAGE was employed in early 1980s. Two-Dimensional electrophoresis was introduced in 1954 by Kolin and this separation technique was later documented as an indispensable tool to separate and characterise cereal proteins, and to obtain intact protein profiles (Galova et al., 2019; Gasparis, 2017; Nałęcz et al., 2009; Pernollet et al., 1989).

SDS-PAGE

The term SDS-PAGE is the combination of SDS (a detergent improving protein resolution), polyacrylamide gel (a synthetic, electrophoretically desirable gel that is thermo-stable, transparent and economic), and electrophoresis (a method separating proteins based on their size and charges) (Pederson, 2008). SDS disrupts the natural secondary and tertiary structures of proteins creating linear peptide chains which can then migrate in the electrophoretic systems based only on their mass and electric charge. When an electric field is applied to the gel, proteins with negative charge migrate across the gel from the cathode (negative electrode) to the anode (positive electrode). The mobility of different proteins depends on their size: the larger biomolecules remain closer to the anode, and thereby protein mixtures are separated by their MW (Gallagher, 2012). The staining and de-staining processes are the last step of the gel electrophoresis which enables the separated protein bands to be visible on the gel. The intensity of the band on the gel indicates the amount of each corresponding protein.

SDS-PAGE studies have investigated the whole protein extracted from oats and their major storage proteins (Camafeita & Méndez, 1998; Comino et al., 2016; Guan et al., 2007; Schalk et al., 2017). The whole protein extracted by the alkaline solution showed two intense bands at MW of 30 kDa – 35 kDa and 20 kDa – 25 kDa along with some other minor bands between 40 kDa – 100 kDa and lower than 15 kDa (Comino et al., 2016; Guan et al., 2007; Schalk et al., 2017). Avenin fractions extracted by ethanol and visible on the gel were between 20 and 35 kDa (Comino et al., 2016; Schalk et al., 2017) which is consistent with the result found by Camafeita and Méndez (1998). Albumins in oats showed a broad protein pattern between 10 kDa to 100 kDa; Globulins had very similar MW to avenins at 25 kDa and 35 kDa while glutens displayed a number of light bands less than 35 kDa (Comino et al., 2016). The MW of wheat gliadin was ranged from 30 kDa to 95 kDa including bands corresponding to ω -, γ -, β - and α - gliadin with ω -gliadin shows the MW of 45 kDa to 95 kDa while other three gliadins show the MW between 30 kDa to 45 kDa (Žilić et al., 2011). Hordein has wider molecular range between 25 kDa to 180 kDa including bands related to B-hordein (25 kDa – 40 kDa), C-hordein (40 kDa to 100 kDa) and D-hordein (140 kDa to 180 kDa) while the globulins and albumins are located at the MW of lower than 20 kDa (Yalçın & Çelik, 2007). SDS-PAGE separation of cereal proteins has its limitations. Some protein isoforms have the same molecular size and are not separated by SDS-PAGE, and the denaturation prior to the electrophoresis prevented the possibility of further characterisation of the protein samples such as determining protein interactions and modifications. Although the SDS-PAGE separation method was invented decades ago, it remains one of the most powerful and economical tools and has been widely utilised in recent studies.

Two-Dimensional Electrophoresis

2-D electrophoresis is another powerful in-gel technique separating oat proteins from a protein mixture. 2-D electrophoresis contains two stages of separation: isoelectric focusing (first dimension) and gel electrophoresis (second dimension). Separation in the immobilised pH gradient strips in the first dimensional electrophoresis system allows the recovery of bands based on the pI of the protein

which is the pH at which each protein is electrically neutral in the electric field. The second phase SDS-PAGE is based on the MW of the uncharacterised proteins. The intact protein profile of the extracted cereal proteins was illustrated by Nałęcz et al. (2009), Chen et al. (2016) and Galova et al. (2019). Considering the recent trend of utilising alkaline solution for extracting whole protein from the cereals and then subjecting the protein to a digestion model or enzymatic cleavage to characterise the bioactive peptides by chromatographic separation, the alkaline extraction may result in the production of protein aggregates with similar MW but different pI values, that potentially alters the bioactive nature of the proteins and derived peptides (Keppler et al., 2020; Zielke et al., 2019). 2-D electrophoresis may not be as precise as the chromatographic separation method and is time-consuming, but it has the edge on providing the visual separation of the extracted proteins which directly shows whether a protein is modified during the extraction. Plus, with the assistance of MS, some modifications of the protein on 2-D electropherogram can be identified. In addition to assessing the protein as a therapeutic food source, 2-D electrophoresis has also been applied in a comparative study of the protein in seeds responding to heat stress acclimation under different moisture conditions (Chen et al., 2016). Twenty one different protein spots were observed in this study before and after heat stress treatment, in which 6 heat shock proteins (chaperones in response to stimulus and PTMs against the incorrect protein folding, accumulation and degradation) with 10% and 16% moisture content were downregulated, 2 ATPases (regulators of ATP synthesis) were downregulated over 50°C at 16% moisture content indicating the slow germination rate and lower vigor of the oat seeds, and an upregulated 12 - S globulin was revealed. 2-D electrophoresis played as an important and efficient tool in this comparative study in comparing the same protein source under different conditions implying the possibility of the future work on obtaining novel peptides derived from modified sources.

1.6.2 Chromatographic Separation Methods

The recent advances of cereal protein proteomics combine high performance liquid chromatography (HPLC) and MS to give a better resolution after the separation, and

increased reproducibility and recovery rate of the sample (Liu et al., 2021; Mason et al., 2020; Schalk et al., 2018). As different modes of chromatography for peptide and protein separation are available, the accurate selection will be important to achieve the target separation based on the features of analytes including their affinity to the stationary phase, hydrophobicity, charge properties, MW, and pI value. So far, the chromatographic methods have been authenticated to characterise digested peptide sample derived from oat proteins whereas 2D-electrophoresis is not the ideal tool in this scenario as the acidic conditions in the digesta and the protein sample prepared with the salt-induced precipitation, the extraction method widely adopted in oat protein preparation, may cause interference during the focussing stage.

Reverse Phase- High Performance Liquid Chromatography (R-HPLC)

R-HPLC is the system with the opposite mechanism to normal phase HPLC. The stationary phase carbon in reverse phase system is non-polar, packed by the silica-based columns, in which the alkyl chain with the length of up to 18 carbons, with pores which are different in sizes exhibiting stable and efficient separation (Dill, 1987). The selection of pore size is based on the size of the protein or peptide analytes. It is also interesting to note that the behaviour of peptides and proteins in R-HPLC is dependent on the retention time of solute in the variety of solvent with different concentration and thus, gradient elution is always required (Snyder et al., 2011). R-HPLC has played an important role in separating the bioactive peptides derived from the cereal proteins and identifying the fractions with the highest bioactive values followed by sequence identification (Baakdah & Tsopmo, 2016; C. Dugardin et al., 2020; Liu et al., 2021; Sun et al., 2019; Vanvi & Tsopmo, 2016). R-HPLC was also used to examine the integrity of synthetic peptides containing the immuno-stimulatory celiac disease epitope for making the standard curve of the Enzyme-Linked Immunosorbent Assays (ELISAs) determining the immunogenicity of avenin epitopes (Mujico et al., 2011).

Ion Exchange Chromatography

Ion exchange chromatography can be either anion or cation exchange chromatography. In this separation technique the electrostatic interactions between the protein and peptides with the charged amino and carboxyl ends, and the

charged packings affects the retention time of the solutes. This technique was used in early research for protein separation. Pernollet et al. (1989) characterised avenin homologues including avenin 3, 5, 6, 7, 8, 9, 10, 11, 13 and 14 separated them using the combination of ion exchange chromatography and R-HPLC. Recently ion exchange chromatography has been preferred in purifying proteins and peptides, providing a good protection for the natural conformation and the bioactive properties of the protein and peptides, and an effective recovery rate (Snyder et al., 2011). Uraipong and Zhao (2018) utilised anion exchange column for isolating the fraction with the highest anti-hypertensive activity derived from intestinal digesta of rice bran protein which exhibited inhibitory effect on α -glucosidase and ACE at 52.43 ± 2 mg acarbose equivalent and 170.13 ± 2.2 nmol captopril equivalent per gram of peptide, respectively. C. Dugardin et al. (2020) selected a 732-cation exchange column for purification of oat globulin hydrolysates and obtained three antioxidant fractions. Fraction C showed the highest hydroxyl radical scavenging activity and DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity (IC_{50} of 1.83 ± 0.03 mg mL⁻¹ and 4.11 ± 0.07 mg mL⁻¹, respectively) and was then identified by electrospray ionization mass spectrometry (ESI-MS/MS) as having five unique antioxidant peptide sequences. This technique was also employed in purifying fractioned rice protein hydrolysates (Thamnarathip et al., 2016).

Size Exclusion Chromatography (SEC)

SEC separates proteins or hydrolysates based on the fractionation range of filtration resins, thus is also given a name as the gel filtration chromatography (Sunilkumar & Tareke, 2019). Theoretically, the separation of SEC is dependent on the pore size and the porosity of the column. The manufacturers provide the selectivity curves of different resins for calibrations corresponding to the specific flow rate, fractionation range and particle size units (Healthcare, 2010). Mason et al. (2020) employed SEC to separate the digesta of canary seed, wheat and oats with the MW of less than 3 kDa and the distribution of all three samples showed 4 major peaks and the similar retention time (peak 1: 23.9 min; peak 2, 26.4 min; peak 3: 26.4 min; peak 4: 35.8 min). As SEC is a time-consuming (long retention time) and complicated technique requiring the evaluation of the chromatogram to identify the fractions, a more rapid

method has been recently adopted in oat protein proteomics. C. Dugardin et al. (2020) separated oat protein hydrolysates into 5 fractions with the MW of < 1 kDa, 1 kDa – 3 kDa, 3 kDa – 5 kDa, 5 kDa – 10 kDa and > 10 kDa by using stirred ultrafiltration units. This separation system is armed with a cut-off membrane which allows solutes above the MW cut-off to be retained in the cell. This rapid method is limited by the volume of the filtration cells.

1.7 Analytic and Bioinformation Tools

MS is the analytical method most used to identify peptide sequences (Gasparis, 2017). In oat proteomics, two different methods for peptide identification by MS were widely adopted: matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) based protein fingerprinting and liquid chromatography-tandem spectrometry (LC-MS/MS) based peptide sequencing. The purified protein fingerprint was analysed using the MALDI-TOF MS based on protein fingerprinting method to characterise the protein fractions (Camafeita & Méndez, 1998; Nałęcz et al., 2009). As the identification of the bioactive peptide sequences increased, the employment of LC-MS/MS and the similar instruments such as the inductively coupled plasma mass spectrometer (ICP-MS) and ESI-MS/MS was found in many recent proteomic studies (Baakdah & Tsopmo, 2016; Chen et al., 2016; C. Dugardin et al., 2020; Mason et al., 2020; Selamassakul et al., 2018; Shittu et al., 2020; Vanvi & Tsopmo, 2016; Walters et al., 2020; Wattanasiritham et al., 2016; P. Zhang et al., 2020). The identification by MS/MS follows different principles, requiring the digestion of the protein, and separation and purification by the chromatographic method. When peptide sample is subjected to MS/MS, peptide is ionised by the first spectrometer for separation based on mass-to-charge ratio (m/z), particular ions from the first spectrometer are fractionated to smaller ions and introduced into the second spectrometer for separation and detection. The sequences were deduced by tracking back to the protein databases for the matched sequences. Tanner et al. (2019) provided the best example of using these two different tools to analyse avenins extracted from oat flours. In this study, the MALDI-TOF mass spectra showed the protein composition of both avenin extract and the prolamin-enriched fraction

from oat flours with 25 peaks ranging from 19.3 kDa to 32.4 kDa while the LC-MS/MS analysed the trypsin digested protein sample and found 276 proteins were identified with 99% protein confidence level showing a high purity of the extracted avenin.

Bioinformatic programs such as BIOPEP are powerful tools for simulating the *in-silico* digestion by various enzymes to predict the sequence and bioactivity of the derived peptides (<https://biochemia.uwm.edu.pl/en/biopep-uwm-2/>). The most acceptable computational tools utilised in cereal proteomics were the combination of the UniProtKB (<https://www.uniprot.org/>), a powerful database which combines the data from Swiss-Prot and TrEMBL databases, and ExPASy (<https://www.expasy.org/>), the program owned by Swiss Institute of Bioinformatics (Gasparis, 2017; Iwona & Dorota, 2018). The use of these tools in combination allowed the theoretical MW and pI values to be computed, based on the peptide sequences and provided further information about the proteins including the function, taxonomy, subcellular location, features of PTMs, expression, domains, complete sequence, and similar proteins, enough to support the analysis of the results obtained from MS. The computational tools were helpful when used with MS to evaluate protein and peptides. Galova et al. (2019) identified the two-dimensional protein maps of oats, wheat, barley, amaranth and buckwheat based on the data computed and retrieved from the UniProtKB databases and ExPASy ProtParam programmes. However, the MW and the pI were computed based on the theoretical amino acid sequences. The spots shown on 2-D electrophoretogram profiles could involve all mature and immature proteins, and the sequence, MW, and pI of which could be altered due to PTMs, and thereby not match the theoretical information retrieved from the databases.

1.8 Recent Studies Investigating Antioxidant and ACE Inhibitory Activity of Cereal Peptides

Oxidation is the main cause of food deterioration; it leads to the production of off flavours and loss of the appearance and nutritional value. The oxidation of lipids and proteins generates free radicals and short-chain fatty acids (SCFAs)-derived off-

putting odours and also leads to the accumulation of the free radicals and excessive generation of reactive oxygen species (ROS) (VIDAL et al., 2018). These free radicals and ROS attack DNA, proteins and membrane lipids inducing cell function disorders and diseases such as cancer, diabetes, sclerosis, neurodegenerative disorders and cardiovascular diseases (Moritani et al., 2017; Wattanasiritham et al., 2016). The food production industry would be perspicacious to make use of antioxidants to inhibit the oxidation and the production of ROS in food. The utilisation and mechanism of in vitro assays (DPPH radical scavenging assay, ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) assay, ferric reducing antioxidant power assay (FRAP), total phenolic content determination assay (TPC), superoxide anion and hydroxyl radical scavenging assay and oxygen Radical Absorbance Capacity assay (ORAC) in cooperation with proteomic methods has been investigated to characterise the antioxidant properties of proteins and peptides derived from cereals (Gong et al., 2021).

ACE is known as a biomarker discovered in human blood relating to the cardiovascular health. As one major part of the Renin-angiotensin system (Thamnarathip et al., 2016) which maintains the homeostasis of cardiovascular system by regulating blood pressure, natriuresis and controlling blood volume, ACE converts metabolites angiotensin I with dipeptide carboxypeptidase into angiotensin II, leading to the narrower blood vessels and increasing blood pressure (Tikellis & Thomas, 2012). It also triggers pathogenic including the systematic dysfunctions in the lungs, eyes, skin, nerves, liver and hearts by growing granulomas (the small tumour-like immune and inflammatory cells of collection) and fibrous tissues referred as sarcoidosis. ACE test is designed for monitoring and diagnosing sarcoidosis (Lieberman, 1975).

Antioxidant and ACE inhibitory agents are two of the most popular commercial food supplements and are also widely used in food fortification. Meanwhile, peptides produced from protein in cereals will benefits human body straight away after digestion.

The studies of the antioxidant and ACE inhibitory effects of the oats, wheat, barley and rice proteins and peptides in recent years were evaluated using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guideline (Moher et al., 2010). All articles and studies were identified based on searching through the Web of Science database dated from January of 2015 to December of 2023. The combination of keywords and phrases in relation to this review article including “oats/wheat/barley/rice protein hydrolysates*”, “oats/wheat/barley/rice peptides*” and “oats/wheat/barley/rice extracts*” plus “characterisation*”, “isolation*”, “purification*”, “identification*”, “fractionation*”, “antioxidant”, “ACE inhibitory” and “anti-hypertension”. Only journal articles characterising cereal proteins and peptides corresponding to antioxidant activity and ACE inhibitory activity obtained using proteomic techniques were included as eligible studies to be discussed in the following sections. In the tables that summarise the studies investigating oat, wheat and barley proteins (Table 1.8.1-Table 1.8.6), studies that focused on bran protein and peptides were highlighted in blue. It should be noted that, from 2015 to 2023, there was no study found investigating the antioxidant and ACE inhibitory activity of barley bran protein and peptides. Rice has been widely investigated than these three cereals. There had been 25 studies found focused on investigating antioxidant peptides and digests derived from wholegrain rice or rice flour and 12 studies focused on ACE inhibitory peptides and digests from wholegrain rice or rice flour. So only studies investigated hydrolysates and peptides of rice bran protein, which met the criteria of selection, was included and summarised in Table 1.8.7- Table 1.8.8. The studies on rice bran peptides would provide results to compare to three other cereals, to help to understand the rationale of the whole thesis .

1.8.1 Oats Derived Peptides

Antioxidant

Six most recent articles detailing antioxidant activities of the protein and peptides derived from oat flours and oat bran, as shown in Table 1.8.1 (C. Dugardin et al., 2020; Esfandi et al., 2019b; Ma et al., 2017; Shittu et al., 2020; Vanvi & Tsopmo,

2016; Walters et al., 2020). Four studies focused on studying the antioxidant activity of oat bran protein hydrolysates. Vanvi and Tsopmo (2016) digested extracted oat bran protein with pepsin and produced 7 fractions after separation by R-HPLC. The ORAC and of fractions ranged from 477.0 to 824.4 $\mu\text{M TE/g}$, their ability to inhibit superoxide activity ranged from 9.7 % to 35.5% and they had from 9.0% to 14%, hydroxyl radical scavenging activity. Overall, 43 peptide sequences were identified in the fraction selected with the highest antioxidant activity. These peptides were derived from 12S globulin, 11S globulin, 12S seed storage globulin, avenin, and gliadin-like avenin proteins. The polar amino acids such as tyrosine, aspartic acid and glutamic acid were believed to contribute to the antioxidant properties of the derived peptides. Esfandi et al. (2019b) utilised Alcalase, Flavourzyme, Protamex and Papain to cleave the Viscozyme-aid and Cellulase-aid extracted oat bran protein isolates to determine the hydrolysates with the highest antioxidant. Viscozyme - Papain hydrolysates showed the highest ORAC value ($417.9 \pm 5.1 \mu\text{M TE/g}$), ABTS scavenging activity ($866.9 \pm 10.6 \mu\text{M TE/g}$) and iron-chelating activity ($75.0 \% \pm 0.4\%$). All hydrolysates were subjected to LC-MS/MS and 92, 171 and 609 peptides were identified, respectively. Most of the peptides were derived from globulins: 89 out of 92 in Viscozyme - Papain hydrolysates, 152 out of 171 in Viscozyme – Protamex hydrolysates, and 446 out of 609 in Cellulase – Papain hydrolysates. Another 177 peptides were derived from avenins in the Cellulase – Papain hydrolysates. Regarding that Viscozyme – Protamex showed lower proteolytic activity (44.0%) than Viscozyme - Papain hydrolysates (69.9%). It was also believed that the large amount of hydrophobic amino acid residues at the C-terminal and the basic amino acids at the N-terminals of the peptide sequences gave the antioxidant properties, stated by Esfandi et al. (2019b). This study evaluated the cytotoxic and cytoprotecting properties of the peptides by treating human hepatic HepG2 cells with these hydrolysates. Viscozyme - Papain hydrolysate was proven to have the strong antioxidant activity (intracellular ROS reduction: 104 %; GSH/GSSG ratio: 8.5; enhancement on antioxidant catalase, glutathione peroxidase and superoxide dismutase: 300%, 100% and 100%, respectively) comparing to other hydrolysates (Esfandi et al., 2019a). A similar study (Walters et al., 2020) investigated the

antioxidant and anti-diabetic activities of oat bran protein concentrates prepared by sonication-facilitated extraction and hydrolysed by Alcalase, Papain and Flavourzyme. SDS-PAGE followed by MS was utilised to characterise the protein profiles of the extracted protein concentrates which was proven to match the sequences of 12S-globulins, 11S-globulins and avenin-types proteins (Walters et al., 2020). SDS-PAGE was utilised to characterise the extracted protein of whole-grain oats aided by Cellulase and Viscozyme showing bands at 19, 20, 27 and 35 kDa (Shittu et al., 2020). It is interesting to note that a band at 60 kDa was shown in the protein concentrates prepared by Viscozyme-aid extraction, this is the MW of the oat allergen protein determined by Galova et al. (2019) and did not show in the SDS-PAGE profile of oat/bran proteins prepared by other extraction methods. Viscozyme could be more specific to breakdown the fibril network bound to this 60 kDa protein type.

Novel antioxidant peptides derived from glutelin and globulins in whole grain oats were characterised by Ma et al. (2017) and C. Dugardin et al. (2020). In these papers, extracted oat protein concentrates were hydrolysed by Alcalase. The hydrolysates were purified by ion-exchange chromatography, separated by either ultrafiltration or R-HPLC, and the fraction with the highest antioxidant activity was subjected to MS for sequence identification. Peptide sequences IRIPIL, FLKPMT, NSKNFPTL, LIGRPIIY and FNDILRRGQLL from globulins and HYNAPAL from glutelins was identified. The hydrophobic and aromatic amino acids in the peptides were believed to contribute to the antioxidant nature of the peptides.

Table 1.8.1 Summarised studies investigating the antioxidant effects of the derived peptide from oats.

Original Protein	Enzymatic Hydrolysis	Methods	Major Findings	References
Whole bran protein	Pepsin	ORAC and superoxide anion and hydroxyl radicals scavenging assay; R-HPLC; MS/MS	<ul style="list-style-type: none"> 7 fractions were obtained after subjecting the hydrolysates to R-HPLC. The fraction with the highest antioxidant activity was selected to subjected to MS resulting in 43 peptides with the MW of 427 Da to 1117 Da originated from globulins and avenins. 	(Vanvi & Tsopmo, 2016)
Whole bran protein	Alcalase; Flavourzyme; Protamex; Papain	ORAC, ABTS and ferric chelation assay; MS	<ul style="list-style-type: none"> The papain hydrolysates derived from Viscozyme-aid extracted oat bran protein isolates showed the highest antioxidant activity with 92 peptides identified mainly from globulins. 	(Esfandi et al., 2019b)
Whole bran protein	Flavourzyme; Papain; Alcalase; Protamex	Lipid peroxidation and ORAC assay; SDS-PAGE	<ul style="list-style-type: none"> Bands determined at 19, 20, 27 and 35 kDa. Viscozyme-aid protein isolate showed the band at 60 kDa. 	(Shittu et al., 2020)

Table 1.8.1 Summarised studies investigating the antioxidant effects of the derived peptide from oats. *Continued*

Original Protein	Enzymatic Hydrolysis	Methods	Major Findings	References
Whole bran protein	Flavourzym; Papain; Alcalase	ORAC and superoxide anion and hydroxyl radicals scavenging assay; SDS-PAGE; MS (trypsin digestion)	<ul style="list-style-type: none"> Major bands shown at 35 kDa and 20 kDa and minor band shown at 50 kDa – 60 kDa (Globulins) 14 peptides were identified in isolate proteins in which four were 12S globulins-type, two were 11S globulins-type, and five were avenins-type proteins. 	(Walters et al., 2020)
Glutelins in the flour	Alcalase	DPPH and hydroxyl radicals scavenging assay; Ion-exchange chromatography; R-HPLC; ESI-MS/MS	<ul style="list-style-type: none"> The glutelin isolates were purified by ion-exchange chromatography and R-HPLC. The fraction with the highest antioxidant level was selected accordingly for purification. The peptide HYNAPAL was identified. 	(Ma et al., 2017)
Globulins in the flour	Alcalase	DPPH and hydroxyl radicals scavenging assay; Ultrafiltration; Ion-exchange chromatography; ESI-MS/MS	<ul style="list-style-type: none"> Fraction (> 10 kDa) with the highest antioxidant activity was subjected to ion-exchange chromatography for purification producing 3 peaks. The fraction obtained by ion-exchange chromatography with the highest antioxidant activity was subjected to MS and five peptides rich in hydrophobic amino acids were detected. 	(C. Dugardin et al., 2020)

ACE Inhibitory Activity

Three recent studies used the combination of *in-silico* methods with *in-vivo* and *in-vitro* methods, as shown in Table 1.8.2 (Bleakley et al., 2017; Iwona & Dorota, 2018; Zheng et al., 2020) to characterise the ACE inhibitory activities of peptides derived from wholegrain oats and oat flours; no study was found to focused on peptide derived from oat bran. A computational simulation of the digestion by papain on selected primary oat proteins was conducted by Bleakley et al. (2017) to predict the ACE inhibitory oat peptides. 11S globulin, 12S globulin, 5 avenin types and 1 albumin type were selected, digested *in-silico* and the predicted peptides were ranked according to PeptideRanker for their inhibitory activities. Six peptides were selected and synthesised, tested at a concentration of 1 mg/mL for ACE inhibition ($48.9\% \pm 7.8\%$ to $97.8\% \pm 21.7\%$). A similar study was conducted by Iwona and Dorota (2018) using the different combinations of pepsin, trypsin and chymotrypsin to digest oat 11S and 12S globulins *in-silico* resulted into 11 – 18 ACE inhibitory peptides with the IC_{50} values of 9.2 mM to 882.13 mM. In the study conducted by Zheng et al. (2020), the novel ACE-inhibitory peptide SSYYPEK (890.4 Da) was obtained. Oat globulins were extracted and hydrolysed by Alcalase, Flavourzyme, pepsin and trypsin accordingly, then the hydrolysates were subjected to gel filtration chromatography producing 6 fractions. The fraction with the highest ACE inhibitory activity (60%) was subjected to R-HPLC to be purified twice to produce the fraction with the highest IC_{50} value (1.05 mg/ml) which was identified by LC-MS/MS and the selected peptide SSYYPEK was synthesised *in-silico* and *in-vitro*. The synthesised peptide showed an *in-vitro* ACE inhibitory IC_{50} value of 98.89 μ M and a significant reduction of systolic and diastolic blood pressure in hypertensive rat model. Molecular docking confirmed strong binding between ACE and the peptide with the T score of 14.74 and 11 hydrogen bonds indicating not only the strong interaction between peptide and the ligand but also the possibility of competitive inhibition.

Table 1.8.2 Summarised studies investigating the ACE inhibitory effects of the derived peptide from oats.

Original Protein	Enzymatic Hydrolysis	Methods	Major Findings	References
Globulins and glutelins from flour	Papain; Fiacin	<i>In-silico</i> papain digestion by BIOPEP; PeptideRanker	<ul style="list-style-type: none"> Peptides obtained by <i>In-silico</i> digestion was selected based on the PeptideRanker score and predicted ACE and DPP-IV inhibitory activity. 9 tri- and quadra-peptides were synthesised and subjected to the ACE and DPP-IV inhibitory assays. The DPP-IV and ACE inhibitory reached 46.3% and 97.8%, respectively. 	(Bleakley et al., 2017)
Globulins from flour	Pepsin; Trypsin; Chymotrypsin A	<i>In-silico</i> UniProtKB and ProtParam program;	<ul style="list-style-type: none"> Amino acid sequences of oat globulins were obtained by using UniProtKB and ProtParam to compute the theoretical physiochemical parameters. 11 – 18 ACE inhibitory peptides were identified with the IC₅₀ values of 9.2 mM to 882.13 mM. 	(Iwona & Dorota, 2018)
Globulins from flour	Alcalase; Flavourzyme; pepsin; trypsin	Gel filtration chromatography; R-HPLC; LC-MS/MS	<ul style="list-style-type: none"> The fraction selected showed the highest IC₅₀ value (1.05 mg/ml) Peptide SSYYPEK was synthesised. The synthesised peptide showed an <i>in-vitro</i> ACE inhibitory IC₅₀ value of 98.89 µM 	(Zheng et al., 2020)

1.8.2 Wheat Derived Peptides

Antioxidant

Eleven recent articles, which investigated the antioxidant peptides derived from wheat protein, are summarised (Table 1.8.3). There is only one paper investigated the ACE inhibitory peptides derived from the wheat bran protein. Zhao et al. (2020) reported that Alcalase, Flavourzyme, papain, neutral enzyme and trypsin digested wheat bran protein and produced hydrolysates with Trolox equivalent antioxidant capacity of $917.11 \pm 5.89 \mu\text{mol TE/g}$, $428.02 \pm 44.00 \mu\text{mol TE/g}$, $344.34 \pm 6.2 \mu\text{mol TE/g}$, $206.33 \pm 5.33 \mu\text{mol TE/g}$ and $783.43 \pm 3.71 \mu\text{mol TE/g}$, respectively. The Alcalase-digested hydrolysates was fractionated by size-exclusion chromatography and ion-exchange chromatography accordingly. Five bioactive peptides were identified in the isolated fraction to be CGFPGHC, QAC, RNF, SSC and WF showing IC_{50} values of Trolox equivalent antioxidant capacity of 0.763 mg/mL , 0.092 mg/mL , 0.877 mg/mL , 0.205 mg/mL , and 0.750 mg/mL , respectively.

The rest 10 studies investigated peptides produced by the hydrolysis of protein from the wheat kernel and germ. Zhang et al. (2019) investigated protein extracted from wheat germ which was digested by Alcalase and fractionated by ultrafiltration membrane (1 kDa, 3 kDa and 5 kDa). The fraction with MW of less than 1 kDa was then fractionated by gel-filtration chromatography with 7 peaks. The fraction with highest DPPH scavenging activity showed the IC_{50} value of 1.17 mg/mL and abundant glutamic acid ($13.76 \pm 0.12 \text{ g/100g}$), phenylalanine ($26.83 \pm 0.09 \text{ g/100g}$) and tyrosine ($18.36 \pm 0.07 \text{ g/100g}$). The fraction was found to mainly consist of tripeptide Gly-Pro-Phe, Gly-Pro-Glu, and Phe-Gly-Glu and showed good permeability through the monolayer of Caco-2 cells on both side of apical compartment and basolateral compartment (0.5-1.0) indicating that the passive diffusion is the predominant transport approach. ABTS assay (Liu et al., 2021) was conducted by on the peptide fraction derived from wheat gluten isolates, hydrolysed by Alcalase and Protamex and prepared by R-HPLC. The fraction with highest ABTS scavenging activity ($1.6 \text{ mmol TE/g sample}$) was identified and 15 peptides candidates were identified for

synthesis. Peptide YQ and RGGY showed the highest ABTS scavenging activity of 11.18 ± 1.02 mmol TE/g sample and 9.04 ± 0.47 mmol TE/g sample. Wheat germ extracts were hydrolysed by protease-K, Alcalase and pepsin and the hydrolysates antioxidant were subjected to R-HPLC (Karami, Peighambaroust, Hesari, Akbari-Adergani, et al., 2019). The fractions with highest ABTS scavenging activity (fractions produced by three enzymes reached scavenging activity of 90%). The peptides identified from these fractions were synthesized. Peptide SGGSYADELVSTAK, MDATAALHYENQK, KELPPSDADW and GNPIPREPGQVPAY showed ABTS scavenging activity of 90% while KELPPSDADW also showed highest DPPH scavenging activity (50%) amongst all peptides. Bromelain, Neutrase and Flavourzyme were used produced hydrolysates from wheat protein isolate to evaluate the protective effects against H₂O₂-induced oxidative damage in C2C12 muscle cells (Chang et al., 2021). The result indicated that 250 mg to 500 mg of wheat protein hydrolysates significantly increased cell availability to 90% (the negative control showed the cell availability of 60%) against H₂O₂ treatment. Wheat hydrolysates produced by *in vitro* digestion and 3 kDa membrane filtration (Mason et al., 2020) showed an overall intermediate antioxidant activity (ORAC: 1.5 mmol TE/mg protein; ABTS: IC₅₀ 100 mg protein/mL; DPPH: IC₅₀ 400 mg protein/mL; iron chelation: IC₅₀ 1.5 mg protein/mL). Karami, Peighambaroust, Hesari, Akbari-Adergani, et al. (2019) indicated that the wheat germ hydrolysates produced by proteinase K showed an overall intermediate antioxidant level (ABTS: IC₅₀ 1.57 mg/mL; DPPH: IC₅₀ 1.83 mg/mL; TAC: 60 mmmol/mL at the concentration of 2mg/mL). The R-HPLC isolated fraction with the ABTS scavenging activity of 90%. Peptide SGGSYADELVSTAK and MDATAALHYENQK were identified and synthesised showing the IC₅₀ value of ABTS scavenging activity of 265.5 ± 8.3 mg/mL and 293.3 ± 6.5 mg/mL. Wheat gluten was treated with different combination of Alcalase, Neutrase and Protamex and then subjected to *in vitro* digestion model (Lim et al., 2022). The significant reduction of DPPH scavenging and ferric reduction power on all hydrolysates was reported indicating the gastrointestinal (GI) enzymes damaged the structure of antioxidant peptides produced by commercial proteases. Tian et al. (2022) utilised size exclusion chromatography to separate wheat germ albumin with MW of < 3 kDa showing

DPPH scavenging activity of 60% at concentration of 2.5 mg/mL and O_2^- clearance rate of 55% at concentration of 5 mg/mL. Wang et al. (2020) synthesised peptide ADWGGPLPH from wheat germ albumin and it showed significant inhibition on glucose-induced oxidative damage in vascular smooth muscle cells by $13.6\% \pm 1.4\%$ at 10 μ M and $20.3\% \pm 4.0\%$ at 20 μ M. Similar study was conducted by Sun et al. (2019) showing the synthesised peptide derived from wheat protein with DPPH scavenging activity of $47.14\% \pm 0.76\%$.

Table 1.8.3 Summarised studies investigating the antioxidant effects of the derived peptide from wheat.

Original Protein	Enzymatic Hydrolysis	Methods	Major Findings	Reference
Bran Protein Isolate	Alcalase, Flavourzyme, papain, neutral enzyme and trypsin	Super anion radical scavenging assay and TAC assay. Gel filtration chromatography; LC-MS/MS	<ul style="list-style-type: none"> Fraction 4-4 showed highest super anion scavenging activity and Trolox equivalent antioxidant activity (70.27% and 3.6 mmol TE/g). The 5 identified peptide showed the IC₅₀ value super anion scavenging activity and Trolox equivalent antioxidant activity ranged from 2.15 mg/mL to 3.27 mg/mL and 0.09 mg/mL to 0.76 mg/mL. Five antioxidant novel peptides (CGFPGHC; QAC; RNF; SSC; WF) were identified. The structure of peptide QAC and SSC were not disrupted during <i>in vitro</i> digestion but showed improved stability. 	(Zhao et al., 2020)
Wheat Gluten Isolate	Alcalase	ABTS assay; Separation using ultrafiltration membranes with MWCO of 1 kDa and nano-filtration; R-HPLC; ESI-MS/MS; R-HPLC-MS/MS	<ul style="list-style-type: none"> Fraction 2-6 showed high ABTS scavenging activity (1.2 – 1.6 mmol TE/g sample). Five peptides derived from these fractions were synthesised and assessed showing high antioxidant power. Peptide LY, PY, YQ, APSY and RGGY were identified. Four novel peptides (LY, YQ; APSY and RGGY) were found to be both rich in antioxidant power and ACE inhibitory power. 	(Liu et al., 2021)
Wheat Germ Protein Extracts	Pepsin, Alcalase and proteinase K	DPPH, ABTS, TAC and Ferric chelation assay; Separation by R-HPLC; R-HPLC-MS/MS	<ul style="list-style-type: none"> Overall, hydrolysates produced by pepsin and Alcalase showed higher antioxidant activity. Identification: KELPPSDADW from pepsin hydrolysis; SGGSYADELVSTAK and MDATAALHYENQK from proteinase K hydrolysis; GNPIPREPGQVPAY from Alcalase hydrolysis. The identified peptides showed combined activity of antioxidant, ACE inhibition and anti-cancer. 	(Karami, Peighambarous t, Hesari, Akbari-Adergani, et al., 2019)

Table 1.8.3. Summarised studies investigating the antioxidant effects of the derived peptide from wheat. *Continued*

Original Protein	Protease	Methods	Major Findings	Reference
Wheat Protein Isolate	Bromelain, Neutrase and Flavourzyme	Cell-based antioxidant assay (MTT assay) using Caco-2 cells	<ul style="list-style-type: none"> Wheat Digesta at a dose of 500 g/mL showed a significant protective effect. Wheat digesta showed good protective effect regarding <i>in vivo</i> H₂O₂- induced oxidative damage. 	(Chang et al., 2021)
Wheat Peptide (sequence unknown; processing method unknown)	/	DPPH and ferric reduction assay.	<ul style="list-style-type: none"> The DPPH scavenging activity and ferric reduction power of wheat peptides were 47.17% ± 0.76% and 0.48% ± 0.02%. Wheat peptide showed intermediate antioxidant power compared to peptide derived from mung bean and corn. 	(Sun et al., 2019)
Wheat Flour	Pepsin-Trypsin Digestion Model	ORAC, TPC, ABTS, DPPH and ion chelation assay; Separation using ultrafiltration membranes with MWCO of 3 kDa;	<ul style="list-style-type: none"> Wheat intestinal digesta < 3 kD showed ORAC (1.5 µmol TE/mg protein), ABTS (IC₅₀: 110 mg/mL), DPPH (IC₅₀: 400 mg/mL), Ferric chelation (IC₅₀: 1.6 mg/mL) and TPC (105 mg FAE/g) Wheat digesta showed intermediate antioxidant power compared to other digesta. 	(Mason et al., 2020)

- “/” in the table is used when the related information was not found.

Table 1.8.3. Summarised studies investigating the antioxidant effects of the derived peptide from wheat. *Continued*

Original Protein	Protease	Methods	Major Findings	Reference
Wheat Germ Protein Extracts	proteinase-K	DPPH, ABTS and TAC assay; Separation by R-HPLC to obtain fractions with bioactivity; R-HPLC-MS/MS	<ul style="list-style-type: none"> The digesta exhibited DPPH (IC₅₀: 1.83 mg/mL); ABTS (IC₅₀: 1.57 mg/mL) and TAC (2mg/mL showed 45 α-tocophenrol equivalent μmol/mL) Two novel peptides (SGGSYADELVSTAK, MDATALHYENQK) were identified to have ABTS scavenging activity. 	(Karami, Peighamardoust, Hesari, Akbari-Adergani, et al., 2019)
Wheat Germ Protein Extracts	Alcalase	DPPH assay; Separation using ultrafiltration membranes with MWCO of 1 kDa; Gel filtration chromatography; HPLC-MS/MS	<ul style="list-style-type: none"> Fraction < 1 kDa was separated into 7 fractions (IC₅₀ value from 1.17 mg/mL to 2.97 mg/mL). Fraction WG-P-4 showed highest DPPH scavenging activity. Peptide GPF, GPE and FGE were identified. Three novel antioxidant peptides were identified. The selected fraction showed to be absorbed from the AP side to BL side was higher than from BL to AP through Caco-2 monolayer <i>in vitro</i> meaning the peptide fraction was able to cross into Caco-2 cell. 	(Zhang et al., 2019)
Wheat Germ Albumin	Microwave-assisted papain hydrolysis	DPPH and super anion radical scavenging assay. Gel filtration chromatography. Separation using ultrafiltration membranes with MWCO of 3, 5 and 10 kDa.	<ul style="list-style-type: none"> 2.5 mg/mL of digesta showed highest DPPH scavenging power of 60% and super anion scavenging activity of 57%. Fraction P4 derived from digesta < 3 kDa showed highest antioxidant power at 2.5 mg/mL (DPPH: 65% and super anion scavenging power: 70%). Hydrolysates with MW of < 3 kDa showed highest overall antioxidant power. The smaller the size of peptide, the stronger the antioxidant activity. 	(Tian et al., 2022)

Table 1.8.3. Summarised studies investigating the antioxidant effects of the derived peptide from wheat. *Continued*

Original Protein	Protease	Methods	Major Findings	Reference
Wheat germ-derived peptide ADWGGPLPH	/	DCF-DA assay to measure intracellular ROS level.	<ul style="list-style-type: none"> High glucose-induced ROS generation was significantly prevented by ADWGGPLPH treatment ($13.6 \pm 1.4\%$ decrease at 10 mM; $20.3 \pm 4.0\%$ at 20 mM) Peptide ADWGGPLPH derived from wheat germ serves as a strong antioxidant beneficial in treatment diabetic vascular dysfunction. 	(Wang et al., 2020)
Wheat gluten isolate	Endo-protease (A, N, P and F). Pepsin-pancreatin digestion model.	DPPH assay. Size-exclusion chromatography.	<ul style="list-style-type: none"> The DPPH scavenging power of wheat gluten digesta was ranged from 25 - 40%. After subjected the digesta to <i>in vitro</i> digestion, the DPPH scavenging power of the intestinal digesta was from 7% to 15%. The <i>in vitro</i> digestion system significantly reduced the antioxidant power of the wheat gluten digesta. 	(Lim et al., 2022)

- “/” in the table is used when the related information was not found.

ACE Inhibitory Activity

Eight recent articles, investigated the ACE inhibitory peptides derived from wheat protein, are summarised in Table 1.8.4. The only study focused on wheat bran protein and hydrolysates was conducted by Zou et al. (2020). In this study, wheat bran protein hydrolysate was prepared by Alcalase was MW of < 1 kDa showed highest ACE inhibitory activity of $84.25 \pm 2.45\%$ compared to other fractions. Fraction < 1 kDa also significantly reduced the blood pressure in spontaneous hypertensive rats by -20 and -35 mmHg. Peptide NL, QL, FL, HAL, AAVL, AKTVF, and TPLTR were determined to be novel anti-hypertensive peptides.

Liu et al. (2021) did similar findings on gluten extracted from wheat flour. Wheat gluten hydrolysate < 1 kDa produced by Alcalase digestion, followed by ultrafiltration membrane and R-HPLC showed ACE inhibition activity of 61.21%-89.36%. Peptide LY, LVS, YQ, APSY and RGGY were corresponding to the ACE inhibitory activity. When wheat germ protein extract was treated by sequential enzymatic hydrolysis by pepsin, Alcalase and proteinase K, peptide SGGSYADELVSTAK, MDATALHYENQK, KELLPPSDADW, SSDEEVREEKELDLSSNE, TVGGAPAGRIVME, VGGIDEVIAK and GNPIPREPGQVPAY were identified (Karami, Peighambaroust, Hesari, Akbari-Adergani, et al., 2019), in which peptide SGGSYADELVSTAK showed lowest IC₅₀ value of ACE inhibition (0.093 μ M). When wheat germ hydrolysate was produced by proteinase K, peptide VALTGDNGHSDHVVF, VDSLLTAAK, MDATALHYENQK, IGGIGTVPVGR and SGGSYADELVSTAK were identified and showed the IC₅₀ value of ACE inhibitory activity ranged from $125.7 \pm 2.3 \mu\text{g/mL}$ to $303.6 \pm 2.47 \mu\text{g/mL}$ (Karami, Peighambaroust, Hesari, Akbari-Adergani, et al., 2019). When wheat protein hydrolysates were produced by Alcalase and proteinase A, peptide IHVTET, AGPCAPNP, APATPSFW, APAPRPPNAP, SAGGYIW and EEAIFLW were identified by P. Zhang et al. (2020) where peptide SAGGYIW with the IC₅₀ value of ACE inhibition of $0.002 \pm 0.000 \text{ mg/mL}$. Khosravi et al. (2022) reported wheat gluten was treated by enzyme extracted from *Biarum bovei*. Hydrolysates with the MW of 3 kDa- 30 kDa and 30 kDa – 100 kDa showed ACE inhibitory activity of $86.03 \% \pm 0.48\%$ and $81.3\% \pm 3.11\%$, respectively. As for the peptides produced by the GI system, wheat protein

intestinal digesta with the MW < 3 kDa showed IC₅₀ value of ACE inhibition activity of about 800 µg/mL (Mason et al., 2020) but wheat protein gastric digesta compared to intestinal digesta showed higher ACE inhibitory power (Camille Dugardin et al., 2020).

Table 1.8.4 Summarised studies investigating the ACE inhibitory effects of the derived peptide from wheat.

Original Protein	Protease	Methods	Major Findings	Reference
Bran Protein Extracts	Alcalase	Separation using ultrafiltration membranes with MWCO of 1, 3, 5, and 10 kDa; R-HPLC-MS/MS	<ul style="list-style-type: none"> • Fraction < 1 kDa showed highest ACE inhibitory activity of $84.25 \pm 2.45\%$. • Peptide NL, QL, FL, HAL, AAVL, AKTVF and TPLTR were identified. • Fraction < 1 kDa showed highest ACE inhibitory activity; The identified peptides with small size showed strong antihypertensive effects in rat models due to its better absorbance rate. 	(Zou et al., 2020)
Gluten Isolate	Alcalase	Separation using ultrafiltration membranes with MWCO of 1 kDa and nano-filtration; R-HPLC; ESI-MS/MS; R-HPLC-MS/MS	<ul style="list-style-type: none"> • Fraction 2-6 showed high ACE inhibitory power (61.21%-89.36%). Five peptides derived from these fractions were synthesised and assessed by ACE inhibition assay showing high inhibitory rate. • Peptide LY, LVS, YQ, APSY and RGGY were identified. • Four novel peptides (LY, YQ; APSY and RGGY) were found to be both rich in antioxidant power and ACE inhibitory power. 	(Liu et al., 2021)
Germ Protein Extracts	Pepsin, Alcalase and proteinase K	Separation by R-HPLC; R-HPLC-MS/MS	<ul style="list-style-type: none"> • Peptide SGGSYADELVSTAK, MDATALHYENQK, KELLPPSDADW, SSDEEVREEKELDLSSNE, TVGGAPAGRIVME, VGGIDEVIAK and GNPIPREPGQVPAY were identified. • Peptide SGGSYADELVSTAK showed lowest IC₅₀ value of ACE inhibition (0.093 μM). • The identified peptides showed combined activity of antioxidant, ACE inhibition and anti-cancer. 	(Karami, Peighambaroust, Hesari, Akbari-Adergani, et al., 2019)

Table 1.8.4 Summarised studies investigating the ACE inhibitory effects of the derived peptide from wheat. *Continued*

Original Protein	Protease	Methods	Major Findings	Reference
Wheat Gluten Isolate	Crude enzyme extract of <i>Biarum bovei</i>	Separation using ultrafiltration membranes with MWCO of 3, 30 and 100 kDa.	<ul style="list-style-type: none"> • Fraction of 3 kDa- 30 kDa and 30 kDa – 100 kDa showed highest ACE inhibitory activity of 86.03 %± 0.48% and 81.3% ± 3.11%. • High hydrophobicity was positively correlated to ACE inhibitory activity. 	(Khosravi et al., 2022)
Two Types of Wheat Protein Isolate WP1 and WP2	Pepsin-Pancreatin Digestion Model	In vitro digestion to obtain crude protein, gastric digesta and intestinal digesta.	<ul style="list-style-type: none"> • Gastric digesta showed higher inhibitory power than intestinal digesta. The gastric digesta of WP1 showed lowest IC₅₀ value of inhibitory activity (60.79 ± 20.18 µg/mL). • Gastric digesta showed higher ACE inhibitory power. 	(Camille Dugardin et al., 2020)
Wheat Flour	Pepsin-Trypsin Digestion Model	Separation using ultrafiltration membranes with MWCO of 3 kDa;	<ul style="list-style-type: none"> • Wheat intestinal digesta < 3 kDa showed highest IC₅₀ value of ACE inhibition (about 800 µg/mL) amongst all samples tested. • Compared to the digesta derived from other samples, wheat intestinal digesta showed the weakest ACE inhibitory power. 	(Mason et al., 2020)

Table 1.8.4 Summarised studies investigating the ACE inhibitory effects of the derived peptide from wheat. *Continued*

Original Protein	Protease	Methods	Major Findings	Reference
Wheat Germ Protein Extracts	proteinase-K	Separation by R-HPLC to obtain fractions with bioactivity; R-HPLC-MS/MS	<ul style="list-style-type: none"> Five peptides were identified and synthesised showing the IC₅₀ value of ACE inhibition ranged from 125.7 ± 2.3 µg/mL to 303.6 ± 2.47 µg/mL. Peptide VALTGDNGHSDHVVF, VDSLTTAAK, MDATALHYENQK, IGGIGTVPVGR and SGGSYADELVSTAK were identified. Novel ACE inhibitory peptides were identified. Peptide with MW ranged from 866 Da to 2.9 kDa showed good ACE inhibitory power. 	(Karami, Peighambardoust, Hesari, Akbari-Adergani, et al., 2019)
Wheat Gluten Isolate	protease A (derived from <i>Pseudomonas aeruginosa</i>), Protamex, Alcalase, the combination of Alcalase and Protamex, the combination of Alcalase and proteinase A and the combination of Protamex and proteinase A	Separation by ultrafiltration membranes with MWCO of 1, 5 and 10 kDa; R-HPLC-MS/MS	<ul style="list-style-type: none"> Hydrolysates (< 1 kDa) by the combination of Alcalase and proteinase A showed the IC₅₀ value of ACE inhibition of 0.21 ± 0.02 mg/mL and was selected for further separation resulting into 7 fractions with the IC₅₀ value of ACE inhibition ranged from 0.04 mg/mL to 0.25 mg/mL. Peptide IHVTET, AGPCAPNP, APATPSFW, APAPRPPNAP, SAGGYIW and EEAIPLW were identified. Six ACE inhibitory peptides were identified. Peptide SAGGYIW showed the lowest IC₅₀ value of ACE inhibition (0.002 ± 0.000 mg/mL). 	(P. Zhang et al., 2020)

1.8.3 Barley Derived Peptides

Antioxidant

Three recent studies have investigated the barley protein peptides and their antioxidant properties (Table 1.8.5). Gumus et al. (2022) investigated peptides extracted from barley sprouts with the MW of < 3 kDa showing IC₅₀ of metal chelation capability of $7.9 \pm 1.03 \mu\text{g}/\text{mL}$ and EC₅₀ (half maximal effective concentration) of cupric ion reducing antioxidant capacity (CUPRAC) antioxidant activity of $26.5 \pm 1.03 \mu\text{g}/\text{mL}$. Peptide SQQENELTSLIVESN and NRFNNASNT were identified to related to antioxidant power. Tok et al. (2021) reported that barley protein extracts digested by trypsin showed antioxidant activity of $2.95 \pm 2.1 \mu\text{M}$ AAE/ μg peptide (CUPRAC). In addition, ultrasonic treatment improved the overall antioxidant activity of barley protein hydrolysates by over 50% (Ikram et al., 2020).

Table 1.8.5 Summarised studies investigating the antioxidant effects of the derived peptide from barley.

Original Protein	Protease	Methods	Major Findings	Reference
Peptide in Barley Sprouts	/	Copper chelation and CUPRAC assay; Separation using ultrafiltration membranes with MWCO of 3 and 10 kDa; R-HPLC; LC-MS/MS	<ul style="list-style-type: none"> Fraction < 3 kDa showed highest antioxidant activity (IC₅₀ of metal chelation: 7.9 ± 1.03 µg/mL; EC₅₀ of CUPRAC antioxidant activity: 26.5 ± 1.03 µg/mL) Peptide SQQENELTSLIVESNNRFNNASNT was identified. The peptide with a high number of amino acids in its structure was identified with good antioxidant activity. 	(Gumus et al., 2022)
Barley Protein Extracts	trypsin	CUPRAC assay and metal chelation assay	<ul style="list-style-type: none"> The hydrolysates showed antioxidant activity of 2.95±2.1×10⁻³ mM AAE/µg peptide (CUPRAC) and 1.69 %/µg peptide (metal chelation) Barley protein had potential of being an antioxidant substance to be incorporated in the diet. 	(Tok et al., 2021)
Highland Barley Spent Grains Protein Extracts	Ultrasound-assisted Alcalase treatment	DPPH, ORAC, FRAP metal chelation, super anion scavenging and hydroxyl scavenging assay	<ul style="list-style-type: none"> Hydrolysates with longer treatment by ultrasound during enzymatic treatment showed significantly higher antioxidant activity. Four-hour ultrasound treatment on hydrolysates showed superior impact in releasing antioxidant peptides. 	(Ikram et al., 2020)

- “/” in the table is used when the related information was not found.

ACE Inhibitory Activity

One recent paper (Yin et al., 2022) was found to investigate the ACE inhibitory peptides from barley protein (Table 1.8.6). Peptide VVTGVGGQ, LPVGP, LLSPP and FPLQPHQP were identified in a Chinese alcohol (Qingke) derived from highland barley showing the IC₅₀ value of ACE inhibitory power ranged from 5.03 ± 0.23 mM to 108.52 ± 3.47 mM.

Table 1.8.6 Summarised studies investigating ACE inhibitory effects of the derived peptide from barley.

Original Protein	Protease	Methods	Major Findings	Reference
Qingke Baijiu (a Chinese alcohol derived from Highland barley)	/	Nano – LC-MS/MS.	<ul style="list-style-type: none"> Identified peptides were synthesised and showed the IC₅₀ value of ACE inhibitory power ranged from 5.03 ± 0.23 mM to 108.52 ± 3.47 mM. Peptide VVTGVGGQ, LPVGP, LLSPP and FPLQPHQP Four novel ACE inhibitory peptides were identified. When the concentration of the identified peptides led to expected ACE inhibitory power, they did not cause proliferation damage to intestinal epithelial cells. 	(Yin et al., 2022)

- “/” in the table is used when the related information was not found.

1.8.4 Rice Derived Peptides

Antioxidant

Twelve studies selected from the database investigated antioxidant peptides derived from RBP (rice bran protein) (Table 1.8.7). Protease 500G was used in a study by Zhang et al. (2014) to hydrolyse heat-stable rice bran. The hydrolysates were

fractioned, and their antioxidant activities were investigated. Fraction HSDRBH-75, a pool of hydrolysates eluted by 75% ethanol, showed the highest DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity and reducing power. All fractions showed protection for lipids. The scavenging activity and reducing power of hydrolysates was shown to be dose-dependent. The hydrolysates prepared by trypsin with an MW of less than 4 kDa showed the most active reducing power (Wang et al., 2017) but the pepsin-trypsin gastro-intestinal (GI) digestion model produced hydrolysates that had a more substantial reducing power compared the pool of peptides with MW between 3-5 kDa (Phongthai et al., 2018). Flavourzyme produced hydrolysates with better reducing power compared to Alcalase and Neutrase (Thamnarathip et al., 2016).

Metal chelation assay determines antioxidant activity by measuring the chelation of copper II or iron II; metals produce oxide anion forming toxic radicals. Alcalase and Protease 500G hydrolysates eluted by 25%, 50%, 75% and 100% ethanol showed the chelation activity of over 20% (Zhang et al., 2014). Similar results were obtained by Zaky et al. (2019). Flavourzyme and the pepsin-trypsin produced hydrolysates with the better metal chelation activity (61.04%) compared to the activity of trypsin-hydrolysates (10%) (Zaky et al., 2019; Zaky et al., 2020). Selamassakul et al. (2018) indicated the bromelain assisted hydrolysis produced hydrolysates from glutenin fraction with the chelation activity of 28% stronger than that of albumin hydrolysates (9.55%). RBP hydrolysed by Alcalase, Flavourzyme, Neutrase, trypsin, bromelain and *in vitro* pepsin-trypsin digestion trial showed a significantly increased ABTS scavenging activity, and the trend was similar to that revealed in the DPPH scavenging assay (Phongthai et al., 2018; Saisavoey et al., 2016; Selamassakul et al., 2018; Thamnarathip et al., 2016; Zaky et al., 2019; Zaky et al., 2020) and TPC assay displaying a dose-dependent manner (Thamnarathip et al., 2016).

Antioxidant activity of rice bran protein hydrolysates (RBPHs) was also investigated by the GSH assay and ORAC assay (Hatanaka et al., 2015; Moritani et al., 2017; Wattanasiritham et al., 2016). GSH levels within cells reflect the cellular redox status and are regulated by the expression of the enzyme γ -glutamylcysteine synthetase (γ -

GCS) and expression levels of Nrf2, NQO1 and HO-1. Moritani et al. (2017) indicated that 5 mg/ml RBPHs prepared with Umamizyme G increased the intracellular GSH levels from around 90 nmol/mg protein to 170 nmol/mg protein in HepG2 cells and 200 nmol/mg protein in COS7 cells after 24 hr treatment. The expression of γ -GCSH and γ -GCSI increased by about 1.5-fold after 8 hr treatment while the expression of NQO1 showed significant elevation of about 1.5-fold at 3 hr and 24 hr, levels of HO-1 increased by roughly 100% at 8 hr and 24 hr. The mRNA level of Nrf2 increased by 50% at 3 hr followed by a rapid decline at 8 hr and 24 hr to about 50% of the initial expression level. ORAC assay measures the total antioxidant activity of slow and fast-acting antioxidant hydrolysates. Wattanasiritham et al. (2016) reported that the ORAC value of hydrolysates (1.-4. μ mol of TE/mg protein) was significantly higher than non-hydrolysed proteins (0.45-2.3 μ mol of TE/mg protein). The ORAC assay was also incorporated to determine the inhibitory effects of RBPHs on DPP-IV activity. DPP-IV is a ubiquitous enzyme that metabolises the insulinotropic hormone, glucagon-like peptide-1 (GLP-1) and leads to postprandial hyperglycaemia (Moritani et al., 2017). RBPHs displayed an ORAC value of $670 \pm 17 \mu$ mol TE/g stronger than that of RBP ($525 \pm 78 \mu$ mol TE/g) (Hatanaka et al., 2015).

Table 1.8.7 Summarised studies investigating the antioxidant effects of the derived peptide from rice.

Original Protein	Protease	Methodology	Major Findings	Reference
Total bran protein	Umamizyme G	Overnight hydrolysis; HepG2 and COS7 cell culture; Measurement of intracellular GSH level; Westblotting; LDH cytotoxicity assay	<ul style="list-style-type: none"> • RBPHs with higher concentration showed better inhibition. • Double GSH level was detected in treated culture; Expression of γ-GCSH and γ-GCSI were promoted by 50% after 8-hour treatment; NQO1 and HO-1 expression level was promoted; <i>Nrf2</i> expression increased by 1.5-fold after 3-hour treatment. 	(Moritani et al., 2017)
Albumin, globulin, glutelin, prolamin	Papain, trypsin	ORAC; R-HPLC; LC-MS/MS	<ul style="list-style-type: none"> • ORAC value of fractions ranged from 0.4 to 24.9nmol of TE; fractions 14, 15 showed the highest antioxidant activity selected to be identified. • Peptides consisting of 6 to 21 amino acids were identified, with MW ranging from 726 to 1578 Da; Peptide in fraction 14 contained more than 50% hydrophobic amino acids and more than two antioxidant sequences. • Denatured RBPHs showed higher antioxidant activity than native RBPHs; Trypsin-hydrolysed albumin RBPHs had the highest antioxidant activity; the hydrophobicity of amino acids contributed to the antioxidant nature of RBPHs. 	(Wattanasiritham et al., 2016)
Total bran protein	Pepsin, trypsin	DPPH, ABTS, metal chelation assay; R-HPLC; amino acid determination	<ul style="list-style-type: none"> • Fractions larger than 10 kDa showed the highest protein content, less than 3 kDa had the highest amino acid content while 3-10 kDa showed highest activity of all three antioxidant assays. • Most tyr was found in the purified fraction followed by His, Ile, Lys, Phe, Thr and Val. • Fraction between 3 to 10 kDa possessed the highest bioactivity; the production of hydrophobic amino acids and aromatic amino acids was associated to the antioxidant activity. 	(Zaky et al., 2020)

Table 1.8.7 Summarised studies investigating the antioxidant effects of the derived peptide from rice. *Continued*

Original Protein	Protease	Methods	Major Findings	Reference
Total rice bran protein	Alcalase 2.4L, Protease 500G	1.8-hour and 2.0-hour hydrolysis; 25%, 50%, 75% and 100% ethanol elution fractionation; R-HPLC; DPPH, FRAP, FIC and test of inhibition of linoleic acid oxidation	<ul style="list-style-type: none"> • Fraction HSDRBH-75 showed the highest value. • The content of hydrophobic amino acids increased by 40%. • Hydrolysed RBPHs with 1kDa showed the highest antioxidant activity 	(Zhang et al., 2014)
Total rice bran protein	Alcalase, Flavourzyme, Neutrase, papain and pepsin-pancreatin	ABTS, DPPH; NO; RAW264.7 cell culture; NO production determination; RNA isolation and iNOS, IL-6, TNF- α mRNA detection by RT-PCR; MF and R-HPLC; LC-MS/MS	<ul style="list-style-type: none"> • Fraction 4 (MW less than 3 kDa) showed the highest antioxidant and inflammatory inhibition activity. • 10 peptide sequences were found with MW from 814 to 1276 Da; Leu, His, Trp and Tyr found to be major amino acids in all peptides. • Peptides less than 3 kDa showed strong antioxidant activity and inhibition activity against NO production induced by LPS in RAW264.7 cells and suppressed the expression of iNOS, IL-6 and TNF-αmRNA. 	(Saisavoey et al., 2016)
Total rice bran protein	Pepsin, trypsin	<i>In vitro</i> digestion; MF; SDS-PAGE; DPPH, metal chelation assay and FRAP; HPLC; MALDI-TOF MS	<ul style="list-style-type: none"> • Fractions less than 3 kDa showed the highest antioxidant activity and outstanding ability to produce aromatic amino acids, except fractions between 3 to 5 kDa showed the highest metal chelating activity. • Major peptides fractions were identified at MW of 609, 944 and 1088 kDa. • <i>In vitro</i> digestion promoted the antioxidant activities which were found to be positively correlated to the amount of tyrosine contents after hydrolysis. 	(Phongthai et al., 2018)

Table 1.8.7 Summarised studies investigating the antioxidant effects of the derived peptide from rice. *Continued*

Original Protein	Protease	Methodology	Major Findings	Reference
Total bran protein	Alcalase, Flavourzyme, Neutrase	Protein assay; 2,4,6-hour hydrolysis; TPC, ABTS, FRAP; SDS-PAGE; IEC	<ul style="list-style-type: none"> Alcalase degraded band at 40 kDa completely after 2 hours; All proteases degraded protein with large MW down to lower than 15 kDa. Alcalase RBPHs: 5 bands from 6 to 36 kDa; Flavourzyme RBPHs: 6 bands from 16 to 64 kDa; 7 bands from 14 to 64 kDa. Alcalase produced the highest protein content; Alcalase showed the greatest extraction efficiency of 56.6% with 30.3% protein content after 6-hour hydrolysis; and highest antioxidant activity after 2 hours of hydrolysis. 	(Thamnarathip et al., 2016)
Total bran protein	Alcalase, Trypsin, Protamax, Flavourzyme	Protein assay; 2,4,6-hour hydrolysis; TPC, DPPH, ABTS, metal chelation assay; SDS-PAGE	<ul style="list-style-type: none"> Protamax and Trypsin RBPHs: 5 to 63 kDa; Alcalase and Flavourzyme RBPHs: < 15 kDa; Bands with 35 kDa and 63 kDa degraded. Protamax produced the highest protein content of hydrolysates; Trypsin hydrolysed RBPHs showed the highest TPC but the least scavenging activity whereas Flavourzyme produced hydrolysates with the least protein content but the highest scavenging activity and metal chelation activity. 	(Zaky et al., 2019)
Total bran protein	Trypsin	DPPH, FRAP; hypertension rats (SHR) model; Gel filtration chromatography, R-HPLC, Q-TOF-MS, Auto Dock 4.0.	<ul style="list-style-type: none"> Hydrolysates with MW < 4 kDa had greater ability to quench DPPH radicals. Peptide YSK was identified. Hydrolysates of MW < 4 kDa showed strong antioxidant at the intracellular level; peptide; YSK showed six hydrogen bonds and ACE residue Glu162, Lys511, Tyr520, Asp415 and Gln281. 	(Wang et al., 2017)

Table 1.8.7 Summarised studies investigating the antioxidant effects of the derived peptide from rice. *Continued*

Original Protein	Protease	Methodology	Major Findings	Reference
Albumin, globulin, glutelin, prolamin	Papain	Protein assay; 30 min hydrolysis; SDS-PAGE; Tyrosinase inhibition assay; copper chelation assay; filtration and R-HPLC; LC-MS/MS	<ul style="list-style-type: none"> Eleven peptides were identified and first eluted fractioned showed highest antioxidant and inhibition activity. Peptides had MW ranged from 1327 to 4819 Da; Gly, Ser and Glu were identified to be the most abundant amino acids in fractions. 	(Phongthai et al., 2016)
Total bran protein	Alcalase, Flavourzyme, Neutrase	Protein assay; 2,4,6-hour hydrolysis; TPC, ABTS, FRAP; SDS-PAGE; IEC	<ul style="list-style-type: none"> Alcalase RBPHs: 5 bands from 6 to 36 kDa; Flavourzyme RBPHs: 6 bands from 16 to 64 kDa; 7 bands from 14 to 64 kDa. Alcalase produced the highest protein content; Alcalase showed the greatest extraction efficiency of 56.6% with 30.3% protein content after 6-hour hydrolysis; and highest antioxidant activity after 2 hours of hydrolysis. 	(Cheetangdee & Benjakul, 2015)
Total bran protein	Alcalase, Trypsin, Protamax, Flavourzyme	Protein assay; 2,4,6-hour hydrolysis; TPC, DPPH, ABTS, metal chelation assay; SDS-PAGE	<ul style="list-style-type: none"> Protamax produced the highest protein content of hydrolysates; Trypsin hydrolysed RBPHs showed the highest TPC but the least scavenging activity whereas Flavourzyme produced hydrolysates with the least protein content but the highest scavenging activity and metal chelation activity. 	(Charoen et al., 2017)

ACE Inhibitory Activity

Six studies revealed the importance of RBPHs on inhibiting ACE actions (Table 1.8.8). Uraipong and Zhao (2016b) treated RBP fractions with Alcales, Neutrase, Flavourzyme and Protamax in order to determine the ACE inhibitory activity of hydrolysates. Protamax- albumin hydrolysates showed relatively high IC₅₀ activity of 5.2 mg protein/mL. Protamax-glutelin, Alcalase-glutelin and Alcalase-albumin hydrolysates showed strong ACE inhibitory activity of 6.2, 8.4 and 9.2 mg protein/mL. RBPHs produced by Flavourzyme showed the lowest activity against ACE activity. The following study of Uraipong and Zhao (2018) simulated *in vitro* digestion with amylase, pepsin, pancreatin and bile extract mimicking the hydrolysis occurring during the oral administration, in the stomach and the small intestine. After the ultrafiltration, digesta lower than 3 kDa showed strong activities except for glutelin and globulin fractions. Authors assumed the synergistic effects between the large and small size peptides was the cause (Uraipong & Zhao, 2018).

Two *in vitro* studies were conducted in 2017 and 2018 (Shobako et al., 2018; Wang et al., 2017), where the trypsin hydrolysates (< 4 kDa) showed strong ACE inhibitory activity with the IC₅₀ value of 3.53 protein mg/mL. This pool of peptides also exhibited the highest scavenging activity against free radicals with the IC₅₀ value of 0.98 mg/mL. The spontaneously hypertensive rat (SHR) model was also used in this study to ensure the pool of peptides remaining biologically active after oral administration. The results showed the declined blood pressure after 2,4,6 and 8 hours of oral administration. The amino acid sequence, Tyr-Ser-Lys, was identified with the MW of 395 Da. Shobako et al. in 2018 also conducted an *in vivo* study to determine the ACE inhibitory activity of the Thermolysin-digested rice bran protein (TRB). The result indicated that hydrolysates reduced the blood pressure of the subject rats from 230 mmHg to 200 mmHg. ACE inhibitory activity was significantly declined found in lung extracts from 300 mU/mg protein to 190 mU/mg protein. RBPHs was reported to significantly lowered the in-plasma ACE level and blood pressure of rats with the high carbohydrate high fat (HCHF) diet (Senaphan et al., 2018). The last study was conducted on 2017 by Pooja et al. Authors used

computational tool (*in silico* approaches) simulating the papain hydrolysis conditions to assess potential ACE inhibitory activity, followed by characterisation and structure determination (Pooja et al., 2017).

Table 1.8.8 Summarised studies investigating the ACE inhibitory effects of the derived peptide from rice.

Original Protein	Protease	Methodology	Major Findings	Reference
Total bran protein	Trypsin	Spontaneously hypertension rats (SHR) model; Gel filtration chromatography, R-HPLC, Q-TOF-MS, amino acid sequencer, Auto Dock 4.0.	<ul style="list-style-type: none"> Hydrolysates of MW < 4 kDa showed strong ACE inhibition at the intracellular level; peptide YSK was isolated; YSK showed six hydrogen bonds and ACE residue Glu162, Lys511, Tyr520, Asp415 and Gln281. 	(Wang et al., 2017)
Total bran protein	Protease G6	<i>In vivo</i> rate model, Aortic pulse wave velocity and hemodynamic measurement; blood biochemical assay; oxidative assessment; west blotting; Morphometric analysis;	<ul style="list-style-type: none"> Lowered blood pressure, ACE activity and ROS production; improved hemodynamic alternation by 40%; treatment lowered the vascular wall thickness. 	(Senaphan et al., 2018)
Globulin, glutelin, prolamin	Pepsin, proteinase K, ficain, papain, bromelain	<i>In silico</i> proteolysis	<ul style="list-style-type: none"> A large number of ACE-inhibitory peptides were released, cleavage at C-terminal. <i>In silico</i> Tys, Ser and Lys were found in abundance; Glutelin fractions, globulin and prolamin were predicted to release 317, 92 and 38 ACE inhibitory fractions. 	(Pooja et al., 2017)

Table 1.8.8 Summarised studies investigating the ACE inhibitory effects of the derived peptide from rice. *Continued*

Original Protein	Protease	Methodology	Major Findings	Reference
Albumin	Alcalase, Protamax, Neutrase, Flavourzyme	Separation using ultrafiltration membranes with MWCO of 3 and 10 kDa; LC-MS/MS	<ul style="list-style-type: none"> • Protamax hydrolysates with MW < 3 kDa showed the strongest ACE inhibition activity. • 39 Peptides in ACE inhibition fraction with sequences including HL, PG, GE, GG and NG. • Hydrolysates with MW < 3 kDa exhibited inhibition activity to both enzymes. 	(Uraipong & Zhao, 2016b)
Prolamin, albumin, albumin, glutelin	Pancreatin	LC-MS/MS; amino acid sequencer	<ul style="list-style-type: none"> • Globulin and glutelin digesta and fractions showed the highest ACE inhibition activity. MW of < 3 kDa displayed higher inhibition activity after ultrafiltration. • GGESEER, GPFNILEQRPR, GEGSSEEEDEGR, SFHDLAEHIR; dipeptides include GE, GG, GP, EK and GH were identified. • Fraction of all four digesta showed higher ACE inhibitory activity after filtration. 	(Uraipong & Zhao, 2018)
Prolamin, albumin, albumin, glutelin	Alcalase, Neutrase, Flavourzyme, Protamax	Separation using ultrafiltration membranes with MWCO of 3 and 10 kDa; anion-exchange chromatography; LC-MS/MS	<ul style="list-style-type: none"> • Fractions of > 10 kDa; 3–10 kDa, and < 3 kDa were obtained and the fraction with lower MW showed better inhibition activity. • 13 peptides were identified from fraction of Alcalase-catalysed glutelin hydrolysates; 6-32 amino acids were identified in isolated. • All hydrolysates possessed inhibitory activity to three enzymes; the bioactivity of albumin and glutelin hydrolysates produced by Protamax and Alcalase were the highest. 	(Uraipong & Zhao, 2016a)

1.9 Protein Extraction Methods from Cereals

Protein extraction is the first step before characterising proteins in cereals, with the intention to isolate one, a few or the whole protein contents from the complexity. Alkaline extraction followed by iso-electric precipitation has been acknowledged as a major accepted approach to extract whole protein content from cereal seeds in order to characterise the bioactive values of derived peptides (Walters et al., 2020; Esfandi et al., 2019b; Shittu et al., 2020; Zhang et al., 2019; Zou et al., 2020; Ikram et al., 2020; Zaky et al., 2020; Phongthai et al., 2018). The yield of oat protein extraction by this method, with the increase of pH, could reach to 76% (Kriger, Kashirskikh, Babich, & Noskova, 2018). Addition of SDS in alkaline extraction solution extracted 95% protein of wheat flour. Alkaline extraction method was developed based on the theory that alkaline solution degrades cellulose and destroys the fibril network to release the protein fractions (Knill & Kennedy, 2003). Soination, microwave and enzymes had been used to assist alkaline extraction aiming for investigating novel proteins and peptides with bioactive potentials (Vanvi & Tsopmo, 2016; Esfandi et al., 2019b; Tian et al., 2022; Shittu et al., 2020). Another method used for extracting whole protein content in oats is to use an extraction buffer containing urea, a reducing agent such as Dithiothreitol (DTT) and 2-mercaptoethanol and a detergent such as 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) (Chen, et al., 2016; Galova, et al., 2019; Nałęcz, et al., 2009). This method usually prepares the oat proteins to be characterised and identified by 2-D electrophoresis and contains the reducing agents and detergents necessary to solubilise proteins and gives high resolution results. The method to extract the separated storage protein in

oats was modified based on Osborne's method (Baladrán-Quintana et al., 2015; Comino et al., 2016). According to the solubility of different storage proteins in different chemical solutions, albumin and globulin fractions, prolamin and glutenins can be extracted by distilled water, saline solution, 70% ethanol and 55% 2-propanol, respectively; this method was used to prepare different protein fractions for the bioactivity determination and confrontational characterisation (Boukid et al., 2019; Wattanasiritham et al., 2016; Comino et al., 2016; Nałęcz et al., 2009).

1.10 Antioxidant and ACE Inhibitory Activity of Bran Protein and Peptides

The number of studies selected by using PRISMA guidelines and focused on investigating the antioxidant and ACE inhibitory protein and peptides from bran and other part of oats, wheat, barley and rice are summarised in Figure 1.10.1 (based on the publications summarised on section 1.8). From 2015 to 2023, studies focused on bran protein and peptides were significantly less than studies investigating wholegrain protein and peptides. The only exception was the number of studies on oat bran antioxidant peptides. Interestingly, most studies focused on bran protein and peptides used commercial enzymes to prepare novel antioxidant and ACE inhibitory peptides. However, these peptides face the challenge of further degradation by GI digestion leading to the loss of bioactivity.

Pepsin-trypsin digestion models, or the combination of other enzymes such as pancreatin, produced digesta which could release resistant peptides to digestion when compared to the peptides produced by the commercial enzymes.

Wattanasiritham et al. (2016) and Phongthai et al. (2016) provided a possible explanation that trypsin degraded proteins after pepsin degradation and larger peptides into peptides with lower MW and free amino acids (FAAs). The electrophoretic profile of rice bran protein after the treatment of pepsin-trypsin

digestion and bromelain hydrolysis showed more intense bands in the low MW range and the bands with higher MW were absent (Selamassakul et al., 2018). Zhang et al. (2014) found that antioxidant peptides were generally in the MW range from 600 Da to 3.6 kDa. Studies above also emphasised the correlation between the degree of hydrolysis and the antioxidant activity; the peak activity of hydrolysates was not at the end stage of the hydrolysis. As for the enzymes, trypsin is an endopeptidase that cleaves at the C-terminal of arginine and lysine. It produces short-chain peptides with positively charged C-terminal lysine and arginine. Proteases G6 cleaves at the C-terminal and produces peptides with aromatic tyrosine, and hydrophobic phenylalanine and tryptophan attached to the ends contributing to the antioxidant activity of digesta. The study by Zhang et al. (2014) determined that the content of the free hydrophobic amino acids such as isoleucine, tyrosine, phenylalanine, proline, leucine, valine and lysine increased about 40% after hydrolysis. The content of free amino acids with high hydrophobicity is positively correlated to the antioxidant activity of the bran protein digesta (Zhang et al., 2019). Also, a positive correlation between the hydrophobicity and antioxidant activity of peptides was determined (Zhang et al., 2014). Similar finding was pointed out by studies investigating peptides and digesta derived from oats, wheat and rice bran (Chang et al., 2021; Esfandi et al., 2019b; Gumus et al., 2022; Karami, Peighambardoust, Hesari, Akbari-Adergani, et al., 2019; Sun et al., 2019; Vanvi & Tsopmo, 2016). Thamnarathip et al. (2016) found that the hydroxyl groups had a high affinity to the aromatic ring. These authors also reported that the increase in the hydrophobicity also increased the presence of hydrophobic peptides at the water-lipid interphase and strengthened the interaction of peptides and free radical at the lipid phase. Based on the findings above, hydrophobic amino acids at C-terminals contribute to the antioxidant properties of the peptides. Granato et al. (2018) questioned the acceptance of the traditional *in vitro* antioxidant assays including DPPH, ABTS, TPC, FRAP and ORAC in future research about bio-active compounds due to the inconsistency between the assays and the lack of epidemiological support. It is an undeniable fact as the *in vitro* screening methods can quickly determine the potential of a bio-active compound out of a large quantity

of samples, which in turn is useful for the determination of the antioxidant activity of RBPHs. These assays can also be used to monitor the tendency of activity during the proteolysis and represent time and be used as cost-efficient tools for such predictive assessments. On the other hand, human digestion and the utilisation of the antioxidant peptides in a living system is a much more complicated metabolic progress and do not involve the participation of all different types of radicals. In this case, cell-based antioxidant assay will be a suitable method (Furger, 2021).

After digestion, cereal protein gastric digesta tended to have high ACE inhibitory activity compared to intestinal digesta (Camille Dugardin et al., 2020). This could be caused by the breakdown of the bioactive polypeptides by trypsin or pancreatin. Peptides containing hydrophobic and positive charged ends are more likely to possess ACE inhibitory actions (Zou et al., 2020). Uraipong and Zhao (2018) inferred that bran hydrolysate with lysine and arginine, which had a positive charge on the C-terminals, were likely to have ACE inhibitory activity. The inference matched the result reported by (Pooja et al., 2017). ACE inhibitory peptides were rich in serine, arginine, lysine, valine, glycine, asparagine and alanine. Within all 9 essential amino acids, isoleucine, leucine, phenylalanine, methionine, threonine and valine are hydrophobic amino acids. Arginine, histidine and lysine are positively charged while aspartic acid and glutamic acid are negatively charged. C-terminal of peptides was believed to regulate ACE inhibitory activity as hydrophobic amino acids were present (Uraipong & Zhao, 2018). By utilising docking stimulation, Wang et al. (2017) investigated the interaction of peptide tyrosine-serine-lysine derived from rice bran at ACE active sites. The overview of the interaction showed six hydrogen bonds between the peptides and ACE residue glutamic acid162, lysine511, tyrosine520, aspartic acid415 and glutamine281 which were featured in three main active sites of ACE (S1, S2 and S3 pocket). The docking study also indicated that the main interaction was established between the peptides and S2 pocket of ACE active sites. Zheng et al. (2020) discovered the peptide serine-serine-tyrosine-tyrosine-proline-phenylalanine-lysine from naked oat protein. This peptide showed strong bindings to ACE active site S1 and could form 11 hydrogen bonds with 8 amino acid residues

including proline407, arginine522, tyrosine523, glutamic acid411, histidine387, glutamic acid384, aspartic acid358 and alanine356. Glutamic acid384 and tyrosine523 belong to the active site S1 indicating a strong competitive inhibition.

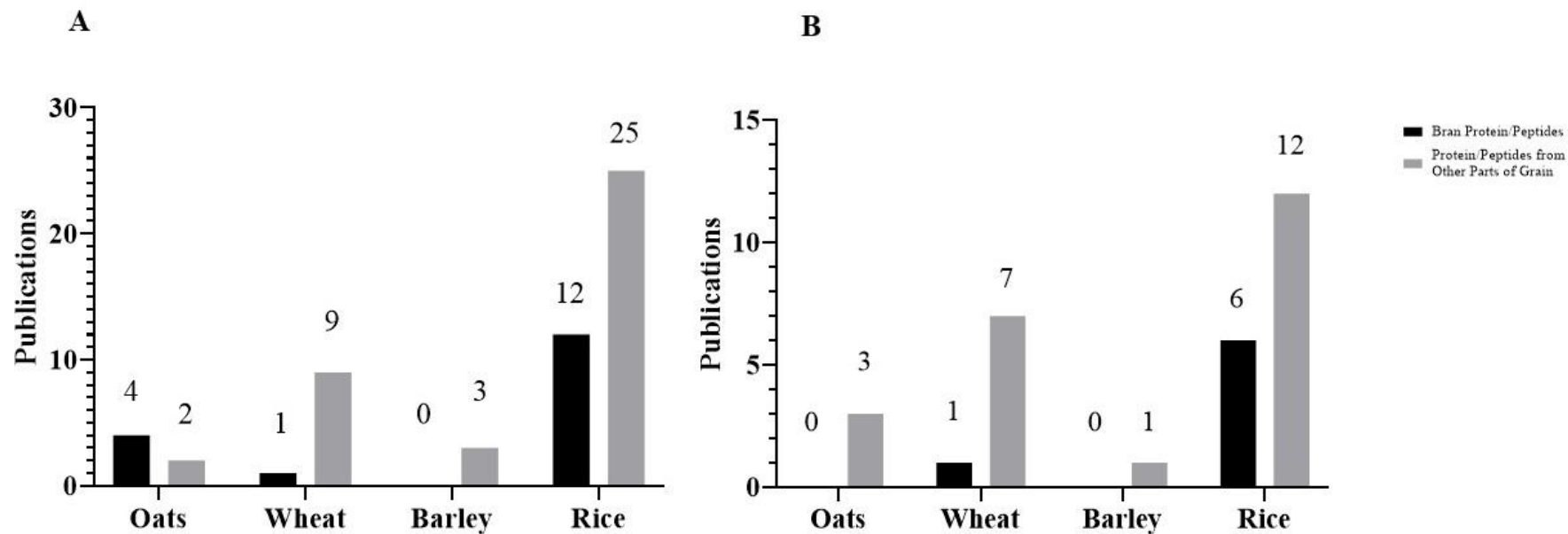


Figure 1.10.1 From 2015-2023, the number of publications focused on investigating antioxidant and ACE inhibitory protein and peptides derived from bran and other parts of oats, wheat, barley and rice. A). Number of studies investigating antioxidant protein/peptides from bran or other parts of these four cereals. B). Number of studies investigating ACE inhibitory protein/peptides from bran or other parts of these four cereals. The black column represents the studies focused on bran protein/peptides while the grey column shows the studies focused on protein/peptides from other parts of cereal grain.

1.11 Bio-accessibility of Cereal Protein and Peptides

Bio-accessibility and bioavailability, by definition, refer to the availability of digested nutrients to be absorbed and digested nutrients enter the circulation in an active form to exert their biological function. Both terms could be used to express the amount of peptides to be used by the body as a therapeutical agent. Novel bioactive peptides have been discovered from different sources but, in fact, are still restricted from clinical application (Zhu et al., 2022). Recent studies are mostly at *in vitro* level (Shobako et al., 2018; Uraipong & Zhao, 2018) and *in vivo* studies have been investigated (Wang et al., 2020; Zou et al., 2020). The developed delivery approaches also pose a challenge to limited absorption of peptides (Li et al., 2021). Bioactive peptides are usually assessed for its integrity and bioactivity after subjecting to GI digestion model. The peptide surviving from the degradation in the stomach and intestine also need to overcome the further degradation triggered by enzymes such as brush boarder proteases (Ozorio et al., 2020). There are four main mechanisms for peptide influx through brush boarder membrane and absorbed by intestine epithelium intact (as shown in Figure 1.11.1): the passive diffusion of peptides between adjacent epithelial cells, the active transport by H⁺- coupled PepT1 and PepT2 transporters, the active transport by Na⁺-coupled SOPT1 and SOPT2 transporters and endocytosis by cell penetrating peptides, as shown in Figure 1.9.1. With the existence of numerous barriers preventing the absorption of peptides, oral administration of peptides could lead to only 1-2% of permeability (Pauletti et al., 1996). Interestingly, recent studies focus on construct bioactive peptides into cyclic form to increase permeability and provide firm structure against degradation (Joo, 2012; Li et al., 2021).

As with the peptides that is not absorbed, there have been studies investigating the interplay of bioactive peptides and gut microbiome in human health (Jaworska et al., 2021; Riaz Rajoka et al., 2021; Zong et al., 2020). Antimicrobial peptides have been known for not only preventing pathogen invasion but also modulating the composition of the microbiome (Jenssen et al., 2006; Ladram & Nicolas, 2016; Zong et al., 2020). Ashaolu (2020) indicated that the hydrolysates of soy protein significantly increased the population of *Bacteroidetes*, *Enterococcus* and *Proteobacteria* and positively shaped the gut microbiome with positive gut microbiome balance index. The study also suggested that peptides may exert biological benefits with the abundant arginine, glutamine and tryptophan which plays important roles

in immune system. Gut microbiome could feed on peptides and amino acids and produce metabolites beneficial to the balance of microbiome (Ashaolu, 2020). Oxidative stress damage could happen to intestine when the system is incapable to eliminate radicals, which led to the destruction of protein, lipid macromolecules and DNA oxidative modification (Cooke et al., 2003; Dalle-Donne et al., 2003; Kadiiska et al., 2005). Microbiome such as *Lactobacillus brevis*, *Bacillus SCo6*, *Akkermansia muciniphila*, *Lactobacillus plantarum* and *Macleaya cordata* are known to prevent oxidative stress (Riaz Rajoka et al., 2021).

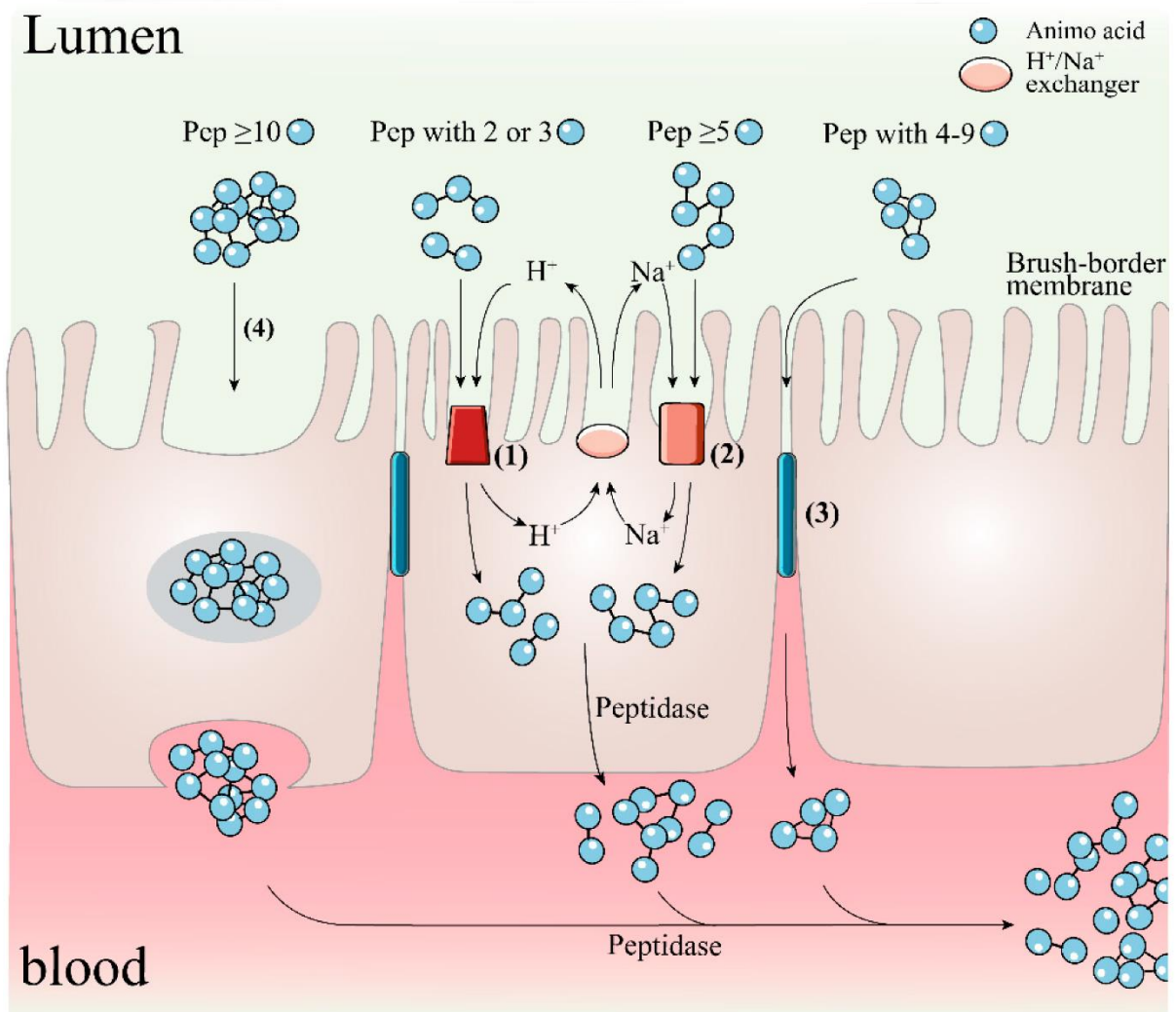


Figure 1.11.1 The influx of peptides with different size. (1) H⁺-coupled PepT1 and PepT 2; (2) Na⁺-coupled SOPT1 and SOPT 2; (3) paracellular; (4) transcytosis (Zhu *et al.*, 2022)

Chapter 2

Experimental Rationale

There have always been unavoidable by-products in the cereal industries including bran, which is a highly nutritional part of the grain. With growing attention in the area of food waste recovery, the by-products can be mixed in animal feeds or used to create new foods as functional ingredients. Many recent studies have focused on characterising plant proteins and their bioactive properties of their derived peptides. However, there has been only limited research on the nutritional and bioactive properties of cereal peptides produced after digestion.

In addition, there has been no study found to investigate anionic intestinal peptides derived from cereal bran or wholegrain cereal as natural antioxidant and ACE inhibitory agents. Most recent studies had focused on investigating the hydrophobic peptides in relation to bioactivity, but after intestinal digestion, a part of intestinal peptides carry negative charge due to the neutral and slightly alkaline environment of the small intestine. Oat bran protein intestinal digesta was selected for further separation and identification to determine the novel bioactive peptides due to its highest overall antioxidant and ACE inhibitory activity and nutritional value.

The current thesis has explored three key research questions, over four experimental themes (Chapter 3, 4, 5, and 6) where studies were conducted in characterising storage proteins in wheat bran, oat bran and wholegrain barley. Then oat bran proteins were hydrolysed via a simulated *in vitro* digestion model and the bioactive peptides were identified and sequenced. Figure 2.1 shows the flowchart illustrating the experimental strategy.

Key Research Questions:

1. Will proteins extracted from oat bran, wheat bran and barley be an alternative source of antioxidant, ACE inhibitory peptides and essential amino acids?

2. Do peptides in oat bran protein intestinal digesta carry antioxidant and ACE inhibitory activity?
3. What are the antioxidant and ACE inhibitory peptides in the oat bran protein intestinal digesta with the potential for use as therapeutic food?

Hypothesis

1. Storage protein in cereal bran is an ideal source of bioactive peptide after digestion.
2. Peptides identified were the reason for antioxidant and ACE inhibitory activity of oat bran protein intestinal digesta.

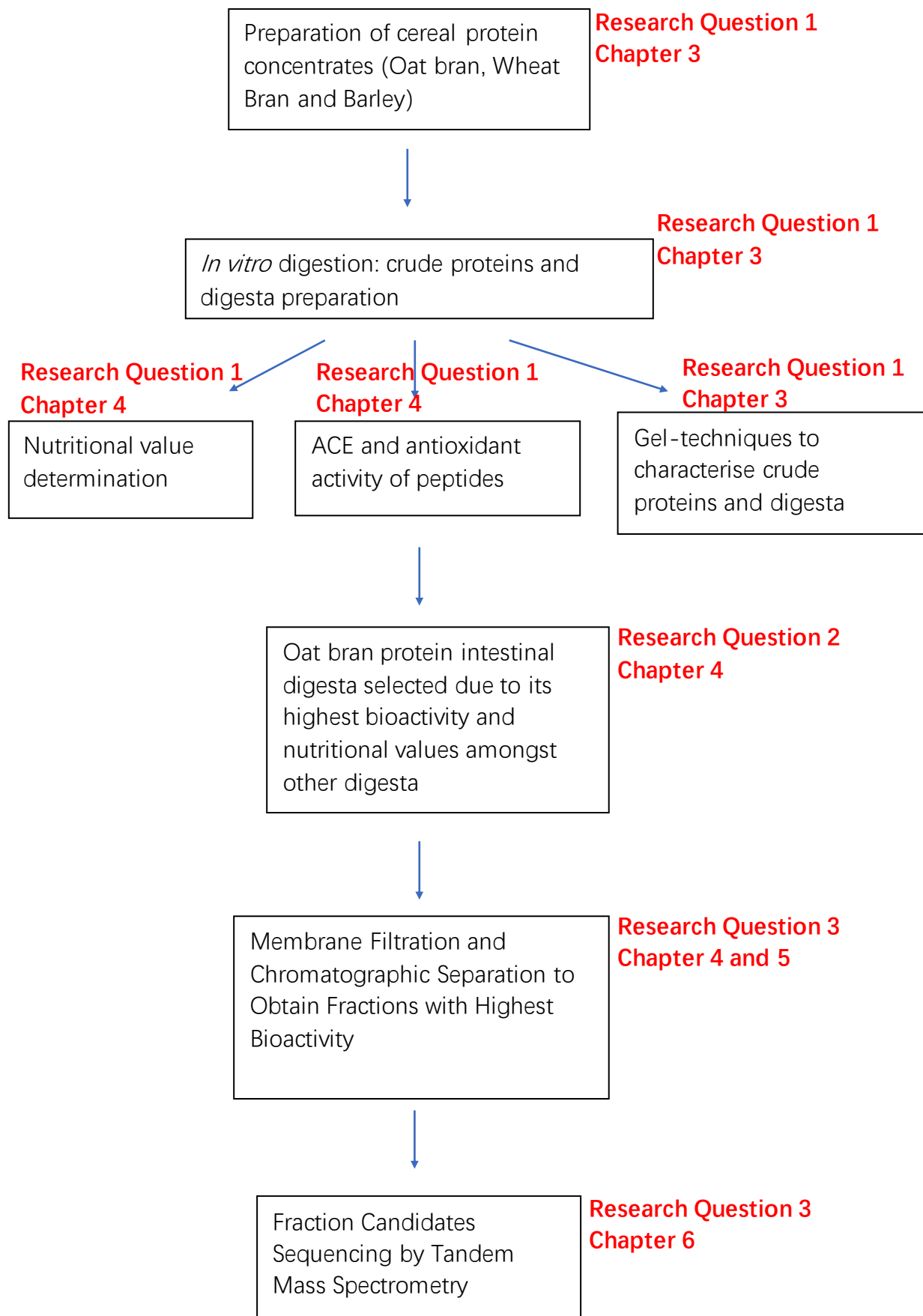


Figure 2.1 Flowchart of the experimental procedures on four main experimental themes (corresponding **Chapters: 3, 4, 5 & 6**).

Experimental theme ONE: Studies of Cereal Protein Concentrates and Digesta (**Chapter 3**)

The aim of this chapter was to establish the protein extraction methods in the laboratory and the *in vitro* digestion model, and then to characterise the proteins and the digesta of oat bran, wheat bran and barley. Proximate analysis was carried out on the protein extracts. The change of protein content and profiles in the digesta and particle size with digestion was determined. The crude proteins and digesta were characterised by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in order to understand the proteolysis of the extracted protein. Oat bran protein concentrate was characterised by two-dimensional electrophoresis, with the aim of identifying storage protein that presents only in bran but could potentially lead to the production of bioactive peptides after digestion.

Experimental theme TWO: Nutritional and Bioactive Properties of the Protein Concentrates and Digesta (**Chapter 4**)

The aim of this chapter was to determine the nutritional and bioactive properties of the peptides obtained before and after the digestion, as these properties contribute to the beneficial outcomes that cereal protein concentrates could bring to the body. Protein digestibility, degree of hydrolysis and the release of the FAAs during digestion were measured to determine the nutritional values of the three types of protein concentrates. Three antioxidant assays (ABTS, TPC and FRAP) assessed the overall antioxidant properties of the digested proteins. The ACE inhibition assay was used to determine the potential regulatory effects of peptides on the cardiovascular system. Total oat bran intestinal digesta and oat bran intestinal digesta with a MW of less than 3 kDa was selected for further analysis due to having the highest nutritional values and bioactivity among all hydrolysates.

Experimental theme THREE: Separation of Bioactive Peptides using Anion Exchange Chromatography (**Chapter 5**)

The aim of this chapter was to isolate anionic peptides from the digesta and to select the fraction with highest antioxidant and ACE inhibitory activity for sequencing. Because oat bran digesta showed overall highest antioxidant and ACE inhibitory activity, oat bran

intestinal digesta with a MW of less than 3 kDa was subjected to anion-exchange chromatography for separation to obtain fractions with highest antioxidant and ACE inhibitory activity. Antioxidant assays including ABTS, ORAC and TPC were employed to determine the mechanism of antioxidant action of negatively charged oat intestinal peptides. FRAP assay was replaced by ORAC assay because intestinal peptides tend to eradicate radicals via transportation of electrons and the donation of protons. ORAC assay was used to confirm this finding from Chapter 5. Six fractions were selected as the candidates due to their highest antioxidant and ACE inhibitory activity.

Experimental theme FOUR: Sequencing and Novel Peptides Determination (**Chapter 6**)

The aim of this chapter was to identify the peptides in the selected fractions and to investigate how the sequences of these anionic peptides could contribute to the antioxidant and ACE inhibitory properties. Six fraction candidates and oat bran intestinal digesta < 3 kDa (7 fractions in total) were subjected to tandem mass spectrometry for sequence identification. Computational tools including PeakView, ProteinPilot, UniProt and BIOPEP were used for peptide sequencing and analysis. All peptides in 6 fractions and the digesta were identified. Also, the most abundant peptides in these 7 samples were identified in order to understand how they are related to the antioxidant and ACE inhibitory activity. Peptides with antioxidant and ACE inhibitory potential were selected for future research.

Chapter 7 General Conclusions and Future Scope

The important results, discussion and conclusions of each chapter will be emphasised in this chapter and relate to the hypotheses and goals of this thesis. Concerns and future directions will be discussed to carry the research to a higher level.

Chapter 3

Studies of Cereal Protein Concentrates and Digesta

3.1 Introduction

In most studies regarding the nutritional and functional features of cereal proteins and peptides, alkaline solution was used to extract total protein from cereals and brans followed by isoelectric precipitation and lyophilisation to obtain the protein concentrates for further analysis such as the analysis of proteomics and the nutritional values of the proteins and peptides (Alu'datt et al., 2012; Alu'datt et al., 2012; Jodayree, 2015; Phongthai et al., 2018; Uraipong & Zhao, 2016b, 2018).

Wheat bran protein contents range between 15.2% - 16.9% (Chalamacharla et al., 2018) and can be separated into major protein groups including the non-prolamin albumin/globulin group, gliadin (a prolamin) and the glutenin (Malik, 2009). Gluten is the mixture of glutenin and gliadin in wheat which gives the unique elastic nature to the bread structure. Oat bran protein constitutes 15% - 18% protein (Duță et al., 2018) with similar storage protein groups but a unique prolamin named avenin. Barley protein ranges from 7% - 25% with four major storage protein groups: albumin, globulin, prolamin and glutelin. Hordein, a unique prolamin in barley, is found only in the endosperm comprising 30% - 50% of total protein, while much less protein is distributed in the husk (Arendt & Zannini, 2013). Bran protein is believed to distribute mostly in aleurone layer of the bran followed by testa and pericarp, respectively (Baladrán-Quintana et al., 2015).

The nutritional efficiency of protein concentrates relies on their digestion in the gastrointestinal tract. The static simulated *in vitro* digestion model used in this study is a reliable alternative to the expensive, challenging, and time-consuming *in vivo* studies. Though the system only partially mimics the complexity of the gastrointestinal system, it is proven to be an effective method in relation to the determination of the digestibility (%), DH%, the change of the protein structure and the release of the bioactive peptides and essential amino acids (Sousa et al., 2020).

The aim of this chapter was to establish the protein extraction methods in the laboratory and the *in vitro* digestion model, and then to characterise the proteins and the digesta of oat bran, wheat bran and barley. Protein extracted by alkaline solution from wheat bran, oat bran, and the whole barley grains were subjected to a simulated *in vitro* pepsin-pancreatin model aiming on characterising extracted storage proteins and peptides derived from them. Proximate analysis was performed on the protein concentrates before *in vitro* digestion. Whole barley grains, instead of the barley bran, were used to prepare protein concentrate for a better characterisation of the specification and complexity of the proteins in barley grains. Barley proteins are mainly found in the starchy endosperm (70-90% of total protein) in which hordein acts as one of the major storage proteins whereas the bran contains mainly albumin and globulins contributing 3-4% and 10-20% to the total protein content in barley, respectively (Šimić et al., 2019). Therefore, the digesta of barley wholegrain was selected as intercomparison of the bioactivity of the digesta to other two cereal samples. After digestion, the protein content of the digesta was determined. The particle sizes of the crude proteins and digesta were also measured. The crude proteins and digesta were subjected to SDS-PAGE to obtain the protein profile of the digesta and determine the characteristics of cereal protein digestion. The oat bran protein extract was analysed by 2-D electrophoresis which was employed to characterise the storage proteins extracted from oat bran.

3.2 Methods

3.2.1 Materials

Wheat and barley seed samples (locally produced, mixed cultivars, harvested February 2019) used in this study were provided by PGG Wrightson Seeds Ltd., Selwyn, New Zealand. The cereals were stored for up to six months at 4°C in a sealed storage organiser before protein extraction. Whole grain oats (Harraways, local South Island oats) were purchased from the local supermarket (New World, Selwyn, New Zealand) and utilised for extraction within a week.

3.2.2 Preparation of Bran Protein Concentrates

Bran Preparation

Wheat, oats, and barley were ground by using Laboratory Mill KT-3310 (PerkinElmer, Shanghai, China). The milled wheat and oats were passed through 300 µm (Endecotts, New

Zealand) sieve to remove the flour. The remaining of wheat bran was comparatively long and slender consisting of yellowish husk pieces and white flakes. Oats brans were flakes with one side of yellowish surface and white the other side. Ground barley kernel was adhering to the husk after milling and smashed to be crumbs instead of becoming powders like wheat and oat kernels did. Wheat bran, oat bran, and barley sample were separately collected in a 500 mL beaker with a foil covered its opening and stored at 4 °C for the extraction.

Alkali Protein Extraction

The wheat bran protein concentrate (WBPC) was prepared according to Fellers, Sinkey, Shepherd, and Pence (1966) with modifications based on Zaky et al. (2019) . The ratio of 200 g of wheat bran to RO H₂O was fixed to 1:10 (w/v) at 23 °C for 60 min with constant stirring. The pH of the mixture was monitored through the whole extraction process to maintain the pH of 10.5 (the adjustment of the pH was made by using 1 mol/L NaOH) for the maximum extraction yield. Centrifugation of 850 × RCF, 15 min at 4 °C was followed to separate and collect solubilised protein in the supernatant. Cheese cloth was employed to filter the extract. The pH of the supernatant was then adjusted to 5.5 by 1 mol/L HCl and the supernatant was placed in the fridge at 4 °C for 60 min allowing full precipitation. The particles in the suspension were separated at 850 × RCF for 15 min and at 4 °C to collect the residue. The resulting precipitated was washed with RO water, lyophilised and stored at -20 °C.

The extraction of oat bran protein concentrate (OBPC) followed the method described by Jodayree (2015) with a slight modification. 200 g of oat bran and 1 mol/L NaCl was mixed in the ratio of 1:8 (w/v). pH was adjusted to 9.5 by 1 mol/L NaOH stirring for 30 min at 23°C. The slurry was centrifuged at 5,000 × RCF for 25 min at 4 °C. Cheese cloth was used to filter the extract. The pH of the supernatant was adjusted to 4.0 by 1 mol/L HCl for 60 min precipitation. Protein precipitates were separated (5,000 × RCF, 25 min, 4°C), collected, washed, lyophilised and stored at -20 °C.

The barley protein concentrate (BPC) was extracted as per the method by Alu'datt et al. (2012). The ratio of 200 g of milled barley and RO water was fixed to 1:10 (w/v) with pH adjusted to 11.0 by 2 mol/L NaOH, stirring for 60 min at 23 °C then centrifuged at 10,000 × RCF, 15 min and 4 °C. After filtering by the cheese cloth, the pH of the supernatant was adjusted to 4.5 with 1 mol/L HCl followed by 60 min precipitation at 4 °C. The protein

residue was centrifuged (10,000 × RCF, 15 min, 4 °C), washed, freeze dried and stored at -20 °C for further analysis.

Protein Content Determination - Dumas Method

Dumas nitrogen determination was performed by using Elementar Rapid-N Max (Hanau, Germany). Sample was subjected to the furnace at 1,000 °C in the presence of pure oxygen gas. The produced gas mixture passed through a reduction chamber at 650 °C to convert nitrogen oxides into N₂ for detection by thermal conductivity detector whereas excessive CO₂ and H₂O produced were separated and disposed. Known weights of glutamic acids were used as the standards to create a calibration curve to translate in N%.

The crude protein content of WBPC, OBPC and BPC (0.3 g in triplicates, respectively) were determined by using Dumas method (1994). Protein yield (%) was calculated based on the weight of extracted protein concentrates and on the protein content of bran samples, by using the following equations (2018).

$$\text{Protein Yield} = (W_p \times P_p) / (W_b \times B_p) \times 100\%$$

where W_p and P_p are weight and protein content of freeze-dried protein concentrates, respectively; W_b and B_p are weight and protein concentrate of bran sample used in the extraction. The protein content was calculated based on the nitrogen conversion factors of 6.31 for wheat bran and 5.83 for oat bran and barley protein concentrate sample (Fujihara et al., 2008).

Fat Content Determination

The crude fat content of samples was determined by modified Soxhlet method (Horwitz, 2010). In general, fat in 3 g of protein concentrate was extracted by 80 mL of hexane (×4 concentrated) using Hot Extraction E-812/E-816 HE (Tec Instrumental S.A., Florida, Argentina). The fat residue was collected and weighted after all hexane was evaporated at 100 °C. The extraction was performed in triplicates.

$$\% \text{ Crude Fat} = \frac{W_f}{W_p} \times 100\%$$

where W_f and W_p are the weight of fat residue dry matter and cereal protein concentrates, respectively.

Total Fibre Content Determination

One gram of protein concentrate (in duplicate) was dispersed into 40 mL MES-Tris buffer (0.05M, pH 8.2). 50 µL Heat-stable α-amylase solution was added to the slurry. The sample was incubated for 30 min at 100 °C in the water bath. Temperature was brought down to 60 °C followed by the addition of 100 µL protease incubating for 30 min. The pH was adjusted between 4.1 - 4.8 by 5 mL 0.561 M HCl solution (5% NaOH or 5% HCl was used when necessary), then 200 µL of amyloglucosidase was added incubating for 30 min at 60 °C. The beaker was covered by aluminum foil during the incubation. The residue buildup on the beaker during the incubation was scraped and washed off into the slurry to minimize bias. 225 mL 95% ethanol was heated up 60 °C and added to the slurry and allowed precipitation for 60 min. A crucible containing 0.1 mg Celite was weighted. The Celite was redistributed and wet by 15 mL 75% ethanol before the suction was applied to allow the Celite to be firmly and evenly distributed onto the fritted glass. The filtration was performed by adding the precipitated sample digest on the Celite followed by washing the precipitate using 30 mL of 78% ethanol, 95% ethanol and acetone successively while suction was applied a vacuum. The sample and crucible were dried overnight in the oven at 103 °C and cooled in desiccator for 1 hr. The weight of the crucible containing fibre residue and Celite was measured to 0.1 mg and substrate the weight of dried crucible and Celite to obtain the weight of fibre residue. Dumas method was used to measure the protein content in one residue of the duplicate while ash analysis (incineration overnight at 525°C, then cooled in desiccator to measure the weight of crucible and ash residue subtracting crucible and Celite weight to get ash content) was used on another. Two blanks were performed simultaneously. The fibre content determination was performed in triplicates.

$$\text{Dietary Fibre}\% = \frac{\left[\frac{R1 + R2}{2} - P - A - B \right]}{\left[\frac{m1 + m2}{2} \right]} \times 100\%$$

where R1 and R2 were weight of fibre residue duplicates; m1 and m2 were weight of sample duplicates; P was the protein content from one fibre residue while A was the ash content from another. B are the average of Blanks.

$$B = \frac{BR1 + BR2}{2} - BP - BA$$

where BR1 and BR2 were weight of blank residue; BP was the blank protein content; BA was the Blank ash content.

3.2.3 *In vitro* Gastrointestinal Digestion

In vitro digestion trial was conducted separately with the sample protein concentrates of wheat bran, oat bran and barley. The method was adopted by Uraipong and Zhao (2018) and Phongthai et al. (2018) with some modifications. The protease to substrate ratio was fixed to 1:100 (w/w) based on the protein content. Pepsin and pancreatin was dissolved separately in 10 mL 0.04 mol/L HCl and 14 mL 0.1 mol/L phosphate buffer (freshly prepared). The pH of the 90 mL of protein sample dispersions (1:90, w/v) were adjusted to 2.0 with 1 mol/L HCl before the pepsin was added. Sample mixtures were maintained stirring for the next 240 min hydrolysis at 37 °C using IKA® RT-15 magnetic multi-stirrer. The gastric phase was initiated by adding 10 mL of pepsin solution to the sample mixture and was continued for 120 min. The gastric phase was ceased by adjusting the pH of the mixture to 8.0 by 1 mol/L NaOH. The intestinal phase began with adding 14 mL of pancreatin solution for another 120 min digestion. Aliquots of hydrolysates (2 mL) were collected with the time interval of 20 min within 240 min. The reaction was stopped by heating the aliquots in a 95 °C water bath for 10 min. After the centrifugation at 5,000 g for 15 min at 4 °C, the supernatant was collected and stored at -20 °C for the further analysis. To each protein concentrate, the digestion was performed in triplicate.

Protein Content Determination – Bradford Method

Bio-Rad protein dye reagent concentrate was used to determine the protein content of the digesta. The dye concentrate was diluted 5 times by using RO water to prepare the working reagent. Digesta collected at all different stage of *in vitro* digestion (10 µL) was combined with 200 µL working reagent in the 96-well plate. Incubation was allowed for 5 mins in the room temperature before the plate was measured on a microplate reader (FLUOstar Omega, BMG Labtech GmbH, Ortenberg, Germany) at the absorbance of 595 nm. Bovine serum albumin (BSA) standard curve was used as the standards to establish the standard curve converting the absorbance readings to protein content in mg/mL. The digesta were assayed in triplicates.

Protein Content Determination – Bicinchoninic acid (BCA) Method

Bradford method measure the soluble protein by binding the protein to the dye fragments while BCA method is based on reduction of copper ions due to the presence of amino acids such as cystine, tyrosine and tryptophan. Different mechanism of protein assays may lead to different results of soluble protein content in the digesta during digestion. Therefore, BCA method was used as the second protein assay to monitor the digestion process. Soluble proteins of digesta were measured using BCA assay method using Pierce™ BCA Protein Assay Kit. Absorption was measured at 570 nm in 96 well microplates using a microplate reader (FLUOstar Omega, BMG Labtech GmbH, Ortenberg, Germany) and BSA standard curve was used. The digesta were assayed in triplicates.

Particle Size Distribution (PSD)

The particle size of protein concentrates before and after the digestion were measured by using the Malvern™ Mastersizer 3000 instrument (Malvern Instruments Ltd, Worcestershire, UK) with Hydro EV Flexible volume wet dispersion. The particle size at 10% (Dx10), 50% (Dx50), and 90% (Dx90) of the volume distribution were calculated from the instrument software.

SDS-PAGE

Analysis was performed by using Pre-cast 4-12% Bis-Tris 10 well gradient gels (Novex™, Carlsbad, CA, USA). 16 µL Hydrolysates at time 0, 20, 40, 80, 120, 140, 160, 200, 240 min equivalent to 40 µg protein were taken and added to 8 µL sample reducing dye (50 mL of LDS sample buffer, 20 mL of sample reducing agent, 30 mL of RO water). Sample mixture was boiled for 5 min at 95 °C in the water bath, followed by centrifugation for 30 secs at 10,000 g before loading. Bolt™ Mini Gel Tank (Life Technologies™, Carlsbad, CA, USA) and 1X MES-SDS Running Buffer were used. 10 µL of Sharp Pre-stained Protein Standard (Novex™, Carlsbad, CA, USA) was loaded into the first well of the gel. The gels were run for 60 min at 175 volts using PowerPac™ Basic Power Supply (Bio-Rad, Albany, New Zealand) and Invitrogen™ X-Cell Surelock Mini-Cell Electrophoresis System (Thermo Fisher Scientific, North Shore City, New Zealand). After the electrophoresis was complete, gels were removed from the cassettes, rinsed twice with RO water, and stained with Coomassie Blue stain (225 mL RO water, 225 mL of methanol, 50 mL of glacial acetic acid, and 0.5 g of Coomassie Blue) for 15 min on a shaker. The gel was then rinsed twice with RO water before adding the de-stain solution (70 mL of RO water, 20 mL of methanol, 10 mL of glacial acetic acid),

left overnight on a shaker. The Gel Doc™ XR with Image Lab™ Software (Bio-Rad, S. No. 721BR15168, USA) was employed for protein bands determination.

3.2.4 Two-Dimensional Electrophoresis Characterising Oat Bran Protein Concentrate

2-D protein profile was prepared by modified method adopted by Galova et al. (2019).

Sample Preparation

One gram of the OBPC was resuspended in 100 mL of RO water. The pH of the mixture was adjusted to pH of 2.0 while heating the mixture up to 37 °C, swirling for 10 min simulating the conditions of oat bran proteins reaching the stomach before the digestion start and allow the proteins to be fully dissolved. The aliquots were collected in the 2 mL Eppendorf tubes centrifuging at 5,000 × RCF for 15 mins. The supernatants were transferred into different 2 mL Eppendorf tubes for dialysis.

Dialysis

Slide-A-Lyzer 12 mL dialysis cassettes with 10,000 MWCO (Thermo Fisher Scientific, North Shore City, New Zealand) was used for dialysis for getting rid of the excessive salt which could interfere with the strip focusing during IEF. The membrane of the cassettes was pre-hydrated for 10 min in RO water. Sample was injected into the cassette without touching the membrane and the excessive air inside of the membrane was removed. RO water was used for buffer exchange. The cassette was placed into 500 mL RO water for dialysis at the room temperature for 3 hr, swirling. Then water was replaced and kept swirling in the fridge (4 °C) overnight. The dialysis was repeated once to minimise the interference of the salts. The salt content of the dialysed sample was estimated by EcoMet™ conductivity/TEMP meter C75 (Istek, Seoul, South Korea). The protein content was determined by both BCA and Bradford methods. The protein content in the dialysed samples were then adjusted to 4 mg/mL, 8 mg/mL and 16 mg/mL in order to visualise all the major and the minor spots of the proteins. RO water, 0.1 M and 0.5 M NaCl solution were used to establish the standards to quantify the salt in the loaded protein sample that could interfere the 1-D focusing stage.

First Dimension, IEF

Immobiline™ 11 cm DryStrip gels Strips pH 3-11 (Cytiva, Marlborough, MA, USA) was rehydrated overnight at room temperature in 215 µL of rehydration buffer (7M urea, 2M

thiourea, 0.4% [v/v] bromophenol blue, 4% [w/v] CHAPS, 0.5% [v/v] Pharmalyte™ 3-10, 0.4% [w/v] DTT). 40 µg and 80 µg of the dialysed protein sample were loaded on the different stripes accordingly. Focusing was performed at 20 °C by using PROTEAN™ IEF cell (Bio-Rad Laboratories, Hercules, CA, USA), current 18 µA per stripe. Focused stripe was used for the second dimension immediately.

Second Dimension, SDS-PAGE

Stripes were collected and transferred to the rehydrate tray for equilibrium with buffer 1 (0.05 Tris, 6M urea, 20% [v/v] glycerol, 2% [w/v] SDS, 1% [w/v] DTT) and buffer 2 (0.05 Tris, 6M urea, 20% [v/v] glycerol, 2% [w/v] SDS, 4% [w/v] IAM) for 15 mins, respectively, on the rocker. Criterion™ TGX™ precast gel (Bio-Rad Laboratories, Hercules, CA, USA) was used for separation at 200 V, 0.04 A and 8 W per gel with 1 × Tris/glycine/SDS buffer (Bio-Rad Laboratories, Hercules, CA, USA). Two drops of bromophenol blue were added to the buffer, which could indicate the complete running of the electrophoresis. The gel was fixed in the solution containing 50% ethanol and 10% glacial acetic acid, followed by being transferred to the staining solution (20% methanol, 10% [w/v] ammonium sulphate, 10% [v/v] phosphoric acid, 0.12% [w/v] Coomassie Blue G250) for 1 hours. RO water was used for destaining the gel. The gel was scanned by Gel Doc XR⁺ with Image Lab Software (Bio-Rad, Hercules, CA, USA).

3.2.5 Statistical Analysis

Data were collected and analysed by one-way ANOVA and Turkey's test using Minitab 19 (Minitab Ltd., Sydney, Australia) for triplicated determination. The results were shown as means ± standard deviation (SD) of the means. Statistical significance was declared as $P < 0.05$.

3.3 Results

3.3.1 Analysis of the Protein Concentrates

Proteins in locally produced cereals proportionally reflects protein characteristics of these cereals in New Zealand. Though, experiments were not conducted on different cultivars of cereals which could lead to biased results, typically, same protein should be found in the protein concentrates. The aim of the extraction was to obtain protein concentrates with over 50% protein. The extraction methods were applied under different conditions to

different sample in order to achieve the target. As extraction methods applied on three samples were based on the same mechanism, similar types of protein are expected in these extracts and therefore the digesta produced after digestion of these protein extracts should be comparable to each other.

WBPC, OBPC and BPC were analysed for their protein, total starch, total fibre and total fat composition (Table 3.3.1). The BPC had the highest protein content of 81.43% while WBPC was 63.51% of protein and OBPC had lowest protein content of 51.19%. The use of the wholegrain of barley for extraction showed higher protein content in the concentrates. The protein content of WBPC, OBPC and BPC were 72.60%, 38.44% and 67.14%. The total starch and fat content of WBPC were 2.18% and 9.79%, respectively, higher than that detected in OBPC and BPC (0.06% and 0.14% of starch content, 2.24% and 1.96% of fat content in OBPC and BPC respectively). A higher total fibre content was determined in OBPC (37.42%) compared to WBPC (24.47%) and BPC (15.61%).

Table 3.3.1. Proximate analysis of the macronutrients composition of protein concentrates.

Protein Concentrates	Total Protein, %	Total Starch, %	Total Fibre, %	Total Fat, %
WBPC	63.51 ± 0.14 ^B	2.18 ± 0.20 ^A	24.47 ± 0.01 ^B	9.79 ± 0.24 ^A
OBPC	51.19 ± 0.53 ^C	0.06 ± 0.00 ^B	37.42 ± 0.00 ^A	2.24 ± 0.00 ^B
BPC	81.43 ± 1.19 ^A	0.14 ± 0.02 ^B	15.61 ± 0.02 ^C	1.96 ± 0.02 ^C

- Starch, fat and fibre content were determined in duplicate; Protein content was determined in triplicate. Data were expressed as Mean ± SD with superscripts in a column wise (upper case alphabet) differ significantly ($P < 0.05$).
- WBPC: wheat bran protein concentrate; OBPC: oat bran protein concentrate; BPC: barley protein concentrate

3.3.2 *In vitro* Digestion - Protein Content of the Digesta

The effectiveness of two protein assays, Bradford and BCA, for determining protein hydrolysis was compared. The protein content of WBPC, OPBC and BPC before digestion was measured by Bradford method (see Figure 3.3.1) after the protein concentrates were suspended and the pH adjusted to 2 and meanwhile, the temperature increased to 37 °C. The protein content were 3.14 ± 0.04 mg/mL, 3.64 ± 0.10 mg/mL and 6.17 ± 0.89 mg/mL, respectively. According to Table 3.3.1, one gram of WBPC, OPBC and BPC in 100 mL gastric juice each should lead to the concentration of 6.35 mg/mL, 5.12 mg/mL and 8.14 mg/mL, showing the yield of detected soluble proteins recovered by the acidic environment of the stomach before digestion to be 49%, 71% and 76% of the total estimated protein. The degradation of protein by pepsin and pancreatin showed a similar pattern for WBPC and OBPC, of which the significant degradation was observed within the first 20 min of gastric digestion and the degradation continued until the end of gastric phase (both protein content dropped to around 2 mg/mL). There was a second significant decrease in the first 20 mins of intestinal digestion when the protein content of WBPC and OBPC decreased to 0.77 ± 0.06 mg/mL 0.42 ± 0.02 mg/mL followed by a slower decline to 0.47 ± 0.01 mg/mL and 0.19 ± 0.02 mg/mL at 240 min, respectively. The significant degradation of BPC happened within the first 20 min of intestinal phase (from 5.28 ± 0.24 mg/mL to 1.43 ± 0.11 mg/mL) whereas the pepsin appeared to have a limited impact on the BPC.

The protein content of the WBPC, OBPC and BPC digesta also determined by BCA method (see Figure 3.3.1) showed different degradation patterns. The protein content of WBPC, OBPC and BPC determined before the digestion were 2.50 ± 0.23 mg/mL, 3.18 ± 0.08 mg/mL and 4.93 ± 0.54 mg/mL showing the recovery of soluble protein to be 39%, 62% and 61%, respectively. During the gastric phase, the BCA method showed an overall increased protein concentration within first 20 min for the three concentrates, and the protein content of BPC digesta increased gradually to 7.51 ± 0.72 mg/mL by the end of the gastric phase. This was followed by a significant reduction in protein content within the first 20 min of intestinal phase for all three protein concentrates. The protein content of WBPC, OBPC and BPC at 240 min were determined to be 1.01 ± 0.05 mg/mL, 0.81 ± 0.16 mg/mL and 4.23 ± 0.12 mg/mL.

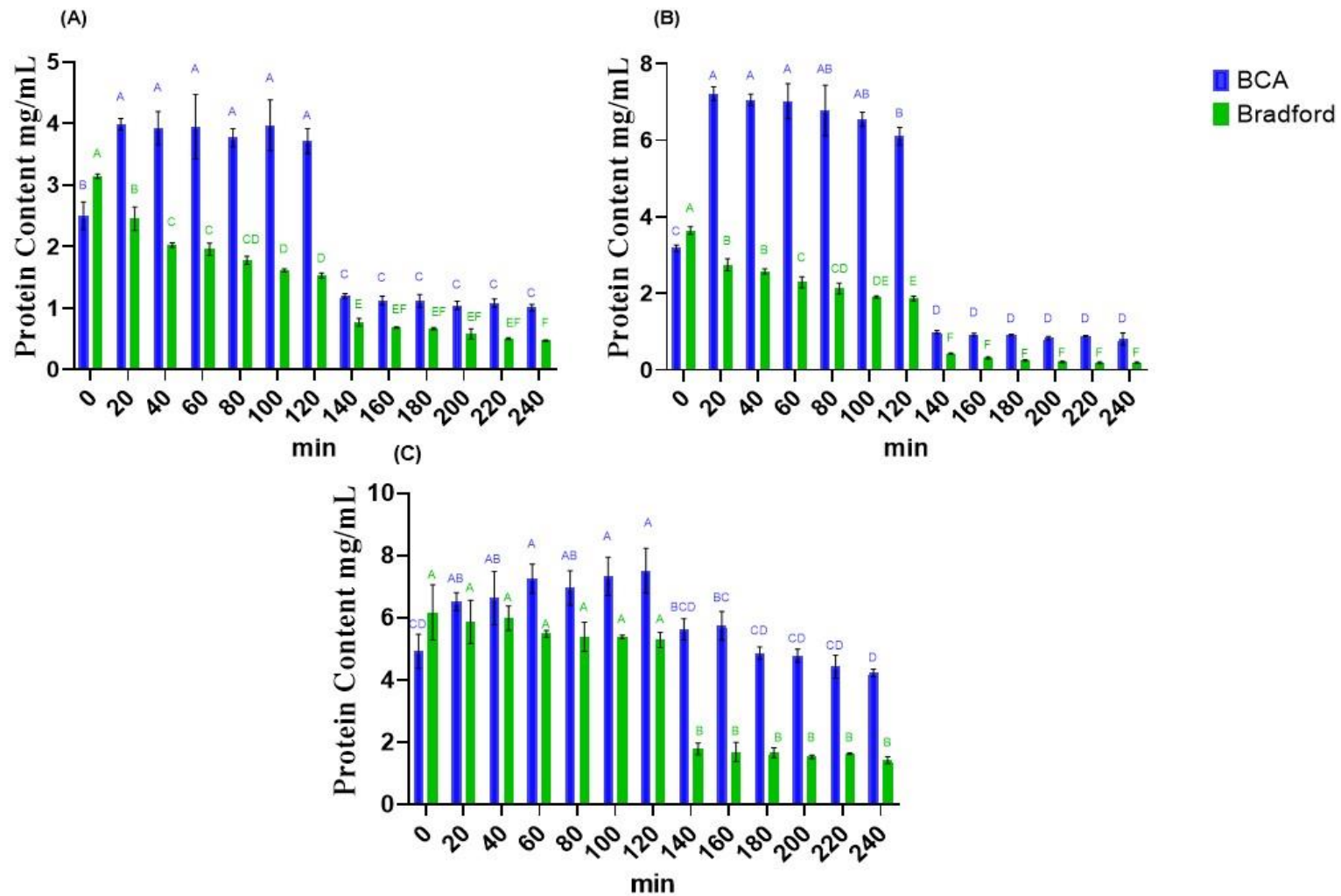


Figure 3.3.1. Protein content of three protein concentrates at different hydrolysis time during 240 min simulated GI digestion, measured by BCA (shown in blue) and Bradford method (shown in green). (A) WBPC (wheat bran protein concentrate); (B) OBPC (oat bran protein concentrate); (C) BPC (barley protein concentrate). All measurements were expressed as Mean \pm SD (n = 3). SD was calculated based on the triplicate of the digestion on each protein sample. Letters above each bar indicated significant difference.

3.3.3 Particle Size Distribution (PSD) Changes during the Digestion

The PSD of the three protein concentrate samples are shown in Table 3.3.2. The two bran protein concentrates showed similar trends. The 50% (Dv50) particle diameter of WBPC was 3.27 μm before the digestion. It increased to 9.40 μm after gastric digestion) and decreased after the intestinal digestion (6.93 μm). The diameter of 50% particles of OBPC (0.82 μm) increased to 48.80 μm and reduced to 14.10 μm at the end of the digestion. The barley protein acted differently. Fifty percent of particle diameters of untreated BPC showed the average size of 9.30 μm . The diameter of the particles decreased to 1.95 μm and increased to 29.40 μm at the end of the digestion.

Table 3.3.2. Particle size distribution of three protein samples at three different stages of digestion.

	WBPC	OBPC	BPC
Digestion Stage	Dv(50) μm	Dv(50) μm	Dv(50) μm
Before Digestion	3.27 \pm 0.09 ^C	0.82 \pm 0.01 ^C	9.30 \pm 0.18 ^B
End of Gastric	9.40 \pm 0.18 ^A	48.80 \pm 0.11 ^A	1.95 \pm 0.03 ^C
End of Intestinal	6.93 \pm 0.05 ^B	14.10 \pm 0.125 ^B	29.40 \pm 0.57 ^A

- Data were expressed as Mean \pm SD with superscripts in a column wise (upper case alphabet) differ significantly ($P < 0.05$, $n = 5$).
- WBPC: wheat bran protein concentrate; OBPC: oat bran protein concentrate; BPC: barley protein concentrate

3.3.4 SDS-PAGE Protein Profile

The protein profiles of SDS-PAGE electrophoretogram for three protein samples subjected to pepsin pancreatin *in vitro* GI digestion are shown in Figure 3.3.2. For WBPC and OBPC, similar patterns of protein bands were obtained before the digestion with the molecular range between 10 kDa and 100 kDa. The identified proteins were named based on literatures (Comino et al., 2016; Moehs et al., 2019; Schalk et al., 2017; Yalçın & Çelik, 2007; Y. Zhang et al., 2020). The patterns of protein before digestion of WBPC and OBPC shared a high similarity, the major bands identified in WBPC included avenin-like protein from albumin and globulin fractions (30 kDa - 35 kDa and 20 kDa – 25 kDa) α -gliadins and γ -gliadins (30 - 35 kDa), and prolamin fractions (30 kDa – 75 kDa). The major bands identified in OBPC were albumin fractions (10 kDa – 80 kDa), globulin fractions and avenins (30 kDa - 35 kDa and 20 kDa – 25 kDa) and glutelins (10 kDa – 37 kDa). As for the BPC, the pattern of protein composition was comparatively more complicated indicating diverse protein fractions were extracted and the major bands included D hordein (125 kDa and 175 kDa), C hordein (40 kDa – 100 kDa), B hordein (22 kDa – 40 kDa), and albumin and globulins (< 22 kDa). Most bands of WBPC and OBPC degraded to lower than 20 kDa within the first 20 min and remained unchanged for the rest 100 min gastric digestion. Further digestion at intestinal phase resulted in the bands becoming less intense or disappearing entirely.

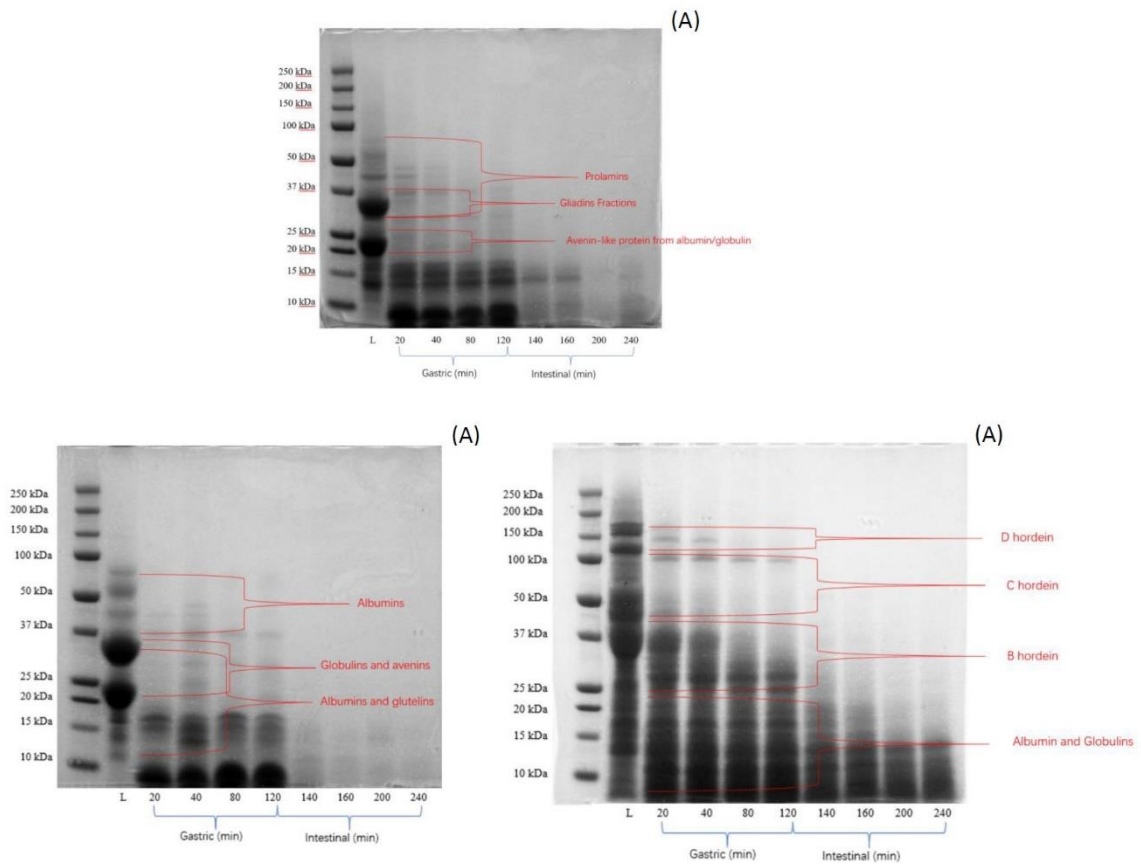


Figure 3.3.2 Protein profiles of (A) WBPC (wheat bran protein concentrate), (B) OBPC (oat bran protein concentrate) and (C) BPC (barley protein concentrate) subjected to *in vitro* pepsin-pancreatin GI digestion (L = protein ladder; lane 3 – 6 are hydrolysates derived from gastric digestion, lane 7 - 10 are hydrolysates from intestinal digestion). 40 µg of samples were loaded to each well.

3.3.5 Two-Dimensional Electropherogram of Oat Bran Protein Concentrate

OBPC was selected for further analysis by 2-D electrophoresis. The sample was resuspended in water with pH of 2 to mimic the pre-digestion stage and to share the same conditions of sample loaded onto the SDS-PAGE. Before subjecting the protein sample to 2-D electrophoresis, it was purified by using dialysis membrane to remove interfering compounds from the analyte. Excessive salt in OBPC sample could be one of the major interferences at the focusing stage. Salt solution was used to increase the yield of protein extraction from oat bran. Salt interference on IEF stage of 2-D electrophoresis was well recorded (Lee & Chang, 2009; Wu et al., 2010): High salt interference may affect the separation of protein by not only increasing the conductivity but causing electrolytic reactions with deleterious effects. Dialysis membrane with 10 kDa was used for purification

because the SDS-PAGE profile (Figure 3.3.2) of pre-digested OBPC showed the major bands at 37 kDa and 25 kDa while most of other minor proteins were located above 10 kDa. The OBPC sample after dialysis showed the conductivity of 20.1 $\mu\text{S}/\text{cm}$, closed to the conductivity of RO water (9.7 $\mu\text{S}/\text{cm}$) and was much lower than the conductivity of 0.1 M and 0.5 M NaCl (20.8 mS/cm and 32.6 mS/cm, respectively).

Figure 3.3.3 shows the profile of 40 μg of extracted bran protein loaded. In total, 29 spots were displayed on the electrophoretogram, with the 16 most intense spots (see Table 3.3.3) labelled. 19 spots were accumulated at the MW of about 36 kDa with pI of 4 to 9 in which 14 spots are comparatively more intense than others. Two major spots are shown at the extreme pI of 11 with the MW of 25 kDa. The minor spots were found outside the clusters of spots. Five minor spots were detected between the MW of 15 to 20 kDa with the pI of about 4 while 1 minor spot was shown at the MW of 15 kDa with pI of about 7 and 2 minor spots were found at the MW of 20 kDa and pI of 9.

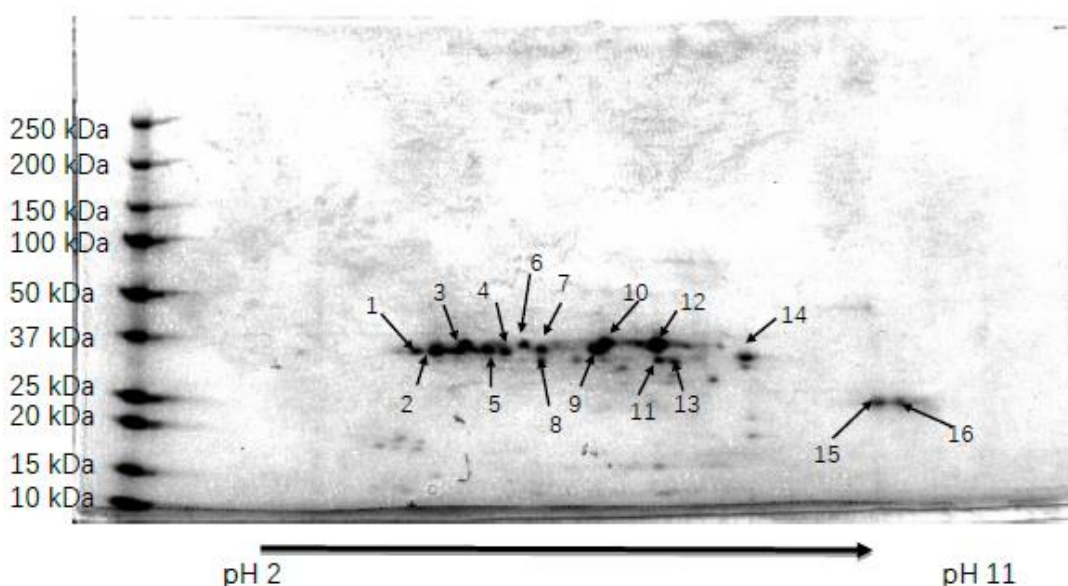


Figure 3.3.3 The protein profile of 40 μg oat bran protein loaded on the 2-D electrophoretogram.

Table 3.3.3 Table of major protein spots from Figure 3.3.4 with estimated MW and isoelectric point.

Major Spot Identified	Mass (kDa)	Estimated Isoelectric Point
1	36.0	4.0
2	36.0	4.2
3	36.0	4.5
4	36.0	5.0
5	36.0	4.2
6	36.0	4.5
7	36.0	5.0
8	35.5	5.0
9	36.0	6.0
10	36.0	6.0
11	35.5	7.0
12	36.0	7.0
13	35.5	7.5
14	35.5	8.5
15	25.0	10.0
16	25.0	10.5

The sample was loaded with twice the amount of protein on the 2-D electrophoresis (see Figure 3.3.4) with the aim of identifying proteins in low abundance which could not be identified in Figure 3.3.3. Figure 3.3.4 compared to Figure 3.3.3 show more intensive accumulation (region A and region B) of the spots listed in Table 3.3.3. Spots in region A and B shows the overlaid spots but not as clearly as Figure 3.3.3 because of horizontal streaking across the gel. The horizontal streaking could be caused by the overload of protein samples leading to insufficient denaturation and solubilisation for IEF and thereby, low resolution. Visible minor spots can be identified under the accumulation at the MW of about 36 kDa to 100 kDa with pI of about 4 to 11 (region C) in Figure 3.3.4, which was not shown in SDS-PAGE profile (Figure 3.3.2.). Region D was also not observed in Figure 3.3.3 showing some minor spots.

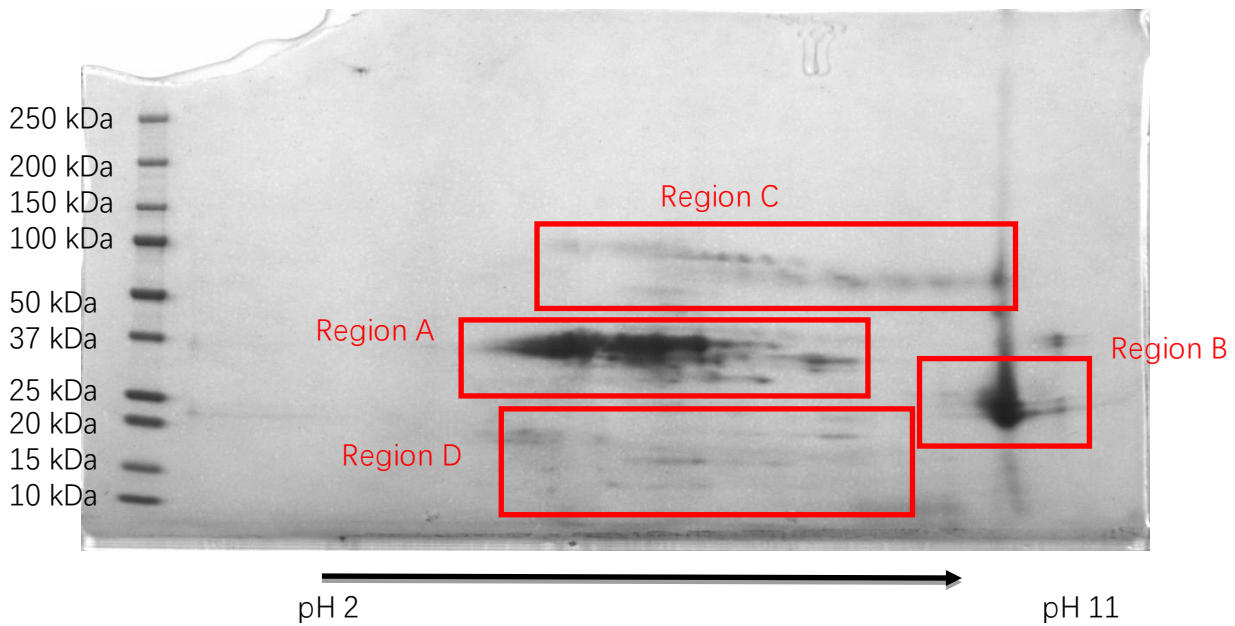


Figure 3.3.4 The protein profile of 80 µg oat bran protein loaded on the 2-D electrophoretogram.

3.4 Discussion

3.4.1 Analysis of the Protein Concentrate

As shown in Table 3.3.1, OBPC showed less protein content and low protein yield. The yield could be diminished due to the 30 min extraction while WBPC and BPC were extracted for 60 min. An alternative would be the use of saline solution for cereal protein extraction, which has been well documented (Karaca et al., 2011; Nadal et al., 2011), but the generation of the chlorinated NaCl/AAs degradation products by the presence of NaCl (Rahn & Yaylayan, 2015) could lower the yield. Alkaline solution extraction of cereal protein is one of the most common methods to extract total protein from cereal products (Qiaoyun et al., 2017) along with emerging techniques where the extraction is assisted by enzymes, sonication, freeze-thaw cycle, high speed blending and hydrothermal cooking, employed at the research level (Amagliani et al., 2017). Theoretically, alkaline solution plays an important role in degrading cellulose and destroys the fibril network to release the protein fractions (Knill & Kennedy, 2003). Different bran protein fractions can be isolated successively based on their solubilities in different extraction solution such as albumins in water, globulins in saline solution and prolamins in alcohol solution according to the Osborne classification (Baladrán-Quintana et al., 2015; Comino et al., 2016). In addition, the low centrifugation

speed (850 × g) could have left the starch and fibre residues suspended in the supernatant causing the starch and fat content of WBPC to be higher. The hydration of ionised hydroxyl of starch in alkaline solution generates electrostatic forces for starch dissolution (Hu et al., 2016). So alkaline solution was used to isolate starch from complexes by precipitation (Palacios-Fonseca et al., 2013). Plus, sodium hydroxide used in extraction has been known to disrupt the secondary structure of protein (Zhao et al., 2014) and lead to the exposure of the sidechains enveloped inside the protein. Alkaline extraction using sodium hydroxide, theoretically, could lead to the oxidation of peptide sidechain and break up the primary structure of protein by releasing amino acids (Liu et al., 2017) and therefore cause the loss of bioactivity after digestion. WBPC could be treated with hexane to remove excessive fat. But this process was not considered because the digestion model used pancreatin which contained lipase at the intestinal digestion stage to reduce the fat content and minimise the influences.

3.4.2 *In vitro* Digestion - Protein Content of the Digesta

BCA and Bradford method were designed for protein quantification but were used to monitor the degradation of proteins during digestion by determining peptide content in the digesta. Pepsin opens the structure of the protein molecules and breaks them down into larger peptide fractions while pancreatin reduces the larger peptides into small peptides and FAAs for absorption. The Bradford method relies on the anionic and hydrophobic bindings of Coomassie blue to the arginine and lysine residues and, to lesser extent, histidine and aromatic residues (Kruger, 2009). Plus, Bradford method has a limitation of binding proteins less than 3 kDa (Biochrom, n.d.; OZbiosciences, n.d.). The BCA method, by definition, determine the protein content by developing purple colour through the reduction of copper ions because of the presence of peptide backbones and the amino acids in the protein including cysteine, tyrosine and tryptophan (He, 2011). Therefore, different degradation patterns were expected to be seen by using these two methods for peptide quantification and the more suitable method was selected for peptide quantification in future experiments. According to the different patterns of Figure 3.3.1, in the first 120 min, some proteins in WBPC and OBPC could reduce to peptides below the detective range of Bradford method whereas the content of detective peptides in BPC digesta could remain to be dynamically unchanged (significant increase within the first 120 min was witnessed by using BCA method for barley digesta). Based on the same rationale, the increase of readings by BCA method

indicated the break-up of the protein structure and the exposure of detective peptides and amino acid residues. With the digestion reached to the end of intestinal stage, low protein content in the digesta indicated that peptides were reduced to undetected size while the most of peptides containing reductant amino acids were degraded into FAAs. Kapoor et al. (2009) modified BCA method for quantification of peptides by using trypsin to cleave the known BSA standards to establish the standard curve. Compared to Bradford method, BCA method was more suitable to quantify the small peptides whereas Bradford method has advantage to measure the content of peptide and proteins with larger molecular size.

3.4.3 Particle Size Distribution Changes during the Digestion

The mechanism of the change of particle size during the digestion is not clear. The proximate analysis shows the excessive fat content in WBPC. The pancreatin (contains lipase) used in the intestinal digestion may act to breakdown large fat globules into smaller ones and fatty acids, fatty acids may conjugate with long chain peptides (Malina & Shai, 2005) leading to the increase in the 50% particle sizes. The increase of the particle size during the digestion could imply the aggregation of proteins to other compounds. The aggregation of plant protein with phenolics (Keppler et al., 2020) and fibre (Zielke et al., 2019) as well as protein-protein aggregation (Singh, et al., 2014) was discussed in other studies. Aggregates of protein during the digestion could potentially reduce the bio-accessibility of protein by decreasing the release of the peptide and amino acids. An extensive study may be recommended by determining the protein content of the residues at each stage of the digestion. In-gel techniques may be used to identify the proteins that are involved in the aggregates. The structure of the aggregates could also be visualised by using confocal scanning microscopy.

3.4.4 SDS-PAGE Protein Profile

The results are consistent with the digestibility and DH% of WBPC and OBPC showing that small protein fractions and polypeptides produced by pepsin hydrolysis at acidic conditions were further digested into small peptides and FAAs by intestinal proteases at alkali conditions. The degradation by pepsin of BPC continued for the first 120 min showed obvious elimination of some bands. During the intestinal digestion, bands at 100 kDa disappeared and most bands had a MW of less than 25 kDa. The bands at the end of the intestinal phase were intensive, in contrast to the other two gels, indicating undigested

protein or protein fractions resistant to digestion. The bands could correspond to barley hordein, the allergic protein composed of proline- and glutamine-rich polypeptide which are resistant to further hydrolysis (Wahab et al., 2016). The in-gel digestion followed by MS may identify the resistant proteins on the gel. In three gels, no new band with high MW appeared during the 240 min digestion implying that three protein concentrates contained no protein exceeded the detection size range of the gel (> 250 kDa). Besides, Figure 3.3.2 (A and B) shows two thick bands between 30 kDa - 35 kDa and 20 kDa – 25 kDa which has a potential of the overlay of different protein fractions that were not separated by 1-dimensional electrophoresis. For a better identification and characterisation of proteins obtained by alkaline extraction, 2-dimensional electrophoresis may be recommended with a second separation based on iso-electric focussing on protein fractions with the similar MW but different iso-electric points.

3.4.5 Two-Dimensional Electropherogram of Oat Bran Protein Concentrate

Two-dimensional protein profile of the oat bran protein concentrate prepared by alkaline extraction and isoelectric precipitation was presented for the first time aiming to characterise the potentially unidentified proteins located in the bran which could lead to novel peptides. The identification of spots was based on comparing the 2-D gel of oat bran proteins with the 2-D profiles obtained from the studies investigating oat proteins. The SDS-PAGE profile of extracted oat globulins indicates the position of globulins at MW of about 37 kDa and 20 kDa; extracted oat avenins are located at the same range and appear to be overlaid with globulin bands but predominately located between 20 kDa and 30 kDa (Comino et al., 2016; Tanner et al., 2019). Based on the rationale above, the identified 16 spots are estimated to be globulins while the avenins are the minor spots located in the region D in Figure 3.3.3 and 3.3.4. Sixteen identified protein spots listed in Figure 3.3.4 were presented in other studies investigating proteins in oat kernels using 2-D electrophoresis (Balážová et al., 2012; Galova et al., 2019). By using the small protein ladder, protein spots between 6 – 116 kDa were shown on their gels and focused over the range of pI 4 -11. Same accumulation of spots was identified at the MW of 36 kDa and pI of 4 to 9. The major spot 15 and 16 in Figure 3.3.3 were not shown in the profile with focusing over pH 3 -11 but were presented in the profile with focusing over pH 6 -11. These two spots were identified as trypsin/ α -amylase inhibitor proteins which were also found in wheat and barley (Nałęcz et al., 2009). Besides, more abundant minor spots were identified in their gels between 10 kDa

to 25 kDa compared to the region D in overloaded profile in figure 3.3.4. Similar area full of minor spots of proteins were revealed in the studies of Nałęcz et al. (2009) and Gasparis (2017) characterising protein extracted from oat flours. This may imply that less oat avenins were extracted in OBPC, meaning the oat bran could be a healthier source to prepare for plant-sourced protein concentrate for people with celiac disease to consume. Region C in Figure 3.3.5 was also found in the studies of Galova et al. (2019) and Balážová et al. (2012), identified to be corresponding to the allergen proteins.

Overall, the spots identified from protein extracted from oat bran matches the 2-D profile of protein extracted from oat flours, which means the protein map of oat flour and oat bran are very similar. There is no particular spot that should be identified by MS given the purpose was to find the novel protein which produced novel bioactive peptides.

In the study of Galova et al. (2019) and Gasparis (2017), selected bioinformatic tools were recommended as the identification technique after 2-D electrophoresis. Selected physicochemical data of avenin extracted from the resource of the UniProtKB (Swiss-Prot and TrEMBL) and ExPASy ProtParam database was presented in the study Gasparis (2017) for interpreting the spots found on 2-D electrophoretogram. Based on the above rationales, Gasparis (2017) identified Avenin-E (theoretical pI of 7.44 and MW of 21 kDa), a celiac immunoreactive protein, which could be spots at MW of 15kDa – 25 kDa and pI of 7 - 8. Table 3.4.1 and Table 3.4.2 were generated including all theoretically physicochemical data of oat avenin and globulins extracted from UniProtKB and ExPASy ProtParam database. However, the spots shown on the electropherograms of OBPC (Figure 3.3.4 and Figure 3.3.5) did not match the extracted data. UniProtKB and ExPASy ProtParam database contains sequence and physicochemical information derived mostly from various projects and research literatures, but the MW and pI of proteins on 2-D electropherograms could be affected by the conditions of protein extraction and the interpretation on pH gradient scale. Moreover, many entries of protein sequence information were theoretically developed based on genome sequencing, which is suitable for interpreting MS spectrum based on protein sequence. The MW and pI of protein subunits can be altered by post-translational modification. Therefore, there are limitations with identifying protein spots on 2-D gel using theoretical pI and MW generated from protein databases.

Table 3.4.1 The theoretical physiochemical data of all oat avenin and related fragments retrieved from UniProtKB and ExPASy ProtParam database (Gasparis, 2017).

No.	Accession Number	Protein Name	Sequence Status	Length (AA)	MW (kDa)	pI	Notes
1	P80356	Avenin-3	Complete	201	23.28	6.06	Nutrient reservoir
2	Q09114	Avenin-E	Complete	182	17.08	8.33	Celiac prolamin
3	Q09097	Avenin-F	Fragment	43	4.96	/	Celiac immunoreactive protein 2
4	Q09095	Avenin-A	Fragment	36	4.14	/	Celiac immunoreactive protein 1
5	P27919	Avenin	Complete	186	21.14	8.19	Avenin precursor
6	I4EP64	Avenin	Fragment	224	25.94	6.06	Nutrient reservoir
7	F4MJY2	Avenin	Fragment	161	18.52	/	/
8	Q09072	Avenin	Complete	203	23.54	7.44	Nutrient reservoir
9	Q09071	Avenin	Complete	162	18.44	8.66	Nutrient reservoir
10	I4EP64	Avenin	Fragment	224	25.94	6.06	Nutrient reservoir
11	I4EP85	Avenin	Complete	190	21.91	8.64	Nutrient reservoir
12	I4EP86	Avenin	Complete	190	21.91	8.64	Nutrient reservoir
13	I4EP88	Avenin	Complete	223	25.86	7.71	Nutrient reservoir
14	I4EP78	Avenin	Complete	193	22.31	8.64	Nutrient reservoir
15	F4MJY5	Avenin	Fragment	198	22.72	/	Nutrient reservoir
16	F4MJY6	Avenin	Fragment	202	23.25	/	Nutrient reservoir
17	F4MJY0	Avenin	Fragment	198	22.82	/	Nutrient reservoir
18	F4MJY4	Avenin	Fragment	187	21.53	/	Nutrient reservoir
19	F2Q9W4	Avenin	Fragment	214	24.63	/	Nutrient reservoir
20	F4MJY3	Avenin	Fragment	204	23.23	/	Nutrient reservoir
21	F2Q9W5	Avenin	Fragment	271	31.61	/	Nutrient reservoir
22	F4MJY1	Avenin	Fragment	221	25.60	/	Nutrient reservoir
23	F2Q9W3	Avenin	Fragment	222	25.73	/	Nutrient reservoir
24	Q2EPY2	Avenin	Complete	193	22.26	6.06	Nutrient reservoir
25	L0L5H3	Gliadin-like avenin	Complete	162	18.44	8.83	Nutrient reservoir
26	L0L8A9	Gliadin-like avenin	Complete	181	21.03	7.48	Nutrient reservoir
27	L0L833	Gliadin-like avenin	Complete	170	19.46	8.66	Nutrient reservoir
28	L0L6J5	Gliadin-like avenin	Complete	192	22.17	6.06	Nutrient reservoir
29	L0L8A0	Gliadin-like avenin	Complete	169	19.33	8.66	Nutrient reservoir
30	L0L8B6	Gliadin-like avenin	Complete	180	20.89	7.48	Nutrient reservoir

Table 3.4.1. The theoretical physiochemical data of all oat avenin and related fragments retrieved from UniProtKB and ExPASy ProtParam database (Gasparis, 2017). (Continue.).

No.	Accession Number	Protein Name	Sequence Status	Length (AA)	MW (kDa)	pI	Notes
31	L0L6J0	Gliadin-like avenin	Complete	262	30.79	6.01	Nutrient reservoir
32	L0L6J7	Gliadin-like avenin	Complete	162	18.44	8.66	Nutrient reservoir
33	L0L6K5	Gliadin-like avenin	Complete	182	21.34	7.48	Nutrient reservoir
34	L0L8A4	Gliadin-like avenin	Complete	170	19.46	8.66	Nutrient reservoir
35	L0L5H5	Gliadin-like avenin	Complete	195	22.60	6.61	Nutrient reservoir
36	L0L4J7	Gliadin-like avenin	Complete	193	22.47	6.08	Nutrient reservoir
37	L0L845	Gliadin-like avenin	Complete	190	22.08	6.87	Nutrient reservoir
38	L0L5I0	Gliadin-like avenin	Complete	210	22.49	6.06	Nutrient reservoir
39	L0L6J0	Gliadin-like avenin	Complete	262	30.79	6.01	Nutrient reservoir
40	L0L5G8	Gliadin-like avenin	Complete	204	23.83	6.01	Nutrient reservoir
41	L0L4I8	Gliadin-like avenin	Complete	231	27.06	7.72	Nutrient reservoir
42	L0L841	Gliadin-like avenin	Complete	181	21.00	7.48	Nutrient reservoir
43	L0L6K1	Gliadin-like avenin	Complete	189	22.03	7.48	Nutrient reservoir
44	L0L4J1	Gliadin-like avenin	Complete	190	22.04	6.87	Nutrient reservoir
45	L0L837	Gliadin-like avenin	Complete	170	19.46	8.66	Nutrient reservoir

Table 3.4.2. The theoretical physicochemical data of all oat globulins and related fragments retrieved from UniProtKB and ExPASy ProtParam database (Gasparis, 2017).

No.	Accession Number	Protein Name	Sequence Status	Length (AA)	MW (kDa)	pI	Notes
1	P14812	Globulin 2 12S acidic chain	Complete	293	33.21	5.7	Nutrient reservoir
1	P14812	Globulin 2 12S basic chain	Complete	201	22.76	9.3	Nutrient reservoir
2	P12615	Globulin 1 12S acidic chain	Complete	293	33.19	6.66	Nutrient reservoir
2	P12615	Globulin 1 12S basic chain	Complete	201	22.71	9.42	Nutrient reservoir
3	O49257	12S globulin	Complete	448	50.78	9.44	Nutrient reservoir
4	O49258	12S globulin	Complete	491	55.58	8.49	Nutrient reservoir
5	Q38780	11S globulin	Complete	503	56.67	9.20	Nutrient reservoir
6	Q38779	11S globulin	Complete	527	59.12	9.26	Nutrient reservoir
7	Q38781	12S globulin	Fragment	313	35.72	/	Nutrient reservoir
8	Q27Q38	11S globulin	Fragment	31	3.51	/	/

3.5 Conclusion

The physicochemical properties of WBPC, OBPC and BPC and their digesta were characterised in Chapter 3. The protein content of WBPC, OBPC and BPC were $63.51\% \pm 0.14\%$, $51.19\% \pm 0.53\%$ and $81.43\% \pm 1.19\%$, respectively. The degradation of three cereal protein concentrates were monitored during in vitro digestion by measuring the protein content of collected digesta and subjecting the digesta to SDS-PAGE. The most significant degradation was witnessed in the first 20 min of gastric and intestinal digestion. Bradford method and BCA method were applied to determine the peptide content of digesta. BCA method, compared to Bradford method, was more reliable to measure the content of peptides in the digesta whereas Bradford method was suitable to monitor the degradation of proteins. The gels showed that globulin and avenin were dominating proteins in WBPC and OBPC while BPC comprised of different subunits of hordeins. Plus, the digesta of BPC at

240 min showed evidence of resistant peptides. 2-D electrophoretogram showed the intact protein map of oat bran protein concentrates prepared by alkali extraction and isoelectric precipitation. 16 major proteins in related to globulin and avenin and two regions of minor proteins were observed. The protein map of OBPC was identical to the protein map of protein extracted from oat flours. No spot of interest was identified which could be a possible source of novel peptides, thus no spot was excised and subjected to MS for identification. The results of PSD showed the signs of aggregation of peptides, possibly with fibre and fat, but further speculation and investigation was not made as it was not relevant to the bioactive values of derived peptides.

Chapter 4

Nutritional and Bioactive Properties of the Protein Concentrates and Digesta

4.1 Introduction

Many studies have focused in recent years on the functional and nutritional properties of proteins in the cereal and the bran (Alu'datt et al., 2012; Balandrán-Quintana et al., 2015; Van den Broeck et al., 2016) and the bioactive attributes of peptides derived from the hydrolysis of these proteins (Shobako et al., 2018; Walters et al., 2020; Yang et al., 2020). In cereal science, only limited studies focused on evaluating the nutritional and bioactive properties of digesta produced by *in vitro* digestion (Sánchez-Velázquez et al., 2021; Zaky et al., 2019; Uraipong & Zhao, 2018).

Simulated *in vitro* digestion model has been believed to be a reliable alternative to the expensive, challenging, and time-consuming *in vivo* studies. Though the system only partially mimics the complexity of the gastrointestinal (GI) system, it is proven to be a gold standard in relation to the determination of the digestibility (%), degree of hydrolysis (DH%), the change of the structure and the release of the bioactive peptides and essential amino acids (Sousa et al., 2020

The chapter details the digestibility, DH% and the profile of the released FAAs of oat bran protein concentrate (OBPC), wheat bran protein concentrate (WBPC) and barley protein concentrate (BPC) after digestion are presented. The antioxidant activity and ACE inhibitory activity of the digesta at each stage of the digestion was measured in order to discover the beneficial properties of the cereal proteins after digestion. Three different approaches (ABTS radical scavenging assay, TPC and FRAP assay) were employed in this chapter to determine the antioxidant potential of protein and digesta through three different mechanisms. These three colorimetric methods examine the antioxidant activity by different mechanism: ABTS assay determines the activity of peptides to eradicate ABTS⁺ radical; TPC assay determines the content of phenol-like peptides in the digesta; FRAP assay assesses the activity of peptides to react with ferric ions (Munteanu & Apetrei, 2021). Membrane filtration with 3,000 MWCO (molecular weight cut-off) was used to separate oat bran intestinal digesta < 3

kDa and > 3 kDa. The antioxidant and ACE inhibitory activity of these two fractions were determined.

4.2 Methods

4.2.1 Materials

Wheat bran, oat bran and barley protein digesta before digestion, at gastric phase and at the end of intestinal digestion were obtained after *in vitro* digestion shown in Chapter 3.

4.2.2 Chemicals and Reagents

O-phthalaldehyde (OPA), serine, L- phenylalanine, ABTS, Trolox, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 2 N Folin-Ciocalteu reagent, ACE from rabbit lung (#A6778, St. Louis, Sigma-Aldrich Corporation, Missouri, USA), hippuryl-L-histidyl-L-leucine, hippuric acid, captopril, pyridine, benzene sulfonyl chloride, and Bradford dye reagent were purchased from Thermo Fisher Scientific (North Shore City, New Zealand).

4.2.3 Digestibility

Protein content in the hydrolysates were determined by the Bradford method (Kruger, 2009). To determine the digestibility of hydrolysates at different times within 240 min digestion, the equation established by Almeida et al. (2015) was used with a slight modification.

$$\% \text{Digestibility} = 1 - (P_h - P_b) / P_s \times 100\%$$

where P_h and P_b represent the protein content in the hydrolysates and in the blank, respectively. P_s represents the protein content in the aliquot obtained at time 0 when pH of the slurry was adjusted to pH 2 at 37°C. At the time, protein concentrates were visually solubilised. Along with the digestion on the samples, sets of blanks used same amount of RO water replacing samples were conducted while the conditions for experiments remained the same. The protein content determined in the aliquots collected from blanks were P_b .

4.2.4 Degree of Hydrolysis

DH% of hydrolysates at different time within 240 min digestion were determined according to the method by Nielsen et al. (2001). In detail, OPA reagent (7.620 g $\text{Na}_2\text{B}_4\text{O}_7$, 200 mg SDS, 160 mg OPA, 176 mg DTT in 200 mL) mixed with the hydrolysates in the ratio of 10: 1 and incubated

for 2 min at the room temperature before measuring at 340 nm in the FLUOstar Omega microplate reader (BMG LABTECH® by Alphatech Systems Ltd, Auckland, New Zealand). Serine was used as the standard. The calculation of DH% is based on the equation below.

$$\text{DH\%} = h / h_{\text{tot}} \times 100$$

Where h is the peptide bonds cleaved during the hydrolysis, h_{tot} represents the total peptide bonds in the protein sample.

Determination of h:

$$\text{Serine-NH}_2 = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{standard}} - \text{Abs}_{\text{blank}}} \times 0.9516 \text{ meqv/L} \times 0.1 \times 100 / X \times P$$

where serine-NH₂ = meqv serine NH₂/g protein; X = g sample; P = protein % in sample; 0.1 is the sample volume in liter (L). h is then: $h = (\text{serine-NH}_2 - \beta) / \alpha$ meqv/g protein, where α and β and h_{tot} are specific constant values for gluten, which was mostly relevant to the proteins in this study (Nielsen et al., 2001).

4.2.5 Free Amino Acids Analysis

The free amino acid analysis of the digested protein samples was conducted by using HPLC separation of derivatised amino acids (Bhat et al., 2019). The protein hydrolysates obtained before and after digestion were filtered into a 2 ml HPLC vial using a 0.45 μm syringe. Analysis of *amino* acid profile in the sample was completed with o-phthalaldehyde-3-mercaptopropionic acid (OPA/3MPA) for the primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for proline. An HPLC 1100 series (Agilent Technologies, Waldbronn, Germany) was employed for the analysis of amino acid composition with a fluorescence detector and an autosampler. ACE® 3 μm C-18 (150 mm \times 4.6 mm) HPLC column (Analytic Shop, Munich, Germany) was pre-set to 40 °C. The HPLC mobile phase A (10 mM sodium phosphate buffer, pH 7.5 with 0.8% tetrahydrofuran (THF)) and mobile phase B (50% methanol, 50% acetonitrile) was used for the analysis. The gradient elution started at 0-40% buffer B for 14 minutes, then was up to 50% buffer B for 6 minutes and eventually up to 100% buffer B over 4 minutes and maintained at 100% buffer B for 5 minutes. The detector

was started at 335nm/440nm (excitation/emission) for 26 min and changed to 260nm/315nm (excitation/emission) to detect proline.

4.2.6 ABTS Radical Scavenging Activity

According to Esfandi et al. (2019b), 7 mM ABTS stock solution (9.5 mL) was prepared and mixed with 100 mM Potassium Persulfate stock solution (245 μ L), then made up to 10 mL with RO water. The ABTS reagent was prepared 12 to 16 hrs prior to the experiment for developing colour, covered with foil. Fresh ABTS reagent was prepared each time and diluted with PBS (pH of 7.4) to obtain an absorbance of 0.706 ± 0.02 at 734 nm using a 96-well microplate reader. Digesta and Trolox standards (prepared by 70% methanol) were transferred into a 96-well microplate reader and mixed with ABTS reagents in a ratio of (1:10 v/v). After 5 mins incubation, the absorbance was measured at 734 nm. The ABTS scavenging activity was expressed as μ M Trolox equivalents (TE)/g of hydrolysate.

4.2.7 Measurement of Total Phenolic Concentration

The content of phenol-like peptides in the digesta was measured according to Lu et al. (2018). Digesta and gallic acid standards (20 μ L) were mixed with 100 μ L of 0.2 N Folin-Ciocalteu reagent and 80 μ L of 7.5% (w/v) sodium carbonate. Incubation was allowed for 2 hours at room temperature in a dark place. The absorbance was measured by using the microplate reader at the absorbance of 760 nm. The results were expressed as 'mg gallic acid equivalents per gram weight'.

4.2.8 Ferric Reducing Antioxidant Power

The ferric reducing power of the digesta was measured based on Prasad et al. (2010) with slight modification. Fresh working solution of FRAP reagent was prepared by mixing 300 μ M Acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM FeCl_3 at a ratio of 10:1:1 (v/v/v). Digesta and iron (II) sulphate standard solution (20 μ L) were transferred into the 96-microplate plate mixing with 200 μ L of the working reagents and incubated for 2 hrs at 37 $^\circ\text{C}$. Microplate reader was used to measure the absorbance at 593 nm. The results were calculated and reported as ' μ mol Fe^{3+} /g sample'.

4.2.9 Angiotensin 1-converting enzyme (ACE) Inhibition Activity

Inhibition of angiotensin 1-converting enzyme (ACE) was measured using the colorimetric assay described by Jimsheena and Gowda (2009). Hydrolysate samples (32 μ L) were added to 5 μ L of ACE with the activity of 1 U/mL. The mixture was incubated at 37°C for 10 minutes. Then 13 μ L of 5 mM hippuryl-L-histidyl-L-leucine substrate solution (made with 50 mM sodium borate buffer, 0.3 M NaCl, pH 8.2) was added to each of tube to start the reaction. 1 M HCl was added to cease the reaction after one hour incubation at 37°C. Pyridine (100 μ L) and benzene sulfonyl chloride (50 μ L) were added to each tube followed by vigorous shake on the vortex for 10 seconds for colour development and cool on ice immediately. Two hundred μ L of the mixture was transferred to a 96-well microplate and was measured at 410 nm using a plate reader (415-2080-FLUOstar Omega, Ortenberg, Germany). The negative control contained 32 μ L of 50 mM sodium borate buffer (0.3 M NaCl, pH8.2) and the positive control contained 32 μ L of captopril (10 μ M). Hippuric acid standards were made in the 50 mM sodium borate buffer (0.3 M NaCl, pH 8.2). The percentage of ACE inhibition was calculated based on the equation shown below.

$$ACE\ inhibition\ \% = 100 - \frac{THA}{CHA} \times 100$$

Where THA is the concentration of hippuric acid in samples and standards and CHA is the concentration of hippuric acid in the negative controls. Digesta was adjusted to 1 mg/mL before the assay.

4.2.10 Membrane Filtration

The intestinal digesta was separated by Amicon® Ultra-15 Centrifugal Filter Units with 3,000 MWCO (Merck, North Shore City, New Zealand) into two fractions (digesta < 3 kDa and digesta > 3 kDa). Fraction with MW of < 3 kDa was concentrated to 1 mg/mL by using SpeedVac vacuum concentrator (Thermo Fisher Scientific, Auckland, New Zealand) at 20 °C. Fraction with MW of > 3 kDa was diluted to 1 mg/mL by using 50 mM phosphate buffer (pH, 8). 100 μ L of each fraction was allocated into 200 μ L Eppendorf tube and stored at -20 °C for analysis.

4.2.11 Statistical Analysis

Data were collected and analysed by one-way ANOVA and Turkey's test using Minitab 19 (Minitab Ltd., Sydney, Australia) for triplicated determination. The results were shown as

means \pm standard deviation (SD) of the means. Statistical significance was declared as $P < 0.05$.

4.3 Results

4.3.1 *In vitro* Protein Digestibility and DH% of Hydrolysates

As shown in Figure 4.3.1, the digestibility and DH% of three cereal proteins shared the same increasing tendency during 240 min digestion. The digestibility of WBPC and OBPC increased dramatically from 0% to 25% within the first 20 min gastric digestion and steadily increased to about 50% at the end of gastric digestion whereas BPC increased to 30% at the end of gastric phase. The digestibility of three proteins soared within the first 20 min of the intestinal digestion and steadily rose to about 90% (70% for BPC). Overall, the pattern of DH% for three proteins was similar to that of digestibility. At the end of the digestion, the DH% of WBPC, OBPC and BPC reached to 50%, 40% and 60%, respectively. In addition, the DH% of three proteins at gastric digestion were observed to increase to about 20% in the first 20 min and plateaued until the beginning of the intestinal digestion.

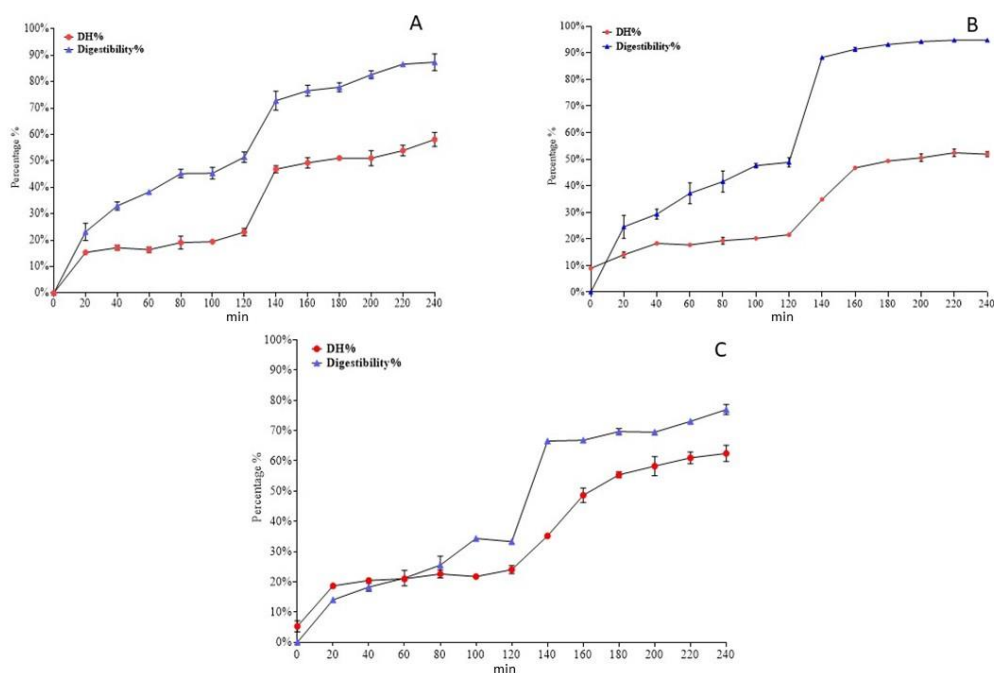


Figure 4.3.1 The DH% and digestibility of three protein concentrates at different hydrolysis time during 240 min GI digestion. (A) WBPC (wheat bran protein concentrate); (B) OBPC (oat bran protein concentrate); (C) BPC (barley protein concentrate). All measurements were expressed as Mean \pm SD (n = 3). SD was calculated based on the triplicate of the digestion on each protein sample.

4.3.2 Free Amino Acids (FAAs) Analysis

The determination of FAAs released during the digestion by HPLC is based on the principle that OPA reacts with primary amino acid groups with the presence of the thiol groups emitting fluorescent light that can be detected by the fluorescence detector of HPLC. The release on increase of total FAAs before digestion and after pepsin hydrolysis was not significantly observed ($P < 0.05$) in WBPC (236.57 $\mu\text{mol/L}$ to 298.09 $\mu\text{mol/L}$) as illustrated in Table 4.3.1. A significant increase was observed before and after gastric digestion in OBPC and BPC (95.45 $\mu\text{mol/L}$ to 211.53 $\mu\text{mol/L}$ and 79.06 $\mu\text{mol/L}$ to 140.09 $\mu\text{mol/L}$), respectively. Then the release of FAAs of WBPC, OBPC and BPC increased dramatically at the end of the intestinal digestion to 4552.24 $\mu\text{mol/L}$, 6844.19 $\mu\text{mol/L}$ and 3465.78 $\mu\text{mol/L}$, respectively.

Table 4.3.2 shows free amino acid profiles at before digestion, after gastric digestion and after intestinal digestion. The content of all amino acids was shown to be increased after gastric digestion. Amino acids such as asparagine, glutamine, histidine, arginine and phenylalanine were observed to increase greatly after intestinal digestion.

Table 4.3.1 The efficacy of the *in vitro* pepsin-pancreatin digestion model on the total free amino acids (FAAs) released from three protein concentrates at the end of three stages of digestion.

	WBPC ($\mu\text{mol/L}$)	OBPC ($\mu\text{mol/L}$)	BPC ($\mu\text{mol/L}$)
Before Digestion	236.57 \pm 5.40 ^{aB}	95.45 \pm 1.40 ^{bC}	79.06 \pm 2.30 ^{cC}
End of Gastric	298.09 \pm 26.40 ^{aB}	211.53 \pm 0.00 ^{bB}	140.09 \pm 10.30 ^{cB}
End of Intestinal	4552.24 \pm 47.9 ^{bA}	6844.19 \pm 25.40 ^{aA}	3465.78 \pm 14.40 ^{cA}

- Data were expressed as Mean \pm SD in replicate with superscripts in a column wise (upper case alphabet) and in a row wise (lower case alphabet) differ significantly ($P < 0.05$, $n = 2$).
- WBPC: wheat bran protein concentrate; OBPC: oat bran protein concentrate; BPC: barley protein concentrate

Table 4.3.2 The free amino acids (FAAs) profile of three protein concentrates at the end of three stages of *in vitro* digestion.

FAAs	WBPC (µg/g)			OBPC (µg/g)			BPC (µg/g)		
	Before Digestion	End of Gastric	End of Intestinal	Before Digestion	End of Gastric	End of Intestinal	Before Digestion	End of Gastric	End of Intestinal
Asp	300.60 ± 9.01 ^C	380.04 ± 1.26 ^B	500.37 ± 14.25 ^A	182.51 ± 4.94 ^C	267.26 ± 1.04 ^B	411.55 ± 9.10 ^A	121.50 ± 6.54 ^C	172.85 ± 6.50 ^B	263.93 ± 4.42 ^A
Glu	487.27 ± 9.73 ^C	677.96 ± 1.16 ^B	1.22×10 ³ ± 22.47 ^A	294.26 ± 21.26 ^C	396.85 ± 5.46 ^B	1.27×10 ³ ± 18.68 ^A	253.95 ± 7.95 ^C	436.15 ± 4.16 ^B	876.27 ± 5.60 ^A
Cystein	46.37 ± 1.34 ^B	44.84 ± 7.63 ^B	150.16 ± 11.83 ^A	ND	30.76 ± 1.18 ^B	372.00 ± 64.84 ^A	ND	42.27 ± 1.64 ^B	126.82 ± 1.49 ^A
Asn	689.31 ± 29.54 ^C	788.35 ± 5.41 ^B	1.76×10 ³ ± 395.32 ^A	574.67 ± 7.23 ^C	634.02 ± 2.06 ^B	4.79×10 ³ ± 249.27 ^A	94.14 ± 0.07 ^C	108.91 ± 1.46 ^B	571.49 ± 12.50 ^A
Ser	481.43 ± 12.58 ^B	481.10 ± 2.32 ^B	822.85 ± 16.22 ^A	355.09 ± 5.34 ^B	361.04 ± 1.12 ^B	951.76 ± 1.02 ^A	206.31 ± 5.97 ^B	216.89 ± 6.64 ^B	642.03 ± 14.33 ^A
Gln	218.63 ± 9.44 ^B	160.64 ± 0.23 ^C	3.75×10 ³ ± 78.71 ^A	14.84 ± 0.28 ^C	17.98 ± 0.05 ^B	6.92×10 ³ ± 33.97 ^A	13.29 ± 0.90 ^B	11.85 ± 1.08 ^C	3.12×10 ³ ± 27.11 ^A
His	48.38 ± 7.33 ^B	56.20 ± 0.04 ^B	1.62×10 ³ ± 48.87 ^A	16.36 ± 0.91 ^C	20.00 ± 0.01 ^B	2.15×10 ³ ± 5.15 ^A	ND	ND	1.15×10 ³ ± 16.01 ^A
Gly	24.71 ± 1.18 ^C	31.68 ± 2.96 ^B	126.85 ± 2.48 ^A	7.92 ± 0.44 ^C	12.02 ± 0.03 ^B	126.68 ± 10.12 ^A	3.69 ± 0.48 ^C	6.46 ± 0.53 ^B	65.76 ± 2.86 ^A
Thr	166.39 ± 5.82 ^C	224.36 ± 0.75 ^B	661.44 ± 15.38 ^A	41.18 ± 0.47 ^C	59.56 ± 0.70 ^B	743.80 ± 1.16 ^A	15.37 ± 3.80 ^B	17.56 ± 0.11 ^B	519.80 ± 5.56 ^A
Arg	290.52 ± 2.19 ^C	301.76 ± 1.10 ^B	35.41×10 ³ ± 112.48 ^A	42.87 ± 0.34 ^C	53.08 ± 0.11 ^B	68.11×10 ³ ± 394.67 ^A	19.26 ± 3.64 ^B	16.69 ± 0.21 ^B	9.94×10 ³ ± 57.57 ^A
Ala	165.41 ± 6.03 ^C	188.70 ± 0.28 ^B	1.11×10 ³ ± 9.68 ^A	30.97 ± 0.348 ^C	47.50 ± 0.17 ^B	1.16×10 ³ ± 12.18 ^A	16.42 ± 1.75 ^C	27.69 ± 1.86 ^B	8.15 ± 2.30 ^A
Tau	1.97 ± 0.08 ^C	5.12 ± 0.20 ^B	13.20 ± 0.05 ^A	12.95 ± 0.02 ^C	14.18 ± 1.07 ^B	20.04 ± 0.95 ^A	ND	3.23 ± 0.92 ^A	3.24 ± 0.06 ^A
Tyr	111.57 ± 0.86 ^C	135.54 ± 4.85 ^B	14.03×10 ³ ± 163.78 ^A	26.90 ± 4.25 ^B	26.54 ± 13.80 ^B	32.43×10 ³ ± 210.21 ^A	4.90 ± 3.11 ^C	14.25 ± 0.45 ^B	9.30×10 ³ ± 31.39 ^A
Val	202.57 ± 3.65 ^C	246.66 ± 7.01 ^B	2.02×10 ³ ± 3.75 ^A	42.56 ± 0.92 ^C	153.07 ± 0.29 ^B	3.30×10 ³ ± 17.62 ^A	10.51 ± 7.63 ^C	57.86 ± 2.88 ^B	1.87×10 ³ ± 9.93 ^A
Met	84.62 ± 1.16 ^C	134.69 ± 10.86 ^B	1.31×10 ³ ± 0.80 ^A	2.59 ± 1.87 ^C	62.39 ± 5.17 ^B	1.80×10 ³ ± 1.15 ^A	1.99 ± 2.89 ^C	52.81 ± 1.27 ^B	1.11×10 ³ ± 0.72 ^A
Trp	73.33 ± 0.23 ^B	109.35 ± 48.24 ^B	3.50×10 ³ ± 36.66 ^A	110.89 ± 0.40 ^C	246.91 ± 1.99 ^B	6.65×10 ³ ± 70.60 ^A	146.78 ± 0.02 ^C	263.44 ± 3.26 ^B	3.26×10 ³ ± 151.79 ^A
Phe	242.45 ± 3.38 ^C	343.64 ± 68.68 ^B	17.37×10 ³ ± 99.37 ^A	20.97 ± 4.52 ^C	205.84 ± 0.97 ^B	35.68×10 ³ ± 282.95 ^A	21.92 ± 4.26 ^C	117.09 ± 2.64 ^B	10.89×10 ³ ± 28.61 ^A
Ile	78.29 ± 1.03 ^C	214.83 ± 76.84 ^B	1.86×10 ³ ± 21.89 ^A	13.83 ± 0.26 ^C	702.48 ± 2.56 ^B	4.22×10 ³ ± 23.83 ^A	13.05 ± 3.39 ^C	80.73 ± 11.92 ^B	2.08×10 ³ ± 92.01 ^A
Lys	500.50 ± 7.83 ^C	842.61 ± 282.25 ^B	5.65×10 ³ ± 23.48 ^A	54.25 ± 1.43 ^C	1.34×10 ³ ± 0.29 ^B	6.74×10 ³ ± 2.57 ^A	37.00 ± 0.54 ^C	346.44 ± 143.50 ^B	3.36×10 ³ ± 15.98 ^A
Leu	274.73 ± 6.40 ^C	447.22 ± 139.64 ^B	18.07×10 ³ ± 40.90 ^A	17.42 ± 5.89 ^C	300.51 ± 18.19 ^B	32.64×10 ³ ± 119.64 ^A	26.43 ± 6.14 ^C	194.82 ± 6.45 ^B	13.96×10 ³ ± 34.16 ^A
Pro	410.84 ± 3.08 ^C	427.34 ± 4.53 ^B	860.48 ± 24.11 ^A	504.59 ± 16.84 ^C	597.69 ± 1.80 ^B	1.78×10 ³ ± 80.28 ^A	266.04 ± 4.24 ^A	256.99 ± 10.47 ^A	568.58 ± 130.97 ^A

- Data were expressed as Mean ± SD with superscripts in a column wise (upper case alphabet) differ significantly ($P < 0.05$, $n = 2$). ND = no data.
- WBPC: wheat bran protein concentrate; OBPC: oat bran protein concentrate; BPC: barley protein concentrate
- The unit of the FAAs was expressed as µg of released amino acid in 100 mL of digestion dispersion/g of total protein added before the digestion

4.3.3 Antioxidant Activity of Crude Proteins and Digesta

The digesta of OBPC, WBPC and BPC were subjected to antioxidant assays without adjustment of concentration, which shows the antioxidant power of digesta produced by 1 gram of each type of protein concentrate. As shown in Figure 4.3.2, the antioxidant activity of three protein concentrates at before and after digestion was presented. Overall, the antioxidant activity of three protein concentrates determined by ABTS, TPC and FRAP assay was significantly increased after digestion. Oat bran gastric digesta showed the highest ABTS scavenging activity of 2074.32 ± 4.93 Trolox equivalents $\mu\text{M/g}$ sample followed by oat intestinal digesta with ABTS scavenging activity of 1899.04 ± 18.52 Trolox equivalents $\mu\text{M/g}$ sample. The digesta derived from WBPC showed moderate ABTS scavenging activity ranged from 602.43 ± 0.56 Trolox equivalents $\mu\text{M/g}$ sample to 830.49 ± 10.18 Trolox equivalents $\mu\text{M/g}$ sample. The oat bran gastric digesta and intestinal digesta also showed the highest TPC value of 249.99 ± 7.72 gallic acid equivalents mg/g sample and $226.63.07 \pm 9.05$ gallic acid equivalents mg/g sample, respectively while wheat bran gastric and intestinal digesta showed decent TPC value (160.68 ± 12.45 gallic acid equivalents mg/g sample and 171.40 ± 4.71 gallic acid equivalents mg/g sample, respectively). Interestingly, the FRAP value of three protein concentrates was lower after digestion, decreased from the range of $450.47 \pm 27.01 - 590.87 \pm 17.33$ $\mu\text{M Fe}^{3+}/\text{g}$ sample to the range of 38.79 ± 7.74 to 361.25 ± 5.28 $\mu\text{M Fe}^{3+}/\text{g}$ sample. Barley intestinal digesta showed highest ferric reducing power (361.25 ± 5.28 $\mu\text{M Fe}^{3+}/\text{g}$) among three intestinal digesta.

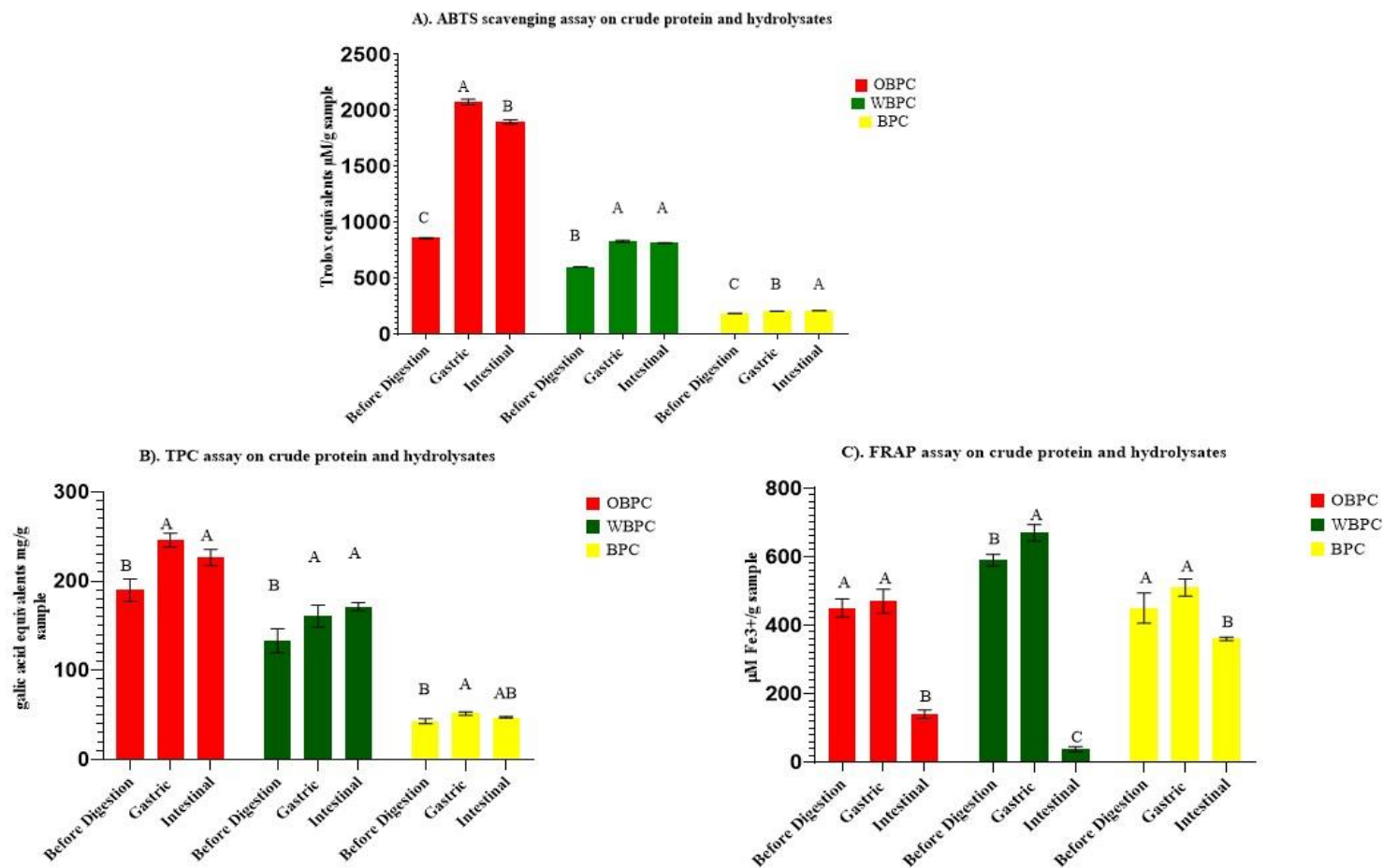


Figure 4.3.2 Antioxidant activity of oat bran protein concentrates (OBPC), wheat bran protein concentrates (WBPC) and barley protein concentrates (BPC) and their hydrolysates after gastric and intestinal digestion.

4.3.4 ACE Inhibitory Activity of Crude Proteins and Digesta

The concentration of digesta were not adjusted showing the ACE inhibitory power of digesta produced by 1 gram of protein concentrate. Figure 4.3.3 shows that the ACE inhibitory power of all three protein digesta are significantly higher than that of crude proteins. The ACE inhibitory of OBPC and BPC reached to about 80% while intestinal digesta showed slightly lower number at around 75%. BPC showed the inhibitory power of $72.11\% \pm 2.35\%$ at gastric phase and $79.50\% \pm 1.29\%$ at intestinal phase, which was the only protein showed higher inhibitory power at intestinal phase. In addition, OBPC showed inhibitory power of $22.12\% \pm 6.65\%$ before digestion but after digestion, it showed the inhibitory power of $84.65\% \pm 2.82\%$ at gastric phase and $74.86\% \pm 0.27\%$ at intestinal phase, showing a great elevation of ACE inhibitory activity after digestion.

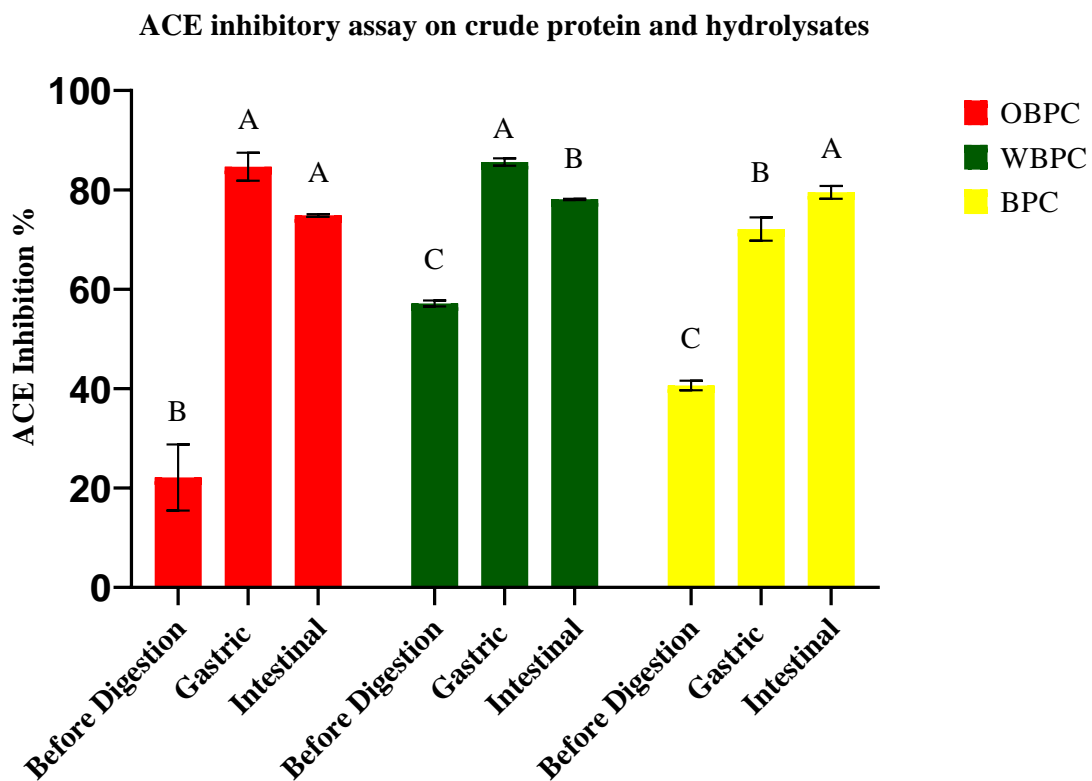


Figure 4.3.3 ACE inhibitory activity of oat bran protein concentrates (OBPC), wheat bran protein concentrates (WBPC) and barley protein concentrates (BPC) and their hydrolysates after gastric and intestinal digestion.

4.3.5 Antioxidant Activity and ACE Inhibitory Activity of Oat Bran Protein Intestinal Digesta Separated by 3,000 MWCO Membrane

Heating up to stop enzymatic reaction is an universal method used in many articles. There are other practical ways to stop the enzymatic reaction including adjustment of pH to denature enzymes, addition of enzyme inhibitors, precipitation everything by adding Trichloroacetic acids (TCA) and membrane filtration. Adjusting pH not just also denatures peptides but also could lead to possible precipitation once it reaches the isoelectric point of the peptides. Enzyme inhibitors could introduce foreign matters which does not belong to the *in vitro* digestion system, and it is hard to see if the inhibitors are going to lead to false results to the further analysis. TCA precipitation again introduced foreign matters disrupting H-bondings leading to conformational change. Membrane filtration seems to cause no harm to the structure of peptides but is not a practical method as it takes long time. Adjustment of temperature may cause the change of the peptide structure but peptides with lower than 3 kDa in MW were basically 10 amino acids long, this method seems to lead to least influence on them.

Table 4.3.3 showed the antioxidant and ACE inhibitory activity of oat bran intestinal digesta with MW of < 3 kDa and > 3 kDa and with concentration of 1 mg/mL. The TPC value and ABTS scavenging activity of digesta < 3 kDa (165.65 ± 2.63 gallic acid equivalents mg/g sample and 1038.32 ± 10.52 Trolox equivalents $\mu\text{M/g}$ sample, respectively) were significantly higher than the digesta > 3 kDa (70.41 ± 3.62 gallic acid equivalents mg/g sample and 744.43 ± 12.04 Trolox equivalents $\mu\text{M/g}$ sample, respectively). Both digesta < 3 kDa and > 3 kDa showed weak ferric reduction power (67.34 ± 18.52 $\mu\text{M Fe}^{3+}/\text{g}$ sample and 70.86 ± 2.15 $\mu\text{M Fe}^{3+}/\text{g}$ sample, respectively). As for the ACE inhibitory power, digesta < 3 kDa ($80.35\% \pm 0.97\%$) also showed higher inhibitory power compared with digesta > 3 kDa ($51.87\% \pm 1.94\%$).

Table 4.3.3 The antioxidant and ACE inhibitory activity of oat bran protein intestinal digesta separated by 3,000 MWCO membrane.

	< 3 kDa	> 3 kDa
ABTS Trolox equivalents µM/g sample	1038.32 ± 10.52 ^A	744.43 ± 12.04 ^B
TPC gallic acid equivalents mg/g sample	165.65 ± 2.63 ^A	70.41 ± 3.62 ^B
FRAP µM Fe3+/g sample	67.34 ± 18.52 ^A	70.86 ± 2.15 ^A
ACE Inhibition %	80.35 ± 0.97 ^A	51.87 ± 1.94 ^B

- Data were expressed as Mean ± SD in replicate with superscripts in a column wise (upper case alphabet) differ significantly ($P < 0.05$, $n = 3$).

4.4 Discussion

4.4.1 *In vitro* Protein Digestibility and DH% of Hydrolysates

The *in vitro* pepsin-pancreatin digestion model is a widely accepted process to mimic the enzyme and pH conditions of human GI system determining the dynamics of the digestion system and observing the bioavailability of the variety of nutrients released. *In vitro* digestion systems are believed to be useful in replacing *in vivo* assays, considering the high cost and the ethical objection due to the animal use (Sousa et al., 2020; Tavano et al., 2016). The pepsin-pancreatin system quantifies the digestion by evaluating the digestibility, DH% and the release of the essential amino acids while digestibility and DH% reflect the degradation of the protein and the hydrolysis of peptide bonds, respectively, and the analysis of FAAs has the advantage of reflecting the release of the FAAs whereas the consumption of essential amino acids by gut microbiota affects the true faecal nitrogen digestibility *in vivo* (Tavano et al., 2016).

Figure 3.3.1 shows the digestibility and DH% of three protein samples during digestion. The digestibility of WBPC and OBPC reached 90% and the digestibility of BPC reached 80%, the results are consistent with previous publications (Büchmann, 1979; Pettersson et al., 1996).

Tomé (2013) also indicated that the digestibility of the protein sourced from plants was about 70% to 90%. High digestibility and DH% of protein indicated that protein was completely degraded to small peptides and amino acids which are absorbed in the distal duodenum and ileum (Fuquay et al., 2011). The extraction process was assumed to alter the confrontation of protein fractions and form aggregates (Joye, 2019) but the result showed the digestibility was not affected by the processing.

The overall DH% of three protein concentrates showed similar patterns in which DH% increased to 20% within the first 20 min gastric digestion and then plateaued until the beginning of the intestinal digestion, after which it continued to show a significant increase ($P < 0.05$) to about 50% to 60%. Uraipong and Zhao (2018) employed a similar pepsin-pancreatin model to digest rice bran protein concentrates extracted by alkaline solution and the result of DH% and digestibility were about 34% and 70% - 80%, respectively. Pepsin has the preferential cleavage site next to phenylalanine, tyrosine, tryptophan and leucine in position P1 and P1'. Pancreatin is a commercial enzyme combination with a number of different proteases extracted from porcine pancreas, ideally having a better range of cleavage sites compared to the pepsin. Gallegos Tintoré et al. (2015) observed that the DH% increased dramatically right after the treatment of pancreatin after pepsin digestion. Malomo et al. (2015) also found the DH% of pepsin on hemp seed protein was about 4% to 7% whereas the combination of pepsin and pancreatin increased the DH% to 28%. The results may imply that pepsin plays an important role in breaking peptide bonds of large protein fractions in the digestion system, followed by further degradation by pancreatin to reduce the size of small protein fractions and long chain peptides into small peptides and amino acids.

During the gastric digestion, it is interesting to note that the digestibility of three protein concentrates continue to rise whereas the DH% of them are comparatively static. The digestibility of the protein was calculated based on monitoring and determining the soluble protein content which was gradually reduced during the digestion comparing to the soluble protein content before digestion. Bradford method worked well at this scenario as Coomassie blue dye binds to protein forms the protein-dye complexes at acidic pH, developing blue colours (Sapan et al., 1999). This article also suggested Coomassie blue dye-binding method was not sensitive to quantify peptides with MW of less than 3 kDa because

short chain peptides will not form the protein-dye complexes that cause the colour change. As protein was gradually degraded during the digestion, the digestibility increased accordingly. DH% measures the percentage of the cleaved peptide bonds. Therefore, as pepsin is specific to peptide next to phenylalanine, tyrosine, tryptophan and leucine and acts to break open proteins during the gastric digestion, DH% did not increase as dramatically as intestinal digestion when most peptide bonds broken producing the large number of small peptides. In addition, OPA method which determined the DH% in this paper is sensitive to the ϵ -amino acid such as lysine but not to cysteine and glycine and the high sensitivity of this method occurs while abundant of the FAAs and small peptides were presented (Nielsen et al., 2001). As Table 4.3.2 indicated, most of the amino acids including lysine released into the environment during the intestinal digestion, which may explain the plateau of DH% during gastric digestion of three protein concentrates.

4.4.2 Free Amino Acids (FAAs) Analysis

The release of the FAAs was one of the most important parameters to determine the extension of the digestion and the bioavailability of the essential amino acids in the GI tract (Bhat et al., 2019; Tavano et al., 2016). The FAAs profile was shown in Table 4.3.2, providing the comprehensive information of the release of FAAs at each stage of the digestion which could be used as the reference materials for the nutritional studies of cereal protein concentrates. All amino acids showed a significantly higher values ($P < 0.05$) at the end of intestinal digestion compared to the previous two stages. Amino acids like arginine, tyrosine, phenylalanine, and leucine had a tremendous amelioration in all three samples. A significant higher value of aspartic acid, glutamic acid and valine were observed before and after gastric digestion ($P < 0.05$). Phenylalanine, leucine, and valine are essential amino acids.

Phenylalanine released by one gram of OBPC after intestinal digestion could be sufficient for the daily requirement of the adult (30 – 40 mg) (Kurpad et al., 2006), though consumption of only one gram of OBPC could lead the deficiency in other amino acids. However, the release of essential amino acids such as histidine, isoleucine and methionine were nowhere near the recommended daily intakes (Kubala, 2019). Cereal protein is considered less healthy than meat protein because of the unbalanced composition of amino acid profiles (Price & Welch, 2013). For example, lysine is the essential amino acid needs to be obtained from food but is the first limiting amino acids in wheat based on the Food and Agriculture Organization/World Health Organization (Hussain et al., 2004). One gram of WBPC, OBPC

and BPC released 5.63 mg, 6.74 mg and 3.36 mg of lysine respectively, at the end of the digestion. The lysine content in wheat, oats and barley are 1.5 mg, 2.6 mg and 2.5 mg per gram of the seeds, respectively (Mäkinen et al., 2017; Munck, 1972). Even though lysine released from protein concentrates was doubled comparing to the lysine level in original cereals but still, the concentrates were deficient in lysine. Likewise, as the second limiting amino acids in cereals, about four times more threonine (6 mg - 7 mg) was released from per gram of protein concentrate after the digestion comparing to the lysine content (about 2 mg - 3 mg) in cereals. The daily requirement of lysine and threonine was about 13 mg/ kg and 7 mg/kg of muscle tissue gain (roughly 1 g and 0.5 g per day, respectively), for the adult while 4 - 5 times more was needed for children from 2 to 12-year age (Allowances, 1989). Consumption of the protein concentrates prepared by alkaline extraction only would not be sufficient to meet the daily requirements. For example, a heaping scoop comes with the commercial protein supplement used at gym has a capacity of about 30 g, 30 g of oat bran protein concentrate will only provide roughly 200 mg of lysine and 20 mg of threonine, which are far from the suggestion of the daily requirement. Plus, the nutritional value of lysine released could be over-estimated as lysine could be damaged during the process of the extraction and digestion, The determination of the quantity of the reactive lysine in the intestinal digesta could be adopted to provide additional information regarding the nutritional value of the FAAs (Torbatinejad et al., 2005). Moreover, hydrolysate aliquots for HPLC to determine FAAs were not precipitated by trichloroacetic acid or perchloric acid and the generation of abundant short chain peptides at the end of the digestion might lead to a higher value. The bioavailability of short peptides was limited by their sizes as, generally, the peptide transport system only allows peptides less than tetra-peptides to be permeable unless the presence of intestinal peptide transporters specific for substrate (peptide) bindings (Shen & Matsui, 2019; Webb Jr et al., 1992).

4.4.3 Antioxidant Activity of Crude Proteins and Digesta

ABTS scavenging power of cereal proteins and digesta

ABTS assay had been widely used to measure the antioxidant activity of cereal protein digesta which donates hydrogen ions or electrons to quench ABTS⁺ radicals (Esfandi et al., 2019b; García-Casas et al., 2022; Karami, Peighambaroust, Hesari, Akbari-Adergani, et al., 2019; Liu et al., 2021; Saisavoey et al., 2016; Zaky et al., 2020). The increased ABTS

scavenging activity of digesta derived from OBPC, WBPC and BPC indicated that the digestion produced polypeptides with lower molecular size but higher proton or electron donation capability. Amongst the hydrolysates, gastric and intestinal digesta of OBPC showed the highest ABTS scavenging activity (2074 ± 5 Trolox equivalents $\mu\text{M}/\text{g}$ sample and 1899 ± 18 Trolox equivalents $\mu\text{M}/\text{g}$ sample, respectively). The number is consistent with a recent study (Sánchez-Velázquez et al., 2021) which investigated the antioxidant power of protein extracts derived from oat flours and its gastric and intestinal digesta. In this study, the ABTS power of non-cooked oat protein intestinal digesta reached to about 2200 Trolox equivalents $\mu\text{M}/\text{g}$ sample. Esfandi et al. (2019b) indicated that the oat bran protein isolate (prepared by using Cellulase and Viscozyme to promote extraction yield) digested by Flavourzyme, papain and Protamex showing ABTS scavenging activity ranged from 137 ± 5 $\mu\text{M TE}/\text{g}$ to 870 ± 10 $\mu\text{M TE}/\text{g}$. The oat intestinal digesta produced by this *in vitro* pepsin-pancreatin digestion model showed almost twice the ABTS scavenging activity compared to the hydrolysates prepared by commercial enzymes. The ABTS scavenging power of wheat gastric and intestinal digesta was about 800 $\mu\text{M TE}/\text{g}$ which was equivalent to about 50% of scavenging power of oat intestinal digesta. Liu et al. (2021) indicated that the wheat gluten isolate digested by Alcalase and Protamex showed higher activity at about 1200 $\mu\text{M TE}/\text{g}$. Hydrophobic amino acids has been believed to extensively contribute to the antioxidant power, especially the radical scavenging power, of peptides derived from cereals (C. Dugardin et al., 2020; Esfandi et al., 2019b; Karami, Peighambaroust, Hesari, Akbari-Adergani, et al., 2019; Sánchez-Velázquez et al., 2021; Tok et al., 2021; Vanvi & Tsopmo, 2016; Zhang et al., 2014). Table 4.3.1 showed the total FAAs released after digestion in three cereal intestinal digesta while oat bran intestinal digesta showed the highest content (6844 ± 25 $\mu\text{mol}/\text{L}$). As shown in table 4.3.2, oat bran intestinal digesta contained abundant hydrophobic amino acids including alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine and tryptophan whereas wheat bran and barley intestinal digesta were comparatively deficient in valine, leucine, isoleucine, proline and phenylalanine.

TPC content of cereal proteins and digesta

High phenol content has been believed to correspond to high antioxidant capability (Brand-Williams et al., 1995; García-Casas et al., 2022; Supawong et al., 2019; Thamnarathip et al., 2016; Zaky et al., 2019). Amongst the digesta derived from three protein extracts, oat bran gastric and intestinal digesta showed highest TPC value of 250 ± 7 gallic acid equivalents

mg/g sample and 227 ± 9 gallic acid equivalents mg/g sample, respectively. The number is significantly higher than the wheat bran and barley gastric digesta (161 ± 12 gallic acid equivalents mg/g sample and 52 ± 2 gallic acid equivalents mg/g sample, respectively) and intestinal digesta (171 ± 5 gallic acid equivalents mg/g sample and 47 ± 1 gallic acid equivalents mg/g sample, respectively). Mason et al. (2020) showed that the oat digesta < 3 kDa produced by *in vitro* digestion model with membrane filtration had the TPC value of about 90 ferulic acid equivalents mg/g sample. Alrahmany and Tsopmo (2012) reported that the phenol extract (by water-methanol 1:1 v/v) from oat bran had the TPC value of 0.5 gallic acid equivalents mg/g sample. The significant higher TPC value found in oat gastric and intestinal digesta may imply that the phenolic compounds in oat bran was released effectively along with proteins. It had been reported that the peptide in the digesta could interact with phenol compounds to exert higher antioxidant power (Hernández-Jabalera et al., 2015). Mason et al. (2020) also found that the wheat digesta < 3 kDa had the TPC value of about 100 ferulic acid equivalents mg/g sample. Ferulic acid is one of the most abundant phenolic acids found in wheat and has been used as the standards in quantifying wheat total phenol content, but the result derived from ferulic acid is not comparable to ones obtained from gallic acids (Tian et al., 2021). There was no study found directly investigating the TPC value of barley protein or its derivatives, García-Casas et al. (2022) found a whey-based beverage with barley as the additional ingredient was reported to have TPC value of about 2.5 gallic acid equivalents mM/kg sample after digestion. Digesta derived from other cereals such as rice was reported to have TPC value of 20 - 80 gallic acid equivalents mg/g sample (Thamnarathip et al., 2016; Zaky et al., 2019). These numbers reported in the literatures were certainly lower. Table 4.3.2 shows that the tyrosine was one of the most abundant amino acids released after digestion in all three digesta. Karami, Peighambardoust, Hesari, Akbari-Adergani, et al. (2019) and Vásquez-Villanueva et al. (2016) stated that the phenolic hydroxyl in tyrosine could contribute to high antioxidant power.

Ferric reducing power of cereal proteins and digesta

Ferric reduction assay is based on the mechanism of electron transfer, working at the acidic environment to suppress proton transfer, increase redox potential and reduce Fe^{3+} to Fe^{2+} (Cerretani & Bendini, 2010). The reduction of ferric reduction power from gastric digesta to intestinal digesta was also observed by Sánchez-Velázquez et al. (2021) and García-Casas et al. (2022). It may suggest that FRAP assay was not an ideal antioxidant assay for observing the antioxidant power of intestinal digesta due to the basic pH environment.

4.4.4 ACE Inhibitory Activity of Crude Proteins and Digesta

The level of ACE plays a crucial role in regulating blood pressure and the blood glucose level (Thamnarathip et al., 2016). The inhibition power of oat bran and wheat bran gastric and intestinal digesta shared the similar value at about 85% and 76%, respectively. The inhibitory activity increased from 72% in barley gastric digesta to 79% in its intestinal digesta. The opposite trend could be caused by the acidic environment of gastric digesta that may inhibit the activity of ACE. The reduced inhibition could also be caused by degradation of ACE inhibitory polypeptides and the release of more short-sequence peptides. It is also worthy to note that the protein concentration of three types of intestinal digesta were not adjusted before assay. Since oat bran and wheat bran intestinal digesta had similar protein concentration whereas barley protein concentrate had 4 times more protein content compared to the other two digesta, the specific ACE inhibitory power of barley gastric and intestinal digesta were comparatively lower. Darewicz et al. (2022) reported that the protein extracted from oat kernel showed the ACE inhibitory power of 88% after gastric and intestinal digestion. A similar study conducted by Camille Dugardin et al. (2020) indicated that the IC_{50} value of ACE inhibitory activity of the intestinal digesta derived from oat protein isolate was 99 $\mu\text{g}/\text{mL}$. In this article, it also reported that the IC_{50} value of ACE inhibitory activity of the wheat intestinal digesta was from 60 mg/mL to 270 mg/mL depending on the grading of grains. ACE inhibitory activity of hydrolysates < 1 kDa derived from wheat protein was reported to be 60% to 90% (Liu et al., 2021; Zou et al., 2020). The IC_{50} value of inhibition power of peptides derived from barley protein was reported to be 5 mM to 108 mM (Yin et al., 2022).

4.4.5 The Antioxidant and ACE Inhibitory Activity of Oat Bran Intestinal Digesta Separated by 3,000 MWCO Membrane

Based on the overall higher antioxidant and ACE inhibitory activity, oat bran intestinal digesta was selected for further analysis. Gastric digesta was not selected as the antioxidant and ACE inhibitory peptides would be absorbed in the small intestine to benefits the human body. Before the intestinal digesta was subjected to R-HPLC-MS/MS for sequencing, membrane filtration with 3,000 MWCO centrifugal tube was applied to separate the digesta to the fractions with MW of < 3 kDa and > 3kDa. As shown in table 4.3.3, fraction digesta < 3 kDa showed significantly higher antioxidant activity (ABTS: 1038.32 ± 10.52 Trolox equivalents $\mu\text{M}/\text{g}$ sample; TPC: 165.65 ± 2.63 gallic acid equivalents mg/g sample), except

for the ferric reducing power. Nevertheless, the FRAP assay was not included in the further analysis in Chapter 5 as table 4.3.3, again, proved that FRAP assay did not reflect the actual reducing power of hydrolysates due to the basic environment. Higher ACE inhibitory activity ($80.35\% \pm 0.97\%$) in digesta < 3 kDa was also observed. It has been observed in many studies that peptide derived from cereal proteins with smaller molecular size (less than 3 kDa in most cases) tended to have higher antioxidant and ACE inhibitory activity due to the higher exposure of hydrophobic amino acids (Gumus et al., 2022; Liu et al., 2021; Mason et al., 2020; Phongthai et al., 2018; Tian et al., 2022; Vanvi & Tsopmo, 2016; Wang et al., 2015; Zhang et al., 2019).

4.5 Conclusion

The hydrolysis of WBPC, OBPC and BPC subjected to simulated *in vitro* pepsin-pancreatin digestion showed high digestibility and DH% indicating a great potential of the generation of short chain peptides and essential amino acids in human digestion system. The unbalanced amino acids profile indicates the shortage of the essential amino acids that can be supplied by the consumption of these three cereal samples. As for the bioactivity of the proteins after digestion, oat bran intestinal digesta showed overall higher antioxidant and ACE inhibitory power compared with wheat bran and barley digesta and was therefore selected for membrane filtration to prepare sample for ion-exchange chromatography in Chapter 5 and MS sequencing in Chapter 6. Oat bran intestinal digesta with MW of < 3 kDa was then selected to be the candidate for chromatography and sequencing due to the higher antioxidant and ACE inhibitory activity.

Chapter 5

Separation of Bioactive Peptides Derived from Oat Bran using Anion Exchange Chromatography

5.1 Introduction

Liquid chromatography (LC) is a technique which separates molecules in liquid mobile phase as a result of interacting with the solid stationary phase. Fast protein liquid chromatography (FPLC) is used to separate proteins, peptides and nucleic acids from biological extracts using column chromatography. The FPLC system is suitable for preparative purification applications. Ion exchange chromatography (IEX) is a type of chromatographic technique used to isolate anion or cation protein/peptides from hydrophobic peptides and from one another based on the affinity of protein/peptides to the column and the electrical charge that peptides carry. Anion exchange chromatography uses a positively charged resin with an affinity for macromolecules with net negative surface charge. Conversely, cation exchange chromatography has negatively charged resin for positively charged macromolecules. The separation of the target protein/peptides could be achieved by changing the salt gradient, pH of the buffer and the nature of the resin. Ion exchange chromatography has the advantage of separating the pool of anionic/cationic peptide from each other and from hydrophobic peptides.

In the last two decades, numerous plant bioactive peptides have been isolated for peptidomic studies using IEX (Bamdad et al., 2015; Li & Aluko, 2010; Ma et al., 2020; Ma et al., 2017; Mei et al., 2013; Suetsuna & CHEN, 2002; Uraipong & Zhao, 2018; Wang et al., 2014). As a critical step in the sequential purification, IEX mostly has been employed before the final purification of R-HPLC followed by the peptide sequence identification by MS.

Anion exchange chromatography has been used to isolate anionic peptides derived from cereal intestinal digesta (Phongthai & Rawdkuen, 2020; Uraipong & Zhao, 2018) and tryptic peptides (Afdhal et al., 1990; Boichenko et al., 2013; Naqash & Nazeer, 2012). Cation exchange chromatography has also been used to isolate cationic antimicrobial peptides (Ditsawanon et al., 2023; Y. Wang et al., 2021; Zhao et al., 2012).

Although hundreds of peptides are produced in oat protein digesta (Esfandi et al., 2019b), there is no report on characterising the bioactive peptides of oat bran protein concentrates obtained by the alkaline extraction subjected to *in vitro* digestion. Therefore, the aim of the present study was to fractionate the digesta into groups of peptides similar in their charge properties by using anion exchange chromatography system and to characterise oat intestinal fractions regarding their antioxidant activity and ACE inhibition power. In the previous chapter, the peptide pool of < 3 kDa showed significantly higher antioxidant and ACE inhibitory activity. Hence, peptides of < 3 kDa oat intestinal digesta were used in the current study.

5.2 Methods

5.2.1 Materials

Oat bran intestinal digesta < 3 kDa was prepared by subjecting oat bran protein concentrate (prepared in Chapter 3) to the *in vitro* digestion followed by membrane filtration by centrifugal tubes with 3000 MWCO.

5.2.2 Chemicals and Reagents

BSA was purchased from ThermoFisher (Christchurch, New Zealand). The HiTrap Q-FF column and the HiTrap SP-FF column was purchased from Cytiva (Marlborough, Massachusetts, USA). The column was packed with Q Sepharose Fast Flow strong anion exchange resin (bed volume: 5 mL; binding capacity: 50 mg human serum albumin/mL 20 mM Tris-HCl, pH 8.2 at 1 ml/min). The FPLC unit used in the study was ÄKTA Pure Protein Purification System with a fixed wavelength at 280 nm operated by UNICORN™ software (Cytiva, Marlborough, Massachusetts, USA).

5.2.3 Dialysed Peptides Preparation for Chromatography

Dialysis with the Snakeskin Dialysis Tubing (Thermo Fisher, Waltham, Massachusetts, USA), 10,000 MWCO was utilised during the preparation of protein concentrates. Protein concentrates was made by using the method shown in Chapter 3, section 1.2.2 with slight modification. After alkali extraction, extracted protein solution was dialysed at room temperature overnight with RO water, the pH of which was adjusted to 10. The purpose of dialysis is to remove the small particles produced during *in vitro* digestion which could falsely increase the antioxidant and ACE inhibitory activity of the intestinal peptides such as phenol

compounds. The acid precipitation was followed for proteins to be precipitated then followed by freeze-drying. The oat intestinal digesta were obtained after *in vitro* digestion (Chapter 3, section 1.2.3). The intestinal peptides < 3 kDa was collected by membrane filtration (Chapter 4, section 1.2.8).

5.2.4 Ion Exchange Chromatography

Preparation

The cleaning protocol for FPLC and the used column was adopted according to Merck (n.d.). The FPLC unit was sanitised by warm water, 20% ethanol, 1M NaOH solution and RO water accordingly. The HiTrap Q-FF column was used in lactoferrin isolation previously and thereby was washed in advance every time before the experiment. To ensure no residue from the previous experiments in the column, the column was washed accordingly by 2 column volume of 2M NaCl, 4 column volume of 1M NaOH, 2 column volume of 2M NaCl and 2 column volumes of distilled water. The column was then thoroughly washed by injecting pepsin solution (1 mg/mL in 0.5 M NaCl, 0.1 M acetic acid) and leaving it overnight at 37 °C. Two column volume of RO water was used to rinse the column until the UV-baseline and eluent pH were stable then 4 column volumes of 70% ethanol or 30% isopropanol was injected. RO water was used to rinse the column until the UV-baseline and eluent pH were stable before the storage buffer was applied until pH and conductivity value reached the required value. The column was washed by 2 column volume of 2M NaCl, 4 column volume of 1M NaOH, 2 column volume of 2M NaCl and 2 column volumes of distilled water before every experiment. The same preparation method was applied to the HiTrap SP-FF column utilised for isolating cationic peptides in the unbound peptides eluted from the washing stage by using anion exchange chromatography.

Testing the viability of the column, using BSA and 1M NaCl

BSA was used as a trail representative protein sample for testing the integrity of the used anion column. Twenty-two milligrams of BSA (pre-conditioned by 0.05M phosphate buffer, pH8) was loaded to a HiTrap Q-FF column at a flow rate of 0.25 mg/mL. Phosphate buffer was used aiming on having the matched solution as oat intestinal digesta. Phosphate buffer has been used to as the buffer to isolate cationic peptides (Chan & Li-Chan, 2007; Y. Wang et al., 2021). Buffer A (0.05 M phosphate buffer, pH8; conductivity: 4.3 mS/cm) was used to wash the column till the pH and conductivity dropped to a stable value. BSA was then eluted

with 0.05 M phosphate buffer containing a gradient up to 1M NaCl (buffer B, pH8) at 1 mL/min. The reason to start off by using gradient separation was to enhance the detectability and peak resolution. The fractionation process by FPLC was monitored by measuring the conductivity (mS/cm) and UV absorbance (at 280 nm) of the eluent. Fractions (1.5 mL/fraction) were collected and pooled corresponding to each individual peak in the chromatograms.

Fractionation of anion peptide form < 3 kDa oat intestinal peptide

I. A gradient separation using 1 NaCl & 0.05 M phosphate buffer

The < 3 kDa peptides were thawed and applied at a flow rate of 0.25 mL/min to a HiTrap Q-FF column, which was pre-equilibrated with 0.05M phosphate buffer (pH of 8). A total of 24 mg of < 3 kDa peptide were applied to 5 mL anion exchange column (×2). After unbound peptides had been washed off with the 0.05M phosphate buffer (pH of 8) at 1 mL/min, bound (anion) peptides were then eluted with 0.05M phosphate buffer (pH of 8) containing a gradient up to 1M NaCl at 1 mL/min. The fractionation process by FPLC was monitored by measuring the conductivity and UV absorbance at 280 nm of the eluent. Fractions (1.5 mL/fraction) were collected and pooled corresponding to each individual peak in the chromatograms. Fractions were stored at -20 °C for further analysis. All further assays were conducted within 2 freeze-thaw cycles in order to prevent the degradation of bioactivity of collected peptides. Negatively charged HiTrap SP-FF column was used on detecting cationic peptides potentially presented in the unbound peptides eluted at the washing stage. The unbound peptide solution collected from the washing stage was collected and loaded onto the HiTrap SP-FF column, and the same conditions as above was applied, except for that the gradient was adjusted to be up to 2M NaCl.

II. A gradient separation using 1 M NaCl & 20 mM Tris-HCl buffer (pH of 8).

The < 3 kDa peptides were thawed and applied at a flow rate of 0.25 mL/min to a HiTrap Q-FF column, which was pre-equilibrated with 20 mM Tris-HCl buffer (pH of 8; conductivity: 0.80 mS/cm). A total of 24 mg of < 3 kDa peptide were applied to 5 mL anion exchange column (×2). After unbound peptides had been washed off with the 20 mM Tris-HCl buffer (pH of 8) at 1 mL/min, bound (anion) peptides were then eluted with 20 mM Tris-HCl buffer (pH of 8) containing a gradient up to 1M NaCl at 1 mL/min. The fractionation process by FPLC was monitored by measuring the conductivity and UV absorbance at 280 nm of the eluent. Fractions (1.5 mL/fraction) were collected and pooled corresponding to each individual peak

in the chromatograms. Fractions were stored at -20 °C for further analysis. All further assays were conducted within 2 freeze-thaw cycles in order to prevent the degradation of bioactivity of collected peptides.

III. A stepwise separation using 0.2M, 0.5M & 2M NaCl with 20mM Tris-HCl buffer (pH of 8)

The stepwise gradient has been used in purifying target protein and peptides (Ng & Yoshitake, 2010). As the oat intestinal digesta is a mixture of peptides with different nature, isocratic separation has the advantage of providing improved separation of pools of peptides at different conductivity. The < 3 kDa peptides were thawed and applied at a flow rate of 0.25 mL/min to a HiTrap Q-FF column, which was pre-equilibrated with 20 mM Tris-HCl buffer (pH of 8). A total of 35 mg of < 3 kDa peptide were applied to 5 mL anion exchange column (×2). After unbound peptides had been washed off with the 20 mM Tris-HCl buffer (pH of 8) at 1 mL/min, bound (anion) peptides were then eluted with 20 mM Tris-HCl buffer (pH of 8) containing a stepwise gradient of 0.2 M, 0.5 M and 2M NaCl at 1 mL/min. The fractionation process by FPLC was monitored by measuring the conductivity and UV absorbance at 280 nm of the eluent. Fractions (1.5 mL/fraction) were collected and pooled corresponding to each individual peak in the chromatograms. Fractions were stored at -20 °C for further analysis. All further assays were conducted within 2 freeze-thaw cycles in order to prevent the degradation of bioactivity of collected peptides.

5.2.5 ORAC assay

ORAC assay was adopted based on Esfandi et al. (2019b) and Jodayree et al. (2012) with slight modification. Fluorescein solution (4.19 µmol/L) prepared by adding 1.58 mg fluorescein in 1 L of phosphate-buffered saline (PBS) buffer (75 mmol/L, pH 7.4) and then was diluted by 52 times to obtain 0.08 µmol/L solution of fluorescein. 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) (0.414 g) (Sigma-Aldrich, St. Louis, Missouri, United States) was dissolved in 10 mL PBS to a concentration of 153 mM. Glutathione (0.1 mg/mL, made by PBS buffer) was used as a positive control. Five Trolox standard were prepared (7.5, 12.5, 25, 50 and 100 µM) to establish the standard curve. The assay was performed in black 96-well microplates containing 120 µL fluorescein, 20 µL of PBS buffer for the blank, 20 µL of glutathione, 20 µL standard, or 20 µL hydrolysates. The assay was carried out in triplicates. Pre-incubation was required for 20 mins at 37°C and immediately followed by the addition of 60 µL AAPH solution and the kinetic measurement for 60 min with the intensity measured every 60 sec by 96-well microplate reader (FLUOstar Omega, BMG

Labtech GmbH, Ortenberg, Germany). Excitation and emission wavelengths were 485/20 and 528/20 nm, respectively. The ORAC value was calculated based on the net area under the curve (AUC) of standards and samples (Gheldof & Engeseth, 2002). The net AUC is calculated by subtracting the AUC of blank from that of a sample.

The AUC of each sample was calculated as

$$AUC = 0.5 + f_1/f_0 + \dots + f_i/f_0 + \dots + f_{59}/f_0 + 0.5(f_{60}/f_0)$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i .

The ORAC value was calculated as

$$ORAC = (AUC_{sample} - AUC_{blank}) / (AUC_{rolox} - AUC_{blank})$$

5.2.6 Statistical Analysis

Data were collected and analysed by one-way ANOVA and Turkey's test using Minitab 19 (Minitab Ltd., Sydney, Australia) for triplicated determination. The results were shown as means \pm standard deviation (SD) of the means. Statistical significance was declared as $P < 0.05$.

5.3 Results

5.3.1 Control Separation of Bovine Serum Albumin

The chromatogram in Figure 5.3.1 showed BSA was fully bound to the column and the detection of BSA in the fraction started at the elute volume of 52.88 mL and the conductivity of 9.79 mS/cm, peaked at 57.35 mL and the conductivity of 20.75 mS/cm. The conductivity of Buffer A (0.05 M phosphate buffer, pH8) was 4.3 mS/cm, while 1M NaCl (buffer B, pH8) at around 120 mS/cm. With the gradient remained at 100% buffer B for 20 mL and dropped down to 0% buffer, the conductivity changed accordingly without the observation of new peak. Due to the limit of 96-wells fraction collector, later fractions were not collected.

The fractions collected were assayed by BCA method (refer to Chapter 3, section 1.3.2) and the total protein content in the fraction was 21.56 ± 0.78 mg which was equivalent to the loaded weight of BSA (22mg). It is safe to say that the column was in full function and capable of being used in fractionating oat intestinal digesta.

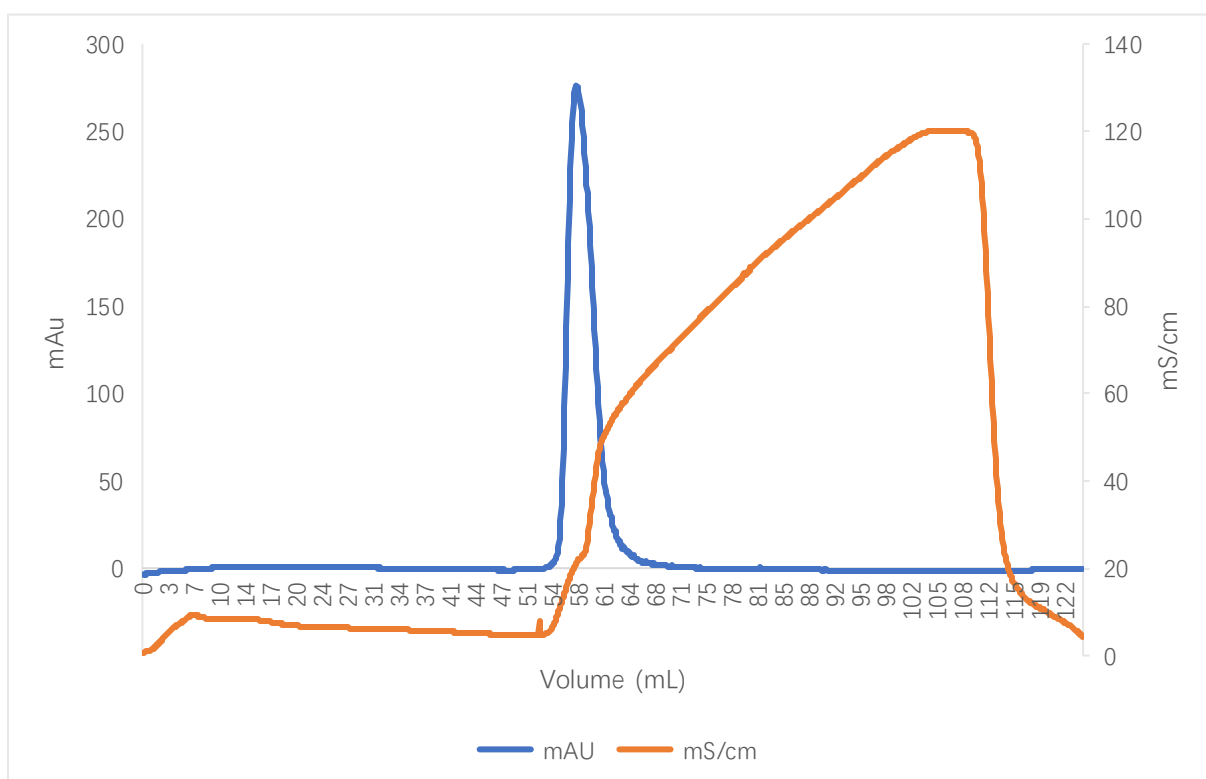


Figure 5.3.1 Chromatograms of bovine serum albumin (BSA) (22 mg) on the loading and fractionation by using gradient flow to 100% buffer B (0.05 M phosphate buffer+ 1M NaCl, pH 8). Conductivity (the orange line, mS/cm) and the absorbance at 280 nm of the eluent (blue line. Abs) in FPLC chromatogram was monitored.

5.3.2 Fractionation of anion peptide form < 3 kDa oat intestinal peptide using a 0-1M NaCl gradient in 0.05 M phosphate buffer

The chromatogram of isolating anion intestinal peptides was shown in Figure 5.3.2. The chromatogram showed a large portion of peptides in the digesta was eluted during the washing stage. The elution of the anionic peptides started at the volume of 58.15 mL and the conductivity of 12.99 mS/cm and peaked at 55.87 mL and the conductivity of 32.20 mS/cm. However, the BCA assay did not pick up any protein/peptide readings in the corresponding eluted fractions relating to the second peak shown during gradient separation. Peptide content in the fractions collected from the washing stage was measured by BSA assay and showed the amount of 20.73 mg indicating the second peak could be a false reading.

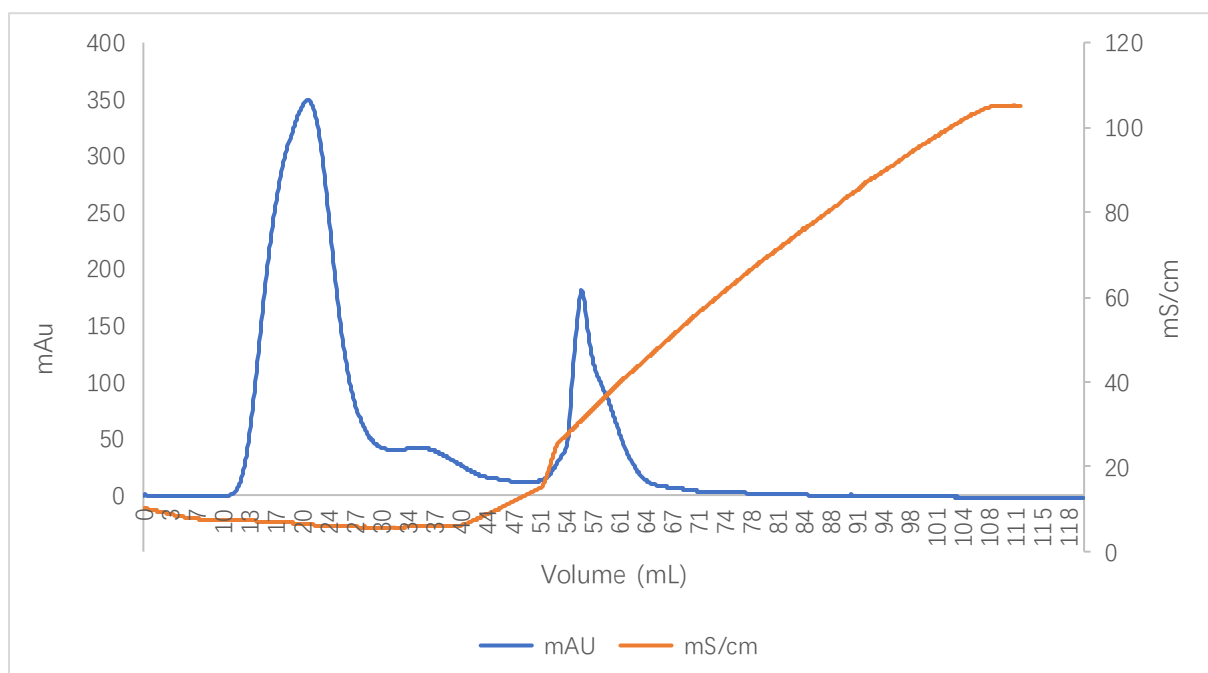


Figure 5.3.2 Chromatograms of oat intestinal peptide < 3kDa (digesta < 3 kDa) (24 mg) on the loading and fractionation by using gradient Flow to 100% buffer B (0.05 M phosphate buffer+ 1M NaCl, pH 8). Conductivity (the orange line, mS/cm) and the absorbance at 280 nm of the eluent (blue line. Abs) in FPLC chromatogram was monitored. Due to the limit of 96-wells fraction collector, exceeded fractions were not collected. With the gradient remained at 100% buffer B for 20 mL and dropped down to 0% buffer, the conductivity changed accordingly without the observation of new peaks.

Cation exchange chromatography was used to determine the cationic peptides possibly presented in the unbound peptides collected from the washing stage during anion exchange chromatography. One major peak was eluted during the washing stage (Figure 5.3.3) and neither a significant peak was found, nor the presence of peptide in the fractions collected during the gradient separation.

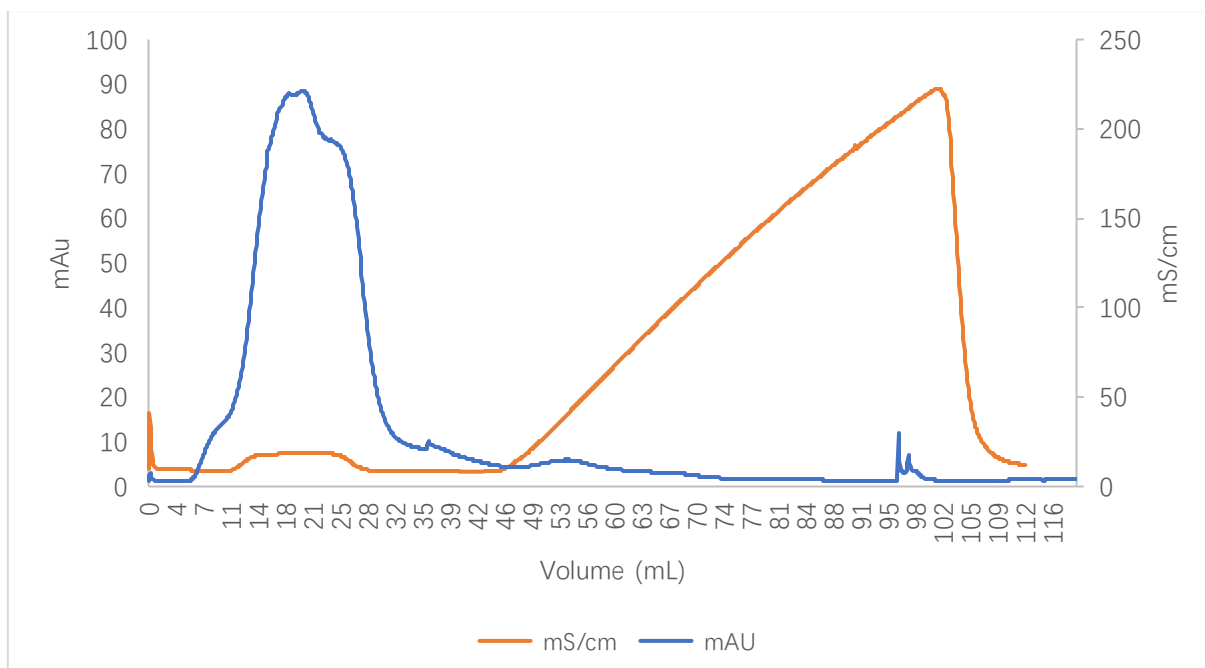


Figure 5.3.3 Chromatograms of unbound peptide (20.73 mg) loaded on HiTrap SP-FF column and fractionated by using gradient flow to 100% buffer B (0.05 M phosphate buffer+ 2M NaCl, pH 8). Conductivity (the orange line, mS/cm) and the absorbance at 280 nm of the eluent (blue line. Abs) in FPLC chromatograms was monitored. Due to the limit of 96-wells fraction collector, exceeded fractions were not collected.

5.3.3 Fractionation of antioxidant anionic peptides form < 3 kDa oat intestinal peptide using a 0-1M NaCl gradient in Tris-HCl buffer

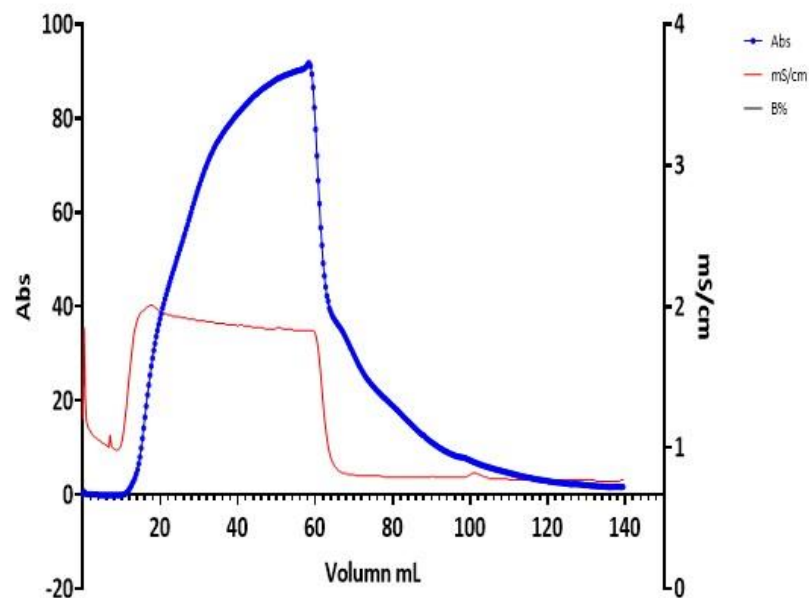
Figure 5.3.4 contains Figure 5.3.4-A and Figure 5.3.4-B which separately shows the elution of unbound peptides at washing stage and bound anionic peptide at linear gradient with unit on x-axis and y-axis with different scale in order to present the chromatogram with better resolution. First peak was observed during the washing stage and the second major peak was observed at the gradient elution. The first peak at the washing stage showed the absorbance reading increased with peptide sample loaded to the column indicating the loss of the peptide and declined dramatically to the baseline when the loading of sample was finished when the column was washed by only buffer A.

The digesta (24mg proteins in total) were loaded, and the degree of unbound peptides was assessed where the result (Figure 5.3.4-A) showed 13.17 mg amount unbounded (conductivity: 1.78 mS/cm), while 9.38 mg peptides were eluted during the gradient (Figure 5.3.4 –B). The yield of anion peptides isolated from the digesta was 39%. Peptides eluted between fraction 10 and fraction 20 are related to the first major peak during gradient

separation shown in Figure 5.3.4-B. Following the first peak, continuous peaks were observed, but as shown in Figure 5.3.4-B, comparatively low peptides content were found in fraction 20 to fraction 30 indicating that the continuous peaks could be artefacts. So, there were two real and significant peaks during the separation between fraction 10 to fraction 20 and between fraction 21 to fraction 30 as suggested in Figure 5.3.4-B.

The criteria for selecting fraction candidates were based on the observation of the significant antioxidant power and the peptide content (over 0.2 mg/mL). Eleven fraction candidates were selected and their ABTS scavenging power, oxygen radical absorbance capacity and the content of phenolic peptides were shown in Figure 5.3.5-C, Figure 5.3.5-D and Figure 5.3.5-E, respectively. ABTS scavenging assay and ORAC assay share the similar principle that the protons and electrons of antioxidant peptide being donated to the radicals (Esfandi et al., 2019b). The scavenging power of the selected fractions to $ABTS^+$ and ROO^- radicals were both expressed as Trolox equivalent μM per gram (μM TE/g). The range of ABTS scavenging power ranged from $12.77 \pm 6.28 \mu M$ TE/g to $275.16 \pm 1.48 \mu M$ TE/g. The fraction 17 (eluted at conductivity of 6.44 mS/cm) and fraction 18 (eluted at conductivity of 7.15 mS/cm) showed the highest ABTS scavenging power $267.45 \pm 10.44 \mu M$ TE/g and $275.16 \pm 1.48 \mu M$ TE/g at the conductivity of 6.44 mS/cm and 7.14 mS/cm, respectively, corresponding to the tail of the first peak on the chromatogram. The fractions relating to the second peaks showed the ABTS scavenging power between $98.80 \pm 23.30 \mu M$ TE/g to $168.35 \pm 7.90 \mu M$ TE/g, eluted at the conductivity between 11.47 mS/cm to 14.80 mS/cm. As for the scavenging power of ROO^- radicals, fraction 22 to fraction 25 corresponding to the second peak showed the highest activity ranged from $103.00 \pm 2.93 \mu M$ TE/g to $170.00 \pm 15.41 \mu M$ TE/g whereas fraction 17 and fraction 18 showed the radical scavenging activity of $96.00 \pm 2.96 \mu M$ TE/g and $114.00 \pm 2.03 \mu M$ TE/g. The classic TPC assay was a method mainly used to measure the content of the phenol compounds estimating the antioxidant power. The reduction capacity of TPC was expressed as gallic acid equivalent microgram per milligram (gallic acid equivalent $\mu g/mg$). Fraction 17 and 18 showed the highest TPC of 34.69 ± 2.94 gallic acid equivalent $\mu g/mg$ and 27.54 ± 3.17 gallic acid equivalent $\mu g/mg$ while the fraction 22 showed the lowest TPC of 8.81 ± 2.07 gallic acid equivalent $\mu g/mg$. FRAP assay was conducted on the fractionated anion peptides but the result was negative.

A). Peptide Sample that was not bound to the column and washed off by 20 mmol Tris-HCl Buffer (pH 8)



B). Separation by Using the Gradient Flow to 50% Buffer B (20 mmol Tris-HCl + 2M NaCl, pH 8)

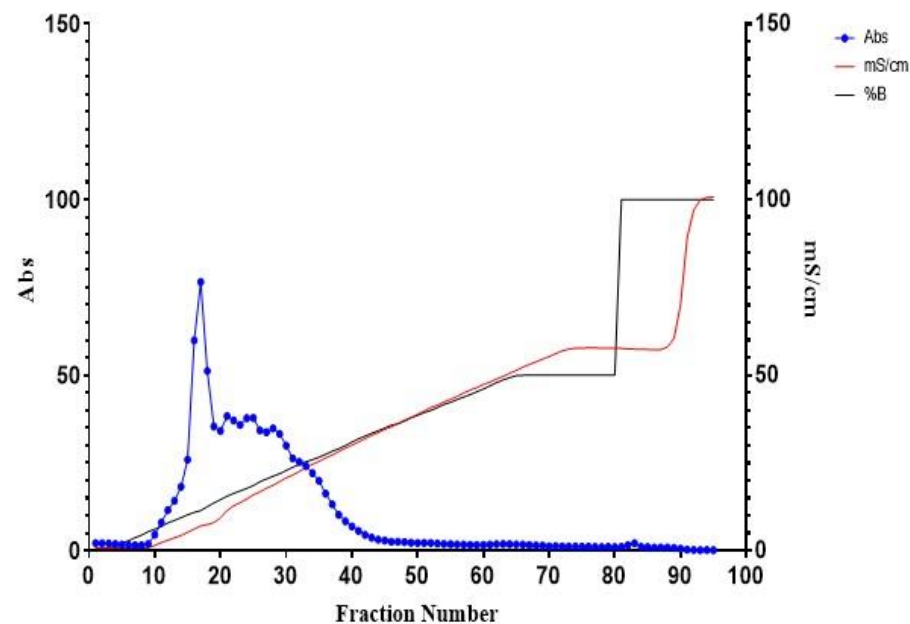


Figure 5.3.4. Chromatograms of peptides (24 mg) on the initial loading phase (A) and fractionation using gradient flow to 50% buffer B (20 mmol tris-HCl + 2M NaCl, pH 8) (B). Conductivity (the red line, mS/cm) and the absorbance at 280 nm of the eluent (blue line. Abs) in FPLC chromatograms was monitored. The black line represents the gradient of NaCl in the mobile phase. Due to the limit of 96-wells fraction collector, exceeded fractions were not collected. With the gradient remained at 100% buffer B for 20 mL and dropped down to 0% buffer, the conductivity changed accordingly without the observation of new peaks.

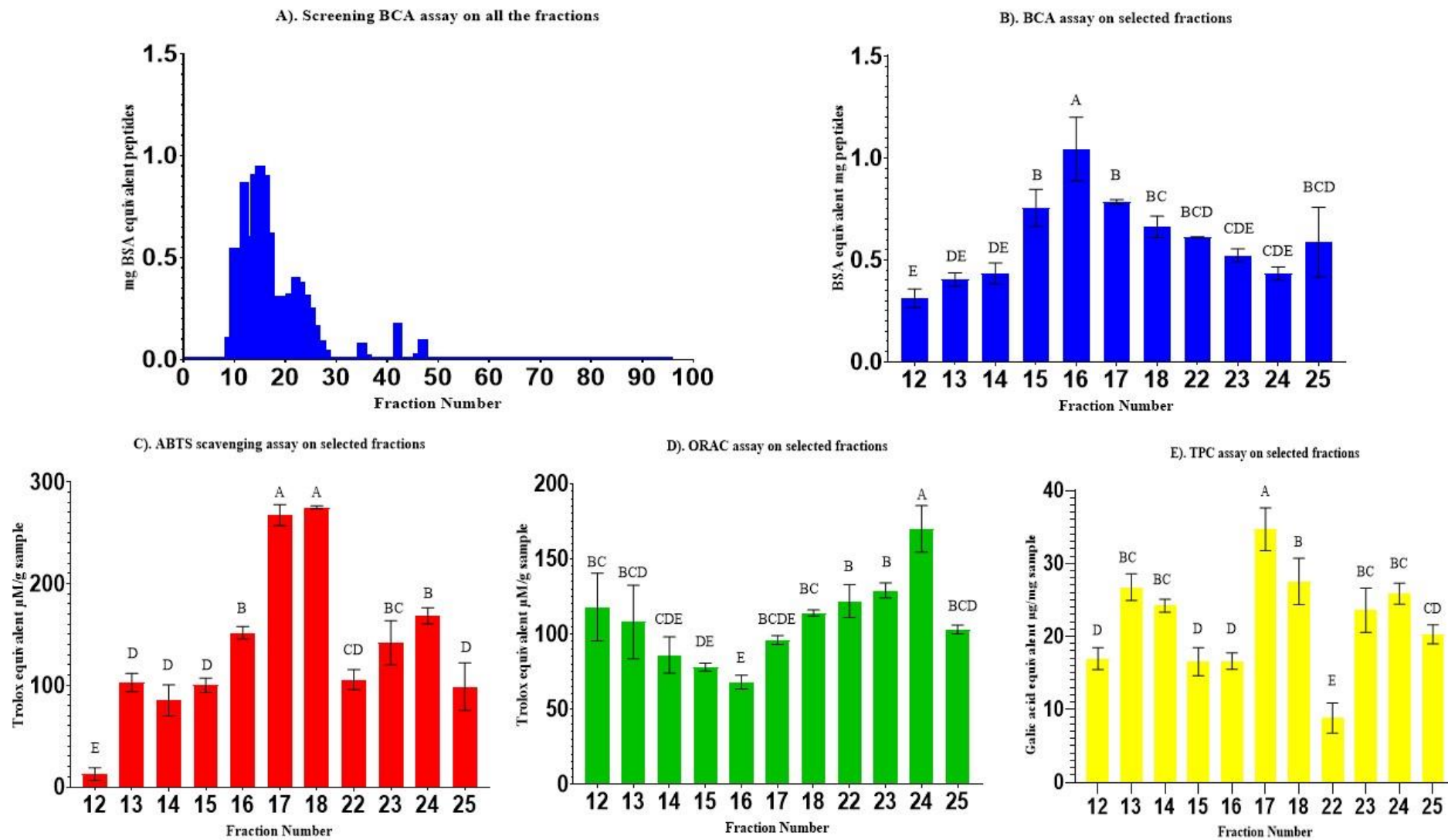


Figure 5.3.5. Protein content of fractions collected by stepwise isocratic elution and the antioxidant capacity of the selected fractions . Data are means \pm SEM (n=3) and different letters indicate significant difference between means (P < 0.05).

5.3.4 Fractionation of antioxidant anionic peptides from < 3 kDa oat intestinal peptide using a stepwise gradient of 0.2M, 0.5M and 2M NaCl with Tris-HCl buffer.

The selected fractions showing significant antioxidant activity in Figure 5.3.6 and Figure 5.3.7 were observed to have the conductivity ranging from 2.39 mS/cm to 14.80 mS/cm. Hervas et al. (2006) indicated that 0.2 M of NaCl solution had the conductivity about 19 mS/cm. The linear regression curve of the concentration of NaCl solution against conductivity (positively correlated) established by Widodo et al. (2018) showed that 0.2 M NaCl solution had the conductivity of 30 mS/cm. Hence, the second separation used 10% stepwise isocratic gradient (0.2M NaCl) aimed to obtain most peptides with highest antioxidant activity, the 25% stepwise isocratic gradient (0.5M NaCl) to obtain the peptides with higher affinity to the column compared to 0.2M NaCl, and 100% stepwise isocratic gradient (2M NaCl) to wash the column and ensure that all the bound peptides were eluted. As Figure 5.3.6 and Figure 5.3.7-A showed, peptides mostly eluted at the beginning of the gradient. Two major peaks were observed at 10% stepwise isocratic gradient and 25% stepwise isocratic gradient, respectively. In total, 17.4 mg of peptide were detected in the fractions corresponding to the first peak showing the binding of anion peptide reached to 49.71% which was also total anion peptides production yield because the second major peak did not show peptide content in the corresponding fractions.

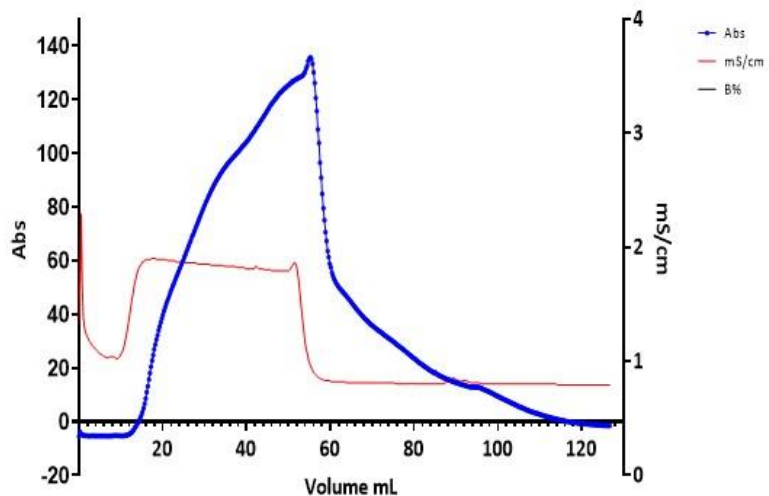
Seven fractions including fraction 7 to fraction 16 were selected as the candidates due to their peptide content and antioxidant activity (Figure 5.3.7 -A, B, C and D). The ABTS⁺ and ROO⁻ scavenging power were ranged from 84.80 ± 0.66 µM TE/g to 218.05 ± 6.08 µM TE/g and 31.76 ± 5.54 µM TE/g to 155.83 ± 13.57 µM TE/g, respectively. The total phenolic peptide content ranged from 14.29 ± 0.66 gallic acid equivalent µg/mg to 26.46 ± 1.75 66 gallic acid equivalent µg/mg.

Overall, fraction 15 and 16 eluted at 13.70 mS/cm and 13.74 mS/cm, respectively, showed significantly higher antioxidant power among all the selected 7 fractions. Fraction 15 and 16 showed the ABTS scavenging power of 218.05 ± 6.08 µM TE/g and 193.31 ± 10.47 µM TE/g, the ROO⁻ radical scavenging power of 117.14 ± 6.44 µM TE/g and 155.83 ± 13.57 µM TE/g and the total phenolic peptide content of 25.55 ±

0.93 gallic acid equivalent $\mu\text{g}/\text{mg}$ and 26.46 ± 1.75 gallic acid equivalent $\mu\text{g}/\text{mg}$, respectively. The result of FRAP assay on the fractions was negative again. It is worthy to note that fraction 12 in Figure 5.3.7 showed significant antioxidant activity too, with the ABTS scavenging power of 210.43 ± 5.76 $\mu\text{M TE}/\text{g}$, the ROO^- scavenging power of 76.43 ± 6.67 $\mu\text{M TE}/\text{g}$ and the phenolic peptide content of 24.37 ± 2.68 gallic acid equivalent $\mu\text{g}/\text{mg}$.

Based on the rationale of selecting the fractions with highest overall antioxidant activity, fraction 17 and 18 in Figure 5.3.5 and Figure 15 and 16 in Figure 5.3.7 were selected to be subjected to R-HPLC-MS/MS for sequencing in Chapter 6.

A). Peptide Sample that was not bound to the column and washed off by 20 mmol Tris-HCl Buffer (pH 8)



B). Separation by Using the Stepwise Isocratic Flow (10%, 25% and 100%) Buffer B (20 mmol Tris-HCl + 2M NaCl, pH 8)

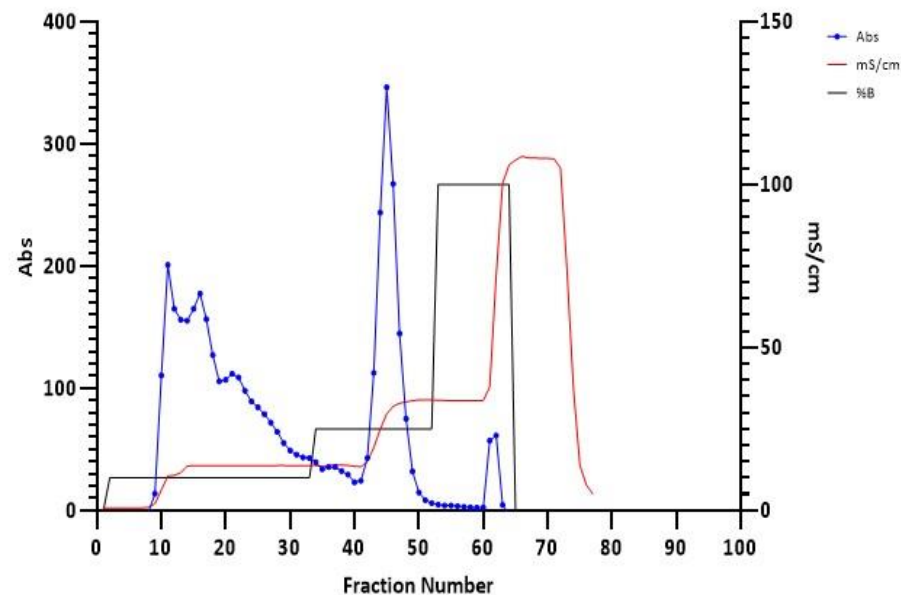


Figure 5.3.6. Thirty-five mg peptide separated by using the stepwise isocratic flow (10%, 25% and 100%) buffer B (20 mmol tris-HCl + 2M NaCl, pH 8). The red and blue lines in FPLC chromatograms stands for conductivity and the UV absorbance at 280 nm of the eluent, respectively. The black line represents the gradient of NaCl in the mobile phase.

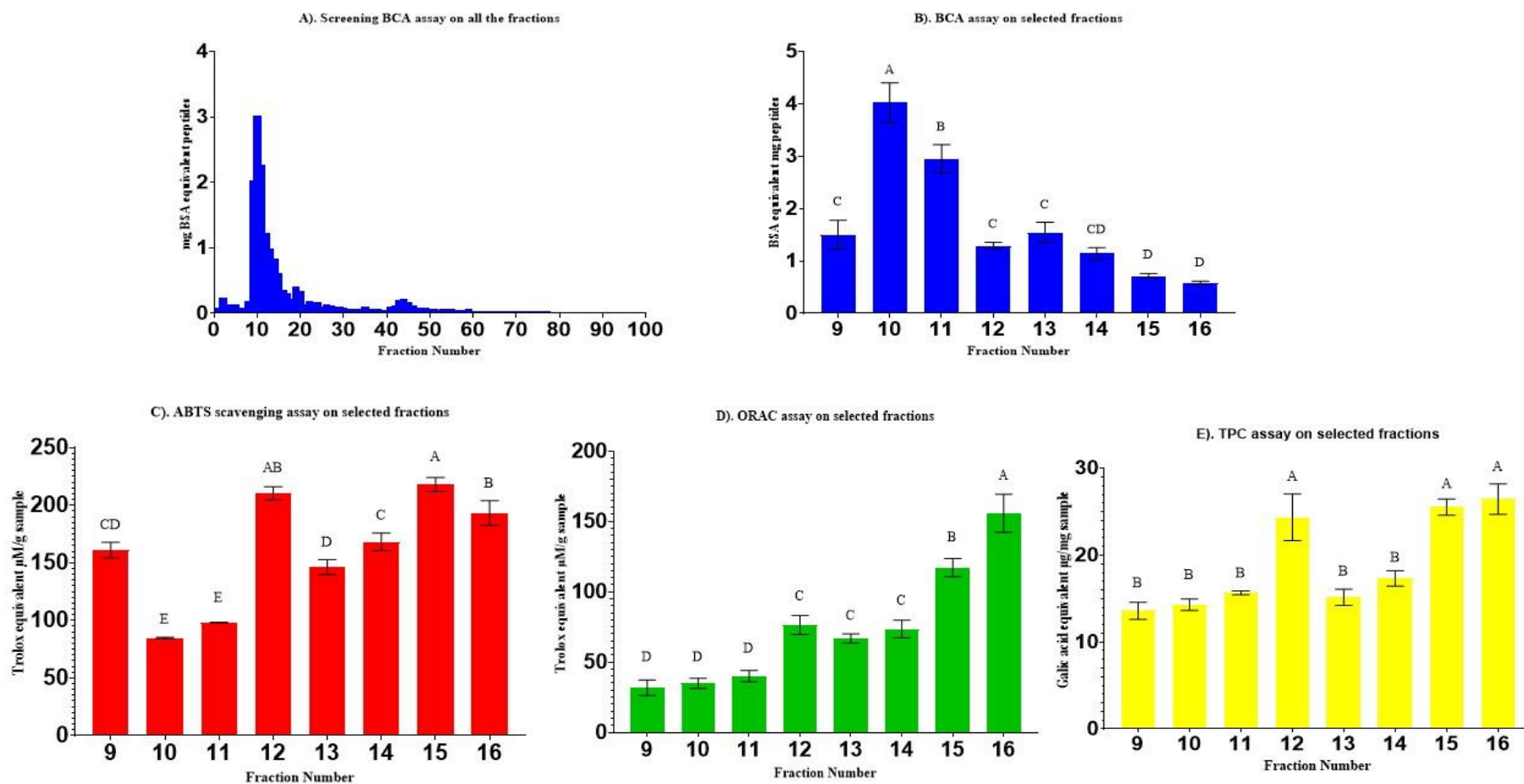


Figure 5.3.7. Protein content of fractions collected by stepwise isocratic elution and the antioxidant capacity of the selected fractions. Data are means \pm SEM (n=3) and different letters indicate significant difference between means ($P < 0.05$)

5.3.5 Fractionation of ACE inhibitory anionic peptides form < 3 kDa oat intestinal peptide using a stepwise gradient of 0.2M, 0.5M and 2M NaCl

The stepwise isocratic elution of 35 mg anion peptides was also designed to determine the fraction with the highest ACE inhibition activity. The fraction with peptide content higher than 1 mg/mL were selected. As the fractions were all obtained from the first major peak according to the chromatogram, fraction 9 and fraction 10 were combined and adjusted to 1 mg/mL for ACE inhibition assay as they eluted at the very beginning of the elution corresponding to the first minor peak. Fraction 11 and fraction 12 were fraction eluted between the first and second small peaks, while fraction 13 and fraction 14 were corresponding to the second minor peaks. The combination of fraction 9 and 10 showed the highest ACE inhibition activity whereas the combination of fraction 13 and 14 showed the lowest inhibition power.

As shown in Table 5.3.1, the ACE inhibition activity of the selected anion peptide fractions ranged from $17.43\% \pm 1.36\%$ to $36.80\% \pm 4.62\%$. Fraction 9 (eluted at conductivity of 2.10 mS/cm) and 10 (eluted at conductivity of 6.28 mS/cm) showed the highest ACE inhibitory power and were thereby selected for sequencing.

Table 5.3.1. The ACE inhibition activity of selected fractions obtained from stepwise isocratic elution.

Fraction Number	ACE Inhibition Activity
Fraction 9, 10	36.80 ± 4.62^A
Fraction 11, 12	29.42 ± 0.27^B
Fraction 13, 14	17.43 ± 1.36^C

- Fraction 9 and 10, fraction 11 and 12, and fraction 13 and 14 were combined, respectively and adjusted to 1 mg/mL for peptide quantification.
- Data are means \pm SEM (n=3) and different letters indicate significant difference between means ($P < 0.05$).

5.4 Discussion

5.4.1 Elution of the Anionic Peptides

BSA was used to test the integrity and the capability of the used positively charged resin. Similar separation was observed by Beck et al. (2020) and Kumar et al. (2022) using anion exchange chromatography to isolate BSA. One sharp peak was seen in both studies peaked at the NaCl concentration of about 200 to 250 mM equivalent to the conductivity of about 20 mS/cm.

Figure 5.3.3 showed the only peak appeared in the cation exchange chromatogram implying that the detachment could be caused by the hydrophobic end of the peptides because no cationic peptide was successfully isolated. In Chapter 4, the free amino acid profile of the oat intestinal digesta showed the abundance of alanine ($1.16 \times 10^3 \pm 12.18 \mu\text{g/g}$), valine ($3.30 \times 10^3 \pm 17.62 \mu\text{g/g}$), leucine ($3.26 \times 10^4 \pm 119.64 \mu\text{g/g}$), isoleucine ($4.22 \times 10^3 \pm 23.83 \mu\text{g/g}$), proline ($1.78 \times 10^3 \pm 80.28 \mu\text{g/g}$), phenylalanine ($3.56 \times 10^4 \pm 282.95 \mu\text{g/g}$), methionine ($1.80 \times 10^3 \pm 1.15 \mu\text{g/g}$) and tryptophan ($6.65 \times 10^3 \pm 70.60 \mu\text{g/g}$), which could comprise the hydrophobic end of peptide. The highly abundant peptides with hydrophobic ends were also found to be presented in oat intestinal digesta < 3 kDa in Chapter 6, section 6.3.2.

Phosphate buffer was initially used for chromatographic separation of anionic peptides in oat intestinal digesta as phosphate buffer was the matched buffer used in the *in vitro* digestion mimicking the intestinal conditions. Studies has indicated that phosphate buffer was not compatible to anion exchange chromatography as phosphate ion strongly interacted with positively charged anion exchange resin and competed to the target peptides (Vajda et al., 2016). Bio-Rad (n.d.) indicated that phosphate buffer contains wrong counter ions, phosphate ions, which have high affinity to positively charged anion resin leading to the loss of interested anion peptides. Tris-HCl buffer has been used in studies isolating anion intestinal peptides (Phongthai & Rawdkuen, 2020; Uraipong & Zhao, 2018). Tris-HCl buffer was thereby adapted in replace of phosphate buffer, in order to maximise the production of anionic peptide. Successfully isolation was achieved as shown in Figure 5.3.4 and Figure 5.3.6 when Tris-HCl buffer was used.

5.4.2 Antioxidant Activity of Anionic Peptides

Esfandi et al. (2019b) determined ABTS scavenging activity of the peptides derived from the oat bran protein with the viscozyme-assisted extraction and papain digestion to be $866.9 \pm 10.6 \mu\text{M TE/g}$, highest among the other fractions. Comparing to the highest ABTS scavenging power of fraction 18 ($275.16 \pm 1.48 \mu\text{M TE/g}$) obtained, fraction 18 from gradient separation showed significantly lower radical scavenging activity. Vanvi and Tsopmo (2016) indicated that pepsin-hydrolysed oat peptide showed ORAC values varying from $300 \mu\text{M TE/g}$ to $700 \mu\text{M TE/g}$. The fraction obtained after R-HPLC in this study also showed the highest ORAC value of $824 \mu\text{M TE/g}$ which was also significantly higher than the selected fraction ($170.00 \pm 15.41 \mu\text{M TE/g}$). As it was illustrated in Chapter 4, the ABTS scavenging activity and phenolic peptide content of oat intestinal digesta were $1558.48 \pm 15.23 \mu\text{M TE/g}$ and 185.99 ± 7.43 gallic acid equivalent $\mu\text{g/mg}$ respectively, which were much higher compared to the candidate fractions. Munteanu and Apetrei (2021) and Apak et al. (2016) indicated that the mechanism of antioxidant based on hydrogen donation (HAT-based) to oxidase radicals or electron transfer (SET-based) to reduce radicals. As the overall antioxidant activity of the fractionated anion peptides showed significant lower value compared to the value of oat intestinal digesta and the digesta obtained by using various enzymes, it would be safe to conclude that the power of either donating hydrogen or electron by anion peptides was weak. Plus, Apak et al. (2016) indicated that ORAC and ABTS assays were HAT based and HAT/SET mixed based assays, respectively while FRAP assay was SET based assay. Based on the negative results of FRAP assay on anion peptide fractions and the positive results of ORAC and ABTS assay, a deduction could be made that the antioxidant mechanism of anion peptides works mainly by donating hydrogen ions whereas these anion peptides carry extra electron but not H ions leading to the weak antioxidant activity. However, there are studies investigating the antioxidant activity of the anion peptides from different food component showing significant antioxidant activity ranged from 30% -70% DPPH radical scavenging activity (Chi et al., 2015; Wang et al., 2019). To provide the verification, peptides will be sequencing in chapter 6 and compared with the novel anion peptides with significant antioxidant

power from other food sources. The identification of N- and C- terminal of the anion peptides could contribute to explore the mechanism of antioxidant peptides. There is no recent study investigating peptides and how they related to the value of phenolic content. The mechanism of TPC assay on antioxidant peptides relies on the peptide-bound copper ions contribute to the reduction of Folin reagent (phosphomolybdic acid) showing the featured blue colour. The amino acids that are involved in this reaction are tryptophan, tyrosine, cysteine, cystine and histidine (Rodger & Sanders, 2017). Hernández-Jabalera et al. (2015) indicated that the antioxidant of digesta could be derived from the peptides-phenolics interactions.

5.4.3 ACE Inhibitory Activity of Anionic Peptides

The ACE inhibition activity of the selected anion peptide fractions was ranged from $17.43\% \pm 1.36\%$ to $36.80\% \pm 4.62\%$ and was lower than the inhibitory activity of oat intestinal digesta ($78.09\% \pm 0.13\%$) and the <3 kDa oat intestinal digesta ($80.35\% \pm 0.97\%$) illustrated in Chapter 4. Zheng et al. (2020) used mixed enzymes including Alcalase, Flavourzyme, pepsin, and trypsin to hydrolyse extracted oat globulin concentrates (the extraction protocol was very similar to my method), the hydrolysates were fractioned by R-HPLC and subjected to ACE inhibition assay. The inhibitory activity of seven fractions varied from about 20% to 70%. The ACE inhibition power of the selected anion peptide fraction was also comparatively lower compared to the fractionated hydrophobic peptides established in this article. Uraipong and Zhao (2018) observed that bioactive peptide (after *in-vitro* digestion) derived from rice bran protein showed significant ACE inhibition power. The fraction produced by anion exchange chromatography with highest ACE inhibition power exhibited the activity of 170 nmol captopril equivalent g⁻¹ peptide, higher than the crude protein (160 nmol captopril equivalent g⁻¹ peptide). Bleakley et al. (2017) showed that the extracted oat protein from seven different strains exert ACE inhibition power by between 86% and 96%. Camille Dugardin et al. (2020) indicated the 50% inhibition of the ACE activity (IC₅₀) of oat intestinal digesta was 99 µg/mL. The mechanism of the ACE inhibitory peptides based mainly on the bindings of the peptides to reduce the enzymatic activity of ACE. The identification of the anion

peptides in Chapter 6 will contribute to explore the ACE inhibitory mechanism of anion peptides.

5.5 Conclusion

The isolation of anionic peptides from oat intestinal digesta < 3 kDa was achieved when phosphate buffer was replaced by Tris-HCl buffer. As for the antioxidant property of fractioned anionic peptides, FRAP assay failed to work on all fractionated anionic peptides. The fraction 17 and fraction 18 (eluted during gradient separation) showed the highest overall antioxidant activity at the conductivity of 6.44 mS/cm and 7.14 mS/cm. The fraction 15 and fraction 16 eluted at 13.70 mS/cm and 13.74 mS/cm during isocratic elution, in addition to fraction 17 and fraction 18 from gradient elution, were selected to be the candidates with overall highest antioxidant activity and were subjected to identification in Chapter 6. Fraction 9 and fraction 10 from isocratic elution were selected for sequencing due to the highest ACE inhibitory activity they showed as the combination. Anionic peptides did not show high antioxidant activity and the reason of which was assumed to be related to the mechanism of anionic peptides to donate hydrogen ions or electrons to the radicals. The sequence of the anion peptides in these six fractions were identified in Chapter 6 and the relation of peptide sequence to the bioactive activity were discussed.

The investigation of the antioxidant anionic peptides derived from the oat intestinal digesta showed the limitation of anionic peptides to scavenge radicals but at the same time, established good potential to be ACE inhibitory agents for human consumption. The future research could focus on isolating the anionic peptides obtained from other cereal sources to understand the complexity and variation surrounding the mechanism of limited antioxidant activity of anionic cereal peptides.

Chapter 6

Peptide Sequencing and Determination of Novel Peptides

6.1 Introduction

Digesta derived from oat protein has been reported to have antioxidant activity and ACE inhibitory power. However, there has been no study carried out to investigate the antioxidant and ACE inhibitory activity of oat peptides produced by using *in vitro* digestion model followed by separation on anion exchange chromatography.

Determining the sequence of anionic peptides could help in understanding why the anionic peptides show lower antioxidant activity and moderate ACE inhibitory power compared to the oat hydrophobic peptides (showed in Chapter 5).

As shown in the 4-12% SDS-PAGE and 2-D gel in Chapter 3, the oat protein concentrate used for digestion was identified to be mainly made of oat globulin and oat avenin. Oat globulins are a major protein group (50% - 80% of the total protein) in oats (Klose & Arendt, 2012). A comprehensive data set including the important elements of the sequence length, definition, source, reference, features and the original sequence of oat globulins are available on UniProt (<https://www.uniprot.org/uniprotkb?query=Globulin%20Avena%20Sativa>). The oat prolamin, avenin, is the second most abundant storage protein (4-15%) in oats (Klose & Arendt, 2012) but may trigger celiac disease. The worldwide prevalence of celiac disease in 2016 was estimated at 1.4% based on serological tests of 275,818 individuals (Gimenez et al., 2017; Tanner et al., 2019). The comprehensive data of oat avenins are available on UniProt (<https://www.uniprot.org/uniprotkb?query=Avenin%20Avena%20Sativa>).

In this chapter, the oat bran intestinal digesta < 3 kDa (digesta < 3 kDa), fraction 17 (G17) and 18 (G18) with the overall highest antioxidant activity from gradient separation (Chapter 5, Figure 5.3.5), fraction 15 (SI15) and 16 (SI16) with the overall

highest antioxidant activity from stepwise isocratic separation (Chapter 5, Figure 5.3.7), and the fraction 9 (SI9) and 10 (SI10) with highest ACE inhibitory from stepwise isocratic separation (Chapter 5, Figure 5.3.7) were subjected to R-HPLC-MS/MS. In each sample, peptides were separated by R-HPLC based on hydrophobicity and then identified by MS/MS. Meanwhile, by using PeakView software, the most intense peptides in each sample were selected for sequencing as they theoretically are related to the bioactivity to the fraction. BIOPEP-UWM was utilised to predict the potential antioxidant and ACE inhibitory peptides derived from oat protein. The novel peptides were identified for future research.

6.2 Methods

6.2.1 Materials

Oat bran intestinal digesta < 3 kDa (prepared in Chapter 4), eluted fractions (selected in Chapter 5) including antioxidant fraction 17, 18 (from gradient elution), antioxidant fraction 15, 16 (from stepwise isocratic elution) and ACE inhibitory fraction 9, 10 (from stepwise isocratic elution) were subjected to tandem MS for peptide identification.

6.2.2 Mass Spectrometry Analysis

The selected fractions were used for peptide sequencing. The sample was prepared by using solid phase extraction (SPE) technique and was then concentrated by using the Savant™ SpeedVac™ Refrigerated Vapor Trap Series (North Shore City, New Zealand) to the final volume of 30 -50 µL. The SPE technique required to load 0.5 ml of 100% methanol, 0.5 ml of 0.1% formic acid, sample supernatant (centrifuge at 16,000 × RCF, 3 min), 1ml of 0.1% formic acid and 0.3 ml of freshly prepared 50% Acetonitrile in 0.1% formic acid accordingly onto 1ml Oasis® 10mg HLB SPE cartridges purchased from Sigma-Aldrich (Auckland. New Zealand).

The digesta injected onto a 0.3x 10mm trap column packed with Reprosil C18 media (Dr Maisch, Ammerbuch, Germany) and desalted for 3 minutes at 15µl/min before being separated on a 0.075 x 200 mm picofrit column (New Objective, Littleton, MA, USA) packed in-house with *Reprosil 3u C18-AQ media* (Dr Maisch, Ammerbuch, Germany). The following gradient was applied at 300 nl/min using a NanoLC 400

UPLC system (Eksigent, Framingham, MA, USA): 0min 1%B; 0.1min 5%B; 17.5min 35%B; 19min 98%B; 21 min 98%B; 21.5min 1%B; 30min 1%B, where A was 0.1% formic acid in water, and B was 0.1% formic acid in acetonitrile.

The picofrit spray was directed into a TripleTOF 6600 Quadrupole-Time-of-Flight mass spectrometer (Sciex, Framingham, MA, USA) for Information Dependent Analysis (IDA), comprising a TOF-MS scan from 300-1600 m/z for 200ms, followed by 30ms MS/MS scans on the 30 most abundant species (m/z 80-1600), using Rolling Collision Energy and High Sensitivity mode, for a total cycle time of 1.15 seconds. The mass spectrometer and UPLC system were under the control of the Analyst TF 1.8 software package (Sciex, Framingham, MA, USA).

The resulting data was searched against an in-house database comprising the Avenae sequences downloaded from Uniprot.org on 24th November 2022, appended with a set of common contaminant sequences (8,374 protein entries in total), using ProteinPilot version 5.0 (Sciex, Framingham, MA, USA). Search parameters were as follows: Sample Type, Identification; Search Effort, Thorough; Cys Alkylation, None; ID Focus: Biological Modifications; Digestion, None. The peptide summary exported from ProteinPilot was further processed using a custom R-Script to remove proteins with Unused Scores below 0.6, eliminate inferior or redundant peptide spectral matches, and to sum the intensities for all unique peptides from each protein. PeakView 2.2 (Sciex, Framingham, MA, USA) was used to manually identify peptides that was not automatically identified by ProteinPilot. Also, PeakView 2.2 listed all peptides in descending order based on intensity. By examining the spectrum, MW and retention time of the peptide, the most abundant peptides in the sample were identified. Spectral matches for different charge states of the same peptide sequences were retained. Due to the lack of enzyme specificity employed, and the short nature of some of the detected peptides, manual sequencing of MS/MS spectra was employed as needed to identify some prominent features.

6.2.3 *In silico* Predicted Antioxidant and ACE Inhibitory Peptide Derived from Oat Globulin

In silico antioxidant and ACE inhibitory peptide derived from oat globulins were analysed using BIOPEP-UMW: Analysis Profile of Potential Biological Activity (<https://biochemia.uwm.edu.pl/en/biopep-uwm-2/>) in order to compare with the amino acid sequences of identified peptides derived from the 7 selected fractions.

6.3 Results

6.3.1 Peptide Summary

The samples selected for proteomic analysis by tandem mass spectrometry were oat bran intestinal digesta < 3 kDa, fraction 17 and 18 selected from gradient separation (G17 and G18) showing the highest activities in three antioxidant assays (ABTS, ORAC, TPC), fraction 15 and 16 selected from stepwise isocratic separation (SI15 and SI16) showing the highest activities in three antioxidant assays (ABTS, ORAC, TPC), and fraction 9 and 10 selected from stepwise isocratic separation (SI9 and SI10) showing the highest ACE inhibitory power. Overall, the total number of identified peptides were 323 in digesta < 3kDa, 115 in G17, 89 in G18, 172 in SI9, 166 in SI10, 68 in SI15 and 30 in SI16 as shown in Appendix A. In Table 6.3.1, peptides presented originated from 10 proteins or polypeptides in digesta < 3kDa, 5 in G17, 7 in G18, 9 in SI9, 8 in SI10, 6 in SI15 and 6 in SI16. The criteria for selection were based on the peptide summary exported by ProteinPilot showing the maximum contribution score (peptide match for parent protein sequence, the highest score is 2) and confidence score (the confidence in the peptide match for MS/MS data, the highest score is 99). Meanwhile, only peptides derived from proteins in *Avena sativa* were included. For example, peptide QQQLLPQ identified in digesta < 3kDa showed the highest contribution and confidence score and was originated from Avenin (I4EP67_9POAL), but this protein was found in *Avena magna* and was thereby excluded. These peptides derived from other species of *Avena* or from other plants were summarised in Table 6.3.2.

Table 6.3.1. Proteins identified in oat bran intestinal digesta < 3 kDa (Digesta < 3 kDa), fraction 17 and 18 selected from gradient separation (G17 and G18), fraction 9, 10, 15, 16 selected from stepwise isocratic separation (SI9, SI10, SI15 and SI16 accordingly), and the number of peptides derived from each protein.

Parent Protein	UniProt Accession Number	Molecular Weight (kDa)	Number of Identified Peptides						
			Digest a < 3 kDa	G17	G18	SI9	SI10	SI15	SI16
12s globulin	O49258_AVESA	58	174	76	48	98	102	13	18
12S seed storage globulin 2	SSG2_AVESA	59	81	4	4	5	3	45	5
11S globulin	Q38780_AVESA	59	43	2	3	4	15	6	2
12S seed storage globulin 1	SSG1_AVESA	59	9	25	23	44	37	0	0
11S globulin	Q38779_AVESA	62	5	7	8	14	3	0	0
Peroxygenase 1	PXG1_AVESA	28	4	0	0	3	2	0	0
Vromindoline 1.3	R4I506_AVESA	17	3	0	0	1	0	0	1
Gigantea	A0A4Y5T8C1_AVESA	126	2	0	0	0	0	0	0
Protochlorophyllide reductase	POR_AVESA	34	1	0	0	0	0	0	0
Avena alpha amylase trypsin inhibitor	A0A1B2LQF1_AVESA	16	1	0	0	0	0	0	0
Vromindoline 2.1	R4I3I7_AVESA	17	0	0	2	2	2	1	0
12s globulin	O49257_AVESA	53	0	1	1	1	2	2	3
Vromindoline 1.2	R4I3J4_AVESA	17	0	0	0	0	0	1	0
Avena alpha amylase trypsin inhibitor-2	A0A1B2LQC9_AVESA	16	0	0	0	0	0	0	1

Table 6.3.2. Proteins identified in 7 samples originated from other *Avena* species or from plants.

Parent Protein	UniProt Accession Number	Origin	Number of Identified Peptides	Peptides
Avenin	I4EP66_9POAL	<i>Avena magna</i>	2	QQQLLPQ; VFIPPQL;
Avena alpha amylase trypsin inhibitor	A0A1B2LQE6_9POAL	<i>Avena magna</i>	1	TPASLPYE
Vromindoline	I2E0Z9_9POAL	<i>Avena longiglumis</i>	4	FGTQQGLIGK; GTQQGLIGK; SIQHELGGF; SIQHELGG;
Vromindoline	I2E0Z7_9POAL	<i>Avena hirtula</i>	1	RAVEHELGGF
Avenin protein (Fragment)	G8ZCT8_9POAL	<i>Avena strigosa</i>	1	VQQQQPF; DELLPEGF
Vromindoline VIN3	W0NPM9_9POAL	<i>Avena strigosa</i>	1	AVQGELGGF
Barley protein Z-like protein	Q9XET7_AVEFA	<i>Avena fatua</i>	1	KADVQSADF
VIP2 protein	Q9M4C5_AVEFA	<i>Avena fatua</i>	1	AAGKEEIGA
Heat shock protein	A0A142DDT6_AGRST	<i>Agrostis stolonifera</i>	1	KVIDVQVQ
Photosystem I P700 chlorophyll a apoprotein A1	A0A2H4HJX8_9POAL	<i>Triplachne nitens</i>	1	FEEWAR
Chloroplast low molecular weight heat shock protein	Q8GV35_AGRST	<i>Agrostis stolonifera</i>	1	KVIDVQVQ

6.3.2 Most Abundant Peptides Identified in Each Sample

R-HPLC separated peptides in the digesta, and the selected fractions derived from anion exchange chromatography and were subjected to MS/MS for sequencing. Theoretically, peptide with abundance may contribute to bioactivity. The intensity of each observed peptide was listed in descending order by using PeakView 2.2 to select the most abundant 10 peptides in each sample. In Table 6.3.3 to Table 6.3.9, the most abundant peptides in 7 samples were identified, their MW and parent proteins determined. The confidence score and hydropathy index were also included. Hydropathy index of the peptide is a number representing the hydrophobic properties of its side chain, calculated by averaging the sum of the hydropathy index of each amino acid in the peptide sequence. The hydropathy index is positively correlated to the hydrophobicity of peptides. The potential ACE inhibitory and antioxidant sequence in the tables are amino acid sequences in each peptide that is predicted to have either ACE inhibitory or antioxidant potential by using BIOPEP-UMW database.

6.3.3 Most Abundant Peptides Identified in Oat Bran Intestinal Digesta < 3 kDa

The 10 most abundant peptide identified in the oat bran intestinal digesta < 3 kDa fraction are listed in Table 6.3.3. All the listed peptides originated from globulin. The number of amino acids ranged from 5 to 13 and the theoretical MW from 568.36 Da to 1555.81 Da. Manual identification was employed for sequencing peptide LIVPQ. Four peptides were determined to show positive hydropathy index.

Table 6.3.3. Amino acid sequence of peptides from oat bran protein intestinal digesta < 3 kDa (Chapter 5). The C- and N- terminals in the determined sequences with potential ACE inhibitory was highlighted in blue and with potential antioxidant activity was highlighted in green while the terminals possess both potential ACE inhibitory and antioxidant power was highlight in red. The in silico predicted bioactive peptides was summarised in Table 6.4.1.

Sample	Sequences	Theoretical Molecular Weight (Da)	Origin	Confidence	Potential ACE inhibitory sequences	Potential Antioxidant sequences	Hydropathy Index
Digesta < 3 kDa	IQSQNDQRGEIIR	1555.81	Globulin	99	RG, GE, EI, IR	IR	-1.36
	GQESGVFTPK	1048.52	Globulin	99	GQ, SG, GV, VF, TP		-0.78
	DVNNNANQLEPR	1382.66	Globulin	99	PR		-1.73
	LHGQNFPIIN	1151.61	Globulin	99	HG, GQ, NF, FP, IL, LN	LH, LHG,	-0.08
	FKQGDVIALPA	1157.65	Globulin	91.9	LK, QG, GD, IA, ALP		0.55
	ALPVDV	612.35	Globulin	50.4	ALP		1.48
	LIVPQ	568.36	Globulin	Manually Identified	PQ		1.48
	WNINAH	753.36	Globulin	93.9	AH	AH	-0.8
	VQMSATRV	890.47	Globulin	96.3			0.33
VQVVNNNGQTV	1170.60	Globulin	94.8	VQV, NG, GQ		-0.16	

6.3.4 Most Abundant Peptides Identified in Antioxidant Fractions Eluted at Conductivity of 6.44 mS/cm and 7.15 mS/cm

The 10 most abundant peptide identified in fraction 17 (G17) and 18 (G18) isolated from gradient separation in chapter 5 are listed in Table 6.3.4 and Table 6.3.5, respectively. All the listed peptides originated from globulin. In G17, the number of amino acids ranged from 4 to 15 and the theoretical MW from 473.25 Da to 1697.78 Da. Peptides identified in G18 contains amino acids ranged from 4 to 16 and the theoretical MW from 473.25 Da to 1844.84 Da. Four peptides were manually identified in G17 while 2 peptides were manually identified in G18. Five peptides in G17 showed positive hydrophathy index while 2 peptides in G18 showed positive hydrophathy index.

Table 6.3.4. Amino acid sequence of peptides from fraction 17 (G17) isolated from gradient separation (Chapter 5). The C- and N- terminals in the determined sequences with potential ACE inhibitory was highlighted in blue and with potential antioxidant activity was highlighted in green while the terminals possess both potential ACE inhibitory and antioxidant power was highlight in red. The *in silico* predicted bioactive peptides was summarised in Table 6.4.1.

Sample	Sequences	Theoretical Molecular Weight (Da)	Origin	Confidence	Potential ACE inhibitory sequences	Potential Antioxidant sequences	Hydropathy Index
G17	NDIL	473.25	Globulin	Manually Identified	IL		0.33
	QAFEPL	703.36	Globulin	52.1	PL, AF, FEP		-0.03
	SQAGITEY	867.40	Globulin	73	GI, AG, EY, TE		-0.49
	LSEALG	588.31	Globulin	Manually Identified	EA, LG		0.78
	SQAGITE	704.33	Globulin	92.8	GI, AG, EY, TE		-0.37
	FEPL	504.26	Globulin	Manually Identified	FEP, PL		0.38
	LSEAL	531.29	Globulin	Manually Identified	EA		1.02
	LQAFEPL	816.44	Globulin	95.3	PL, AF, LQ, FEP		0.51
	AEGQSQSQNLKDEHQ	1697.78	Globulin	99	GQ, EG	KD, LK	-2.1
	QNLKDEHQ	993.45	Globulin	99		KD, LK	-2.6

Table 6.3.5. Amino acid sequence of peptides from fraction 18 (G18) isolated from gradient separation (Chapter 5). The C- and N- terminals in the determined sequences with potential ACE inhibitory was highlighted in blue and with potential antioxidant activity was highlighted in green while the terminals possess both potential ACE inhibitory and antioxidant power was highlight in red. The *in silico* predicted bioactive peptides was summarised in Table 6.4.1.

Sample	Sequences	Theoretical Molecular Weight (Da)	Origin	Confidence	Potential ACE inhibitory sequences	Potential Antioxidant sequences	Hydropathy Index
G18	SQAGITEY	867.40	Globulin	98.2	AG, GI, TE, EY		-0.49
	AEGQSQSQNLKDEHQ	1697.77	Globulin	99	GQ, EG	KD, LK	-2.1
	NDIL	473.25	Globulin	Manually Identified	IL		0.33
	GYNDGDAPVVA	1076.48	Globulin	92	GDAP, GY, AP, DA, GD, DG, YN		-0.2
	QAFEPL	703.35	Globulin	89.2	PL, AF, FEP		-0.03
	FEPL	504.26	Globulin	Manually Identified	FEP, PL		0.38
	NDGDPIVA	870.41	Globulin	98.8	GDAP, AP, DA, GD, DG		-0.02
	FAEGQSQSQNLKDEHQ	1844.84	Globulin	99 or 0.5	GQ, EG	KD, LK	-1.8
	NNRGE ^{EF}	864.37	Globulin	99	GE, RG, EF		-2.3
SQAGVTEY	853.38	Globulin	99	AG, GV, EY, TE		-0.49	

6.3.5 Most Abundant Peptides Identified in Antioxidant Fractions Eluted at Conductivity of 13.70 mS/cm and 13.74 mS/cm

The 8 most abundant peptides identified in fraction 15 (SI15) and 6 most abundant peptides in fraction 16 (SI16) isolated from stepwise isocratic separation in Chapter 5 were listed in Table 6.3.6 and Table 6.3.7, respectively. The exclusion was based on either sequence was not found in the Avenae database or the spectrum could not be interpreted. All the listed peptides originated from globulin. In SI15, the number of amino acids ranged from 6 to 10 and the theoretical MW from 754.34 Da to 1374.59 Da. Peptides identified in SI16 contains amino acids ranged from 6 to 10 and the theoretical MW from 754.34 Da to 1374.59 Da. One peptide was manually identified in SI15 while 2 peptides were manually identified in SI16. All abundant peptides identified in these two fractions showed weak hydrophobicity.

Table 6.3.6. Amino acid sequence of peptides from fraction 15 (SI15) isolated from stepwise isocratic separation (Chapter 5). The C- and N-terminals in the determined sequences with potential ACE inhibitory was highlighted in blue and with potential antioxidant activity was highlighted in green while the terminals possess both potential ACE inhibitory and antioxidant power was highlight in red. The in silico predicted bioactive peptides was summarised in Table 6.4.1.

Sample	Sequences	Theoretical Molecular Weight (Da)	Origin	Confidence	Potential ACE inhibitory sequences	Potential Antioxidant sequences	Hydropathy Index
SI15	SQAGITEY	868.38	Globulin	Manually Identified	GI, AG, EY, TE		-0.49
	FDEQNEQLR	1177.5	Globulin	99	LR		-2.1
	QAGVTEYF	913.39	Globulin	9.9	AG, GV, EY, TE		-0.08
	FDEQNEQL	1021.44	Globulin	90.8			-1.8
	YFDEQNEQFR	1374.59	Globulin	99	FR		-2.12
	FDEQNEQF	1055.46	Globulin	99			-1.93
	DEQNEQL	874.37	Globulin	92.9			-2.46
	WNINAH	754.34	Globulin	96.2	AH	AH	-0.8

Table 6.3.7. Amino acid sequence of peptides from fraction 16 (SI16) isolated from stepwise isocratic separation (Chapter 5). The C- and N-terminals in the determined sequences with potential ACE inhibitory was highlighted in blue and with potential antioxidant activity was highlighted in green while the terminals possess both potential ACE inhibitory and antioxidant power was highlight in red. The *in silico* predicted bioactive peptides was summarised in Table 6.4.1.

Sample	Sequences	Theoretical Molecular Weight (Da)	Origin	Confidence	Potential ACE inhibitory sequences	Potential Antioxidant sequences	Hydropathy Index
SI16	SQAGITEY	868.38	Globulin	Manually Identified	GI, AG, EY, TE		-0.49
	FDEQNEQL	1021.44	Globulin	Manually Identified			-1.8
	YFDEQNEQFR	1374.59	Globulin	84.2	FR		-2.12
	DEQNEQL	874.37	Globulin	92.7			-2.46
	FDEQNEQFR	1211.5	Globulin	99	FR		-2.21
	WNINAH	754.34	Globulin	42.9	AH	AH	-0.8

6.3.6 Most Abundant Peptides Identified in ACE Inhibitory Fractions Eluted at Conductivity of 2.10 mS/cm and 6.28 mS/cm

The 10 most abundant peptide identified in fraction 9 (SI9) and 10 (SI10) isolated from stepwise isocratic separation in Chapter 5 were listed in Table 6.3.8 and Table 6.3.9, respectively. All the listed peptides were originated from globulin, except for peptide PSKEPV identified in SI10, which was derived from protein early flowering 3. In SI9, the number of amino acids ranged from 5 to 15 and the theoretical MW from 584.32 Da to 1674.80 Da. Peptides identified in SI10 contains amino acids ranged from 4 to 15 and the theoretical MW from 473.25 Da to 1791.85 Da. Three peptides were manually identified in SI9 while 4 peptides were manually identified in SI10. Peptides including ALPVDV, LSEAL and LSEALGI showed strong hydrophobicity (hydropathy index > 1).

Table 6.3.8. Amino acid sequence of peptides from fraction 9 (SI9) isolated from stepwise isocratic separation (Chapter 5). The C- and N-terminals in the determined sequences with potential ACE inhibitory was highlighted in blue and with potential antioxidant activity was highlighted in green while the terminals possess both potential ACE inhibitory and antioxidant power was highlight in red. The *in silico* predicted bioactive peptides was summarised in Table 6.4.1.

Sample	Sequences	Theoretical Molecular Weight (Da)	Origin	Confidence	Potential ACE inhibitory sequences	Potential Antioxidant sequences	Hydropathy Index
SI9	ALPVDV	612.35	Globulin	49.5	ALP, LP		1.48
	LQAFEPL	816.44	Globulin	99	PL, AF, LQ, FEP		0.51
	EQQFGQNI	962.45	Globulin	37.9	FG, GQ		-1.33
	VIEPQ	584.32	Globulin	Manually Identified	IEP, PQ		0.02
	LAGNPKEDQQFGQNI	1674.80	Globulin	67.6	LA, AG, FG, GQ, NK, KE		-1.32
	LSEALG	588.31	Globulin	Manually Identified	EA, AL, LG		0.78
	LSEAL	531.29	Globulin	Manually Identified	EA, AL		1.02
	LSEALGI	701.40	Globulin	99	GI, LG, EA, LGI		1.31
	VSQQGPVEH	979.48	Globulin	98.9	GP, GPV, QG, VE, QGP		-0.9
	DVNNNANQLEPR	1382.65	Globulin	99	PR		-1.73

Table 6.3.9. Amino acid sequence of peptides from fraction 10 (SI10) isolated from stepwise isocratic separation (Chapter 5). The C- and N-terminals in the determined sequences with potential ACE inhibitory was highlighted in blue and with potential antioxidant activity was highlighted in green while the terminals possess both potential ACE inhibitory and antioxidant power was highlight in red. The *in silico* predicted bioactive peptides was summarised in Table 6.4.1.

Sample	Sequences	Theoretical Molecular Weight (Da)	Origin	Confidence	Potential ACE inhibitory sequences	Potential Antioxidant sequences	Hydropathy Index
SI10	ALPVDV	612.35	Globulin	50.2	ALP, LP		1.48
	LQAFEPL	816.44	Globulin	98.7	PL, AF, LQ, FEP		0.51
	EQQFGQNI	962.44	Globulin	29.8	FG, GQ		-1.33
	QAFEPLR	703.35	Globulin	72.9	PL, AF, FEP, LR		-0.67
	LSEALG	588.31	Globulin	Manually Identified	EA, AL, LG		0.78
	LSEAL	531.29	Globulin	Manually Identified	EA, AL		1.02
	VIEPQ	584.31	Globulin	Manually Identified	IEP, PQ		0.02
	NDIL	473.25	Globulin	Manually Identified	IL		0.33
	YVFDVNNANQLEPR	1791.85	Globulin	65.2	YV, VF, PR		-1.01
	PSKEPV	655.35	Early flowering 3	92.4	KE		-1.2

6.4 Discussion

6.4.1 General Discussion

Amongst the intestinal digesta < 3 kDa and 6 selected fractions, digesta < 3 kDa was determined to contain most identified peptides (323), which is plausible as the 6 selected fractions were all isolated from it. As shown in Table 6.3.1, most of the identified peptides originated from 12S globulin (O49258_AVESA), followed by 12S globulin 2 (SSG2_AVESA), 11S globulin (Q38780_AVESA), 12S seed storage globulin 1 (SSG1_AVESA) and 11S globulin (Q38779_AVESA), except for fraction SI15 which contained most peptides derived from 12S globulin 2 (SSG2_AVESA). It is interesting that in Chapter 3, 4 - 12% SDS-PAGE and 2-D electrophoresis both indicated that in the original oat bran protein extracts, globulin and avenin were most abundant proteins, but no peptide in the digesta < 3 kDa and 6 selected fractions was identified to be derived from avenin. Only two peptides QQQLLPQ and VFIPPQL in the digesta < 3 kDa with the highest confidence and contribution score were identified to be derived from avenin but in *Avena magna*. It could be safe to assume that the *in vitro* digestion model using pepsin and pancreatin may not cleave avenin into peptides with the MW less than 3 kDa. Avenin is an oat prolamin with high proline profile and is also a possible trigger of celiac disease (Malalgoda & Simsek, 2017). Gilissen et al. (2016) indicated that the peptide epitopes in avenin were predicted to resist trypsin and chymotrypsin digestion, which makes oats a recommended food for celiac disease patients.

Table 6.4.1 summarises antioxidant peptide and ACE inhibitory peptide sequences from oat proteins predicted by using BIOPEP. Pepsin has preferential cleavage site at the C-terminals of phenylalanine (F), leucine (L), tryptophan (W) and tyrosine (Y). Example of peptides generated by the pepsin cleavage could be QVGQSTQYQEGQSTQY, AILSPF, ATRVNL and QLFGQSSTPW. Pancreatin is an extract from the pancreas containing enzymes capable of digesting fat, carbohydrates and proteins. The major proteases it contains are trypsin and chymotrypsin. Trypsin preferentially cleaves at arginine (R) and lysine (K) at C- terminal unless proline

presents at position P1' (next to arginine or lysine in C-terminal direction) which blocks this action except for certain circumstance such as when lysine in P1 and tryptophan in P2 (Keil, 2012; Rodriguez et al., 2008). Chymotrypsin preferentially cleaves at tryptophan, tyrosine and phenylalanine (F) in position P1 (high specificity) and to a lower specificity at leucine, methionine and histidine (H) in position P1 (Keil, 2012). Example of the identified peptides generated by trypsin and chymotrypsin cleavage could be FTPWQSSR, ALGISQQVAQK, QLFGQSSTPW, GQLLIIPQHY and NLKNNRGEEF. However, pancreatin contains other minor levels of proteases such as elastase and carboxypeptidase (Sahin-Tóth & Szabó, 2011) leading to other amino acid residues at C-terminal.

The theory of the positive correlation of hydrophobic amino acids at the C- or N-terminal of peptides in cereals to antioxidant and ACE inhibitory activity has been established in the last decades (Esfandi et al., 2019b; Pooja et al., 2017; Rawlings & Salvesen, 2013; Thamnarathip et al., 2016; Uraipong & Zhao, 2018; Walters et al., 2020; Zhang et al., 2014). Hydrophobic amino acids lysine (G), alanine (A), valine (V), leucine (L), isoleucine (I), proline (P), phenylalanine, and tryptophan were the major components of the listed peptides in Table 6.4.1, except for methionine (M) which was only present in the predicted peptide VM and VHM. In appendix A, many peptides contain arginine, valine, leucine and phenylalanine at N-terminal and C-terminal which makes them potentially bioactive.

Based on hydrophobicity, the length and confidence score of the identified abundant peptides and compared to the other research, peptide LIVPQ in digesta < 3 kDa would be selected for further analysis. Peptides identified in antioxidant anionic fractions including FEPL, LSEAL and SQAGITEY would be selected for further analysis. Abundant peptides in ACE inhibitory fractions including LQAFEPL, ALPVDV and NDIL would be selected for future investigation.

Table 6.4.1. *In silico* predicted bioactive peptides summarised by using BIOPEP.

Predicted Peptide Derived from Oat Globulin with Bioactivity	Dipeptides	Tripeptides	Tetra-peptides	Penta-peptide
Antioxidant Peptide	LH, HL, HH, AY, LY, IY, AH, EL, KD, PW, IR, LK, KP, TY, VY, FC, WG	VHH, YSY, PWQ, LKP, VYV, LAN, LHG, EAK, YLL, SVL, LLY, LRH		
ACE Inhibitory Peptide	RL, IR, LY, VF, YW, RF, VY, HY, FP, PR, LF, FY, AY, GP, PL, IA, IP, AF, AP, LA, KR, RA, GF, FR, IF, VG, GI, GL, AG, HL, GR, FG, DA, GS, GV, GQ, GK, HG, GE, GG, QG, AI, SG, LG, GD, TG, EG, EA, NG, PG, VR, QK, DG, NF, SY, SF, FK, NK, RR, AR, KA, EY, KP, EI, IE, VE, TE, LQ, LN, PT, TQ, AH, PQ, EK, KE, TF, AV, TP, FQ, YV, IL, YH, RG, ST, YN, LR, QP, VM, EF, ER, DR, LP, GY, YL, YP, AA, GH, KG, WG, KF, EV,	FQP, LSP, LLP, PGL, GPV, LKP, VAV, LTF, TNP, LLF, IEP, IEY, LVY, FEP, ALP, LEE, IFL, VQV, AQL, LVQ, LGI, QGP, VHM, NPR, VVL, ILP, PVP, FWN, LPG, VHW, EQR, YLL, LLY	GDAP, RALP	LTFPG

6.4.2 Antioxidant and ACE inhibitory Peptides in Oat Bran Intestinal Digesta < 3 kDa

In Chapter 4, digesta < 3 kDa showed ABTS scavenging power of 1538.222 ± 15.03 Trolox equivalents $\mu\text{mol/L}$ sample and $80.35\% \pm 0.97\%$ ACE inhibitory activity. Appendix A showed many peptides that have hydrophobic amino acids at C-terminal and N-terminal. As shown in Table 6.3.3, 10 most abundant peptides in digesta < 3 kDa were identified, in which all peptides all hydrophobic amino acid at N- terminal except for peptide DVNNNANQLEPR. Hydrophobic amino acids presented at C-terminal of peptides FKQGDVIALPA, ALPVDV, VQMSATRV and VQVVNNNGQTV, which also contain hydrophobic amino acid at N-terminal. Table 6.4.1 showed that peptides IQSQNDQRGEIIR and WNINAH have predicted amino acid sequence with both antioxidant and ACE inhibitory activity at the C-terminal. Peptide LHGQNFILN has predicted antioxidant amino acid sequence at N-terminal. Only peptide VQMSATRV does not match to predicted bioactive peptides shown in Table 6.4.1, which makes it a potential ACE inhibitory peptide though it is not in BIOPEP database. In addition to the relationship between hydrophobicity and bioactivity of peptide, Uraipong and Zhao (2018) indicated that peptides contain hydrophobic and positively charged ends are more likely to possess ACE inhibitory actions. Therefore, peptide GQESGVFTPK, DVNNNANQLEPR and WNINAH may contribute to the ACE inhibitory power of digesta < 3 kDa.

The peptide IQSQNDQRGEIIR, DVNNNANQLEPR, LHGQNFILN, ALPVDV, VQMSATRV, WNINAH and VQVVNNNGQTV were determined to match a part of the antioxidant peptides identified by Esfandi et al. (2019b) contributing to high ABTS and ORAC scavenging activity. Meanwhile, peptide LIVPQ match the peptide LIVPQHY, LLLPQYH and LIVPQHF identified by Vanvi and Tsopmo (2016) contributing to ORAC and superoxide anion and hydroxyl radicals scavenging capacity. The identified peptide did not match the ACE inhibitory cereal peptides identified in other literatures (Bleakley et al., 2017; Shobako et al., 2018; Uraipong & Zhao, 2016b, 2018; Zheng et al., 2020).

6.4.3 Antioxidant Peptide in Fractions Eluted at Conductivity of 6.44 mS/cm and 7.15 mS/cm

Anionic peptide fraction 17 and 18 isolated from anion exchange chromatography with highest ABTS and ORAC scavenging power and TPC value in Chapter 5 were subjected to R-HPLC-MS/MS and 10 most abundant peptides were listed in Table 6.3.4 and Table 6.3.5. Peptide SQAGITEY, QAFEPL, NDIL, FEPL and AEGQSQSQNLKDEHQ were observed in both fractions. Peptides GYNDGDAPVVA, AEGQSQSQNLKDEHQ, LSEAL, LQAFEPL, FAEGQSQSQNLKDEHQ show hydrophobic amino acid at N-terminal while NDIL, QAFEPL, LSEALG, FEPL, LSEAL, LQAFEPL, GYNDGDAPVVA, NDGDPIVA and NNRGEEF show hydrophobic amino acid at C-terminal. Peptide LSEALG, FEPL, LSEAL, LQAFEPL, GYNDGDAPVVA have hydrophobic amino acid at both ends. None of the identified peptides contained predicted antioxidant amino acid sequences shown in Table 6.4.1 which could explain the result of Chapter 5 that the anionic peptide fractions showed lower antioxidant activity comparing to other literatures. In addition, the hydropathy index also suggested that most abundant peptides identified tended to be hydrophilic or slightly hydrophobic. Although, peptide with high percentage of hydrophobic residues such as FEPL and LSEAL (hydropathy index: 0.38 and 1.02, respectively) may still contribute to antioxidant activity of the selected fraction.

Based on the MS data, the most abundant 10 peptides identified in G17 and G18 are completely different from the 10 peptides identified in digesta < 3 kDa. The peptide SQAGITEY, QAFEPL, FEPL, LQAFEPL, GYNDGDAPVVA, NDGDPIVA and NNRGEEF were found in determined antioxidant oat peptides by (Esfandi et al., 2019b). The antioxidant peptide AEGQSQSQNLKDEHQ was also identified by (Vanvi & Tsopmo, 2016). Ma et al. (2017) identified peptide HYNAPAL derived from oat glutelin with high antioxidant activity. Ma et al. (2020) identified peptide IRIPIL, FLKPMT, NSKNFPTL, LIGRPIIY and FNDILRRGQLL originated from oat globulin. These peptides do not match any peptide identified in appendix A. Therefore, it could be safe to conclude that the antioxidant property of anionic oat peptides was developed from

hydrophobic residues, but the sequence of anionic peptides derived from globulin is not suitable for peptides to develop high antioxidant power.

6.4.4 Antioxidant Peptide in Fractions Eluted at Conductivity of 13.70 mS/cm and 13.74 mS/cm

Anionic peptide fraction 15 (SI15) and 16 (SI16) isolated from anion exchange chromatography with highest ABTS and ORAC scavenging power and TPC value in Chapter 5 were subjected to R-HPLC-MS/MS. Eight most abundant peptides in SI15 and 6 most abundant peptides in SI16 were listed in Table 6.3.6 and Table 6.3.7. In these two fractions, some abundant peptides were observed to show poor spectrum or were unable to be manually identified. Fraction SI15 and SI16 showed similar level of antioxidant activity compared to fraction G17 and G18 in Chapter 5. However, only peptide SQAGITEY identified as the most abundant peptide in fraction SI15 and SI16 as well as in fraction G17 and G18. Notably, Peptide WNINAH observed in fraction SI15 and SI16 was the most abundant peptide in digesta < 3 kDa. Other identified rich peptides contained low hydrophobic amino acid content. It is also interesting to note that, except for peptide QAGVTEYF, all the identified peptides match the identified antioxidant peptides determined by Esfandi et al. (2019b). It could be safe to deduce that the peptide WNINAH in these two fractions may play an important role of developing antioxidant activity. In addition, Vanvi and Tsopmo (2016) indicated that, other than hydrophobicity, peptide sequence itself may play an more important role than hydrophobicity in terms of showing high antioxidant activity.

6.4.5 ACE Inhibitory Peptide in Fractions Eluted at Conductivity of 2.10 mS/cm and 6.28 mS/cm

Anionic peptide fraction 15 (SI15) and 16 (SI16) with highest ACE inhibitory power determined in Chapter 5 were subjected to R-HPLC-MS/MS. Ten most abundant peptides in each fraction were listed in Table 6.3.8 and Table 6.3.9. Most peptides in these two fractions show high content of hydrophobic amino acid residues. Peptide ALPVDV, LQAFEPL, EQQFGQNI, LSEALG and LSEAL were identified in both fractions. Except for peptide EQQFGQNI, VSQQGPVEH and PSKEPV, all the identified peptides

have a predicted ACE inhibitory amino acid sequence at either N- or C- terminal. Compared to digesta < 3 kDa, peptide ALPVDV was the most abundant and hydrophobic peptide and was also identified in both fractions but with low confidence score. Peptide NDIL, which was manually identified, was observed to be part of the identified ACE inhibitory peptide FNDILRRGQLL by Ma et al. (2020). In addition, the peptide with hydrophobic amino acids especially with aromatic residues in C-terminals were likely to bind to the active site of ACE (Zheng et al., 2020). Also, Uraipong and Zhao (2018) indicated that peptides contain hydrophobic and positively charged ends are more likely to possess ACE inhibitory actions. It is also worthy to note that Wang et al. (2015) used *in vitro* digestion model to digest oat protein and produced peptide LQAFEPL which is dipeptidyl peptidase IV (DPP4) inhibitory peptide with high binding and inhibitory potential to DPP4 enzyme regarding computational docking. It is also interesting to note that, as well the preference for hydrophobic terminals of the peptides, positively charged sidechains had also been known to exert ACE inhibitory activity (Uraipong & Zhao, 2018; Zou et al., 2020). In Table 6.3.8 and Table 6.3.9, peptide VSQQGPVEH, DVNNNANQLEPR, QAFEPLR and YFVDVNNNANQLEPR had charged amino acid at C-terminal while EQQFGQNI had charged amino acid at N-terminal. As these peptides were exposed in the buffer with pH of 8, the sidechains deprotonated and carried negative charge. These anionic peptides showed highest hydropathy index amongst all identified abundant peptides and have potential to contribute to inhibitory activity of the digesta.

6.5 Conclusion

Novel peptides were identified in the digesta and 6 fractions and were all originated from oat globulin. The most abundant peptides in digesta < 3 kDa tended to have hydrophobic ends and to show positive hydropathy index. The peptides identified in antioxidant fractions tended to be hydrophilic whereas the peptides in ACE inhibitory fractions showed the potential of ACE inhibition caused by both hydrophobicity and negatively charged ends by peptides. Peptides LIVPQ, FEPL, LSEAL, LQAFEPL, ALPVDV, NDIL and SQAGITEY identified in the digesta fraction, and 6

anionic fractions were selected for future investigation. These 7 peptides have potential to be antioxidant and ACE inhibitory agents and could be synthesised and assayed again to validate their antioxidant and ACE inhibitory activity.

Chapter 7

General Conclusion

The current investigation aimed to answer three key research questions where we have successfully characterised storage proteins in the wheat bran, oat bran and barley and identified the antioxidant and ACE inhibitory peptides and their sequences of the oat bran proteins hydrolysed via simulated *in vitro* digestion model.. The novel peptides from oat bran digesta was also identified via anion-exchange chromatography based on comparatively higher overall digestibility, antioxidant and ACE inhibitory activity, followed by amino acid sequences in the selected fraction via R-HPLC-MS/MS. The discovery of novel bioactive peptides in oat bran showed an opportunity of utilising cereal by-products in producing therapeutic agents and a pathway to explore functional compounds in bran.

Key Research Question 1: Will proteins extracted from oat bran, wheat bran and barley be an alternative source of antioxidant and ACE inhibitory peptide and essential amino acids?

Protein extracted from oat bran, wheat bran and barley are an ideal source of antioxidant and ACE inhibitory peptides and essential amino acids after digestion. To investigate the nutritional value and bioactive activity of cereal protein and its digesta, in Chapter 3, protein from oat bran, wheat bran and barley were extracted by alkaline extraction followed by iso-electric precipitation and lyophilisation. The protein, fat, fibre and starch content of the protein concentrates were measured before digestion. The protein content of these concentrates was determined by the Dumas method showing the wheat bran, oat bran and barley protein concentrates had the intermediate protein content from 50 - 80%. The protein content from the extraction of the bran was lower than that of the wholegrain as the strong fibral-protein network needs to be broken for the release of protein. Fibre was observed to

be the second predominant macro-nutrient in the bran protein extracts. Protein content was also measured by BCA and Bradford assays before and during digestion. During 240 min digestion, protein content decreased measured by Bradford method whereas BCA method showed the higher protein content at gastric phase compared to the crude protein and then a dramatic decrease at the start of intestinal phase till the end. The mechanism of two methods indicated that BCA method was more capable to determine the content of peptides, after enzymatic reaction and thereby was selected for peptide quantification for the remaining chapters.

The nutritional value of cereal protein was higher following gastrointestinal digestion, The digestibility of three types of protein increased from 80- 90% while the degree of hydrolysis showed a similar tendency increasing from around 10% to 50 - 70% indicating the protein after digestion would have better bio-accessibility. A dramatic increase in digestibility was observed during intestinal digestion. The results matched the free amino acid profiles which indicated a significant increase of FAAs released at the end of the intestinal digestion compared to that of gastric digestion. The major FAAs in intestinal digesta included glutamic acid, asparagine, glutamine, histidine, aspartic acid, alanine, tyrosine, tryptophan, phenylalanine, leucine and isoleucine. However, the limiting amino acids lysine and threonine were deficient after digestion.

The SDS-PAGE results confirmed the increased digestibility and degree of hydrolysis after digestion. By using this technique, globulin, albumin and avenin fractions were identified in the oat bran protein extracts; prolamins and gliadin fractions were identified in the wheat bran protein extracts; hordein, albumin and globulin were identified in barley protein extracts. Pepsin in the gastric digestion stage cleaved the storage proteins into polypeptides and further degradation was witnessed after intestinal digestion. Barley protein was eventually degraded to be less than 15 kDa indicating the presence of polypeptides resistant to the pepsin-pancreatin cleavage. Two-dimensional electrophoresis was adopted to characterise extracted oat bran

protein. No significant difference was noted between the electrophoretogram of oat bran protein and the protein extracted from the wholegrains.

Key Research Question 2: Do peptides in oat bran protein intestinal digesta carry antioxidant and ACE inhibitory activity?

In oat intestinal digesta, intermediate level of phenol-like peptides was found, and these peptides do not show strong ferric reduction power but show strong scavenging activity against ABTS⁺ radical by transferring electrons and protons. ABTS, TPC and FRAP assays were utilised to measure the antioxidant activity of the crude protein and digesta. A significant enhancement of overall antioxidant activity was observed after digestion while oat and wheat bran gastric digesta showed slightly stronger activity compared to intestinal digesta. Barley intestinal digesta showed significantly weaker activity compared to the gastric digesta. The digesta showed weak ability to reduce ferric ions and intermediate content of phenol-like peptides but it showed strong activity to eradicate ABTS radicals. Three cereal proteins after gastrointestinal digestion all showed strong ACE inhibitory at over 80%. Based on the strongest overall antioxidant and ACE inhibitory activity among three digesta samples, oat intestinal digesta was selected for separation by anion exchange chromatography. Oat intestinal digesta with the MW of less than 3 kDa (digesta < 3 kDa) was isolated first by using centrifugal tubes of 3,000 MWCO. Higher overall antioxidant and ACE inhibitory power of digesta < 3 kDa compared to digesta > 3 kDa indicated that shorter peptides tended to have stronger bioactive activity.

Anionic fractions were isolated from digesta < 3 kDa to investigate their antioxidant and ACE inhibitory activity by using anion exchange chromatography. There have been many papers indicated that hydrophobic peptides could be the reason of the antioxidant and ACE inhibitory properties in the protein hydrolysates. There is no research focused on investigating the antioxidant and ACE inhibitory activity of the negatively charged peptides. Because of the neutral and slightly alkaline

environment in the small intestine, a part of the intestinal peptides nevertheless carries the negative charge.

Anionic peptides showed weak ability against ABTS⁺ and ROO⁻ radicals by transferring electrons and donating protons but showed intermediate ACE inhibitory activity. FRAP assay was replaced by ORAC assay aiming to confirm the proton or electron donation capability of oat peptides in the isolated fractions. Anionic peptide fractions did not show strong overall antioxidant activity. The abundant peptides in the selected fractions sequenced by R-HPLC-MS/MS also indicated low levels of hydrophobic amino acids and simulated potential antioxidant amino acid sequences in the peptides. However, oat anionic fraction showed moderate ACE inhibitory activity of 38%. All peptides in intestinal digesta < 3 kDa and selected anionic fractions were sequenced. The identified sequences showed that the most abundant peptides in the fraction contained potential ACE inhibitory amino acid sequences at either N- or C- terminal. Peptide such as ALPVDV, EQQFGQNI, QAFEPLR and LSEAL showed high hydrophobicity which was related to the inhibition of ACE.

Key Research Question 3: What are the antioxidant and ACE inhibitory peptides in the oat bran protein intestinal digesta with the potential for use as therapeutic food?

This research provides information of antioxidant and ACE inhibitory activity of the protein extracted from oat bran, wheat bran and barley, the digesta derived from them and the characteristics of the protein, digesta and the peptides in it. This knowledge can help to understand the nutritional value and bioactivity of proteins and peptides in wholegrain cereal and cereal bran which can benefit the New Zealand cereal industry. Though the protein extracted from oat bran, wheat bran and barley show imbalanced free amino acid profile after digestion, the high digestibility, the strong antioxidant and ACE inhibitory activity of the bran intestinal digesta confirm that the bran is an ideal source of protein to make protein supplements. There is also the potential of having a commercial peptide supplement in the market based on the bran protein in the pharmaceutical industry. The

negatively charged peptides from oat bran protein intestinal digesta showed weak activity of eradicating radicals by transferring electron and donating proton but intermediate ACE inhibitory activity. This finding covers the unexplored area of how the charge peptides in intestinal digesta behave as an antioxidant or ACE inhibitory agent. Seven novel peptides including LIVPQ, FEPL, LSEAL, LQAFEPL, ALPVDV, NDIL and SQAGITEY were identified and selected for future investigation. To translate the novel amino acids sequence to the therapeutic use, there are many challenges and unsolved problems lying ahead. The storage of bioactive peptides needs to be considered as the terminals and side chains of peptides are exposed and easily oxidised. An unique delivery system needs to be designed for oral administration of bioactive peptides aims on protecting further digestion by digestive enzymes as well as the enzymes within the epithelium To address these problems and the gaps in this research, six recommendations were made and summarised below taking the research to the next level.

Future Research

1. In this study, peptides with potential to be ACE inhibitors were identified. It will be helpful to employ molecular docking to assess the binding affinity of identified novel peptides and the enzyme.
2. Novel peptides need to be synthesised for ACE inhibitory assay to confirm the discovery. The selection criteria will be based on the hydrophobicity, the amino acid sequences and the binding affinity of the peptide candidates.
3. This study confirmed the oat peptides are capable of donating electrons and protons to scavenge radicals (ABTS[•] and ROO[•]). The test range of radicals could expand to superoxide, hydroxyl, hydroperoxyl, hydrogen peroxide, and nitric oxide to examine the scavenging capability of oat peptides with a broader spectrum of radicals.
4. *In vitro* cell line works such as cell-based antioxidant assay and *in vivo* studies could be carried out on the synthesised novel peptides to elucidate the mechanism of the bioavailability of the bioactive peptides.

5. Bioactive peptides could also be beneficial to the gut microbiota and have indirect health benefits. The oat intestinal peptides could be tested on the duodenal microbiome.
6. At the industrial level, the purification for the large-scale production of peptide with a specific sequence has remained problematic. Further work is required in this area as “solid phase peptide synthesis” which has been utilised in the industry has been known to have trouble making peptides with high hydrophobicity.

Appendix A Peptide Identified in Digesta and Anionic Fractions

Appendix A. Peptide sequences of digesta < 3 kDa, G17, G18 and S19 identified using LC-MS/MS and data validated by ProteinPilot. digesta < 3 kDa: Oat bran intestinal digesta < 3 kDa, G17: Fraction 17 isolated from gradient separation (Chapter 5), G18: Fraction 18 isolated from gradient separation (Chapter 5), S19: Fraction S19 isolated from stepwise isocratic separation (Chapter 5).

digesta < 3 kDa	G17	G18	S19
AEGQSQSQNLKDEHQ	AGQSWDQSFN	AGQSWDQSFN	AGNNKEDQQFGQNI
AEGQSQSQNLKDEHQR	AQGQSQSQTIKDEHQ	AQGQSQSQTIKDEHQ	AIYVFDVNNNANQLEPR
AEGQSQSQNLKDEHQRV	ARVQVVNNHGQTV	DEQNEQFR	ANQLEPR
AILSPF	DEQNEQFR	DEQNEQLR	AQGQSKSQNLKDEHQ
ALGISQQA	DEQNEQLR	DGDAPIVAV	ARVQVVNNHGQTV
ALGISQQAQ	DGDAPIVA	DIEEGSSSPVR	AYQPIQSQEGQ
ALGISQQAQK	DQQFGQNI	DQQFGQNI	AYQPIQSQEGQST
ALGISQQAQK	DQQFGQNI	DQSQFAQGQSQTIKDEHQ	CAGVSVIR
ALPVDVLANA	DQSQFAQGQSQTIKDEHQ	DVNNNANQLEPR	DEQNEQFR
ANQLEPR	DRLQAFE	ELVEHQAYQPI	DEQNEQLR
AQGQSQSQTIKDEHQ	DRLQAFEPL	FAQGQSQSQTIKDEHQ	DGDAPIVAV
ARKNIENPQHADTYNPR	DVNNNANQLEPR	FDRLQAFE	DQQFGQNI
ARQNIENPKRADTYNPR	EHQAYQPI	FLKPFVS	DQQFGQNI
ARVQVVNNHGQTV	EHQAYQPIQSQEGQSTQ	GEEFGAFTPK	DQRGEII
ATRVNL	ELVEHQAYQPI	GLLLPQYH	DQSQFAQGQ
AVEHELGG	EYFDEQNEQFR	IEEGSSSPV	DQSQFAQGQSQ
AVEHELGGF	FAQGQSKSQNLKDEHQ	IQSQNDQRGEII	DRLQAFEPL
DEQNEQFR	FAQGQSQTIKDEHQ	IYVFDVNN	DVNNNANQL
DQQFGQNI	FDEQNEQFR	KPTMSQQELVEH	DVNNNANQLEPR
DQSQFAQGQR	FDRLQAF	KPTMSQQELVEHQ	EEFGAFTPK

DQSQFAQQSQSQSTIKDEHQ	FDRLQAFE	LIVPQH	EFLAGNN
DRLQAFEPL	FDVNNNANQLEPR	LKPTMSQQELVEH	EHQAYQPI
DRLQAFEPLR	GLLLPQYH	LKPTMSQQELVEHQ	FAQQQSKSQNLKDEHQ
DRLQAFEPLRQV	GQESGVFTPK	LQAFEPL	FAQQQSQSQTIKDEH
DVNNNANQLEPR	GYNDGDAPVV	LQFLKPT	FAQQQSQSQTIKDEHQ
EALGISQQVAQK	HQAYQPIQS	LQFLKPTM	FDEQNEQFR
EFLAGNNK	HQAYQPIQSQ	LVEHQAY	FDRLQAFEPL
EQQSGNNIFSGL	IEEGSSSPV	LVEHQAYQPIQSQEGQ	FDVNNNAN
EQRGEIIR	IQSQNDQRGEI	MSQQELVEHQ	FDVNNNANQLEPR
FAEQSQSQNLKDEHQ	IQSQNDQRGEII	MSQQELVEHQA	FEFLAEQR
FAQQQSKSQNLKDEHQ	IYVFDVNN	NDGDAPIV	FLKPIVSQ
FAQQQSQSQTIKDEHQ	IYVFDVNNN	NDGDAPIVAI	FLKPTMSQQELVEHQ
FAQQQSQSQTIKDEHQ	KNIENPQHADT	NDGDAPVVA	GEEFGAFTPK
FDAFTPK	KNIENPQHADTY	NDQRGEII	GLLLPQYH
FDEQNEQF	KPTMSQQELVEH	NIENPQHADT	GNNKEDQQFGQNI
FDEQNEQFR	KPTMSQQELVEHQ	NNANQLEPR	GQESGVFTPK
FDRLQAFEPL	KPTMSQQELVEHQA	NNRGEEDFAFTPK	GYNDGDAPVVA
FDVNNNANQLEPR	LAGNNKEDQQFG	NNRGEEDFG	HQAYQPIQSQEGQST
FEFLAEQR	LKPTMS	NNRGEEDFGA	HQAYQPIQSQEGQSTQ
FEPLRQ	LKPTMSQQELVEH	NNRGEEDFGAF	IEPQGLLLPQYH
FEPLRQV	LKPTMSQQELVEHQ	PAGIVHW	IQSQKEQRGEII
FGAFTPK	LQFLKPT	QDIEEGSSSPVR	IQSQNDQRGEI
FKQGDVI	LQFLKPTM	QEAQNLKNRGE	IQSQNDQRGEII
FKQGDVIA	LVEHQAY	QEAQNLKNRGEED	IQSQNDQRGEIIR
FKQGDVIAL	LVEHQAYQPI	QESQNLKNRGEED	IYVFDVNNNANQLEPR
FKQGDVV	LVEHQAYQPIQS	QGQSKSQNLKDEHQ	KEDQQFGQNI
FKQGDVVA	LVEHQAYQPIQSQEGQ	QNLKDEHQ	KNIENPQHADT
FLKPFVSQQGPVEH	LVEHQAYQPIQSQEGQST	QQELVEHQ	KNIENPQHADTYNPR
FLKPFVSQQGPVEHQ	NDGDAPIVAV	QQGPVEH	KPTMSQQELVEHQ

FLKPFVSQQGPVEHQA	NDGDAPVVAI	QQQFQPFQ	KPTMSQQELVEHQA
FLKPIV	NDQRGEII	QQQFQPFQDQ	KPTMSQQELVEHQAYQPI
FLKPIVS	NNANQLEPR	QQQFQPFQDQSQ	LAGNNKEDQQFGQ
FLKPIVSQ	NNNGQTVFNDR	QSFTPWQS	LAGNNKEDQQFGQN
FLKPIVSQQ	NNRGEEF	QSGQSWDQS	LAGNNKEDQQFGQNI
FLKPT	NNRGEEFDAFTPK	QSGQSWDQSFN	LAGNNKEDQQFGQNIF
FLLAGNN	NNRGEEFG	QSQEGQSTQY	LAKDDEGYLPK
FLLAGNNK	NNRGEEFGA	QSQTIKDEHQ	LKPTMSQQELVEH
FPVYIDR	NNRGEEFGAF	QYQEGQSTQ	LKPTMSQQELVEHQ
FTPWQSS	NNRGQESGVF	RALPVDV	LLAGNNKEDQQFGQN
FTPWQSSR	NRGEEFGA	RGEEFGA	LLAGNNKEDQQFGQNI
FTQTGSQSYQDEGESSSTEK	NRGEEFGAF	SFQPYPE	LQAFEPL
FTQTGSQSYQDEGESSSTEKASE	QDIEEGSSSPVR	SGQSWDQSFN	LQFLKPT
GEIIRVT	QEAQNLKNNRGEE	SIQHELGG	LQFLKPTM
GEIIRVTQ	QEAQNLKNNRGEEF	SIQHELGGF	LQFLKPTMSQQELVEH
GISQQAQ	QELVEHQAYQPI	SPFWNINAH	LQFLKPTMSQQELVEHQ
GISQQAQK	QESQNLKNNRGEEF	SQAGIIIEY	LQFLKPTMSQQELVEHQA
GIVHWGYNDGDAPVV	QFRCAGVSVIR	SQAGITE	LSEALGI
GIVHWGYNDGDAPVVA	QNLKDEHQ	SQAGITEYF	LSEALGISQQ
GLLLPQ	QQQFQPFQ	SQAGVTEY	LVEHQAYQPIQS
GLLLPQYH	QQQFQPFQDQ	SQAGVTEYF	LVEHQAYQPIQSQEGQST
GLLLPQYHNAPGL	QQQFQPFQDA	SQEGQSTQY	LVEHQAYQPIQSQEGQSTQ
GLLLPQYHNAPGLV	QSGQSWDQSFN	SQQELVE	MLAKDDEGYLPK
GLVLPQYH	QSQSHLKDEHQ	SQQELVEH	MSQQELVEHQ
GLVLPQYHNAPA	QSQTIKDEHQ	VEHQAYQPIQS	NANQLEPR
GQESGVFTPK	QSWDQSFN	VEHQAYQPIQSQEGQ	NDGDAPIVAV
GQLLIIPQ	QVGQSTQYQEGQ	VEHQAYQPIQSQEGQST	NDQRGEII
GQLLIIPQH	QVGQSTQYQEGQSTQY	VEHQAYQPIQSQEGQSTQ	NIENPQHA
GQLLIIPQHY	QYQEGQSTQ	VIQPQL	NIENPQHADTYNPR

GQLLIVPQ	QYQEGQSTQY	VRSQAGITEY	NNANQLEPR
GQLLIVPQH	QYQEGQSTQYQ	VSQQGPVEH	NNHGQTVFNDIL
GQNFPILN	RVIEPQGL	VYVFDVNN	NNHGQTVFNDILR
GQSFTPW	SGQSWDQSFN	VYVFDVNNNAN	NNNGQTVFNDR
HNAPGL	SPFWNINAH	YFDEQNEQFR	NNRGEEFDAFTPK
HNAPGLV	SQAGIIEY	YQEGQSTQY	NNRGEEFGA
HSVMHM	SQEGQSTQY	YQNAILSPF	NNRGEEFGAF
ILGAPSAPIEK	SQQELVEH	YVFDVNN	NNRGEEFGAFTPK
IQSQKEQRGEII	SQQELVEHQ	YVFDVNNN	NRGEEFDAFTPK
IQSQKEQRGEIIR	SQQELVEHQA	YVFDVNNNAN	PAGIVHW
IQSQNDQRGEI	SQQELVEHQAY	YVFDVNNNANQLEPR	PAGIVHWG
IQSQNDQRGEII	TQYQEGQST		QAYQPIQSQEGQSTQ
IQSQNDQRGEIIR	TQYQEGQSTQ		QAYQPIQSQQEQSTQ
IRGVYDGSL	VEHQAYQPI		QEAQNLKNNRGEEF
IRVSQGLQ	VEHQAYQPIQ		QEAQNLKNNRGEEFDAFTPK
ISRQEAQNL	VEHQAYQPIQS		QFLKPTMSQQELVEHQ
ISRQEAQNLK	VEHQAYQPIQSQEGQ		QGDVVALPA
ISRQEAQNLKNNRGEE	VEHQAYQPIQSQEGQST		QGDVVALPAG
ISRQESQNL	VEHQAYQPIQSQEGQSTQ		QGDVVALPAGI
ISRQESQNLK	VEHQAYQPIQSQEGQSTQY		QNLKDEHQ
IVHWGYNDGDAPVVA	VFDVNNNAN		QPIQSQEGQ
IYVFDVNNNANQLEPR	VIEPQGL		QPIQSQEGQST
KEDQQFGQNI	VIEPQGLLLPQYH		QPIQSQEGQSTQ
KNIENPQHADT	VRSQAGITEY		QPIQSQQEQSTQ
KNIENPQHADTYNPR	VSQQGPVEH		QPIQTQEGQAT
KNNRGEEFDAFTPK	VSQQGPVEHQ		QPIQTQEGQATQ
KNNRGEEFGAFTPK	VSQQGPVEHQA		QQQFQPFQD
KPTMSQQEL	VSQQGPVEHQAY		QQQFQPFQDQ
KPTMSQQELVEHQ	YFDEQNEQFR		QQQFQPFQDQA

KQGDVVALPA	YNDGDPIVAV	QQQFQPFDDAAQ
KTNPNSMVSH	YQEGQSTQY	QQVPVEQQ
KTNPNSMVSHI	YQEGQSTQYQ	QSQNDQRGEI
KTNPNSMVSHIAG	YVFDVNN	QSQNDQRGEII
KTNPNSMVSHIAGK	YVFDVNNN	QSQSHLKDEHQ
KTNPNSMVSHIAGKS	YVFDVNNNAN	QSQTIKDEHQ
KTNPNSMVSQI	YVFDVNNNANQ	QVGQSPQYQEGQ
KTNPNSMVSQIAGK	YVFDVNNNANQLEPR	QVGQSPQYQEGQST
KTNPNSMVSQIAGKT		QVGQSPQYQEGQSTQ
LAGNNKEDQQFGQ		QVGQSPQYQEGQSTQY
LAGNNKEDQQFGQN		QVGQSTQYQEGQ
LAGNNKEDQQFGQNI		QVGQSTQYQEGQSTQ
LAGNNKEDQQFGQNIF		QYQEGQST
LGISQQAQ		QYQEGQSTQ
LGISQQAQK		QYQVGQSPQYQEGQSTQ
LGISQQAQR		QYQVGQSTQYQE
LGLQKGEIGK		RAVEHELGGF
LHGQNFPI		RFAQGQSKSQNLKDEHQ
LHGQNFPILN		RVIEPQGL
LHGQNFPILN		RVIEPQGLLLPQ
LHGQNFPILNLV		SEALGISQQ
LIVPQH		SEALGISQQAQ
LIVPQHYV		SEALGISQQVAQ
LKPFVSQQGPVEH		SPDAVVAL
LKPFVSQQGPVEHQA		SQEGQSTQY
LKPTMSQQELVEH		SQFAQQSQSQSTIKDEHQ
LKPTMSQQELVEHQ		SQNDQRGEII
LLIIPQH		SQQAQRISQNDQRGEI
LLPQYHNAPG		SQQAQRISQNDQRGEII

LLPQYHNAPGL	STQYQEGQST
LLPQYHNAPGLV	TNPNSMVSH
LPAGIVH	TNPNSMVSHIAGK
LPAGIVHW	TQYQEGQSTQ
LQAFEPLRQV	VEHQAYQPI
LQFLKPF	VEHQAYQPIQ
LQFLKPIV	VEHQAYQPIQS
LQFLKPT	VEHQAYQPIQSQ
LQFLKPTMS	VEHQAYQPIQSQEGQST
LQFLKPTMSQ	VEHQAYQPIQSQEGQSTQ
LQFLKPTMSQQ	VFDVNNNAN
LQFLKPTMSQQEL	VFDVNNNANQLEPR
LQFLKPTMSQQELVEH	VIEPQGL
LQFLKPTMSQQELVEHQA	VIEPQGILLPQ
LSEALGI	VIEPQGILLPQYH
LSEALGISQAAQK	VIEPQGLV
LSEALGISQAAQR	VIEPQGLVLPQYH
LTQTGFQS	VNNNANQLEPR
LVEHQAYQPI	VQVVNNH
LVEHQAYQPIQSQEGQST	VQVVNNHGQTV
LVLPQYH	VQVVNNHGQTVFNDIL
MIQGHAQVQ	VQVVNNNGQTV
MSQQELVEH	VQVVNNNGQTVF
NAILSPF	VQVVNNNGQTVFNDR
NANQLEPR	VRSQAGITE
NDGDAPIVAV	VSQQGPVEHQ
NDQRGEIIR	VSQQGPVEHQA
NIENPKRADTYNPR	VSQQGPVEHQAY
NLKNNRGEEF	VSQQGPVEHQAYQPI

NLKNNRGEEFGAFTP	VSQQGPVEHQAYQPIQSQQEQSTQ
NLKNNRGQESGVFTP	VVNNHGQTV
NLVQMSATRV	VYVFDVNNNANQLEPR
NLYQNAIL	YFDEQNEQFR
NNANQLEPR	YQVGQSTQYQEGQSTQ
NNHGQTVFN	YVFDVNNNANQLEPR
NNHGQTVFNDIL	
NNHGQTVFNDILR	
NNNANQLEPR	
NNNGQTVFNDR	
NNNGQTVFNDRL	
NNRGEEFDAFTP	
NNRGEEFGA	
NNRGEEFGAF	
NNRGEEFGAFTP	
NNRGQESGVF	
NNRGQESGVFTP	
NPNSMVSHIAG	
NPNSMVSHIAGK	
NPNSMVSQIAGK	
NRGEEFGAFTP	
NSKNFPIL	
NSKNFPILN	
PAGIVHW	
PAGIVHWG	
PAGIVHWGYNDGDAPVVA	
PFWNINAH	
PNSMVSHIAGK	
PNSMVSQIAGK	

PQYHNAPAL
QAFEPLR
QAFEPLRQV
QESQNLK
QFLKPIVSQ
QFLKPT
QFLKPTMSQQ
QGDVVALPA
QGQLLIVPQ
QGQLLIVPQH
QGQLLIVPQHY
QKEFLAGNN
QKEFLAGNNK
QKEFLAGNNKEDQQFGQN
QKEFLAGNNKR
QLFGQSSTPW
QLLIIPQH
QNAILSPF
QNAILSPFWNINAH
QNAILSPY
QNIENPKR
QNIENPKRA
QNIENPKRADT
QNIENPKRADTYNPR
QNLKDEHQ
QNLKDEHQR
QPIQSQEEQST
QPIQSQEEQSTQ
QPIQSQEGQSTQ

QQFQPF
QQFQPFDDQ
QQFQPFDDQAAQ
QSNDQRGEII
QSQTIKDEHQ
QSQTIKDEHQR
QVGQSPQYQEGQ
QVGQSPQYQEGQSTQ
QVGQSTQ
QVGQSTQYQEGQST
QVGQSTQYQEGQSTQ
QYQEGQSTQ
RALPIDVLA
RALPVDV
RALPVDVLA
RALPVDVLAN
RALPVDVLANA
REQQFGQNI
RKNIENPQHADTYNPR
RVIEPQG
RVIEPQGGLLPQ
RVIEPQGGLLPQYH
RVIEPQGGLLPQYHNAPG
RVIEPQGGLLPQYHNAPGLV
RVIEPQGLV
RVIEPQGLVLPQ
RVIEPQGLVLPQYH
RVIEPQGLVLPQYHNAPA
SATRVNL

SEALGISQQAQK
SEALGISQQAQR
SEKLVGL
SMVSHIAGK
SMVSIAGK
SPFWNINA
SPFWNINAH
SQAGVTEY
SQGLQFLKPF
SQNDQRGEIIR
SQNLKDEHQRV
SQQAAQRIQSQNDQR
SQQAAQRIQSQNDQRGEIIR
SQQVAQKI
TGVSVIR
TNPNSMVS
TNPNSMVSHIAGKS
TNPNSMVSQIAGK
VAQLIQSCYPSK
VDVNNPEGTK
VEHQAYQPI
VEHQAYQPIQS
VEHQAYQPIQSQEGQST
VEHQAYQPIQSQEGQSTQ
VEHQAYQPIQSQEGQSTQY
VFDVNNNANQLEPR
VHHIKQGDVV
VHHIKQGDVVALPA
VHWGYNDGDAPVVA

VIEPQGL

VIEPQGLLLPQYH

VIEPQGLLLPQYHNAPG

VIEPQGLV

VIEPQGLVLPQYH

VLPQYHNAPA

VNLYQNA

VPALLPP

VQMSATR

VQMSATRV

VQMSATRVN

VQVVNNHGQTV

VQVVNNHGQTVF

VQVVNNNGQTV

VQVVNNNGQTVF

VQVVNNNGQTVFNDR

VRSQAGITEY

VSHIAGK

VSQGLQFLKP

VSQGLQFLKPF

VSQGLQFLKPI

VSQQGPVEH

VSQQGPVEHQ

VSQQGPVEHQA

VSQQGPVEHQAY

VSQQGPVEHQAYQPI

VSYIAGKT

VVNNHGQTV

VYILQGR

VYILQGRG
 VYVFDVNNNANQLEPR
 WNINAH SVM
 YFDEQNEQ
 YFDEQNEQF
 YFDEQNEQFR
 YHNAPGL
 YHNAPGLV
 YILQGR
 YQNAILSPF
 YQVGQSTQ
 YVFDVNNNANQLEPR
 YVVLKK

Appendix A. Peptide sequences of SI10, SI15 and SI16 identified using LC-MS/MS and data validated by ProteinPilot. SI10: Fraction 10 isolated from stepwise isocratic separation (Chapter 5), SI15: Fraction 15 isolated from stepwise isocratic separation (Chapter 5), SI16: Fraction SI16 isolated from stepwise isocratic separation (Chapter 5). *Continue*

SI10	SI15	SI16
AGQSWDQSFN	ADTYNPR	AMDESQWH
ALPIDVLA	AEGQSQSQNLKDEHQ	AVEHELGGF
ANQLEPR	AGIIEYFDEQNEQFR	DEQNEQLR
AQGQSQSQTIKDEHQ	AVEHELGGF	DRLQAFEPL
ARVQVNNHGQTV	DEGESSSTEKASE	DVNNNANQLEPR
AVQGELGGF	DEQNEQFR	FDEQNEQFR
AYQPIQSQEGQ	DEQNEQLR	FDEQNEQLR
AYQPIQSQEGQST	DRLQAFEPL	GEEFGAFTP
DEQNEQFR	DVNNNANQLEPR	HWGYNDGDAPVVA
DGDAPIVAV	ELVEHQAY	LQFLKPT

DQQFGQNIF	EQQFGQNI	MSQQELVEH
DQSQFAQGQ	FAEGQSQSQNLKDEHQ	NNRGEEFD
DQSQFAQGQSQSQTIKDEHQ	FAEGQSQSQNLKDEHQQR	NNRGEEFDA
DVNNNANQLEPR	FDEQNEQ	NNRGEEFDAF
EEFGAFTPK	FDEQNEQF	NNRGEEFGAF
EHQAYQPI	FDEQNEQLR	PAGIVHW
ELVEHQA	FDVNNNANQLEPR	PFWNINAH
ELVEHQAYQPI	GEDESSLTN	QDEGESSTEKASE
FAQGQSKSQNLKDEHQ	GEEFGAFTPK	SIQHELGGF
FAQGQSQSQTIKDEHQ	GFQSYQD	SPFWNINAH
FDEQNEQFR	GIVHWGY	SYQDEGESSTEKA
FDEQNEQLR	GLLLPQYH	SYQDEGESSTEKASE
FDRLQAFEP	GYNDGDAPVV	VHWGYN
FDRLQAFEPL	HWGYNDGDAPVVA	VHWGYNDGDAPV
FDVNNNANQLEPR	IQSQNDQRGEI	VHWGYNDGDAPVV
FLKPTMSQQELVEH	KNIENPQHADT	VHWGYNDGDAPVVA
FLKPTMSQQELVEHQ	LIIPQH	WGYNDGDAPVVA
FLKPTMSQQELVEHQA	LQFLKPT	YFDEQNEQLR
FQPFQSQ	MSQQELVEH	YQNAILSPF
GEEFGAFTPK	NDGDAPIVA	YVFDVNNNANQLEPR
GIVHWGYNDGDAPVVA	NDGDAPIVAV	
GLLLPQYH	NNANQLEPR	
HQAYQPIQSQEGQST	NNRGEEF	
HQAYQPIQSQEGQSTQ	NNRGEEFD	
IENPQHADTYNPR	NNRGEEFDA	
IKQGDVVALPA	NNRGEEFDAFTPK	
IQSQKEQRGEIIR	PAGIVHW	
IQSQNDQRGEI	QAGQSWDQSFN	
IQSQNDQRGEII	QDEGESSTEKA	

IQSQNDQRGEIIR	QDEGESSSTEKASE
IYVFDVNNN	QDIEEGSSSPV
IYVFDVNNNANQLEPR	QELVEHQ
KEDQQFGQN	QGDVVALPA
KEDQQFGQNIF	QNLKDEHQ
KNIENPQHADT	QSQTIKDEHQ
KNIENPQHADTY	RALPVDV
KPTMSQQELVEH	SPFWNINAH
KPTMSQQELVEHQ	SPYWNINAH
KPTMSQQELVEHQA	SQAGIIEY
KTNPNMVSCHIAGK	SYQDEGESSSTEKA
LAGNNKEDQQFGQ	VEHQAYQPIQSQEGQST
LAGNNKEDQQFGQN	VEHQAYQPIQSQEGQSTQ
LAGNNKEDQQFGQNI	VFDVNNNANQLEPR
LAGNNKEDQQFGQNIF	VHWGYNDGDAPVVA
LHGQNFILN	VIEPQGL
LKPTMSQQELVEH	VIEPQGLLLPQYH
LKPTMSQQELVEHQ	VQMSATRV
LKPTMSQQELVEHQA	VQVVNNHGQTV
LQFLKPT	VQVVNNNGQTV
LQFLKPTMSQQELVEH	VSQQGPVEH
LQFLKPTMSQQELVEHQ	VSQQGPVEHQ
LQFLKPTMSQQELVEHQA	VSQQGPVEHQA
LSEALGI	YFDEQNEQ
LVEHQAY	YFDEQNEQF
LVEHQAYQPI	YHNAPGLV
LVEHQAYQPIQSQEGQST	YQNAILSPY
LVEHQAYQPIQSQEGQSTQY	YVFDVNNNANQLEPR
MSQQELVEH	

MSQQELVEHQ
MSQQELVEHQA
MSQQELVEHQAYQPI
NDGDPIVAV
NDQRGEII
NIENPQHA
NIENPQHADTYNPR
NKEDQQFGQNI
NKEDQQFGQNI
NNHGQTVFNDIL
NNNGQTVFNDR
NNRGEEF
NNRGEEFDAFTP
NNRGEEFGAF
NNRGEEFGAFTP
PAGIVHW
QDIEEGSSSPVR
QEAQNLKNNRGEE
QEAQNLKNNRGEEF
QESQNLKNNRGEE
QESQNLKNNRGEEF
QFLKPTMSQQELVEH
QFLKPTMSQQELVEHQ
QFLKPTMSQQELVEHQA
QGDVIALPAG
QGDVVALPA
QGQSKSQNLKDEHQ
QGQSQSHLKDEHQ
QKEFLAGNNKED

QNIENPK
QNLKDEHQ
QPIQSQEGQ
QPIQSQEGQST
QPIQSQEGQSTQ
QPIQTQEGQAT
QQFQPF
QQFQPFQ
QQFQPFQDQ
QQFQPFQDQA
QQFQPFQDQAQ
QSGQSWDQSFN
QSQNDQRGEI
QSQNDQRGEII
QSQSHLKDEHQ
QSQTIKDEHQ
QVGQSPQYQEGQSTQ
QVGQSPQYQEGQSTQYQ
QVGQSTQYQEGQSTQ
QVGQSTQYQEGQSTQY
QVGQSTQYQEGQSTQYQ
QYQEGQSTQ
QYQEGQSTQY
QYQEGQSTQYQAGQSQDR
RFAQGQSKSQNLKDEHQ
SEALGISQQ
SGQSWDQSFN
SPFWNINAH
SQAGVTEY

SQNDQRGEII
SQNLKDEHQ
SQQAAQRIQSQNDQRGEII
SQQELVEHQ
STQYQEGQST
STQYQEGQSTQ
TNPNSMVSH
TQYQEGQSTQ
VEHQAYQPI
VEHQAYQPIQ
VEHQAYQPIQS
VEHQAYQPIQSQ
VEHQAYQPIQSQEGQST
VEHQAYQPIQSQEGQSTQ
VEHQAYQPIQSQEGQSTQY
VFDVNNNANQ
VFDVNNNANQLEPR
VIEPQGL
VIEPQGLLLPQ
VIEPQGLLLPQYH
VIEPQGLV
VIEPQGLVLPQYH
VQMSATRV
VQVVNNH
VQVVNNHGQTV
VQVVNNNGQTV
VQVVNNNGQTVFNDR
VSQQGPVEH
VSQQGPVEHQ

VSQQGPVEHQA

VSQQGPVEHQAY

VYVFDVNNNANQLEPR

YFDEQNEQFR

YMLAKDDEGYLPK

YVFDVNNN

YVFDVNNNAN

YVFDVNNNANQ

YVFDVNNNANQLEPR

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