ROLE OF FOOD PROTEIN-DERIVED ZINC-CHELATING PEPTIDES IN HUMAN NUTRITION AND INFLAMMATION

by

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DEDICATION PAGE

I dedicate this thesis to Heavenly God as a sacrifice of praise and thanksgiving.
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ABSTRACT

Food protein-derived peptides possess the intrinsic capacity to form metal complexes, notably physiologically relevant metals such as Zn$^{2+}$. The Zn$^{2+}$-binding property of peptides can be exploited to enhance human zinc bioavailability, as dietary Zn$^{2+}$ can be delivered in form of zinc-peptide complexes. Effective zinc delivery through peptides depends on the gastrointestinal stability of the complexes and zinc release for absorption, which in turn can be influenced by the structural properties, such as net surface charge, of the peptides. An integral study in this project investigated the influence of the structural properties of whey protein hydrolysates produced with Everlase (WPH-Ever) and papain (WPH-Pap) on the gastric stability and dialyzability of their zinc complexes. WPH-Ever had higher zinc-chelating capacity than WPH-Pap due to its higher net negative charge. Moreover, the zinc complex of WPH-Ever was more stable during simulated gastric digestion, which can be attributed to its more negatively charged surface, leading to the formation of stronger zinc complexes. However, the higher zinc-binding of WPH-Ever resulted in lesser total zinc dialyzability, whereas more zinc ions were released from WPH-Pap; this suggests that peptides’ structures can affect the stability of their zinc complexes and zinc accessibility. Zinc-chelating food peptides have the potential to inactivate the zinc-dependent endopeptidase “a disintegrin and metalloproteinase 17” (ADAM17), which is responsible for the release of tumour necrosis factor-α (TNF-α), a therapeutic target in inflammatory diseases. Zinc-chelating peptides derived from whey and rye secalin proteins inhibited ADAM17 enzymatic activity up to 93 and 70%, respectively. Moreover, the secalin peptides quenched ADAM17 fluorescence emission by static mechanism. Molecular docking revealed that the secalin peptides interacted with ADAM17 via zinc cofactor coordination, hydrogen bonds, and hydrophobic interactions. These findings demonstrate that food-derived zinc-chelating peptides are potential anti-inflammatory therapeutic agents.
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CHAPTER 1 INTRODUCTION

1.1 THESIS OVERVIEW

Given the high global prevalence and incidence of chronic health conditions, such as cardiovascular diseases and diabetes, there is an increased interest on the use of natural resources against human diseases and in general health promotion. Such natural agents include dietary components, which can be utilized as therapeutic agents in form of functional foods or nutraceuticals to complement the conventional drug therapies. Moreover, dietary factors greatly contribute to the etiology and exacerbation of many chronic diseases, and disease risks can be modified through dietary alterations (Klop, Elte, & Cabezas, 2013; Key et al., 2004; Steyn et al., 2004; Shikata, Ninomiya, & Kiyohara, 2013). Furthermore, therapies of natural origin are thought to be devoid of side effects or possess low risk, making them suitable for long-term administration compared to their synthetic counterparts.

Peptides derived from food proteins are dietary components that have promising therapeutic application beyond basic nutrition, due to their health-promoting physiological effects including antioxidative, anti-inflammatory, antihypertensive, anticancer, and lipid-lowering (Korhonen & Pihlanto, 2006; Hartmann & Meisel, 2007). Moreover, food peptides or protein hydrolysates are active ingredients of over 10 functional foods on the market (Korhonen, 2009). Bioactive peptides have been shown to not only exhibit single biological effects but also participate in diverse physiological processes, thereby making them multifunctional bioactive agents (Udenigwe & Aluko, 2012). An intrinsic property of peptides that can promote their multiple biological functions is their ability to form metal
coordinate complexes, notably divalent metals such as Zn$^{2+}$, Fe$^{2+}$, Cu$^{2+}$ and Ca$^{2+}$ (Guo et al., 2014), which are physiologically relevant. For instance, a given peptide can sequester Fe$^{2+}$, thereby rendering the metal unavailable for microbial growth and hydroxyl radical-mediated oxidative reactions, and hence serves as both antioxidative and antimicrobial agent, respectively (Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008; Elias, Kellerby, & Decker, 2008). Thus, metal-chelating peptides can be used as therapeutic agents to control and inactivate aberrant physiological processes whereby metal ions play key roles. This project was initiated to explore food protein-derived zinc-chelating peptides as bioactive dietary components of physiological relevance in human nutrition and inflammation.

Zinc is an essential micronutrient with established critical roles in several biological metabolic processes affecting human growth and development such as synthesis of nucleic acid, cell division, and gene expression (Hambidge, 2000). Zinc functions basically as a structural and functional component of cellular proteins, including over 300 metalloenzymes, transcription factors, and hormones (McCall, Huang, & Fierke, 2000). Therefore, zinc is required to be bioavailable in the right amount for the proper functioning of these metabolic activities. Zinc bioavailability is mostly dependent on its dietary absorption; however, zinc absorption can be impeded by the presence of certain components within food matrices. Notably, myo-inositol hexakisphosphate (phytate), found in plant-based foods, forms insoluble complexes with zinc, and hence renders the metal unavailable for intestinal absorption (Kumar, Sinha, Makkar, & Becker, 2010). Moreover, recovering zinc from such complexes is challenging, given the lack of phytase (phytate-hydrolyzing enzyme) in the gut (Lönnerdal, 2000). Dietary zinc absorption can be
enhanced by food supplementation with inorganic zinc salts; however, these salts tend to alter food sensory properties (Salgueiro et al., 2002), thereby making such foods unacceptable for consumption.

However, the zinc-chelating property of food peptides can be exploited to deliver dietary zinc. Peptides possess metal ligands, such as histidine, aspartate, cysteine, glutamate, and serine (or phosphorylated) that can form soluble zinc complexes, and hence prevent the metal complexation with phytates. Thus, food peptides have the potential to enhance the accessibility of zinc from foods, and many zinc-chelating peptides have been identified from different food proteins (Udechukwu et al., 2016). Zinc complexes of peptides intended for nutritional purposes are required to be stable during transit in the gastrointestinal tract, and the stability of the complexes can be affected by peptides’ structures. Metal chelation by peptides is dependent on their structural attributes particularly net surface charge, which depends on the pH of their environment (Elias et al., 2008). Peptides possess a net negative charge at neutral-alkaline pH, and hence can attract and form stable metal complexes. Conversely, the complex formation and stability is impaired at acidic pH due to net positive charges accrued at the peptides’ surface. Thus, zinc-peptide complexes are likely to dissociate at the acidic pH of the stomach, and this can predispose zinc to its absorption inhibitors (Miquel & Farre, 2007). On the other hand, due to their strong metal ligands, peptides can form strong zinc complexes, which is indeed desirable for gastric stability; however, can the strong affinity of these peptides for zinc decrease zinc accessibility from their complexes for intestinal absorption? To date, there is a dearth of information on how the structures of peptides can influence both the stability of their zinc complexes and the release of the bound metal for absorption. Therefore, the
investigation of structure-stability/release relationship of zinc-peptide complexes is one of the novel studies in this project.

As mentioned earlier, zinc maintains the structural and functional integrity of many endogenous proteins, and needs to be available for the activation of many cellular processes. However, certain physiological processes can be over-activated in the presence of zinc, leading to health-detrimental effects. Such biological processes include inflammation, in which zinc has been identified to possess critical functions. Inflammation is an indispensable body defense mechanism against noxious substances, and is mediated by extracellular signaling molecules known as cytokines, which are classified as either pro- or anti-inflammatory, depending on their role in inflammation (Dinarello, 2000). Inflammatory response usually lasts for a short time, and successfully restores tissue homeostasis; however, inadvertent damages can be inflicted on the host when inflammation becomes chronic (Soehnlein & Lindbom, 2010). The adverse effects of inflammation stem from uncontrolled response by pro-inflammatory cytokines, particularly tumour necrosis factor (TNF)-α. TNF-α has been identified as the key mediator of inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, diabetes and atherosclerosis, and hence a prime target of anti-inflammatory therapeutic compounds (Silva, Ortigosa, & Benard, 2010).

One of the therapeutic strategies for controlling the pro-inflammatory response of TNF-α is preventing its activation and release into the blood stream. TNF-α is a 26 kDa membrane protein that exists as an inactive protein (pro-TNF-α) (Palladino, Bahjat, Theodorakis, & Moldawer, 2003). However, it is activated to a 17 kDa soluble protein via extracellular domain cleavage by a zinc-dependent enzyme, “a disintegrin and
metalloproteinase 17” (ADAM17), also known as TNF-α converting enzyme (TACE) (Black et al., 1997). Thus, ADAM17 has been implicated as a therapeutic target in inflammatory diseases, and its enzymatic activity can be regulated or inhibited by chelation of the zinc cofactor at its catalytic site, which is involved in substrate catalysis as well as proper structure formation. Through molecular modelling, several synthetic compounds, featuring zinc-chelating ligands, were shown to interact with ADAM17 zinc cofactor as a mechanism of their ADAM17 inhibitory activity (Murmkar, Giridhar, & Yadav, 2013). Despite their preclinical efficacy, the drug leads have not been validated for therapeutic usage, due to lack of clinical efficacy and toxicity (Moss, Sklair-Tavron, & Nudelman, 2008). However, the zinc-chelating property of natural agents, such as food peptides, can be exploited to mitigate ADAM17 extracellular shedding activity for controlling aberrant TNF-α-mediated inflammatory response. Thus, exploring food-derived zinc-chelating peptides as ADAM17 inhibitors is an integral and a novel research that was executed in this project.

1.2 THESIS OBJECTIVES AND ORGANIZATION

Three studies were conducted in this project. The first study focused on elucidating the effect of the structural properties of whey protein-derived peptides on the gastric stability and dialyzability of their zinc complexes. The second study evaluated the physiological relevance of the zinc-chelating property of food peptides by investigating the ADAM17 inhibitory capacity of zinc-chelating peptides. The peptides were derived from one of the whey protein hydrolysates produced in the first study. Finally, the third study was prompted by the results of the second, and was conducted to understand the molecular interactions
of food peptides with ADAM17. However, the peptides were derived from rye secalin proteins, other than whey.

This thesis is organized in a manuscript format, and consists of six chapters, including the present chapter. Chapter 2 is the literature review, and is divided into two parts; part 2A is a published paper, and reviews the literature on the prospects of enhancing zinc bioavailability with food-derived zinc-chelating peptides; part 2B is a manuscript intended for publication, and reviews the synthetic ADAM17 inhibitors as therapeutic advances to mitigating inflammatory diseases. Chapter 3 is an original manuscript that has been submitted for peer-review, and addresses the first study objective. Similarly, chapters 4 & 5 are original manuscripts submitted for peer-review, and address the second and third study objectives, respectively. Then, the thesis is concluded in chapter 6, followed by the appendix.

1.3 REFERENCES


CHAPTER 2 LITERATURE REVIEW

PART 2A PROSPECTS OF ENHANCING DIETARY ZINC BIOAVAILABILITY WITH FOOD-DERIVED ZINC-CHELATING PEPTIDES

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2A.1 ABSTRACT

Zinc is an essential micronutrient that strongly influences human health and nutrition through its involvement in several biological processes. Zinc functions as structural and functional component of many transcription factors and enzymes that regulate cell growth, gene expression, and immune response, and its deficiency can lead to retarded growth and impaired immune functions. The physiological functions of zinc are dependent on its bioavailability in tissues, which in turn depends on intestinal absorption of dietary zinc. The presence of dietary fibre and phytates impedes intestinal zinc absorption, as they can form insoluble complexes with zinc, decreasing its bioavailability. Peptides derived from food proteins can enhance zinc absorption and bioavailability. Peptides that contain amino acid residues such as cysteine, histidine, serine, aspartate, and glutamate can chelate
divalent metals, including zinc, forming soluble metal coordinate complexes. The structure-function relationship of zinc-chelating peptides and the stability of the peptide-metal complexes to gastrointestinal digestion are critical to their relevance in human nutrition and health promotion.

### 2A.2 INTRODUCTION

The importance of zinc in many life forms has been a subject of interest in the past few decades. Zinc is an essential micronutrient whose role extends from nutritional to the control of key biological processes that affect human growth and development. In order to execute its biological functions, zinc is required to be bioavailable in the right amount, primarily through the absorption of dietary zinc and its transport into cells. However, some well-characterized factors can compromise zinc bioavailability. Although the amount of bioavailable zinc can be decreased in some clinical conditions in which the expression of zinc transporter is down-regulated (DeNiro & Al-Mohanna, 2012), dietary zinc absorption is considered to be the major factor affecting zinc bioavailability.

Zinc absorption can be impeded by the presence of some food matrix components. Myo-inositol hexakisphosphate (phytate) constitutes the major dietary impediment to zinc absorption. This is due to the propensity of phytate to form insoluble complexes with zinc, which renders the metal unavailable for intestinal uptake (Kumar et al., 2010), although this can also be beneficial in limiting absorption and decreasing toxicity during divalent metal overload. Furthermore, the lack of phytate-hydrolyzing enzyme, phytase, in the human gastrointestinal tract makes it challenging to recover free zinc from such complexes (Lönnerdal, 2000). Moreover, the anti-nutritional effect of phytate can lead to zinc
deficiency. Consumption of foods containing large amounts of iron, calcium, and copper also affects zinc absorption due to their competition for common divalent metal-ion transporters and carrier proteins (Cámara & Amaro, 2003; Shenkin, 2008).

Considering the adverse effects of zinc deficiency, it is imperative to improve zinc absorption and bioavailability for optimum health. Food products formulated with zinc salts, e.g. zinc sulphate, have the promise for use as an intervention strategy, but the supplementation can lead to alteration of the physical and sensory properties of the food (Salgueiro et al., 2002). Moreover, the use of multi-mineral supplements is limited by the instability of the complex and their ability to trigger intestinal disorders (Guo et al., 2014). Another measure adopted to mitigate poor zinc absorption involves the degradation of phytate using commercial phytase, or through processing methods such as fermentation, milling, and leavening (R S Gibson, Yeudall, Drost, Mtitimuni, & Cullinan, 1998). However, taking into account that the outlined methods are not adequate for processing all phytate-containing foods, the latter strategy appears to be insufficient in enhancing zinc bioavailability. Thus, there is a need to identify alternative approaches for improving dietary zinc absorption and bioavailability.

Food protein-derived peptides are one of the dietary compounds known to have multiple health benefits. Among the many bioactivities exhibited by peptides is their capacity to form coordinate complexes with divalent metals such as Zn\(^{2+}\), Fe\(^{2+}\), Cu\(^{2+}\) and Ca\(^{2+}\) (Guo et al., 2014). This property enables peptides to function in many respects such as in the prevention or termination of free radical-mediated oxidative reactions facilitated by pro-oxidant transition metals, e.g. Fe\(^{2+}\) and Cu\(^{2+}\) (Elias et al., 2008). The metal-chelating capacity of peptides can also be relevant in human nutrition, particularly for use in the
delivery of dietary metals such as zinc. Hence, peptides can serve as a delivery agent for enhancing dietary mineral bioavailability and minimizing the risk or occurrence of mineral deficiencies. The metal-chelating property of peptides can be attributed to the structural diversity of their constituent amino acid residues. Therefore, understanding the structural requirements for metal chelation by peptides will facilitate their application as dietary mineral carriers. This review discusses the zinc-chelating properties of food peptides in the context of their structure-function relationship and stability of their metal coordinate complexes.

2A.3 OVERVIEW OF THE PHYSIOLOGICAL FUNCTIONS OF ZINC

Zinc is an essential micronutrient with diverse biological functions, and it influences a wide range of physiological processes. Many proteins require zinc either for their activities or stabilization of their structures. Zinc has been identified as the catalytic cofactor for over 300 metalloenzymes (McCall et al., 2000). Many transcription factors and hormones bind zinc in order to form a stable structure (Prasad, 1998). The influence of zinc on most biological processes is attributed to its participation in basic cellular events such as cell division, proliferation, and differentiation, synthesis of nucleic acids, and gene expression (Michael Hambidge, 2000). The catalytic activities of polymerases in DNA replication and transcription are zinc-dependent (Slater, Mildvan, & Loeb, 1971; Shankar & Prasad, 1998). Moreover, impaired physical growth and congenital abnormalities such as hypogonadism are major signs of marginal zinc deficiency (Prasad, 2012). Zinc is critical to the integrity of both innate and adaptive immune systems, due to its significant role in the growth, development, and activation of immune cells such as macrophages, natural killer cells, and
B and T-lymphocytes (Shankar & Prasad, 1998). The immune system is sensitive to zinc deficiency as abnormal low levels of zinc can lead to alterations in cell-mediated immune response. For instance, the activity of cultured natural killer cells was found to be suppressed in the presence of O-phenanthroline, a known strong metal chelator, but was almost completely restored with zinc supplementation (J. I. Allen, Perri, McClain, & Kay, 1983). In addition, downregulated expression of cytokines such as IL-1, IL-2, IL-4, IF-γ, and decreased activation of TNF-α, have been observed in zinc-deficient states (Moulder & Steward, 1989; Salas & Kirchner, 1987). These effects can negatively impact B and T-lymphocytes, as their activities are also influenced by these cytokines (Shankar & Prasad, 1998). Health conditions such as dermatitis, pneumonia, and diarrhea arising from altered immune resistance to infections are also clinical manifestations of severe zinc-deficient states (Prasad, 2012). The biological functions of zinc include a support of the body’s antioxidant defense systems; for instance, zinc is a component of superoxide dismutase, which is one of the body’s antioxidant enzymes (Mertens et al., 2015). Despite these important functions, excessive intake of zinc is associated with health implications such as severe abdominal cramping and diarrhea (Maret & Sandstead, 2006). However, considering the critical role of zinc in health promotion and the fact that the body has limited zinc reserves (Cummings & Kovacic, 2009), adequate zinc intake and absorption is crucial for ensuring physiological zinc homeostasis.

2A.4 STRATEGIES FOR ENHANCING DIETARY ZINC ABSORPTION AND BIOAVAILABILITY

The need to enhance dietary zinc intake dates back to the 1960s when the adverse effects of zinc deficiency on human growth were noted (Prasad, Miale, Farid, Schultet, &
Sandstead, 1963). The prevalence of many diseases associated with zinc deficiency, such as diarrhea, malaria, measles and pneumonia, accounts for 4% of morbidity and mortality in children (Hess, Lönnerdal, Hotz, Rivera, & Brown, 2009; Haider & Bhutta, 2009; Penny, 2013). The risk factors to zinc deficiency include: inadequate zinc intake, poor zinc absorption, and excessive zinc loss in disease conditions such as diabetes (Rosalind S. Gibson, 2012). However, inadequate zinc intake and poor absorption are considered the predominant factors leading to zinc deficiency. The low income populations of developing countries are thought to be the most vulnerable to dietary zinc deficiency due to their inability to afford foods (such as meat and fish) that contain zinc in its absorbable form (Maret & Sandstead, 2006). Such group of people tend to consume mostly plant-based foods, which are likely to contain unavailable zinc due to their phytate content. Furthermore, dietary preferences for exclusively plant-based foods can be another risk factor for zinc deficiency (Maret & Sandstead, 2006). Consequently, food fortification or supplementation have been implemented as means of combating dietary zinc deficiency. The use of inorganic zinc salts, such as zinc sulphate and zinc oxide, was first adopted; however, to date, the outcome has been contradicting and unsatisfactory (Haase, Overbeck, & Rink, 2008). Although supplementation in some cases ameliorates the disorders arising from zinc deficiency, many intervention studies have attributed their ineffectiveness to the poor solubility of the zinc salts, such as zinc oxide, thereby leading to poor absorption (Haase et al., 2008; L. H. Allen, 1998). Secondly, zinc salts can alter sensory and physical properties of foods, which can then affect consumer acceptability of the food (Salgueiro et al., 2002). Multi-mineral supplementation remains unsuccessful, due to the interactions among various nutrients, resulting in reduced bioavailability of some nutrients such as zinc,
to the benefit of others such as iron (Guo et al., 2014). There is interest in the use of amino acids such as histidine and methionine, which can form zinc complexes, to enhance zinc absorption (Wedekind, Hortin, & Baker, 1992). However, methionine has a weak interaction with zinc leading to poor zinc absorption (Guo et al., 2014; Hempe & Cousins, 1989). Attention has been given to other metal chelators, such as ethylenediaminetetraacetic acid, since they can form soluble complexes with zinc; however, results have not been consistent, as decreased zinc absorption has also been observed (Lönnerdal, 2000). The challenges surrounding these means of zinc supplementation have created the need to explore the zinc-chelating property of other food factors in improving zinc bioavailability.

2A.5 FOOD PROTEIN-DERIVED ZINC-CHELATING PEPTIDES

Dietary zinc absorption is thought to be facilitated by protein-rich foods, as proteins can form soluble zinc complexes thereby preventing the complexation of phytates with zinc (Lönnerdal, 2000). However, the improvement of zinc absorption depends on the type of protein as some food proteins, such as casein, have been shown to negatively affect zinc absorption (Sandstrom, Cederbblad, & Lonnerdal, 1983). It was thought that zinc could be entrapped in casein curds, formed under gastric pH, which may be incompletely digested in the intestine thereby decreasing zinc absorption and increasing its fecal excretion (Shen, Robberecht, Dael & Deelstra, 1995). Zinc-binding protein fragments can be used in formulating protein-based zinc supplements or fortificants. The relatively smaller sizes of peptides can enhance their solubility, and digestibility in releasing the bound zinc compared to intact proteins. In addition, iron bioavailability has been improved when
protein hydrolysates are used as delivery agents instead of the intact protein (Hurrell, Lynch, Trinidad, Dassenko, & Cook, 1989). Hydrolysis of proteins releases the peptides and can increase the accessibility of reactive amino acid residues to target systems (Elias et al., 2008). Many zinc-chelating peptides have been identified from different food protein hydrolysates. Tables 1 and 2 provide lists of the food proteins, protease used for their hydrolysis, and sequences of the resulting zinc-chelating peptides. Most of the peptides were isolated from plant proteins and are mainly of low molecular sizes, which can enhance their solubility in aqueous solutions. Moreover, di- and tripeptides can be co-transported intact with bound zinc into the enterocytes via peptide transporter (PepT1) if they arrive at the intestine (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011).

2A.5.1 PRODUCTION AND PURIFICATION OF ZINC-CHELATING PEPTIDES FROM FOOD PROTEINS

Usually, production and purification of zinc-chelating peptides from food proteins follow bioassay-guided procedures (Fig. 1), with a first step of selecting the protein of choice. The food protein source can be selected based on knowledge of their natural property of binding zinc or other divalent metals, or it could be in a bid to valorize underutilized foods or by-products (Udenigwe & Aluko, 2012). Then, the selected protein is hydrolyzed to produce the hydrolysates using the desired protease, which can be of either narrow or broad cleavage specificity. Purification of zinc-chelating peptides from food protein hydrolysates can be accomplished by highly selective chromatographic techniques such as immobilized metal affinity chromatography (IMAC). IMAC is used for purification of compounds, such as proteins, that have an affinity for immobilized metal ions present on the
chromatographic column. The compounds form specific reversible complexes with the metal ions, and are then retained on the column (Block et al., 2009). Changing elution solvent parameters such as ionic strength or pH can weaken the interaction between the complexes, leading to elution of the proteins (Gaberc-Porekar & Menart, 2001). Moreover, the use of IMAC in protein purification can result in high sample recovery because the elution conditions utilizes mild and non-denaturing solvents (Seun, Lin, & Hsu, 2004). In some cases, the zinc-binding peptide fractions obtained from IMAC are subjected to further purification by other techniques, such as size exclusion chromatography or reverse-phase HPLC in order to obtain pure peptides. Following their purification, the sequence of zinc-chelating peptides is often determined using mass spectrometry techniques, such as MALDI-TOF/MS or LC/MS/MS. The identification of the zinc-chelating peptides can provide insight on the amino acid residues that can be involved in zinc-coordination, and facilitates the study of their structure-function relationships.

2A.5.2 EFFECT OF ENZYMATIC HYDROLYSIS ON THE ZINC-BINDING CAPACITY OF PEPTIDES

While protein hydrolysis can produce peptides with improved physicochemical properties and bioactivity, the conditions employed in the hydrolysis can influence the zinc-chelating capacity of the resulting peptides. For instance, the nature of proteases used for hydrolysis can affect the functionality of peptides due to their different cleavage specificities (Tavano, 2013). Zinc-chelating peptides have been derived with a wide range of proteases, including gastrointestinal proteases (pepsin and trypsin), plant proteases (papain), and microbial proteases (Alcalase and Flavourzyme). Serine proteases, Alcalase and trypsin, have released peptides with the highest zinc-chelating capacity irrespective of the protein
substrate (Wang, Zhou, Tong, & Mao, 2011; Wang, Li, & Ao, 2012; Wang, Li, & Ao, 2012). Chen et al. reported a decline in the zinc-binding capacity of oyster protein-derived peptides after 90 min of 5 h hydrolysis (Chen et al., 2013). Similar trends have been observed in peptides derived from rapeseed and sesame proteins (Wang, Li, & Ao, 2012; Xie et al., 2015), and have been attributed to loss of the exposed metal-binding sites during extensive peptide bond cleavage. Moreover, the sulfhydryl groups of cysteine residues, which are known metal-binding sites, can interact with some matrix components during protein hydrolysis leading to their depletion (Mohan, Udechukwu, C. K. Rajendran, & Udenigwe, 2015). These potential limitations need to be considered when developing zinc-chelating peptides for dietary zinc delivery.

2A.6 STRUCTURE-FUNCTION RELATIONSHIP OF ZINC-CHELATING PEPTIDES

The bioactivity of peptides can be dependent on their structural composition, arrangement, and size. Peptides are composed of amino acid residues such as histidine, aspartate, glutamate, cysteine, and serine, whose side chains can serve as ligands for binding transition metals. Moreover, the structural motif of zinc-finger proteins, with high affinity for physiological zinc, has been identified to contain cysteine and histidine residues as the zinc-coordinating ligands (Krishna, 2003). The distinct structures of these amino acid residues suggest that metal-peptide complexes can be formed by electrostatic interactions, coordinate covalent bonds, or a combination of both bonding forces. As shown in Table 2, many of the identified zinc-chelating peptides contain one or a combination of these amino acid residues; however, the peptides exhibited varying zinc-binding capacities due to their different structures. In addition, the N- and C-terminals of peptides can also participate in
the binding interaction (Guo et al., 2014). It should be noted that the availability of these sites for metal-binding is pH-dependent, as peptides possess either a net positive, negative, or neutral surface charge at different pH, which can promote or hinder electrostatic interactions.

Caseinophosphopeptides (CPPs) from milk casein are known for their metal-chelating capacity and have been commercialized in many milk-based infant formula as dietary mineral carriers (Miquel & Farre, 2007). Many of the identified CPPs usually contain a unique cluster sequence of three phosphorylated serine residues and two glutamate residues [Ser(P)-Ser(P)-Ser(P)-Glu-Glu], which is regarded as the major metal-chelating site (Miquel & Farre, 2007). Moreover, one mole of a CPP, Glu-Ser(P)-Ile-Leu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu, can chelate six zinc ions (Reynolds, 1993). In the past few decades, studies on zinc-chelating peptides from food proteins have revolved around CPPs. However, recent efforts are focusing on identifying zinc-binding peptides from other food proteins. A hexadecapeptide, His-Leu-Arg-Gln-Glu-Glu-Lys-Glu-Glu-Val-Thr-Val-Gly-Ser-Leu-Lys, isolated from oyster protein hydrolysates was found to contain prominent metal-chelating sites including the imidazole group of C-terminal histidine and the carboxylate anion of the four glutamate residues (Chen et al., 2013). The presence of these amino acid residues suggests a possible complex formation via coordinate covalent bonds, electrostatic interactions, or both. Fourier transform infrared (FTIR) spectroscopy revealed the existence of binding interaction via carboxylate anion and amino nitrogen atoms (Chen et al., 2013). However, it is unclear whether the carboxylate anion that participated in the complex formation was from the glutamate residues or peptide C-terminal. Likewise, the
location of the amino nitrogen is unclear, although it was reported to be characteristic of an amide, which includes the peptide bonds and glutamine residue side chain.

Plant protein-derived peptides have also displayed zinc-chelating capacity. For instance, the peptides, Leu-Ala-Asn, Ser-Met, and Asn-Cys-Ser, identified in sesame protein hydrolysate exhibited 49%, 56% and 73% zinc-chelating capacity, respectively at estimated concentration of 20 mM (Wang et al., 2012). Despite the presence of Asn in Leu-Ala-Asn and Asn-Cys-Ser, the wide gap in their zinc-binding capacity can be due to the presence of cysteine and serine in the latter, which are better metal-chelators than leucine and alanine. Varying zinc-chelating capacity of peptides due to their diverse structural composition can be clearly observed from the peptides, Ser-Met and Asn-Ser-Met, identified in different proteins (Wang, Li, & Ao, 2012; Xie et al., 2015). The peptides have a similar composition except for the presence of asparagine residue in Asn-Ser-Met, which exhibited higher zinc-chelating capacity than Ser-Met (Xie et al., 2015). Possibly, the asparagine residue in Asn-Ser-Met provided an extra zinc-chelating site, giving rise to coordination of more zinc ions compared to Ser-Met. Divalent metal-chelating ligands do not normally include cationic factors in their structure, to limit electrostatic repulsion. Thus, it is not understood how the cationic peptides, Ala-Arg (34%) and Gly-Lys-Arg (54%), identified from the rapeseed protein hydrolysate were able to chelate zinc (Xie et al., 2015). In addition, the relatively more cationic peptide (Gly-Lys-Arg) had more zinc-chelating capacity than Ala-Arg of a weaker cationic strenght. This indicates that factors other than the side chain amino acid residues are responsible for the zinc-chelating capacity of the peptides. However, the carbonyl group of peptide bonds participates in zinc-coordination (Wang, Li, & Li, 2014). Similarly, X-ray diffraction of a Cu\(^{2+}\) complex of
Phe-Leu revealed that the divalent metal was coordinated by the peptide bond and the C-terminal carboxylate anion, providing more structural sites for stabilizing metal complexes (Sanchiz et al., 2006).

In addition to the peptide composition, the bioactivities of some peptides are enhanced when certain amino acid residues are located either at the N- or C-terminal position (Ismail & Gu, 2010). The presence of histidine residues at the N-terminal position potentiates the metal-chelating capacity of peptides compared to when it is located at the C-terminal (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998). Similarly, the zinc-chelating capacity of the peptide, His-Asn-Pro-Asn-Pro-Gly-Leu-Pro-Tyr-Ala-Ala (91%), derived from wheat germ protein hydrolysate is greater than that of Asn-Ala-Pro-Leu-Pro-Pro-Leu-Lys-His (15%), despite the presence of histidine in both peptides (Megias, Pedroche, Yust, Giron-Calle, Alaiz, Millan, & Vioque, 2007). Aside from the amino acid sequence of the peptides, another plausible explanation to this observation could be the molecular size of the peptides, which is likely to influence their metal-chelating activity (Wang, Li, Wang, & Xie, 2015). Although the mechanism of zinc-binding of peptides has not been clearly investigated, spectroscopic studies of zinc-peptide complexes have shown that the carboxylate anion, sulfhydryl groups of cysteine residues, hydroxyl groups of serine residues, and carbonyl and imino groups of peptide bonds or asparagine are possible zinc coordinating sites (Wang et al., 2014).

2A.7 STABILITY OF ZINC-CHELATING PEPTIDES TO GASTROINTESTINAL DIGESTION
Unlike peptides whose bioactivities require that they should be absorbed and reach the target site intact, further cleavage of metal-bound peptides is desirable in the intestine to release the bound metal for absorption. However, the peptides need to be resistant to cleavage by gastric proteases. This is due to the tendency of their coordination complexes to dissociate as a result of the acidic pH of the stomach, especially when electrostatic interactions are involved in the complex formation. The dissociation of peptide-zinc complexes at acidic pH was demonstrated in the studies of Wang et al., where zinc bound to yak casein hydrolysate had a much greater release rate at a pH of 2 than at slightly acidic and alkaline pH (Wang et al., 2011). The peptides, Ser-Met and Asn-Cys-Ser, derived from sesame protein hydrolysate were resistant to simulated peptic digestion but were further hydrolyzed by pancreatin (Qiao, Zhu, Wang, & Bin, 2006), which can possibly release the bound zinc for intestinal absorption. High molecular weight peptides with high zinc-chelating capacity retained about 85% of their activity after simulated gastrointestinal digestion (Chen et al., 2013). On one hand, this can be advantageous as such peptides can help to prevent the formation of insoluble zinc complexes with inhibitors such as phytates in the gastrointestinal tract. Conversely, the peptides can hinder zinc absorption and bioavailability if their zinc complexes are not absorbed, thereby leading to fecal excretion of zinc. Moreover, the large molecular weight of peptides can decrease intestinal zinc or iron absorption (Sato, Noguchi, & Naito, 1986). In addition, the weak affinity of CPPs for zinc is thought to be favourable for zinc delivery, as zinc can be gradually released in the intestinal lumen for absorption (Etcheverry, Wallingford, Miller, & Glahn, 2005). Therefore, the stability of zinc complexes of peptides to gastrointestinal digestion should be such that the complexes are strongly resistant to gastric conditions but can be dissociated
to release the bound zinc on arrival in the duodenum. There is a dearth of information on
detailed structure-release properties of zinc-chelating peptides intended for nutritional
application.

2A.8 EVIDENCE OF ENHANCED ZINC ABSORPTION AND
BIOAVAILABILITY WITH FOOD PEPTIDES

The dietary form of zinc can influence intestinal zinc absorption. Several studies have
demonstrated that zinc bound to peptides is absorbed more than that in the form of
inorganic zinc salts. The effects of CPPs on zinc bioavailability has been extensively
evaluated both in vitro and in vivo, and reviewed by Miquel and Farre (Miquel & Farre,
2007). Despite the prospects of using these peptides in enhancing zinc bioavailability, some
studies have reported lack of positive effects. This can be attributed to different factors
including the food matrix, methods for zinc measurement, CPP concentration, and the
amount of zinc absorption inhibitors such as phytates, iron, and calcium present in the
administered diet (Miquel & Farre, 2007). For instance, an aqueous matrix may provide
better zinc absorption than a solid matrix (Lönnerdal, 2000). This was demonstrated in the
jejunum of female Sprague-Dawley rat pups where zinc absorption from an aqueous
solution of CPP-zinc complex was 60% higher than from zinc sulphate solution (Pérès et
al., 1998). Some studies have shown the effect of CPPs on zinc bioavailability to be dose-
dependent, as a sharp decrease in both zinc-binding to CPP and uptake by Caco-2 cells was
observed with increasing CPP concentration from 14 to 72 µM (Hansen, Sandstrom, &
Lonnerdal, 1996). Thus, it is very important to monitor the concentration of CPPs to be
administered since high concentration can reduce the amount of zinc absorbed due to
saturation of intestinal zinc transporters (Lönnerdal, 2000). Furthermore, standard methods
of zinc detection should be established to ensure accurate comparison of results. Besides CPPs, a few peptides from other food proteins have been evaluated for their effect on zinc bioavailability. In Caco-2 cells, zinc was readily absorbed from a peptide derived from wheat germ protein than in the form of zinc sulphate (Zhu et al., 2015). Zinc bound to yak casein hydrolysate was readily released under in vitro intestinal pH than zinc in form of zinc acetate, and this suggests a possible enhancement of zinc absorption and bioavailability (Wang et al., 2011). Considering that the efficacy of current zinc supplements on zinc bioavailability is not well established in vivo, the effect of zinc-chelating peptides needs further evaluation in human clinical trials in order to establish their clinical relevance for nutritional purposes. Such study will also provide information on the amount of peptide-zinc chelate to use to avoid overload, considering the negative effects of excess zinc on the immune system (Ibs & Rink, 2003).

2A.9 CONCLUSION AND FUTURE DIRECTIONS

The zinc-chelating capacity of food protein-derived peptides has provided a promising means of mitigating zinc nutritional challenges in humans. Food peptides are natural and appear to possess low risk of side effects, which can offer them an edge over conventional zinc compounds used as dietary supplements. Although there are some correlations between the structure and zinc-chelating capacity of peptides, their structure-function relationship is still unclear. Most studies have focused on identifying zinc-chelating peptides from food proteins with little attention given to the underlying zinc-binding chemical mechanisms. Therefore, future studies should endeavor to elucidate the
mechanism of formation of the peptide-zinc complexes. Studying the mechanism will provide insights on (i) the particular ligands participating in zinc chelation, and (ii) the chemical nature of the peptide interaction with zinc. For instance, if a peptide has three possible zinc-binding sites, it would be necessary to know if zinc binds separately to the individual sites or if the binding is a synergistic action of the different binding sites, inter- or intramolecularly. In addition, it is imperative to determine the binding affinity of zinc-chelating peptides in order to gain insight on the strength of their complexes. This is important because the zinc-peptide complexes should be at least stable for effective zinc delivery. Understanding of their binding mechanisms will facilitate the commercialization of peptides as either zinc supplements or zinc-fortified functional foods. Furthermore, this would help in determining the amount of peptides for use in food formulation in order to avoid excessive zinc intake, due to co-transportation with the chelating peptides, and associated deleterious effects. Moreover, the efficiency of intestinal zinc absorption can decrease with zinc overload due to saturation of the intestinal zinc transporter. Moving forward, human clinical trials will be necessary to confirm the efficacy of the food peptides in enhancing zinc bioavailability or attenuating the adverse effects of zinc deficiency.

2A.10 REFERENCES


### Table 2A.1. Zinc-chelating food protein hydrolysates

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protease (hydrolysis duration, h)</th>
<th>Concentration (%, w/v)</th>
<th>Zinc-chelating capacity (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yak casein</td>
<td>Papain (6)</td>
<td>n/a</td>
<td>39</td>
<td>Wang et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Pepsin (6)</td>
<td></td>
<td>45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trypsin (6)</td>
<td></td>
<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alcalase (6)</td>
<td></td>
<td>59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flavourzyme (6)</td>
<td></td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Rapeseed</td>
<td>Alcalase (6)</td>
<td>2.0</td>
<td>85</td>
<td>Xie et al., 2015</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>Flavourzyme (6)</td>
<td>1.0</td>
<td>55</td>
<td>Zhu et al., 2015</td>
</tr>
<tr>
<td></td>
<td>Papain (6)</td>
<td></td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alcalase (6)</td>
<td></td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>Oyster</td>
<td>Pepsin (5)</td>
<td>0.8</td>
<td>5.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Chen et al., 2013</td>
</tr>
</tbody>
</table>

<sup>a</sup>Represents µg of zinc chelated per mg protein hydrolysate; n/a, concentration was not reported

### Table 2A.2. Zinc-chelating peptides and their food protein sources

<table>
<thead>
<tr>
<th>Food protein source</th>
<th>Protease used</th>
<th>Peptide sequence</th>
<th>% Zinc chelating capacity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oyster</td>
<td>Pepsin</td>
<td>His-Leu-Arg-Gln-Glu-Glu-Lys-Glu-Glu-Val-Thr-Val-Gly-Ser-Leu-Lys</td>
<td>n/a</td>
<td>Chen et al., 2013</td>
</tr>
<tr>
<td>Sesame</td>
<td>Trypsin</td>
<td>Leu-Ala-Asn Ser-Met Asn-Cys-Ser</td>
<td>49.0</td>
<td>Wang et al., 2012</td>
</tr>
<tr>
<td>Rapeseed&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Alcalase</td>
<td>Ala-Arg Gly-Lys-Arg Glu-Pro-Ser-His Asn-Ser-Met</td>
<td>34.7</td>
<td>Xie et al., 2015</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>Alcalase</td>
<td>Asn-Ala-Pro-Leu-Pro-Pro-Leu-Lys-His&lt;sup&gt;b&lt;/sup&gt; His-Ala-Pro-Asn-Pro-Gly-Leu-Pro-Tyr-Ala-Ala&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.2</td>
<td>Zhu et al., 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>56.1</td>
<td></td>
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<td></td>
<td>54.5</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>82.1</td>
<td></td>
</tr>
</tbody>
</table>

Peptide concentrations of 50 µM<sup>a</sup>, 9 µM<sup>b</sup>, and 8 µM<sup>c</sup>; n/a, % zinc-chelating capacity was not reported
Fig. 2A.1 Bioassay-guided purification and identification of zinc-chelating peptides from food protein hydrolysates
CHAPTER 2 LITERATURE REVIEW

PART 2B THERAPEUTIC APPROACH TO INFLAMMATORY-MEDIATED DISEASES: A REVIEW OF ADAM17 INHIBITORS

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2B.1 ABSTRACT

A disintegrin and metalloproteinase 17 (ADAM17) is an enzyme responsible for releasing the soluble form of membrane-anchored proteins. Its role includes the release of tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)), whose over-activation is implicated in the pathogenesis and progression of inflammatory diseases, thus making it an important target for anti-inflammatory therapies. Many therapeutic agents that strongly inhibit TNF-\(\alpha\) have been successfully developed. One approach for reducing TNF-\(\alpha\) level during inflammation is by targeting its activation pathway, including the inhibition of ADAM17 enzymatic activity. This has become a promising therapeutic approach since ADAM17 is overexpressed and activated in a number of life-threatening diseases. Despite the number of compounds that has been developed against ADAM17, none has passed the second phase of clinical trials.
for approval as a drug due to the challenge of producing desired physiological effects or lack of selectivity. This review discusses the structure-function relationship and selectivity of known ADAM17 inhibitors, and the prospects of their clinical application as anti-inflammatory agents.

2B.2 INTRODUCTION

The human immune system elicits an inflammatory response upon recognition of cell injury or foreign substances such as pathogens or chemical irritants. Inflammation is a non-specific response involving tightly regulated cascade of events that function to destroy the antigens and repair the damaged cells, thereby maintaining tissue homeostasis (Soehnlein & Lindbom, 2010). The inflammatory response is mediated by extracellular signaling molecules known as cytokines, which are classified as either pro- or anti-inflammatory, depending on their role in inflammation (Dinarello, 2000). A balanced activity of both groups of cytokines is crucial to achieving a successful inflammatory response (Palladino et al., 2003). Inflammation is an indispensable body defense mechanism; however, it can lead to inadvertent host damages when uncontrolled. The adverse effects of inflammation originate from dysregulated activities of pro-inflammatory cytokines, especially tumour necrosis factor (TNF)-α. Since its identification as the key mediator of inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, diabetes and atherosclerosis, TNF-α has received notable attention as a prime target of therapeutic compounds intended for use against inflammatory-mediated diseases (Silva et al., 2010).
Clinical approaches to disease treatment or management usually involve identification of the activated molecular signaling pathways and specific molecules with critical role in the disease pathogenesis or progression. Initial therapeutic strategies to inhibit TNF-α pro-inflammatory response in diseases were aimed at blocking its synthetic and signal transduction pathways. For instance, blockade of nuclear factor (NF)-κB (the transcription factor in TNF-α synthetic pathway) activation was viewed as a potential avenue since, in addition to TNF-α, it also upregulates the expression of other pro-inflammatory cytokines and mediators of inflammatory response (Palladino et al., 2003; Silva et al., 2010; K. Newton & Dixit, 2012). Moreover, US FDA approved the usage of bortezomib (Velcade™) as an adjunct therapy for multiple myeloma (Mateos & San Miguel, 2007); bortezomib is a peptide-based drug that prevents the activation of NF-κB by inhibiting 26S proteasome, which mediates the phosphorylation of IκB (inactive form of NF-κB) (Karin, 1999; Mateos & San Miguel, 2007). A milestone in the research on anti-TNF-α therapies was the discovery of etanercept (Enbrel™, a soluble TNF-α receptor II linked to Fc portion of immunoglobulin G), infliximab (Remicade™, a mouse-human chimeric anti-human TNF-α antibody), and adalimumab (Humira™, a human anti-human TNF-α antibody). These are protein-based drugs that effectively prevent TNF-α from transducing a signal by either serving as a decoy receptor (etanercept) or binding to it (infliximab and adalimumab), and have received US FDA approval for the treatment of inflammatory diseases such as rheumatoid arthritis and Crohn’s disease (Furst et al., 2001; Taylor, 2003). Their therapeutic usage has been remarkably successful; however, they are associated with serious health implications, notably increased incidence of re-activation of latent tuberculosis and high risk of lymphoma (Palladino et al., 2003; Furst et al., 2001).
Consequently, strict precautions are highly recommended before commencing treatment, thereby posing a restriction to their usage. Furthermore, their high cost and parenteral mode of administration, which is deemed inconvenient, also contribute to their clinical usage limitations (Shaw, Nixon, & Bottomley, 2000). Despite the shortcomings, the positive outcome of these therapies clearly outlines the value of TNF-α inhibition in immune-mediated disorders, and this has been a priority in the search for anti-inflammatory therapies. In addition, subsequent drug developments have sought both active and orally bioavailable molecules, which are desirable over the injectable anti-TNF-α therapies (Hasegawa, Takasaki, Greene, & Murali, 2001).

While alternative strategies are being sought to antagonize TNF-α, recent endeavors are focused on controlling its level in the blood by targeting its activation pathway. TNF-α is expressed as a 26-kDa inactive membrane-bound pro-TNF-α, which is then activated to a 17 kDa soluble protein via extracellular domain cleavage by the enzyme, a disintegrin and metalloproteinase 17 (ADAM17), also known as TNF-α converting enzyme (TACE) (Black et al., 1997). Inhibiting ADAM17 enzymatic activity is considered to be an effective upstream therapeutic approach for modulating TNF-α levels and forestalling its pro-inflammatory effects, which also include induction of the expression of other pro-inflammatory cytokines and mediators (Newton et al., 2001). To date, the research on ADAM17 inhibitors has yielded several compounds that effectively inhibit the enzyme. However, none of the inhibitors has been approved for therapeutic usage due to their lack of efficacy in clinical trials or risk of toxicity. In this review, we discuss the structure, function and potential of known ADAM17 inhibitors, with highlights on challenges and prospects of their clinical application as anti-inflammatory agents.
ADAM17 is a type 1 zinc-dependent transmembrane protein belonging to the ADAM family of metalloproteinases and the metzincin group of zinc metalloproteases (Seals & Courtneidge, 2003). The ADAM family of proteases function biologically in cell adhesion and ectodomain shedding of membrane-anchored proteins and their receptors (Gooz, 2010). The structure of ADAM17 consists of multiple domains (Fig. 1) with distinct functions (Edwards, Handsley, & Pennington, 2009). The prodomain consists of a free cysteine residue which coordinates the zinc cofactor present at the catalytic site via a cysteine switch mechanism, and hence keeps the enzyme in an inactive state (Joaquin Arribas & Esselens, 2009). Moreover, the isolated prodomain has been reported to selectively inhibit the enzyme (Gonzales et al., 2004). Apart from its inhibitory capacity, the prodomain also acts as a molecular chaperone to protect the enzyme from proteolytic degradation during transport in the secretory pathway (Milla, Gonzales, & Leonard, 2006). Following the prodomain is the metalloprotease or catalytic domain, which executes the most relevant physiological functions of the enzyme and is activated by the removal of the prodomain by furin-like pro-protein convertase (Endres et al., 2003). The catalytic domain consists of a highly conserved zinc-binding motif, HEXXHXXGXXH (X can be any amino acid residue) which coordinates zinc with the three histidine residues (Seals & Courtneidge, 2003). The disintegrin domain interacts with integrins, which are \( \alpha \) and \( \beta \) chains heterodimeric adhesion receptors with ligand-binding extracellular domain (Hynes, 2002; White, 2003). The outcome of this interaction is not clearly understood; however, isolated ADAM17 disintegrin domain has been reported to inhibit integrin-mediated cell migration.
by binding α5β1, which is one of its cognate integrins (Huang, Bridges, & White, 2005). The cysteine-rich domain of ADAM17 regulates some of its catalytic activity to some extent as it is involved in substrate recognition; for instance, it is required for the cleavage of L-selectin and interleukin-1 receptor II (Reddy et al., 2000).

Over fifty substrates have been identified for ADAM17 including cytokines (such as pro-TNF-α), growth factors (such as transforming growth factor-α), receptors (such as TNFR, interleukin-1RII, and Notch), adhesion molecules (such as L-selectin) and amyloid precursor protein (APP) (Scheller, Chalaris, Garbers, & Rose-John, 2011). The substrates play a central role in various physiological processes and are diverse in both their structures and functions. ADAM17 substrates lack a homology among the primary sequence of their cleavage sites, and this makes it difficult to fully understand how the enzyme recognizes its substrates (Joaquín Arribas & Esselens, 2009). In fact, mutation of residues at the cleavage site or its vicinity does not hinder the shedding of some substrates (J Arribas, López-Casillas, & Massagué, 1997; Althoff et al., 2001). The diverse range of substrates cleaved by ADAM17 implicated it as a therapeutic target in a broad spectrum of diseases where the substrates have aberrant functions (Joaquín Arribas & Esselens, 2009), notably in immune disorders such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, stroke, and endotoxic shock (associated with dysregulated TNF-α signaling), in cancer pathogenesis and progression (linked to over-activated EGFR signaling pathway), and in Alzheimer’s disease (resulting from the accumulation of amyloid-β peptide in the form of amyloid plaques in brain tissues) (Pruessmeyer & Ludwig, 2009). Therefore, developing inhibitors against ADAM17 is such an appreciable idea as its inhibition would have a great significance in a diverse range of pathologies.
2B.4 ADAM17 INHIBITORS

The basic principle behind ADAM17 inhibition is chelation of the zinc cofactor present in the active site, which is expected to impair the enzyme’s catalytic activity. Thus, the identified inhibitors were tailored for zinc chelation. The design of ADAM17 inhibitors has also explored peptidomimetics to yield peptide-based inhibitor compounds bearing strong zinc-chelating ligands such as hydroxamic acids and other alternative ligands (Barlaam et al., 1999). In general, the non-peptidic inhibitors are thought to have better oral absorption and metabolic stability compared to their peptide-based counterparts. In this review, the inhibitors are categorized into early and later groups of inhibitors. Each category depicts several developmental stages as well as challenges encountered in the discovery of therapeutic ADAM17 inhibitors.

2B.4.1 EARLY INHIBITORS

The early ADAM17 inhibitors are compounds that were developed to inhibit matrix metalloproteases (MMPs). Like ADAM17, MMPs belong to the class of zinc metalloproteases and are among the list of therapeutic targets in inflammatory diseases (Close, 2001; Nagase, Visse, & Murphy, 2006). The rationale for using MMP inhibitors against ADAM17 is due to the similar catalytic site of both metalloproteases (Moss, White, Lambert, & Andrews, 2001). The inhibitors include marimastat (1), prinomastat (2) and CGS 27023A (3), whose structures feature hydroxamic acid as the zinc-chelating ligand, and their development was primarily intended for cancer treatment (Brown, 2000). Their ADAM17 inhibitory capacity has been validated in a number of preclinical studies; for instance, oral administration of marimastat dose-dependently inhibited lipopolysaccharide
(LPS)-induced TNF-α production in mice model of sepsis and arthritis (Tsuji et al., 2002). Similarly, half maximal inhibitory concentrations (IC\textsubscript{50}) of 7 and 16 µM were observed \textit{in vivo} for marimastat and prinomastat, respectively (Barlaam et al., 1999). The efficacy of these compounds in the early phases of clinical trials indicated their potentials for use as drugs against inflammatory diseases; however, further trials were discontinued due to lack of selectivity, which led to tendonitis, a musculoskeletal side effect (Brown, 2000). The adverse effect is thought to result from non-specific inhibition of MMPs (particularly MMP1), which are required for normal physiological matrix turnover (Aranapakam et al., 2003). However, the ability of these compounds to inhibit ADAM17 is promising and encourages further research in developing selective ADAM17 inhibitors.

\begin{align*}
\text{(1)} & \\
\text{(2)} & \\
\text{(3)} & 
\end{align*}

\textbf{2B.4.2 LATER INHIBITORS}
Given the setback faced with the early inhibitors, identification of selective ADAM17 inhibitors has become the Holy Grail in this research field. Conversely, other perspectives maintained that dual MMP/ADAM17 inhibitors would be superior to the selective types considering the pathogenic role of MMPs in cancer and cartilage degradation in rheumatoid arthritis. However, dual inhibitors would be fully embraced only if they do not interfere with the normal physiological functions of MMP-1. The observed efficacy of the early inhibitors set the pace especially as their structures serve as templates for designing the later inhibitors. Initially, the development of selective ADAM17 inhibitors proved difficult, considering the active site similarities of MMPs and ADAM17, until the emergence of detailed structural information for both metalloproteases. Although the proteases have similar catalytic site, the shape and size of their S1' hydrophobic pockets are different, with a larger and deeper pocket found in ADAM17 compared to MMPs (Maskos et al., 1998). In addition, the S1' and S3' sites of ADAM17 are interconnected (Fig. 2) (Maskos et al., 1998). This suggests that inhibitors with large substituents at the P1' position can establish strong interactions with the S1' subsite of ADAM17. In fact, docking of marimastat on the active site of ADAM17 showed that there was no perfect fit between the S1' subsite of the enzyme and the P1' isobutyl group of the inhibitor (Moss et al., 2001), and this probably accounted for the lack of selectivity of marimastat for ADAM17. This evidence further confirms the requirement for large P1' substituents in order to maintain an enzyme-inhibitor structure stabilization. The identification of differences in the structural morphology of MMPs and ADAM17 became the landmark basis of developing selective ADAM17 inhibitors. Optimization of the P1 and P1' positions
on the inhibitors for the corresponding hydrophobic pockets of ADAM17 has given rise to several classes of inhibitors with high affinity for the enzyme.

2B.4.2.1 HYDROXAMATE CLASS OF INHIBITORS

This class of later ADAM17 inhibitors also possess a hydroxamate group as their zinc-chelating ligand. Depending on the distinct scaffolds in their structures, they can be subcategorized into succinate-based, macrocyclic, γ-lactam and the sulfonamide series.

2B.4.2.1.1 SUCCINATE-BASED ADAM17 INHIBITORS

With the knowledge of the structural discrepancies for achieving selectivity, a series of compounds were synthesized in a bid to improve the ADAM17 inhibitory activity of marimastat. In the new compounds, the \(-\text{OH}\) group at the P1 position of marimastat was substituted with bulky groups (sulfonamides, ethers, and thioethers) represented by compounds 4, 5 and 6, respectively (Barlaam et al., 1999). Although the structural modifications were successful in improving selectivity and potency as shown by the inhibitory effect on the isolated enzyme, they appear to be less effective towards inhibition of soluble TNF-\(\alpha\) release in human whole blood (Barlaam et al., 1999). The decreased effect was found to be more pronounced in the ether and thioether series compared to the sulfonamide series. Further optimization of the sulfonamide series to maximize interactions with the enzyme’s subsites yielded heterocyclic compounds represented by compound 7, with better activity in both the isolated enzyme and whole blood systems (Barlaam et al., 1999).
More attempts to improve the selectivity of marimastat substituted the P1-OH group with a methylene group, and replaced the P1' isobutyl group with bulkier groups such as phenyl or substituted phenyl groups. These modifications resulted in compounds, represented by compounds 8, 9 and 10, with about 2 folds increase in ADAM17 inhibitory activity and selectivity over MMP-1, MMP-2 and MMP-3 (Kottirsch, Koch, Feifel, & Neumann, 2002). Compound 10 was the best in the series and had 10- and 20-fold increase in inhibitory activities against ADAM17 and soluble TNF-α release in human peripheral blood mononuclear cells, respectively, compared to the activity of marimastat. Moreover, concentrations as low as 3-10 mg of the derivatives per kg body weight produced up to 79% inhibition of soluble TNF-α release in rats whereas 30 mg marimastat/kg body weight was needed to give a similar effect.
Moreover, Xue et al. made extensive structural modifications on CGS 27023A (3) to improve its selectivity and potency in inhibiting ADAM17, thus giving rise to several 5- and 6-membered cyclic succinates (Xue et al., 2003). The 6-membered series were generally more active than their 5-membered counterparts, and the striking structural differences are the cyclohexyl and the P1' quinolinyl groups. Among all the compounds, the ADAM17 inhibitory activity (IC$_{50}$, 8 nM) of compound 11, as well as its selectivity (5000-folds more over a wide range of MMPs) is promising, but its efficacy in vivo can be a limitation given its low activity (IC$_{50}$, >50 µM) in a whole blood assay. Further optimization of the quinoline moiety and the cyclohexyl ring led to the identification of an orally bioavailable compound, IM-491 (12), which inhibited ADAM17 in the whole blood assay with lower IC$_{50}$ of 20 nM compared to the parent compound (Xue et al., 2003). However, the central amide bond present in compound 12 was unstable under simulated gastric fluid condition, leading to the release of the aniline residue, which is potentially mutagenic. The amide group was further replaced with a sulfone, which drastically reduced the inhibitory capacity against soluble TNF-α release (Xue et al., 2004). Therefore, further preclinical development of this compound was discontinued.
Later, GlaxoSmithKline group developed GW 3333 (14), which is a dual inhibitor and exhibited \textit{in vitro} ADAM17 inhibitory activity with IC\textsubscript{50} of 40 nM (Conway et al., 2001). Although the compound has promising activity against ADAM17, the inhibitory effect was greater for MMPs (ranging from IC\textsubscript{50} of 4-20 nM) including MMP-1, indicating a better selectivity for MMPs over ADAM17. The activity of GW 3333 was further demonstrated in a rat model of LPS-induced arthritis where a dose-dependent inhibition (IC\textsubscript{50}, 1 mg/kg body weight) of soluble TNF-\(\alpha\) release was observed (Conway et al., 2001). In addition, the compound produced a long-lasting action with a half-life up to 12 h. However, it had poor oral bioavailability due to poor solubility. Thus, two structural modifications were adopted to address the solubility issue as well as to enhance its selectivity. First, incorporating the side chain of arginine at the P2' position was thought to enhance its solubility; and secondly, nitro or sulfonyl groups (electron withdrawing groups) covalently bonded to arginine would create a neutral surface charge on the compound, thereby enabling its compatibility with the plasma membrane. These modifications yielded nitro-arginine and sulfonyl-arginine compounds represented by GW4459 (15) and compound 16, respectively, both with better solubility and selectivity for ADAM17 (IC\textsubscript{50}, 4.3-49 nM) over MMPs (IC\textsubscript{50}, 8.1 to >2500 nM) (Rabinowitz et al., 2001). However, the enhanced solubility of the compounds did not translate into a better oral bioavailability as they showed no effect on soluble TNF-\(\alpha\) release when administered orally. This suggests that solubility may not have contributed to the poor oral bioavailability of the parent compound and its derivatives; it is likely the compounds were not absorbed intact \textit{via} the oral route.
Inhibitory capacity against the release of soluble TNF-α from human peripheral mononuclear blood cells was reported for two dual inhibitors, PKF242-484 (17) and PKF241-466 (18), developed by Novartis with IC\textsubscript{50} of 56 and 141 nM, respectively (Trifilieff, Walker, Keller, Kottirsch, & Neumann, 2002). In mice model of LPS-induced lung inflammation, the compounds had no effect on immune cell activation but they inhibited the release of soluble TNF-α, suggesting that they may not have perturbed immune cell signaling events, which is a good sign of specificity and targeted activity. Structure-function relationship was observed with PKF242-484, possessing an –OMe moiety, having a better activity than PKF241-466, which has a methyl moiety instead. The methoxy group can possibly contribute in zinc chelation by PKF242-484, or other interactions such as hydrogen bond formation with the enzyme’s catalytic site residues leading to better inhibitory activity. This is supported by previous studies where the introduction of a methoxy group produced better ADAM17 inhibitors (Kottirsch et al., 2002). Furthermore, these inhibitors are desirable for further drug development given the
similarity of their inhibitory effects irrespective of their mode of administration (parenterally or orally).

A high selectivity for ADAM17 (IC\textsubscript{50}, 5.2 nM) over different MMPs (IC\textsubscript{50}, 25-500 nM) was also reported for Ro 32-7315 (19) in enzyme isolated systems (Beck et al., 2002). It has a similar structure as other succinate hydroxamates but features a sulfonyl hydrazide group in place of the usual P2' amino acid. Its oral administration gave a dose-dependent inhibition of LPS-induced soluble TNF-\(\alpha\) release in a rat model of arthritis (42-93%) and in the blood of healthy human volunteers (69%). The efficacy and pharmacokinetic profile of Ro 32-7315 makes it a strong candidate for clinical studies as well as elucidation of its molecular mechanisms of action.
2B.4.2.1.2 MACROCYCLIC ADAM17 INHIBITORS

Strategic developments to refine the potency as well as selectivity of the succinate-based compounds for ADAM17 inhibition gave rise to the macrocyclic class of inhibitors. Their design involves linking the P1 and P2' positions of succinate-based MMP inhibitors to form macrocycles, which are thought to have better metabolic stability and cell permeability owing to their ring rigidity (Xue et al., 2001). Furthermore, the design of these inhibitors took advantage of the large S1’ site of ADAM17 by incorporating several bulky groups at the corresponding P1’ position. For instance, compound 20 with a trimethoxyphenyl P1’ substituent exhibited selectivity for ADAM17 with an IC₅₀ of 7.6 nM, which is many folds less than that for MMPs, particularly MMP-1 (Holms et al., 2001). Xue et al. developed potent inhibitors, SL422 (21) and SP057 (22), possessing glycine and N-methylamide residues at P3’ and P4’ positions, respectively, and the glycine residue was shown to be critical to their inhibitory capacity (Xue et al., 2001). Although these compounds have promising ADAM17 inhibitory activity, they are very active towards a wide range of MMPs, including MMP-1 and MMP-13. Furthermore, they have shorter half-lives and lower oral bioavailability, which can be as a result of their larger molecular sizes compared to other ADAM17 inhibitors.
2B.4.2.1.3 γ-LACTAM ADAM17 INHIBITORS

The Bristol-Myers-Squibb group sought after selective ADAM17 inhibitors and developed a number of inhibitors containing γ-lactam scaffolds (Duan et al., 2002). They synthesized several compounds, represented by compound 23, whose inhibitory activity relies on the substituent at the R position and supports the requirement of large groups at the P1' position in order to achieve selectivity. For instance, low selectivity and high IC<sub>50</sub> value were obtained when R was smaller groups but the introduction of larger moieties such as a 3,5-bis-trifluoromethyl benzyloxy group resulted in higher selectivity and ADAM17 IC<sub>50</sub> ranging from 2-7 nM. However, the potency of these compounds was not reproduced in whole blood assay (IC<sub>50</sub>, >50 µM), and this was attributed to their high protein-binding
capacity and impermeability in cells (Duan et al., 2002). However, several modifications
gave rise to compounds with substantially enhanced ADAM17 inhibitory activities in
whole blood assay. Particularly, compound 24, known as IK682, bears a (2-methyl-4-
quinoliny1) methoxy group at the P1' position and has an IC$_{50}$ of 0.35 µM in the whole
blood assay and ADAM17 inhibition constant (Ki) of 0.56 nM, which is over 5000 times
lower than that observed for MMPs. Another feature of this compound is its good
pharmacokinetics reflected by its oral bioavailability in rats (41%) and Beagle dogs (32%).
The focus on γ-lactam ADAM17 inhibitors was heightened with the discovery of BMS-
561392 (25), which inhibited the activity of isolated enzyme and TNF-α release in whole
blood assay with IC$_{50}$ of 0.2 and 90 nM, respectively (Grootveld & McDermott, 2003).
When administered orally to rats, it blocked LPS-induced TNF-α release with EC$_{50}$ of 6
mg/kg body weight and was also orally bioavailable (Qian et al., 2007). BMS-561392
demonstrated a high level of efficacy and tolerance in clinical trials; however its therapeutic
potential was disproved by its hepatotoxic effects, leading to its withdrawal from Phase II
clinical trials (Moss et al., 2008).
2B.4.2.1.4 SULFONAMIDE ADAM17 INHIBITORS

These compounds possess sulfone groups in their structures, and a crystal structure showed that one of the oxygen atoms of the sulfone moiety participates in hydrogen bonding with the amide hydrogen of Leu164 in MMPs (Duan et al., 2002). Many compounds have been identified in this class and are a part of ADAM17 inhibitors that were tested in clinical trials. Levin et al. explored various aryl groups in the design of sulfonamide inhibitors and obtained compounds bearing anthranilic acid scaffolds as the most active in the series, with their general structure represented as compound 26 (Levin et al., 2001). The bromine in the compounds was found to be needed in maintaining inhibition as its removal or replacement resulted in a 6-fold loss of activity. Despite the promising inhibitory capacity exhibited by the compounds \textit{in vitro}, they failed to reproduce a similar effect when evaluated in mice models, and this was attributed to their poor oral bioavailability. Thus, their structures were then optimized to improve oral bioavailability, selectivity and efficacy. It was suggested that long substituents, such as acetylenic groups, will run across the linear interconnecting S1'-S3' tunnel in ADAM17 to suitably occupy the hydrophobic pockets but will not be accommodated in MMPs (Levin et al., 2002). This postulation was validated with the design of compounds represented by 27, bearing a butynloxy group at the P1' position. Compound 27 is two times more potent and 100 times more selective than the parent compound 26 (Levin et al., 2002). In a mice model of inflammation, oral administration of compound 27 at 50 mg/kg body weight completely inhibited soluble TNF-\(\alpha\) production after one hour, and this was observed to last for 6 h.
Therefore, long P1' substituent can be considered an important structural feature of ADAM17 inhibitors and many subsequent studies have designed highly potent and selective inhibitors with the incorporation of a butynyloxy group. For instance, compound 28 was shown to have an IC$_{50}$ value of 17 nM and a 50-fold higher selectivity over MMP-1 compared to the parent compound whose IC$_{50}$ was 120 nM (DasGupta, Murumkar, Giridhar, & Yadav, 2009). Similarly, Nelson et al. synthesized compounds (represented by 29) bearing a benzodiazepine scaffold, which exhibited high potency and selectivity when substituted with butynyloxy group, in comparison to the parent (Nelson, 2002). Findings from this study clearly illustrates the incompatibility of MMPs and inhibitors possessing butynyloxy groups and a preferential inhibition of ADAM17 by these compounds.
The concept of incorporating butynyloxy groups was extended to the development of highly potent sulfonamide inhibitors bearing thiomorpholine scaffolds, whose therapeutic potential has been evaluated in clinical trials. One of the compounds is TMI-1 (30), which is a strong dual MMP/ADAM17 inhibitor. Preclinical studies showed it had low IC₅₀ of 3-26 nM for ADAM17 and MMP inhibition, and strongly inhibited TNF-α production in different arthritic models (Zhang et al., 2004). TMI-2 (31) is an analogue of TMI-1 with the thiomorpholine moiety replaced with a hydroxyl group. This compound exhibited an improved in vitro potency and selectivity than TMI-1, although both have a similar effect on cellular TNF-α production (Levin et al., 2003). Another thiomorpholine compound, TMI-05 or Apratastat (32), derived by replacing the methyl group present at the P1' position of TMI-1 with a hydroxyl group, was found to be a potent dual MMP/ADAM17 inhibitor that also inhibited TNF-α release in mouse collagen-induced arthritic model (Shu et al., 2011). In Phase I clinical trials, no risk of side effects was observed for Apratastat; however, further therapeutic development was discontinued following its lack of efficacy in Phase II trial (Thabet & Huizinga, 2006).
Moderately active inhibitors have been also obtained using piperazine scaffolds but these compounds have short half-life (Letavic et al., 2003). Compound 33 was the best in the series with IC\textsubscript{50} of 6, 3 and 1600 nM for ADAM17, MMP-13 and MMP-1, respectively. Moreover, 4,4-piperidine-β-sulfone inhibitors developed with the incorporation of butynyloxy groups showed higher selectivity and potency for ADAM17 (Lombart et al., 2007). Particularly, compound 34 had an IC\textsubscript{50} value of 1.5 nM for ADAM17 inhibition and over 200-fold better selectivity than MMP-2 & 13.

![Chemical structures of compounds 32, 33, and 34](image)

**2B.4.2.1.5 B-BENZAMIDO ADAM17 INHIBITORS**

In a bid to eliminate the potential mutagenicity of the 4-(2-methylquinolin-4-ylmethoxy) aniline moiety present in IM-491 (12), Duan et al. reversed the central amide bond, giving rise to highly potent, selective and orally bioavailable β-benzamido inhibitors (Duan et al., 2008). The most promising compound (35) in the series inhibited ADAM17 activity with an IC\textsubscript{50} less than 1 nM, and exhibited more than 30,000-fold selectivity over a wide range of MMPs. Ott et al. explored 2-substituted benzimidazolemethylphenyl group as an
alternative P1' substituent in place of 4-(2-methylquinolin-4-ylmethoxy) phenyl group, which has always been used for achieving selectivity (Ott et al., 2008). This led to the discovery of more potent and selective compound (36) with excellent pharmacokinetic profile, reflected by a 99% oral bioavailability in rats (Ott et al., 2008). This compound suppressed LPS-induced soluble TNF-α release in mice model of inflammation with EC\textsubscript{50} of 1.9 mg/kg body weight, and this effect lasted for 5 h post-administration.

![Chemical structures](image)

(35) (36)

2B.4.2.2 NON-HYDROXAMATE ADAM17 INHIBITORS

From the onset, hydroxamates were extensively utilized as strong zinc-chelators in the design of ADAM17 inhibitors. Moreover, a good number of the hydroxamate-based compounds have shown appreciable potency and selectivity over a wide range of MMPs. Despite the progress made in identifying these inhibitors, they are not yet seen as ideal inhibitors partly due to their hydroxamate moiety. Under gastric conditions, hydroxamates are rapidly metabolized via O-glucuronidation and amide bond hydrolysis releasing hydroxylamine, which can cause methemoglobinemia (Gilmore et al., 2007). This rapid hydroxamate metabolism is reflected by the generally low oral bioavailability of the
hydroxamate-based ADAM17 inhibitors. This has led to efforts to develop ADAM17 inhibitors with non-hydroxamate zinc-binding ligands. Several studies have shown that some non-hydroxamate inhibitors are more active and orally bioavailable than the hydroxamates. For instance, Kamei et al. reported that reverse hydroxamate (N-formyl-N-hydroxyamino) compounds possess better ADAM17 inhibitory activity and selectivity over MMPs compared to their hydroxamate counterparts. Particularly, compound 37 inhibited ADAM17 activity with IC$_{50}$ of 2 nM and has over 2000-fold higher selectivity for the enzyme (Kamei et al., 2004).

Furthermore, pyrimidinetrione has been previously used as a zinc-chelating ligand to achieve MMP inhibition. Apart from its zinc-chelating role, pyrimidinetrione was shown from a crystal structure of MMP-9-inhibitor complex to also interact with residues in the active site vicinity via hydrogen bonding, and similar interaction would be expected for ADAM17 (Duan et al., 2005). Using the pyrimidinetrione MMP-9 inhibitor as a lead compound, Duan et al. replaced the phenoxyphenyl P1' substituent with 4-(2-methylquinolin-4-ylmethoxy)phenyl and obtained compound 38, which had a moderate ADAM17 inhibitory activity (IC$_{50}$, 1 µM) and high selectivity over MMPs compared to the lead compound (Duan et al., 2005). Replacement of the methyl group on the pyrimidine ring with N-methylpiperazine group in 38 dramatically improved its potency depicted as compound 39, which had an IC$_{50}$ of 0.091 µM. In a bid to further enhance the activity of
compound 39, an amide linkage was introduced between its pyrimidine and benzene ring to form compound 40, which was more selective and had IC$_{50}$ of 0.026 µM in inhibition ADAM17 activity (Duan et al., 2007). However, this compound was weakly active (IC$_{50}$, > 50 µM) towards inhibition of TNF-α production in a whole blood assay (Duan et al., 2007). These findings imply that the strong inhibitory effect observed in vitro may not always be reproduced in vivo.

Through comprehensive modelling and structural optimizations, compounds bearing hydantoin as the zinc-chelating ligand were produced and these compounds exhibited high potency and selectivity (Sheppeck, Gilmore, Yang, et al., 2007; Yu, Tong, et al., 2010; Yu, Guo, et al., 2010). For instance, compound 41 inhibited ADAM17 activity with Ki of 6 nM (Yu, Guo, et al., 2010). To maximize its interactions with the enzyme’s S1 pocket, a pyridine molecule was substituted on the phenyl group of its hydantoin ring to yield compound 42, with a Ki of 0.8 nM (Yu, Tong, et al., 2010). One important finding about inhibitors of this type is that the stereochemistry of the hydantoin ring is critical to their
selectivity and inhibitory capacity. This was evident in the study by Sheppeck et al. as the most active compound (43) was synthesized in the (5R,6S)-trans form leading to IC$_{50}$ of 11 nM for ADAM17 inhibition and high selectivity compared to MMPs (Sheppeck, Gilmore, Yang, et al., 2007). However, in the (5S,6R)-trans form, the activity was lower with IC$_{50}$ of 900 nM. The latter stereochemistry causes an outward orientation of the quinolinyl P1' group rather than projecting it into the S1' pocket of the enzyme. Modification of hydantoin into ligands such as triazolones and imidazolones obtained very potent and selective compounds, 44 and 45, respectively (Sheppeck, Gilmore, Tebben, et al., 2007). Their IC$_{50}$ for ADAM17 inhibition were 34 and 9 nM, respectively, whereas the IC$_{50}$ observed with different MMPs were over 3000 nM.
A number of inhibitors bearing tartaric acid as the zinc-chelating ligand has also been developed (Dai et al., 2011; Li et al., 2010; Rosner et al., 2010). Similarly, their activity is determined by the conformation of the tartrate moiety as only the L-stereoisomer is active. Rosner et al. reported that compound 46 had Ki of 400 nM but several modifications to increase interactions with the non-prime sites of ADAM17 gave rise to compound 47, which inhibited the enzyme with lower Ki of 11 nM (Rosner et al., 2010). However, these compounds were observed to have low inhibitory capacity against soluble TNF-α release in a whole blood assay.

Furthermore, thiols have been used to develop ADAM17 inhibitors since they contribute to specificity for MMP inhibition (Freskos et al., 1999). Thus, Bandarage et al. designed potent inhibitors, exemplified by compound 48, bearing thiols as their zinc-binding ligand.
Compound 48 inhibited ADAM17 with Ki of 28 nM and showed a high selectivity over MMP-2, 7, 8, 9 and 13. Modelling studies revealed that the thiol group strongly binds to the catalytic zinc while the butynyloxy group interact with the S1'-S3' as expected [75].

2B.5 HIGHLIGHT OF THE STRUCTURE-FUNCTION RELATIONSHIP OF ADAM17 INHIBITORS

Drug design usually employs molecular modelling techniques that aid the understanding of complex molecular interactions, and hence facilitate the development of effective therapeutics. In the research on ADAM17 inhibitors, different modelling techniques have been used to identify the binding modes of the inhibitors to the crystal structure of ADAM17 (Sarkate et al., 2015). Three distinct molecular interactions, viz. (1) zinc coordination, (2) hydrogen bonding and (3) interaction with S1' hydrophobic pocket, have been characterized as key factors to achieving both potency and selectivity. The active site interactions, especially co-factor zinc chelation, differ between the hydroxamate and non-hydroxamate groups of inhibitors. The hydroxamates coordinate zinc with the oxygen atom of their hydroxyl and carbonyl groups (Fig. 3), and hence are bidentate ligands (DasGupta et al., 2009). The hydroxamates play a dual role as they can also form hydrogen bonds with the side chain of Glu406, which further stabilizes the complex. The X-ray crystal structure
of IK682 (24) bound to ADAM17 (Fig. 4) is a typical representation of hydroxamate inhibitor interaction with the enzyme (Niu et al., 2006). Apart from the interaction with zinc and Glu406, the NH group of the hydroxamate moiety in IK682 also forms hydrogen bonds with the backbone carbonyl of Gly349. The carbonyl group of the pyrrolidinone ring also interacts via hydrogen bonding with the amide hydrogen of Leu348 and Gly349. The phenyl group forms an aromatic stacking with His405 while the quinolinyl moiety runs through the S1’ and S3’ hydrophobic tunnel.

Pyrimidinetrione moieties are also bidentate ligands and usually form two hydrogen bonds; one is formed between the carbonyl group at the 4-position of the pyrimidine ring and the amide hydrogen of Leu348, while the other is formed between its C2 carbonyl group and Glu406 (Sheppeck, Tebben, et al., 2007). The X-ray crystal structure of compound 41 bound to ADAM17 active site (Fig. 5) reveals that hydantoins form monodentate zinc complexes with one of their amide nitrogen, while the second amide nitrogen interacts with the carbonyl oxygen of Gly349 (Yu, Tong, et al., 2010). The phenyl group of the hydantoin forms hydrophobic interactions with the S1 subsite comprised of Leu350, Lys315 and Val314, while the P1’ indazolinone moiety interacts with the S1’ hydrophobic pocket. As shown in Fig. 6A, tartrates coordinate zinc in a tridentate fashion with their two hydroxyl groups and the carbonyl located at the non-prime side (Rosner et al., 2010). The molecular interactions of tartrate-based inhibitors are clearly illustrated by the crystal structure of compound 46 bound to ADAM17 active site (Fig. 6B). The carbonyl oxygen at the prime side is involved in hydrogen bonding with the amide hydrogen of Leu348 and Gly349. The ethylthiophene molecule forms hydrophobic and a pi-stacking interaction with the S1’ residues and His405, respectively. With the identification of the different binding modes.
that exist among the different groups of the inhibitors, it can be concluded that active site residues such as Glu406, His405, Leu348 and Gly349 are relevant for ADAM17 inhibition. Therefore, interaction with these residues would be regarded as the minimum requirement for any inhibitor designed for binding ADAM17 active site.

2B.6 CONCLUSION AND FUTURE DIRECTIONS

ADAM17 inhibitors have been shown to be promising candidates for inhibiting TNF-\(\alpha\) release towards the treatment of inflammatory diseases. Exclusive ADAM17 inhibitors has remained elusive given the similarities in the active site of ADAM17 and MMPs. While some ADAM17 inhibitors are active towards a broad spectrum of MMPs, a good number of them show considerably little to no effect on different MMPs, including MMP-1, which suggests potential low risk of side effects. Current understanding of the structure-function relationships of the known ADAM17 inhibitors has also helped in the design of potent, selective and safer active site inhibitors. However, developing allosteric inhibitors of ADAM17 can provide insight on other enzyme-inhibitor interactions that can enhance selectivity and limit toxicity. Moreover, natural compounds can also be explored for ADAM17 inhibition as a possible mechanism of their anti-inflammation health benefits. For instance, peptides from natural sources (e.g. food) have similar building blocks as tissue inhibitors of metalloproteinases, and can be more desirable ADAM17 inhibiting agents as they are generally thought to pose lower risk of side effects compared to synthetic compounds.

2B.7 REFERENCES


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2B.9 Figures

**Fig. 2B.1** A graphical representation of the domain structures of ADAM17. ■ Prodomain coordinated to the active site zinc ■ Metalloproteinase domain ■ Disintegrin domain ■ Cysteine-rich domain ■ Epidermal growth factor-like domain ■ Transmembrane domain ■ Cytoplasmic tail; figure was modified from Scheller et al.

**Fig. 2B.2** Solid surface representation of the catalytic domains of TACE (left) and MMP-3 (right) showing the differences in the S1' structure and the interconnected S1' and S3' tunnel; figure was obtained from Maskos et al. [36] with permission from XXX.
Fig. 2B.3. Zinc-binding mechanism of hydroxamate-containing inhibitors; figure was obtained from Sarkate et al. [76] with permission from XXX.

Fig. 2B.4. Structure of IK682 bound to the active site of ADAM17.
Fig. 2B.5. X-ray crystal structure of compound 41 bound to the active site of ADAM17.

Fig. 2B.6. Zinc-binding mechanism of tartrates (A) and crystal structure of compound 46 bound to the active site of ADAM17 (B).
CHAPTER 3  INFLUENCE OF STRUCTURAL AND SURFACE PROPERTIES OF WHEY-DERIVED PEPTIDES ON ZINC-CHELATING CAPACITY, AND IN VITRO GASTRIC STABILITY AND BIOACCESSIBILITY OF THE ZINC-PEPTIDE COMPLEXES

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3.1 ABSTRACT

Gastrointestinal stability of zinc-peptide complexes is essential for zinc delivery. As peptides’ surface charge can impact the stability of their metal complexes, we evaluated the zinc-chelating capacity and stability of zinc complexes of whey protein hydrolysates produced with Everlase (WPH-Ever; $\zeta$-potential, -39 mV) and papain (WPH-Pap; $\zeta$-potential, -7 mV) during simulated digestion. WPH-Ever had lower amount of zinc binding amino acids residues, but had higher zinc-chelating capacity than WPH-Pap, attributable to its highly anionic surface charge for electrostatic interaction with zinc. Zinc release during peptic digestion was lower for WPH-Ever, indicating higher gastric stability. However, over 50% of zinc remained bound in both complexes after digestion. FTIR spectroscopy suggests the involvement of COO\textsuperscript{-}, C-O of aspartate/glutamate, and C-OH of
serine/threonine R-groups in zinc-peptide complexation. These findings indicate that strong zinc chelation can promote gastric stability and impede intestinal release, for peptides intended for use as dietary zinc carriers.

3.2 HIGHLIGHTS

- Whey protein hydrolysates, WPH-Ever and WPH-Pap, had high and low net negative surface charge, respectively.
- Zinc-chelating capacity of the hydrolysates depended on their negative surface charge magnitude.
- Zn-WPH-Ever was more stable during gastric digestion than Zn-WPH-Pap.
- Less than 50% of the bound zinc was bioaccessible from both complexes.
- Peptides’ structures can enhance gastric stability of their zinc complexes and also decrease zinc bioaccessibility.

3.3 INTRODUCTION

Zinc is an integral component of the human body that is involved in several biological processes as a catalytic cofactor of over 300 metalloenzymes, and provides structural integrity to proteins such as transcription factors and hormones (McCall, Huang, & Fierke, 2000). It functions in cellular events such as gene expression, cell division, differentiation and proliferation (Hambidge, 2000). Zinc is required for the development and activation of immune cells including macrophages, B- and T-lymphocytes; hence, individuals who are deficient in zinc have high susceptibility to infections (Prasad, 2012). The availability of zinc for its biological roles is mostly dependent on its intestinal absorption, which in turn
depends on its release from food matrices. The major challenge to zinc bioavailability is poor absorption due to the heterogeneous nature of food. Myo-inositol hexakisphosphate (phytate), present in particularly plant-based foods, forms insoluble complexes with zinc, and hence makes it unavailable for absorption (Hansen, Sandstrom, & Lönnerdal, 1996; Kumar, Sinha, Makkar, & Becker, 2010). Moreover, the accessibility of zinc from such complexes is difficult due to the absence of phytase (phytate-hydrolyzing enzyme) in human digestive tract (Lönnerdal, 2000). Biological zinc is required to be constantly replenished, given that it is primarily obtained from the diet and the body has limited zinc stores (Cummings & Kovacic, 2009). Therefore, alternative measures are sought to promote zinc bioavailability by improving its bioaccessibility, which is the amount that is available for absorption from ingested food (Etchevery, Grusak, & Fleige, 2012).

Food-derived peptides are promising agents that can serve as dietary zinc carriers due to their zinc-chelating capacity (Udechukwu, Collins, & Udenigwe, 2016). The structures of peptides feature metal ligands such as His, Cys, Asp, Glu, and Ser (or phosphorylated) that can form soluble zinc complexes, thereby enhancing the mineral absorbability and preventing its complexation with phytates. Many zinc-chelating protein hydrolysates and peptides have been produced from different food proteins, and there is evidence that zinc is absorbed better from zinc-peptide complexes than from inorganic zinc salts (Udechukwu et al., 2016). The intestinal release of zinc bound to peptides during gastrointestinal (GI) digestion is desirable for absorption; however, the stability of zinc complexes at the gastric phase is an important factor for mineral delivery. Zinc released from zinc-peptide complexes during gastric digestion can be competitively bound by
phytates on entering the duodenum, thereby decreasing its bioaccessibility (Miquel & Farrre, 2007).

The stability of zinc-peptide complexes can be affected by the pH of their environment. This is because most of the peptidic ligands, including the terminal amino and carboxylate anion, Asp, Glu, and His, respond to pH changes, leading to association or dissociation of their metal complexes. These ligands are deprotonated at neutral-alkaline pH, which then increases electrostatic attraction with metal ions, thereby strengthening the complexes (Kallay, Varnagy, Micera, Sanna, & Sovago, 2005). Conversely, an acidic environment causes the protonation of these residues and tends to weaken the attractive forces. Consequently, zinc-peptide complexes are liable to dissociate at the acidic pH of the stomach, which can eventually lead to inaccessibility of the metal due to the formation of insoluble complexes with absorption inhibitors. For instance, a previous study reported that zinc bound to yak casein hydrolysates was released more at pH of 2 than at slightly acidic or alkaline pH (Wang, Zhou, Tong, & Mao, 2011). Structural properties of peptides such as net surface charge can influence the stability of their metal complexes. Moreover, the ability of peptides to interact with metal ions can be dependent on their net charge, such that high negative charges can enhance metal ion affinity and reinforce metal complexes (Elias, Kellerby, & Decker, 2008). Therefore, this study was conducted to investigate the role of the net surface charge of whey protein hydrolysates on their zinc-chelating capacity and stability of their zinc complexes during simulated gastric and intestinal digestion. In vitro zinc bioaccessibility from the peptide complexes, measured as zinc dialyzability, was also evaluated.
3.4 MATERIALS AND METHODS

3.4.1 MATERIALS

Bovine whey protein isolate was purchased from Bulk Barn Foods Ltd (Truro, NS, Canada); proteases, 8-anilino-1-naphthalene sulfonic acid (ANS) and O-phthalaldehyde were purchased from Sigma-Aldrich (Oakville, ON, Canada); 4-(2-pyridylazo) resorcinol (PAR), sodium dodecyl sulphate (SDS), dithiothreitol (DTT), and zinc sulphate heptahydrate (ZnSO$_4$.7H$_2$O) were purchased from Fischer Scientific (Ottawa, ON, Canada).

3.4.2 WHEY PROTEIN HYDROLYSIS

Aqueous suspensions of bovine whey protein isolate (5% w/v) were hydrolyzed with proteases of different cleavage specificities viz. papain, bromelain, ficin, Alcalase, Flavourzyme, Neutraste, Esperase, Everlase, Protamex, and Savinase, at enzyme-to-substrate ratio of 1:100. The hydrolysis was carried out for 5 h at the optimum conditions of the proteases (65 °C, pH 7.0 for papain; 37 °C, pH 6.5 for bromelain; 37 °C, pH 7.0 for ficin; 55 °C, pH 8.0 for Alcalase and Savinase; 50 °C, pH 7.0 for Flavourzyme and Neutraste; 60 °C, pH 8.0 for Esperase and Everlase; 50 °C, pH 6.5 for Protamex). Hydrolysis was terminated by adjusting the reaction mixtures to pH 4.0 using 1 M HCl, followed by centrifugation at 10,000 × g for 10 min. Then, the supernatants were adjusted to pH 7.0 using 1 M NaOH, and they were freeze-dried to obtain the hydrolysate powders, which were stored at -20 °C. Free amino group content of the hydrolysates was determined by the O-phthalaldehyde (OPA) method (Nielsen, Petersen, & Dambmann, 2001), and taken
as a measure of the degree of hydrolysis and amount of peptides per unit mass of the protein hydrolysates.

3.4.3 DETERMINATION OF SURFACE HYDROPHOBICITY, ZETA POTENTIAL, AND PARTICLE SIZE

Surface hydrophobicity (so) of the hydrolysates was determined by a fluorescence method using ANS. Samples were diluted with 0.01 M phosphate buffer (pH 7.0) to concentrations ranging from 0.015 to 0.0009%. Then, fluorescence was measured at the excitation and emission wavelengths of 390 and 470 nm, respectively. The slope of the fluorescence vs. concentration plot was taken to be the surface hydrophobicity (Paraman, Hettiarachchy, Schaefer, & Beck, 2007). Zeta (ζ) potential and the mean particle size of the protein hydrolysates were determined using a Zetasizer Nano Series Nano-ZS (Malvern Instruments Ltd., Malvern, UK). The samples were dispersed in deionized water and the measurements were obtained in a disposable capillary cell after equilibration for 120 s at 25 °C. Two hydrolysates were then selected for subsequent analysis according to their ζ-potential, such that one was of the highest magnitude (most negatively charged, WPH-Ever) and the other of the lowest magnitude (least negatively charged, WPH-Pap).

3.4.4 ANALYSIS OF AMINO ACID COMPOSITION

A complete amino acid profile of WPH-Ever and WPH-Pap was analysed at the Sick Kids Proteomics, Analytics, Robotics & Chemical Biology Centre (SPARC BioCentre, Toronto, ON, Canada) as reported by Mohan and Udenigwe (2015). The amino acid composition of the hydrolysates was calculated as mole%.
3.4.5 DETERMINATION OF ZINC-CHELATING CAPACITY

The zinc-chelating capacity of WPH-Ever and WPH-Pap was determined using the method described by Jakob et al. (2000). The assay principle is based on the reaction of 4-(2-pyridylazo)resorcinol with free zinc ions to form a red coordination complex. The samples and reagents were prepared in 40 mM HEPES-KOH buffer (pH 7.5). Then, 250 µL of the samples was mixed with 125 µL each of 8 mM DTT and 250 µM ZnSO₄·7H₂O, at a final concentration of 1 mg/mL. The blank experiment contained all assay components except the samples, which were substituted with an equal volume of the buffer. Ethylenediaminetetraacetic acid (EDTA) was used as a positive control. The mixtures were incubated at 37 °C for 10 mins, followed by the addition of 25 µL of 2 mM PAR and absorbance measurement at 500 nm. The percentage zinc-chelating capacity was calculated as \[ \frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}} \times 100 \], where A is absorbance.

3.4.6 PREPARATION OF THE ZINC-PEPTIDE COMPLEXES

WPH-Ever and WPH-Pap (10 mg/mL) were dissolved in 50 mM phosphate buffer (pH 7.0) containing ZnSO₄·7H₂O (50 µM). The mixtures were equilibrated at room temperature for 1 h under stirring. Thereafter, the unbound zinc was removed by dialysis for 5 h using a 500 Da molecular weight cut-off semipermeable dialysis membrane (Spectrum Laboratories, CA, USA), and the retentates (containing the chelate) were collected. A portion of the retentates was freeze-dried to obtain the intact chelate powders (Zn-WPH-Ever and Zn-WPH-Pap).

3.4.7 FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY OF THE ZINC-PEPTIDE COMPLEXES
Chemical interactions in Zn-WPH-Ever and Zn-WPH-Pap were determined by attenuated total reflectance (ATR) FTIR spectroscopy using an FTIR spectrometer (Spectrum One, Perkin Elmer, CT, USA). The spectra were recorded in the region of 4000 to 600 cm$^{-1}$ as an average of 16 scans, and significant shifts in absorption bands were used to deduce the possible zinc-chelating sites.

3.4.8 DETERMINATION OF GASTRIC STABILITY AND ZINC DIALYZABILITY UNDER SIMULATED GI DIGESTION

Zinc dialyzability was determined using the method described by Miller et al. (1981), with modifications. The zinc-peptide solutions from section 2.6 were maintained at pH 2.0 and 37 °C, and then digested with pepsin for 1 h at enzyme-to-peptide 1:100. This was followed by dialysis to remove free zinc in order to determine the gastric stability of the complexes. Then a portion of the retentates was freeze-dried to obtain the peptic digest powders. The other portion was adjusted to the optimum conditions for pancreatin (pH 7.5; 40 °C) and further digested for 3 h. Thereafter, the solutions were dialyzed and the retentates were freeze-dried to obtain the peptic-pancreatic digest powders. The zinc content of Zn-WPHEver, Zn-WPHPap and their peptic and peptic-pancreatic digests was analysed by atomic absorption spectroscopy using AA240FS VARIAN Fast Sequential Atomic Absorption Spectrometer (Agilent Technologies, CA, USA). Zinc-free hydrolysates were used as controls, and the gastric stability was determined by calculating the amount (%) of zinc released after hydrolysis with pepsin. Zinc dialyzability (%), a measure of bioaccessibility, was also determined by calculating the total amount of zinc released after the simulated GI digestion.
3.4.9 STATISTICAL ANALYSIS

All experiments, except protein hydrolysis and amino acid analysis, were done in triplicate, and results were expressed as mean ± standard deviation. Significant differences ($P < 0.05$) between results were determined by one-way analysis of variance followed by a Holm–Sidak multiple comparison test. The statistical analyses were done using SigmaPlot 12.1 (Systat Software, San Jose, CA, USA).

3.5 RESULTS AND DISCUSSION

3.5.1 HYDROLYSIS OF THE WHEY PROTEIN HYDROLYSATES

Hydrolysis is known to improve the bioactivity of food proteins due to the release of the encrypted peptides, with enhanced structural properties that can increase their molecular interactions in both food and biological systems. Also, peptides within protein hydrolysates can act synergistically to produce a more pronounced effect than their parent proteins. As shown in Fig. 1A, the whey protein hydrolysates contain more free amino groups than the intact protein (65 Serine mequiv NH$_2$/g protein), indicating the occurrence of hydrolysis and exposure of amino acid residues whose side chains can participate in zinc coordination. Moreover, the metal-chelating capacity of food proteins, including whey, has been noted to increase after enzymatic hydrolysis (Kim et al., 2007; C. Wang, Li, & Ao, 2012; Wu, Liu, Zhao, & Zeng, 2012). However, the exposed zinc-binding sites can be lost during extensive peptide bond cleavage, thereby leading to a decrease in the binding capacity of the end product (Xie et al., 2015; Zhu, Wang, & Guo, 2015). Thus, the duration of protein hydrolysis can be optimized in the development of zinc-chelating peptides for nutritional
purposes, in order to obtain hydrolysates that are rich in zinc-chelating protein fragments. The protein hydrolysates prepared with Alcalase, Flavourzyme and Savinase have the highest degree of hydrolysis (DH) whereas those prepared with bromelain, Neutrase and Protamex have the lowest DH. This is due to the different cleavage specificities of the proteases, which can impact the structural properties and behaviour of the protein hydrolysates (Tavano, 2013).

3.5.2 STRUCTURAL PROPERTIES OF THE PROTEIN HYDROLYSATES

The whey protein hydrolysates have varying amounts of hydrophobic amino acid residues on their surface, as indicated by their different surface hydrophobicity (Fig. 1B). So was observed to inversely correlate with DH, and this has been established for protein hydrolysates (Paraman et al., 2007). So determines the solubility of peptides in aqueous environments, which is important in evaluating the accessibility of zinc to its peptide binding sites. Moreover, all the whey protein hydrolysates possess a net negative surface charge at neutral pH, as determined by their \( \zeta \)-potential, with WPH-Ever and WPH-Pap having the highest and lowest net negative charge values, respectively (Fig. 2A). The varying magnitudes of the hydrolysates’ \( \zeta \)-potential (-39 to -7 mV) suggests that their particles will possess different levels of stability in aqueous solution. Conversely, the protein hydrolysates had similar mean particle diameter, except for WPH-Alc and WPH-Pap (Fig. 2B). WPH-Pap had a mean particle diameter (2248 nm) that is over five folds higher than most of the others, despite having a relatively higher DH. This can be due to the low electrostatic repulsion at WPH-Pap surface, reflected by low magnitude \( \zeta \)-potential, which can facilitate intermolecular aggregation leading to larger peptide particles. The different structural properties of the whey peptides can limit or promote their matrix
interactions. For instance, they can exhibit different affinities for metal ions and the stability of their metal complexes can be influenced by their charge magnitude. Phosphoserine residues of caseinophosphopeptides form strong iron complexes that can withstand changes in pH, due to the highly negatively charged phosphate groups (Pérès et al., 1999). The zinc chelating capacity of the whey protein hydrolysates and stability of the zinc-peptide complexes are expected to vary due to their different surface charges and particle sizes.

3.5.3. AMINO ACID COMPOSITION

The amino acid composition of food proteins and peptides is a major determinant of their functionality and bioactivity. Qualitative and quantitative information on the potential zinc-chelating ligands in WPH-Ever and WPH-Pap can be derived from their amino acid profiles. Amino acid residues whose side chains have been reported to bind divalent metals include Asp, Glu, His, Ser, Cys, Asn, and Gln (Guo et al., 2014). These residues constitute about 40% of the amino acids in WPH-Ever and WPH-Pap; however, they significantly occurred at varying proportions in both hydrolysates (Table 1). WPH-Pap had more of the zinc-chelating amino acids (43%) than WPH-Ever (38%), and the samples had similar total amounts of cationic amino acids (12% and 14%, respectively), which can impede zinc chelation due to electrostatic repulsion. Based on this information, both samples can chelate zinc but WPH-Pap is expected to have higher capacity to chelate zinc.

3.5.4. ZINC-CHELATING CAPACITY OF THE PROTEIN HYDROLYSATES

The zinc-chelating capacity of the whey protein hydrolysates is shown in Fig. 3. WPH-Ever exhibited about 2-fold higher zinc chelating capacity than WPH-Pap, despite having
lower amount of zinc-chelating amino acid residues. This indicates that amino acid composition cannot be used as the only predictor of the zinc-chelating capacity pattern of food proteins and peptides. Moreover, peptides practically exist in heterogeneous environments where they interact with themselves and other matrix components. It is apparent that the higher magnitude \( \zeta \)-potential of WPH-Ever played a major role in the zinc chelation by enabling the attraction of more zinc ions to the highly anionic surface of the peptides. In fact, the zinc-chelating capacity of the whey protein hydrolysates in this study (Fig. 3) had a significantly strong negative relationship with their \( \zeta \)-potential (Fig. 2A) with Spearman’s rank order correlation \( r_s = -0.733 \) (\( P=0.0131, n=10 \)). Whey protein hydrolysates have been reported to bind divalent metals such as calcium and iron (Kim et al., 2007; Zhao, Huang, Cai, Hong, & Wang, 2014), but this study is the first to report their zinc-chelating capacity and the importance of their net surface charge in determining zinc affinity.

### 3.5.5. CHARACTERIZATION OF THE ZINC-PEPTIDE INTERACTIONS BY FTIR

Infrared spectroscopy was used in this study to gain insight on the functional groups in WPH-Ever and WPH-Pap that could be involved in zinc coordination. We found that there was no substantial difference between the IR spectra of WPH-Ever and WPH-Pap, and also between their zinc-bound forms. This suggests that the zinc-binding ligands in both hydrolysates are similar in nature. However, some differences were observed in both band position and intensity between the spectra of zinc-free and zinc-bound forms of each hydrolysate. The wave number of the significant band shifts in the FTIR spectra of WPH-Pap and Zn-WPH-Pap are summarized in Table 2. Contrary to previous findings (X. Wang et al., 2011; D. Chen et al., 2013; C. Wang, Li, & Li, 2014), it appears that the amide bonds
did not contribute to the coordination, as no significant shift was observed in the -NH (3275 cm\(^{-1}\)), amide I (1644 cm\(^{-1}\)), and amide II (1543 cm\(^{-1}\)) bands of the spectra. However, the fingerprint region of both spectra provides substantial information on the participating ligands. The COO\(^{-}\) symmetric stretch at 1400 cm\(^{-1}\) shifted to 1394 cm\(^{-1}\) in Zn-WPH-Pap, indicating the formation of COO-Zn (Reddy, Radhika, & Manjula, 2005; Lin et al., 2015). In WPH-Pap, the absorption bands at 1314 and 1246 cm\(^{-1}\) were assigned to the hydrogen-bonded and free forms of C-O bonds of Asp and Glu side chains, respectively (Barth, 2007). The appearance of these bands in Zn-WPH-Pap at lower wavenumbers of 1145 and 1067 cm\(^{-1}\), respectively, is an evidence of interaction between these residues and zinc. Furthermore, the bands, 1180 and 1076 cm\(^{-1}\), arising from the stretching vibrations of the C-OH bond of Ser or Thr side chains, were respectively found at 938 and 858 cm\(^{-1}\) in the complex, suggesting the formation of C-O-Zn (Chaud et al., 2002). Zinc chelation by the oxygen atoms causes a reduction in the electron density of the C-O bonds, which consequently decreases the bond strength and stretching frequency. The spectral findings show that the carboxylate anion of peptide C-terminal, Asp and Glu side chains, and the hydroxyl group of Ser and Thr residues are involved in zinc chelation. Moreover, these groups have been suggested to be key metal-chelating ligands in food protein hydrolysates and peptides (Lv et al., 2009; Wang, Li, & Li, 2014; Carrasco-Castilla et al., 2012).

3.5.6 GASTRIC STABILITY AND ZINC DIALYZABILITY

The behaviour of the metal-peptide complexes in a given matrix can be dependent on their structural attributes. The higher negative charges of WPH-Ever can confer stability on its metal complex compared to WPH-Pap. We found that zinc was released from the zinc-peptide complexes after simulated gastric digestion, and this is not surprising since
metal complexes are likely to disintegrate at the acidic gastric pH. In addition, the proteolytic action of pepsin may have contributed to further peptide hydrolysis and dissociation of their zinc complexes. However, zinc release was significantly higher for Zn-WPH-Pap than for Zn-WPH-Ever (Table 3), which indicates that the latter was more stable at the gastric phase. This can be due to the differences in the surface charge, particle size and strength of the zinc complexes of both hydrolysates. A previous study found that Fe$^{3+}$ complexes of casein hydrolysates, which was expected to dissociate under the acidic pH of the stomach, remained intact after peptic digestion (Chaud et al., 2002). The molecular size of peptides can also influence the release of metal ions from their complexes. Smaller-sized peptides are thought to easily reach and bind their metal targets compared to those of larger sizes. However, both WPH-Ever and WPH-Pap possess similar DH and amount of peptide per unit mass (Fig. 1A), although differences in their particle diameters can greatly influence their solubility and functional group accessibility. Moreover, WPH-Ever could have greater resistance to peptic cleavage than WPH-Pap, due to its smaller particle size (M. Chen & Li, 2012). Zinc release from both complexes increased further with pancreatic digestion, and remained higher for WPH-Pap than for WPH-Ever (Table 3). This confirms that WPH-Ever formed a stronger zinc complex than WPH-Pap. A similar study found that zinc-peptide complexes were largely unaffected during a simulated gastric digestion but were mostly released after hydrolysis with pancreatin (Wang, Li, Wang, & Xie, 2015). Based on net charge, the peptides would be expected to retain their zinc chelating capacity at pancreatic pH; therefore, the release of zinc under this condition can be attributed to further cleavage by the pancreatic enzymes. Zinc release at this digestion phase is desirable for intestinal absorption. Taken together,
zinc dialyzability is significantly higher for WPH-Pap than for WPH-Ever, indicating that the zinc delivered via the former is more bioaccessible (available for absorption) if these peptides were to behave similarly in vivo. However, it can be observed that more than half of the zinc bound to WPH-Ever and WPH-Pap were retained in the complexes after simulated GI digestion. This indicates that the structures of peptides can affect the release of the bound zinc for intestinal absorption. If translated in vivo, the fate of the zinc ions is not particularly certain as they can be absorbed by passive or carrier-mediated transport, and can also be co-transported with the bound peptides into the enterocytes (Pérès et al., 1999).

3.6 CONCLUSION

This study demonstrates that whey proteins are a source of zinc-binding peptides that can be used as delivery agents to promote zinc nutrition in humans. For the first time, higher magnitude of the net negative surface charge of the peptides in solution was associated with higher zinc-chelating capacity. Particularly, WPH-Ever exhibited a greater capacity to bind zinc compared to WPH-Pap due to its relatively higher negative surface charge, despite having a lower content of metal-chelating amino acid residues. The FTIR spectra of the zinc complexes of WPH-Ever and WPH-Pap indicated that they have common zinc-chelating ligands, although this did not determine the release of zinc from their complexes during simulated GI digestion. The zinc complexes of the hydrolysates are gastric stable, especially Zn-WPH-Ever, given that little amount of zinc was released during peptic digestion. The whey protein hydrolysates can be used to improve zinc bioaccessibility as shown by the amount of dialyzable zinc, which was higher for WPH-Pap. The high affinity
between the peptide ligands and zinc appears to limit the total zinc dialyzability from the complexes, and this was more pronounced in Zn-WPH-Ever. This indicates that high zinc-binding affinity can concurrently enhance the gastric stability and reduce bioaccessibility of the metal delivered via the peptide ligands. This suggests the need to understand the physiological fate of the released zinc and those bound to the peptides at the intestinal digestion phase.

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The authors do not have any existing conflict of interest in this study.

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Nutrition, 130(5), 1378S–1383S.


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Fig. 3.1. (A) Free amino group content, and (B) surface hydrophobicity of whey protein hydrolysates (WPH) produced with Alcalase (Alc), bromelain (Brom), Esperase (Esp), Everlase (Ever), Ficin (Fic), Flavourzyme (Flav), Neutrase (Neu), Papain (Pap), Protamex (Prot), and Savinase (Sav); bars in each chart with different letters represent significantly different mean values with P<0.05.

Fig. 3.2. (A) Zeta (ζ)-potential, and (B) mean particle diameter of the whey protein hydrolysates; bars in each chart with different letters represent significantly different mean values with P<0.05.

Fig. 3.3. Zinc chelating capacity of the whey protein hydrolysates and EDTA; bars with different letters represent significantly different mean values with P<0.05.
Table 3.1. Amino acid composition (mole%) of whey protein hydrolysates produced with Everlase (WPH-Ever) and papain (WPH-Pap).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>WPH-Ever</th>
<th>WPH-Pap</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>9.98</td>
<td>11.20</td>
</tr>
<tr>
<td>Glx</td>
<td>18.80</td>
<td>21.97</td>
</tr>
<tr>
<td>Ser</td>
<td>5.44</td>
<td>6.06</td>
</tr>
<tr>
<td>Gly</td>
<td>1.62</td>
<td>1.76</td>
</tr>
<tr>
<td>His</td>
<td>1.80</td>
<td>2.15</td>
</tr>
<tr>
<td>Arg</td>
<td>2.25</td>
<td>2.53</td>
</tr>
<tr>
<td>Thr</td>
<td>7.20</td>
<td>8.45</td>
</tr>
<tr>
<td>Ala</td>
<td>4.69</td>
<td>5.06</td>
</tr>
<tr>
<td>Pro</td>
<td>6.13</td>
<td>7.38</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.45</td>
<td>3.04</td>
</tr>
<tr>
<td>Val</td>
<td>5.59</td>
<td>5.42</td>
</tr>
<tr>
<td>Met</td>
<td>2.37</td>
<td>1.87</td>
</tr>
<tr>
<td>Ile</td>
<td>5.52</td>
<td>4.76</td>
</tr>
<tr>
<td>Leu</td>
<td>9.84</td>
<td>7.41</td>
</tr>
<tr>
<td>Phe</td>
<td>3.13</td>
<td>2.21</td>
</tr>
<tr>
<td>Lys</td>
<td>9.69</td>
<td>7.06</td>
</tr>
<tr>
<td>Cys</td>
<td>2.52</td>
<td>1.68</td>
</tr>
</tbody>
</table>

Asx, Asp + Asn; Glx, Glu + Gln
Table 3.2. Wavenumber (cm$^{-1}$) of band shifts observed in FTIR spectra of WPH-Pap and Zn-WPH-Pap.

<table>
<thead>
<tr>
<th>Functional groups</th>
<th>WPH-Pap</th>
<th>Zn-WPH-Pap</th>
</tr>
</thead>
<tbody>
<tr>
<td>COO$^-$ symmetric stretch</td>
<td>1400</td>
<td>1394</td>
</tr>
<tr>
<td>C-O bonds of Asp and Glu</td>
<td>1314</td>
<td>1145</td>
</tr>
<tr>
<td></td>
<td>1246</td>
<td>1067</td>
</tr>
<tr>
<td>C-OH bonds of Ser/Thr</td>
<td>1180</td>
<td>938</td>
</tr>
<tr>
<td></td>
<td>1076</td>
<td>858</td>
</tr>
</tbody>
</table>

Table 3.3 Zinc release and dialyzability of zinc-peptide complexes after simulated gastric (peptic) and gastrointestinal (peptic-pancreatic) digestion of WPH-Ever and WPH-Pap.

<table>
<thead>
<tr>
<th>Whey protein hydrolysate</th>
<th>Zinc released after peptic digestion (%)</th>
<th>Zinc released after peptic-pancreatic digestion (%)</th>
<th>Zinc dialyzability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WPH-Ever</td>
<td>6.74 ± 0.05$^b$</td>
<td>32.09 ± 2.34$^b$</td>
<td>38.83 ± 2.38$^b$</td>
</tr>
<tr>
<td>WPH-Pap</td>
<td>10.49 ± 0.02$^a$</td>
<td>36.49 ± 0.59$^a$</td>
<td>46.53 ± 0.89$^a$</td>
</tr>
<tr>
<td>P value</td>
<td>P&lt;0.005</td>
<td>P&lt;0.004</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Numbers in each column with different superscript letters represent significantly different mean values.
Fig. 3.1. Udechukwu, Downey, Udenigwe
Fig. 3.2. Udechukwu, Downey, Udenigwe
Fig. 3.3 Udechukwu, Downey, Udenigwe
CHAPTER 4 ADAM17/TACE INHIBITION BY ZINC-CHELATING PEPTIDES DERIVED FROM WHEY PROTEIN HYDROLYSATE PRODUCED WITH ESPERASE

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4.1 ABSTRACT

“A disintegrin and metalloproteinase 17” (ADAM17), or tumour necrosis factor (TNF)-\(\alpha\)-converting enzyme, is a therapeutic target in inflammatory diseases. Compounds possessing zinc-chelating ligands inhibit ADAM17 via coordination of the zinc cofactor present at its catalytic site. In this study, zinc-chelating peptides were derived from whey protein hydrolysate produced with Esperase (WPH-Esp). WPH-Esp was ultrafiltered using a 1 kDa molecular weight cut-off membrane. Peptides with high zinc-binding affinity were isolated from the ultrafiltrate (<1 kDa peptide fraction) using immobilized metal affinity chromatography (IMAC-Zn\textsuperscript{2+}). The peptides exhibited a high zinc-chelating capacity (up to 83\%) compared to the ultrafiltrate and WPH-Esp, with maximum chelating capacity of 40 and 23\%, respectively. Moreover, the high zinc-binding capacity of the peptides was shown to be a mechanism for ADAM17 inhibition, as they produced the highest inhibition of up to 93\% at the lowest concentration. LC/MS/MS analysis revealed the presence of 25
peptides, rich in strong peptidic ligands such as His, Asp, and Glu, and thereby explains their relatively high zinc-chelating capacity and affinity for the enzyme. The peptides were identified as protein fragments of bovine β-lactoglobulin, α-lactalbumin, serum albumin, β- and k-casein, osteopontin-k, and folate receptor-α. This study demonstrates that food-derived zinc-chelating peptides can be used in controlling inflammation as therapeutic ADAM17 inhibitors.

**Keywords:** inflammatory diseases; TNF-α; ADAM17; inhibition; zinc-chelating whey peptides; immobilized metal affinity chromatography

### 4.2 INTRODUCTION

Therapeutic advances to chronic inflammatory diseases, such as rheumatoid arthritis, aim at inhibiting tumour necrosis factor-α (TNF-α), due to its excessive pro-inflammatory immune response (Silva, Ortigosa, & Benard, 2010). The adverse inflammatory reactions of TNF-α can be mitigated by (i) inhibiting its synthesis via the transcriptional or translational mechanisms, (ii) inactivation of its signalling pathway through a specific binding by inhibitors, and (iii) preventing pro-TNF-α activation, which downregulates TNF-α level in the blood stream (Nelson and Zask, 1999). Anti-TNF-α biologics such as etanercept, adalimumab, and infliximab specifically bind TNF-α, thereby hindering the cytokine from transducing a signal (Palladino, Bahjat, Theodorakis, & Moldawer, 2003). These drugs have been successfully used for the treatment of rheumatoid arthritis and Crohn’s disease, but their usage is limited by high risk of serious infections and malignancies, high production cost, and intravenous mode of administration (Furst et al.,
2001; Taylor, 2003; ; Shaw, Nixon, & Bottomley, 2000). However, their efficacy validated TNF-α as a strong disease target, and stimulated the current research focus on inhibiting TNF-α through its activation pathway.

TNF-α exists on the cell membrane in an inactive form (pro-TNF-α); however, the active protein is released by removal of the extracellular domain by a zinc-dependent endopeptidase known as ‘a disintegrin and metalloproteinase 17’ (ADAM17) or TNF-α-converting enzyme (TACE). Thus, ADAM17 is an important upstream therapeutic target for combating TNF-α-mediated pathologies. The zinc cofactor in ADAM17 catalytic site is a specific target for the enzyme inhibition, given its critical role in substrate catalysis and proper structure folding. Several synthetic compounds inhibit ADAM17 by zinc coordination (among other mechanisms) through their zinc-chelating ligands (Murumkar, Giridhar, & Yadav, 2013). However, the therapeutic application of the inhibitors is mostly impeded by their inability to produce clinical efficacy or their adverse side reactions due to off-target activity (Moss, Sklair-Tavron, & Nudelman, 2008).

Food protein-derived peptides are potentially TACE inhibitors due to their zinc-chelating capacity, which is mostly attributed to the side chains of amino acid residues such as His, Asp, Glu, Cys, and Ser (Udechukwu et al., 2016). Moreover, food peptides have demonstrated regulatory functions in inflammation (Chakrabarti, Jahandideh, & Wu, 2014). Bioactive peptides are primarily produced as a mixture of structurally diverse protein fragments within protein hydrolysates, where they can individually or synergistically exert their biological activities. However, highly potent bioactive peptides can be enriched from protein hydrolysates by means of bioassay-guided procedures, given that the structural requirements for the interaction and inactivation of the physiological
disease targets are known. For instance, the cationic property of peptides can be exploited to deactivate the negatively-charged calmodulin (CaM), in relation to regulating the metabolic activities of CaM-dependent enzymes such as nitric oxide synthase; and the cationic peptides can be processed from protein hydrolysates by cation-exchange chromatography (Udenigwe & Aluko, 2012b). Moreover, peptides rich in hydrophobic amino acid residues for angiotensin-1-converting enzyme inhibition can be obtained using reverse phase-high performance liquid chromatography (Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000; Segura-Campos, Chel-Guerrero, & Betancur-Ancona, 2011). Similarly, since zinc chelation is an important structural requirement for ADAM17 inhibition, bioactive peptides can be tailored to possess strong affinity for ADAM17 catalytic site zinc cofactor. Thus, the objectives of this study were to (i) strategically derive strong zinc-chelating peptides from whey proteins, and (ii) investigate the ability of the peptides to inhibit ADAM17 enzymatic activity.

4.3 MATERIALS AND METHODS

4.3.1 MATERIALS

Bovine whey protein isolate was purchased from Bulk Barn Foods Ltd (Truro, NS, Canada); proteases, 8-anilino-1-naphthalene sulfonic acid (ANS) and O-phthalaldehyde were purchased from Sigma-Aldrich (Oakville, ON, Canada); and 4-(2-pyridylazo) resorcinol (PAR), sodium dodecyl sulphate (SDS), dithiothreitol (DTT), and zinc sulphate heptahydrate (ZnSO₄·7H₂O) were purchased from Fischer Scientific (Ottawa, ON, Canada).
4.3.2 STRATEGIC PRODUCTION OF THE ZINC-CHELATING PEPTIDES

4.3.2.1. WHEY PROTEIN HYDROLYSIS

Aqueous suspensions of bovine whey protein isolate (5% w/v) were hydrolyzed with proteases of different cleavage specificities viz. papain, bromelain, ficin, Alcalase, Flavourzyme, Neutrase, Esperase, Everlase, Protamex, and Savinase, at enzyme-to-substrate ratio of 1:100. The hydrolysis was carried out for 5 h at the optimum conditions of the proteases (65 °C, pH 7.0 for papain; 37 °C, pH 6.5 for bromelain; 37 °C, pH 7.0 for ficin; 55 °C, pH 8.0 for Alcalase and Savinase; 50 °C, pH 7.0 for Flavourzyme and Neutrase; 60 °C, pH 8.0 for Esperase and Everlase; 50 °C, pH 6.5 for Protamex). Hydrolysis was terminated by reducing the pH of the reaction mixtures to 4.0 using 1 M HCl, followed by centrifugation at 10,000 × g for 10 min. Then, the pH of the supernatants was adjusted to 7.0 using 1 M NaOH, and they were freeze-dried to obtain the hydrolysate powders, which were stored at -20 °C. The free amino content of the hydrolysates was analysed using the O-phthalaldehyde (OPA) method (Nielsen, Petersen, & Dambmann, 2001), and used as a measure of degree of protein hydrolysis.

4.3.2.2. CHARACTERIZATION OF THE WHEY HYDROLYSATES BY SURFACE HYDROPHOBICITY, ZETA POTENTIAL, AND PARTICLE SIZE

Surface hydrophobicity (So) of the hydrolysates was determined by a fluorescence method using ANS. Samples were diluted with 0.01 M phosphate buffer (pH 7.0) to concentrations ranging from 0.015 to 0.0009%. Then, fluorescence was measured at the excitation and emission wavelengths of 390 and 470 nm, respectively. The slope of the fluorescence vs. concentration plot was taken as the surface hydrophobicity (Paraman, Hettiarchechy, Schaefer, & Beck, 2007). Zeta (ζ) potential and mean particle size were analysed using a
Zetasizer Nano Series Nano-ZS (Malvern Instruments Ltd., Malvern, UK). The samples were dispersed in deionized water and the measurements were obtained in a disposable capillary cell after equilibration for 120 s at 25 °C.

4.3.2.3 DETERMINATION OF ZINC-CHELATING CAPACITY

The whey protein hydrolysates were screened for zinc chelation as previously reported (Jakob et al., 2000). The assay principle is based on the reaction of 4-(2-pyridylazo) resorcinol with free zinc ions in solution to form a red coordination complex. The samples and reagents were prepared in 40 mM HEPES-KOH buffer (pH 7.5). Then, 250 µL of the samples (at final concentrations of 0.1-1 mg/mL) was mixed with 125 µL each of 8 mM DTT and 250 µM ZnSO$_4$.7H$_2$O. The blank experiment contained all assay components except the samples, which were substituted with an equal volume of the buffer. The mixtures were incubated at 37 °C for 10 mins, followed by the addition of 25 µL of 2 mM PAR and absorbance measurement at 500 nm. The percentage zinc-chelating capacity was calculated as 

\[
\frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}} \times 100,
\]

where A is absorbance. Then, the best three zinc-chelating hydrolysates, WPH-Everlase (Ever), Esperase (Esp), and Savinase (Sav), were selected for further processing of the zinc-chelating peptides.

4.3.2.4 PEPTIDE FRACTIONATION BY MEMBRANE ULTRAFILTRATION

The peptides in the selected whey hydrolysates (10 mg/mL) were fractionated by passage through an ultrafiltration membrane of 1 kDa molecular weight cut-off, using an Amicon Stirred Cell (EMD Millipore Corporation, Darmstadt, Germany) under nitrogen gas at 40 psi. The permeates (<1 kDa peptide fraction) and the retentates (>1 kDa peptide fraction) were collected after 4 h, and freeze-dried to obtain the powders. The molecular weight
peptide fractions were screened for their zinc-chelating capacity, and the best zinc-chelating lower molecular weight peptide fraction, which was that of WPH-Esp, was chosen for the subsequent experiment.

4.3.2.5 IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY (IMAC)

Peptides with strong zinc-binding affinity in the <1 kDa fraction of WPH-Esp were isolated on a 1 mL HiTrap IMAC Sepharose 6 Fast Flow prepacked column (GE Healthcare, Chicago, Il, USA). The column was loaded on a peristaltic pump (Thermo Fisher, Ottawa, ON, Canada), and washed with 5 column volume (CV) of deionized water. Then, it was charged with 0.1 M ZnSO$_4$. 7H$_2$O, and the unbound zinc was washed off with 5 CV of water. This was followed by column equilibration with 5 CV of 0.02 M sodium phosphate/0.5 M NaCl buffer (pH 7.2). Sample (20 mg/mL) was prepared in the phosphate buffer, and passed through the column at 2.5 mL/min to bind the immobilized zinc. Thereafter, the column was washed with 10 CV of the phosphate buffer to remove the unbound peptides. The bound peptides were recovered using pH method of elution with 10 CV of sodium acetate buffer (pH 4). The solution of the bound peptides was freeze-dried to obtain the powder, and the zinc-chelating capacity was determined.

4.3.2.6 DESALINATION OF THE IMAC-DERIVED ZINC-CHELATING PEPTIDES

The bound peptide fraction was desalted on a C18 hydrophobic column (Chromatographic Specialties, Brockville, ON, Canada). The column resin was activated with 100% acetonitrile, and equilibrated with the binding buffer, 0.1% trifluoroacetic acid (TFA) in water. The sample was prepared in the binding buffer, and passed through the column, which was then centrifuged for 2 min at 110 × g to facilitate salt removal. Thereafter, the
column was washed twice with the binding buffer to remove residual salt, followed by peptide elution using an aqueous solution of 0.1% TFA and 70% acetonitrile. Residual organic solvent was removed from the peptide sample using a nitrogen evaporator.

4.3.2.7 IDENTIFICATION OF THE ZINC-CHELATING PEPTIDES BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS/MS)

LC-MS/MS analysis was conducted on an Orbitrap analyser (Q-Exactive, ThermoFisher, San Jose, CA, USA) outfitted with a nanospray source and EASY-nLC nano-LC system (Thermo Fisher, San Jose, CA, USA) by the Mass Spectrometry Facility at SPARC BioCentre. The peptides were dissolved in 0.1% formic acid and loaded onto a PepMap RSLC EASYSprayC18 column (75 \( \mu \)m x 50 cm, 2 \( \mu \)m particles) (Thermo Fisher) at a pressure of 800 bar. Peptides were eluted over 60 min at a flowrate of 250 nl/min using a 0–35% acetonitrile gradient in 0.1% formic acid. The eluted peptides were then introduced into the Q-Exactive mass spectrometer (Thermo Fisher) by nano-electrospray. The instrument consisted of one MS full scan (400–1500 m/z) in the Orbitrap mass analyser with an automatic gain control (AGC) target of 1e6, maximum ion injection time of 120 ms and a resolution of 70,000, followed by 10 data-dependent MS/MS scans with a resolution of 17,500, an AGC target of 1e6, maximum ion time of 120 ms, and one microscan. The intensity threshold to trigger a MS/MS scan was set to 1.7e4. Fragmentation occurred in the HCD trap with normalized collision energy set to 27. The dynamic exclusion was applied using a setting of 10 s. Data analysis was performed using PEAKS® software (Bioinformatic Solutions, Waterloo, ON, Canada).

4.3.3 ADAM17 INHIBITION ASSAY
ADAM17 inhibitory capacity of WPH-Esp, the <1 kDa peptide fraction, and the IMAC-derived zinc-chelating peptides was determined at 0.1-1 mg/mL. All samples were prepared in 25 mM Tris-HCl buffer (pH 8.0). In a 96-well black microplate, 25 µL of 0.4 ng/µL rh ADAM17 was mixed with equal volumes of the sample and 10 µM ZnSO₄·7H₂O. The positive control experiment contained all components except the sample, which was substituted with an equal volume of the buffer. The reaction mixtures were equilibrated at 37 °C for 5 mins followed by the addition of 25 µL of 40 µM ADAM17 fluorogenic peptide substrate. Fluorescence was measured at the excitation and emission wavelengths of 320 and 405 nm, respectively for 5 mins at every 30 seconds. ADAM17 enzymatic activity was determined as the rate of release (R) of the fluorescent reaction product. Percentage inhibition was calculated as \((R_0-R/R_0) \times 100\), where \(R_0\) and \(R\) are the reaction rates in the absence and presence of the peptides, respectively.

### 4.3.4 STATISTICAL ANALYSIS

Assays were conducted in triplicate, and results were expressed as mean ± standard deviation. Significant differences \((P<0.05)\) between results were determined by one-way analysis of variance, followed by a Holm–Sidak multiple comparison test. The statistical analyses were done using SigmaPlot 12.1 (Systat Software, San Jose, CA, USA).

### 4.4 RESULTS AND DISCUSSION

#### 4.4.1. STRATEGIC DEVELOPMENT OF THE ZINC-CHELATING WHEY PEPTIDES

#### 4.4.1.1 PEPTIDE RELEASE FROM WHEY PROTEINS
Conventionally, bioactive peptides are produced by enzymatic hydrolysis of food proteins. The food protein precursors can be selected based on the knowledge of their tendency to release peptides that can exhibit the desired bioactivity (Udenigwe & Aluko, 2012a). Whey proteins have not been reported to bind zinc; however, they are potential sources of zinc-chelating peptides due to their capacity to bind other divalent metals, notably calcium (α-lactalbumin, β-lactoglobulin) and iron (lactoferrin) (Walzem et al., 2002; Krissansen, 2007). Moreover, peptidic fragments of whey proteins are better iron and calcium chelators than the intact proteins (Kim et al., 2007; Rui, 2009). The release of potentially zinc-chelating peptides in this study is shown by the higher content of free amino groups of the whey protein hydrolysates (Fig. 1A in chapter 3), compared to the parent protein (65 Serine mequiv NH₂/g protein). Protease-induced protein hydrolysis usually yields hydrolysates with different structural properties, due to the different cleavage specificity of the proteases, and this eventually impact the molecular interactions of the peptides (Tavano, 2013). For instance, protein hydrolysates prepared with Alcalase and trypsin are thought to possess better antioxidative and ACE-inhibitory capacity, which can be attributed to the release of small peptides that can easily access their molecular targets (A. Pihlanto-Leppälä, 2000; Sarmadi & Ismail, 2010). Thus, the use of a broad spectrum of proteases in this study would enable an optimum selection of protein hydrolysate with high structural compatibility for zinc-binding and ADAM17 inhibition. Moreover, the hydrolysates are likely to behave differently given their varying degree of hydrolysis as indicated by the free amino group content (Fig. 1A in chapter 3).

4.4.1.2. STRUCTURAL PROPERTIES OF THE WHEY PROTEIN HYDROLYSATES
The molecular interactions of peptides in a given matrix can be greatly influenced by their structural properties. For instance, the ability of peptides to form coordinate complexes with metal cations is mostly a function of their net surface charge (Elias, Kellerby, & Decker, 2008). Thus, evaluation of the structural properties such as surface charge, particle size, and surface hydrophobicity (So) of the whey protein hydrolysates can provide an important guide to the selection of peptides with high zinc-binding affinity for ADAM17 inhibition. The protein hydrolysates possess a negatively charged surface as shown by their ζ-potential (Fig. 2A in chapter 3), which is favourable for electrostatic interaction with zinc ions. However, zinc chelation by the hydrolysates is likely to occur at varying strength and capacity due to their different magnitude ζ-potential (-39 to -7 mV), with WPH-Ever and WPH-Pap having the highest and least negative charges, respectively. On the other hand, the hydrolysates, except WPH-Alc and WPH-Pap, had similar mean particle diameter (Fig. 2B in chapter 3). Particularly, the mean particle diameter (2248 nm) of WPH-Pap is extremely greater than most of the other hydrolysates, despite its relatively higher DH. This can be explained by its very low magnitude ζ-potential, which may have promoted electrostatic repulsion at the surface, resulting in the formation of larger peptide particles by intermolecular aggregation. Moreover, the different surface hydrophobicity of the hydrolysates (Fig. 1B in chapter 3) can impact their solubility and accessibility to zinc ions in aqueous solutions.

4.4.1.3. ZINC-CHELATING CAPACITY OF THE WHEY PROTEIN HYDROLYSATES

The whey protein hydrolysates exhibited significantly dose-dependent zinc-chelating capacity (Fig. 1), which had a strong negative relationship with their ζ-potential (Fig. 2A
in chapter 3), as shown by the Spearman’s rank order correlation coefficient, \( r_s = -0.733 \) (P=0.0131, n=10). The hydrolysates produced with Alcalase, Everlase, Esperase, and Savinase had the best zinc-chelating capacity (especially at 1 mg/mL); hence they are likely to contain better ADAM17 inhibitory peptides based on zinc chelation. Some studies have reported the release of potent zinc-chelating peptides with broad specific proteases, including Alcalase (Wang, Zhou, Tong, & Mao, 2011; Zhu, Wang, & Guo, 2015). Although WPH-Alc demonstrated similar chelating capacity, WPH-Ever, Esp, and Sav were chosen for further peptide processing. Everlase, Esperase, and Savinase are rarely used in peptide production compared to Alcalase, which has been extensively utilized in bioactive peptide discovery. Thus, these proteases can be explored for their potential to release bioactive peptides that can be used for anti-inflammatory therapies. Moreover, WPH-Ever, Esp, and Sav are likely to have better interaction with ADAM17 than WPH-Alc, given their higher negative charges and smaller peptide particles (Figs. 2A & 2B in chapter 3).

4.4.1.4. MOLECULAR WEIGHT FRACTIONS OF WPH-EVER, ESP, AND SAV

Protein hydrolysis usually yield peptides of broad molecular weight range. Therefore, downstream processing of bioactive peptides usually incorporates membrane ultrafiltration to isolate peptides of specific molecular sizes, depending on the intended application (Korhonen & Pihlanto, 2006). For instance, higher molecular weight peptides are preferable for functional properties such as gelation (Ismail & Gu, 2010). On the other hand, smaller-sized peptides are more desirable for functional food formulation than larger peptides, as they can withstand further gut proteolysis and permeate the intestinal cells into the blood stream (Gardner, 1988; Sarmadi & Ismail, 2010). However, more potent
bioactive peptides are sometimes embedded in higher molecular weight peptide fractions, which can pose challenges to absorption and bioavailability. The peptides in WPH-Ever, Esp, and Sav were separated into higher (>1 kDa) and lower (<1 kDa) molecular weight fractions, with the aim of selecting the peptide fraction (preferably <1 kDa) with the best zinc-chelating capacity. As shown in Fig. 2, the zinc-chelating capacity of the peptide fractions of WPH-Esp (2A), Ever (2B), and Sav (2C) increased dose-dependently. The chelating capacity of the >1 kDa fractions is significantly higher than that of the <1kDa fractions for WPH-Ever (at 0.5 and 1 mg/mL) and WPH-Sav (at all concentrations). This suggests that more zinc-binding ligands are incorporated in the larger peptides of WPH-Ever and WPH-Sav. Conversely, the zinc-binding sites appear to be equally distributed between the peptide fractions of WPH-Esp (Fig. 2), and indicates that both fractions are likely to have similar interaction with ADAM17 zinc cofactor. Therefore, the <1 kDa fraction of WPH-Esp was chosen as the best candidate for isolation of the zinc-chelating peptides.

### 4.4.1.5. AFFINITY PURIFICATION OF THE ZINC-BINDING PEPTIDES IN <1 KDA FRACTION OF WPH-ESP

Peptides with high metal-binding affinity can be selectively enriched from protein hydrolysates using immobilized metal affinity chromatography (IMAC). Besides its high selectivity, IMAC is considered suitable for isolation of metal-binding proteins and peptides, given its mild and non-denaturing elution conditions, resulting in high sample recovery (Gaberc-Porekar & Menart, 2001). The separation of peptides on IMAC columns is based on their interaction with immobilized metal ions (Porath, 1992). Peptidic metal ligands, such as Asp, Glu, and His, are deprotonated at neutral-basic pH conditions, and
hence form reversible metal complexes, which are retained on the column (Block et al., 2009). Different elution methods can be used to recover the metal-bound peptides, with pH elution being the most commonly employed. At low pH, typically 3.5-4.0, the protons of the peptidic metal ligands are reconstituted, leading to the dissociation of the peptides from the metal complexes, and are then eluted (Cheung, Wong, & Ng, 2012). Using this approach, peptides with higher metal-chelating capacity (including zinc) than the parent hydrolysates were isolated from different food proteins (Torres-Fuentes, Alaiz, & Vioque, 2011; Chen et al., 2013; Zhu et al., 2015). As shown in Fig. 3A, the chelating capacity of the isolated zinc-chelating peptides (69-83%) is many folds higher than that of WPH-Esp (1-23%) and the <1 kDa peptide fraction (2-40%), and therefore demonstrates an efficient peptide separation. Thus, highly potent zinc-chelating peptides were successfully developed in this study, and these peptides would be expected to exhibit better ADAM17 inhibition via zinc cofactor coordination. The procedures and considerations taken for the production and processing of the zinc-chelating peptides are shown in Fig. 6.

4.4.1.6. IDENTIFICATION OF THE ZINC-CHELATING PEPTIDES

The zinc-chelating peptides were identified to understand their structure-function relationship. LC/MS/MS analysis revealed the presence of 25 peptides, with molecular weight ranging from 0.6-2 kDa (Table 1), and therefore suggests that the peptides are likely to be absorbed intact on oral administration. About 54% of the peptides correspond with protein fragments of bovine β-lactoglobulin (10), α-lactalbumin (1), and serum albumin (1), while the remaining proportion were found in the sequence of other bovine milk proteins viz. β- (9) and kappa (1)-casein, osteopontin-k (1), and folate receptor-α (1). It is possible that the non-whey protein fragments were co-isolated with the whey proteins.
Also, four peptides contain oxidized methionine residues; this may have occurred during protein isolation, hydrolysis, or storage, and the safety of such modified peptides on consumption is not known. However, the structural features of the identified peptides clearly explain their high zinc-chelating capacity. The side chains of His, Asp, and Glu residues have been shown to be strong peptidic metal ligands. Moreover, high content of these residues is correlated with the metal-chelating capacity of peptides (Storcksdieck genannt Bonsmann & Hurrell, 2007; Torres-Fuentes et al., 2011; Carrasco-Castilla et al., 2012). These amino acid residues were present in most of the zinc-chelating peptides identified in this study, occurring frequently and in clusters within the peptide sequences (Table 1). The presence of N-terminal His residues in HHKAEPGPEDSLHEQ, HQPHQPLPPTVMFPPQ, KEMPFPKYPVEPF may have enhanced the zinc chelation, as His residue at this position is thought to potentiate the metal-chelating capacity of peptides (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998; Zhu et al., 2015). Moreover, these peptides can easily reach their targets such as ADAM17, considering their relatively small sizes. HHKAEPGPEDSLHEQ shares some structural similarities (such as peptide length and His composition and position) with ADAM17 catalytic site zinc structural motif, HEXXHXXGXXHD (X can be any amino acid residue), and hence suggests that it may have similar affinity for the zinc cofactor, leading to inhibition.

4.4.2. ADAM17 INHIBITION BY THE ZINC-CHELATING PEPTIDES, WPH-ESP, AND <1 KDA PEPTIDE FRACTION

The zinc-chelating peptides, <1 kDa peptide fraction, and WPH-Esp significantly (P<0.001) inhibited ADAM17 enzymatic activity in the order of their zinc-binding capacity (Figs. 3A and 3B). This demonstrates the feasibility of using strong zinc-binding food
protein fragments against ADAM17. In fact, synthetic compounds possessing ligands with higher affinity for zinc are better inhibitors than those with lower zinc-binding affinity (Sheppeck et al., 2007). However, the inhibitory effect of the zinc-chelating peptides and their precursors was observed to decrease with increasing concentration (Fig. 3B), this suggests that the peptides are likely to be more active at lower concentrations. Numerous studies on food-derived zinc-chelating peptides were focused on their application as dietary zinc carriers for enhancing zinc bioavailability (Udechukwu et al., 2016). However, this study is the first to demonstrate that the zinc-binding property of peptides can be exploited in mitigating human diseases, particularly those mediated by ADAM17.

4.5 CONCLUSION

Indeed, food-derived peptides possess the structural features that can enable them to interact and inactivate molecular disease targets. Findings from this study indicate that post-hydrolysis processing of food peptides can enhance their biological activities. Particularly, zinc-chelating peptides derived from WPH-Esp using immobilized metal affinity chromatography possessed strong zinc-chelating capacity compared to their parent hydrolysate and <1 kDa molecular weight fraction. Moreover, the relatively high ADAM17 inhibitory capacity of the zinc-chelating peptides suggests the health-promoting effect of whey-derived zinc-binding peptides in TNF-α-mediated inflammatory diseases.

ACKNOWLEDGEMENTS

This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) through a Discovery Grant Program (RGPIN 435865-2013), and Canada Foundation for Innovation (CFI) through Infrastructure Grant.
4.6 REFERENCES


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Evolution Create Functionalities for Mammalian Nutrition: What We Know and What We May Be Overlooking. *Critical Reviews in Food Science and Nutrition*, 42(4), 353–375.


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**Fig. 4.1.** Zinc-chelating capacity of the whey protein hydrolysates at 0.1 (A), 0.25 (B), 0.5 (C), and 1 (D) mg/mL; bars with different letters represent significantly different mean values with P<0.05.

**Fig. 4.2.** Zinc-chelating capacity of the molecular weight fractions of WPH-Esp (A), WPH-Ever (B), and WPH-Sav (C); bars with different letters represent significantly different mean values with P<0.05.

**Fig. 4.3.** Zinc-chelating (A) and ADAM17 inhibitory capacity (B) of WPH-Esp, <1 kDa fraction, and the zinc-chelating peptides; bars with different letters represent significantly different mean values with P<0.05.

**Fig. 4.4.** Production and processing of zinc-chelating peptides from whey protein hydrolysate produced with Esperase.
<table>
<thead>
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<th>Zinc-chelating peptide sequence</th>
<th>Molecular weight (Da)</th>
<th>Bovine protein and accession number</th>
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Fig. 4.1. Udechukwu & Udenigwe
Fig. 4.2. Udechukwu & Udenigwe
**Fig. 4.3.** Udechukwu & Udenigwe
Whey proteins

Enzymatic hydrolysis

Ultrafiltration with 1 kDa molecular weight cut-off membrane

Isolation of zinc-chelating peptides by Immobilized metal affinity chromatography

Identification of zinc-chelating peptides by LC/MS/MS

- Determination of structural properties and zinc-chelating capacity of the hydrolysates
- Selection of best three zinc-chelating hydrolysates
- Determination of zinc-chelating capacity of the filtrates (< 1 kDa) and retentates (> 1 kDa)
- Selection of the best zinc-chelating ultrafiltrate

Fig. 4.4. Udechukwu & Udenigwe
CHAPTER 5 INHIBITION OF ADAM17/TACE ACTIVITY BY ZINC-CHELATING RYE SECALIN-DERIVED TRIPEPTIDES AND ANALOGUES

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5.1 ABSTRACT

“A disintegrin and metalloproteinase 17” (ADAM17), or tumour necrosis factor (TNF-\textgreek{a}) converting enzyme, is an upstream target for mitigating TNF-\textgreek{a}-mediated inflammatory diseases. ADAM17 can be inhibited by chelation of its catalytic site zinc cofactor, which is required for substrate catalysis and structure stabilization. In this study, rye secalin-derived tripeptides (CQV and QCA) and analogues (QCV and QVC) exhibited zinc-chelating capacity (~35% at 0.5 \textmu M) and dose-dependently inhibited ADAM17 activity with up to 70\% inhibition observed at 5 \textmu M. Moreover, ADAM17 intrinsic fluorescence emission was quenched with increasing peptide
concentration via the static mechanism, with CQV producing the highest quenching constants, Ksv and Kq. Molecular docking revealed that the peptides interacted with ADAM17 active site residues, mostly occupying the S1 and S1' subsites; CQV had the shortest distance to the zinc cofactor and lowest binding energy. The peptides coordinated the zinc cofactor through their C-terminal carboxylate anions for QCV, QVC and CQV, and peptide bond carbonyl for CQV. CQV also had more hydrogen bonding with the N, O and H atoms of ADAM17 active site residues but, unlike the other peptides, this did not involve the peptidyl sulphhydryl groups. Interaction with ADAM17 S1' hydrophobic pockets suggests a possible selectivity of the peptides for ADAM17, and their promise as bioactive candidates for controlling inflammation.

**Keywords:** inflammatory diseases; ADAM17; TNF-α; secalin; bioactive peptides; zinc chelation; fluorescence quenching; molecular docking

### 5.2 INTRODUCTION

Aberrant immune response to harmful stimuli can result in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease, which contribute to the global disease burden. A popular therapeutic strategy for treating or managing inflammatory disorders has involved the inhibition of tumour necrosis factor (TNF)-α, a pro-inflammatory cytokine whose over-activation is implicated in inflammatory disease onset and progression. Different avenues for controlling physiological levels of TNF-α have been studied, and one of them targets the pathway leading to its activation. TNF-α is expressed on the cell membrane as an inactive 26-kDa pro-TNF-α, which is then activated via a proteolytic cleavage of the extracellular domain by a
zinc-dependent endopeptidase known as “a disintegrin and metalloproteinase 17” (ADAM17) or TNF-α-converting enzyme (TACE)\(^5\). This makes ADAM17 a therapeutic target in inflammatory diseases, and its inhibition is viewed as an upstream target for modulating TNF-α level in the blood\(^6\). Moreover, ADAM17 is a potential target in other chronic diseases notably in cancer, where it is overexpressed leading to the activation of epidermal growth factor receptor (EGFR) signalling pathway by processing EGFR ligands such as transforming growth factor-α\(^7,8\). ADAM17 enzymatic activity can be inhibited by targeting the zinc cofactor present at its catalytic site, which plays a critical role in substrate catalysis and structure stabilization. Several synthetic compounds possessing a zinc-binding ligand inhibit the enzyme, and molecular modelling studies revealed that the ligands coordinate with the catalytic site zinc\(^9\). The attraction of these compounds to the zinc cofactor also enhances their binding with the enzyme through hydrogen bonds and hydrophobic interactions\(^9\). However, these synthetic drug leads have yet to be endorsed for therapeutic usage mostly due to their low bioavailability, lack of clinical efficacy, or potential toxicity\(^10\).

Food proteins are natural sources of zinc-chelating peptides\(^11\), which can be explored for ADAM17 inhibition. Moreover, food-derived peptides have exhibited anti-inflammatory activities in different models of inflammation\(^12\). The structural arrangement of amino acids in peptides can be such that it can mimic ADAM17 cleavage sequence (Ala76-Val77) of pro-TNF-α or their neighbouring residues, thereby promoting enzyme-inhibitor interaction. Moreover, some synthetic ADAM17 inhibitors possess a sulphydryl (-SH) group as the zinc-binding ligand, and this can interact with the zinc cofactor leading to disruption of enzymatic activity\(^13\). Notably, cysteiny1 SH group is an important zinc-binding ligand especially in endogenous zinc-finger proteins\(^14\). In addition, the C-terminal carboxylate anion, and the carbonyl and imino groups of peptide bonds
can also participate in zinc coordination\textsuperscript{15,16}. Therefore, the objectives of this study were to determine the zinc-chelating capacity and ADAM17 inhibitory activity of cysteine-containing rye secalin tripeptides and analogues, and to understand the interactions of the peptides with ADAM17 active site using molecular docking.

5.3 MATERIALS AND METHODS

5.3.1 MATERIALS

4-(2-pyridylazo) resorcinol (PAR), dithiothreitol (DTT) and zinc sulphate heptahydrate (ZnSO\textsubscript{4}.7H\textsubscript{2}O) were purchased from Fischer Scientific Co. (Ottawa, ON, Canada); deuterium oxide and recombinant human (rh) ADAM17 expressed in insect cells were purchased from Sigma-Aldrich (Oakville, ON, Canada); and ADAM17 fluorogenic peptide substrate III was purchased from R&D Systems (Minneapolis, MN, USA).

5.3.2 PEPTIDE PRODUCTION

The signal peptide (f1-19) of rye secalin (UniProtKB/TrEMBL accession number Q9FR41) was removed and the protein chain (f20-455) was then digested \textit{in silico} with proteinase K (E.C.3.4.21.14) using ExPASy PeptideCutter. Two cysteine-containing tripeptides, CQV (f324-326) and QCA (f343-345), were identified as possible bioactive compounds and then synthesized at 95% purity by GenScript Inc. (Piscataway, NJ, USA). The position of the cysteine residue in CQV was rearranged to obtain the analogues, QCV and QVC, which were also synthesized for structure-function relationship studies.

5.3.3 DETERMINATION OF ZINC-CHELATING CAPACITY
The tripeptides were evaluated for zinc-chelating capacity as described\textsuperscript{17}. The assay principle is based on the reaction of 4-(2-pyridylazo) resorcinol with free zinc ions to form a red coordination complex. Solutions of the peptides and reagents were prepared in 40 mM HEPES-KOH buffer (pH 7.5). Then, 250 µL of each sample was mixed with 125 µL of 8 mM DTT and 125 µL of 250 µM ZnSO\textsubscript{4}.7H\textsubscript{2}O. Final peptide concentrations were 0.005, 0.05, 0.5 and 5 µM. In the blank experiment, the samples were substituted with an equal volume of the buffer. The mixtures were incubated at 37 °C for 10 min. Thereafter, 25 µL of 2 mM PAR was added to the reaction mixtures to bind free zinc, followed by absorbance measurement at 500 nm. Zinc-chelating capacity was calculated as: 
\[ \frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}} \times 100 \]
where A is absorbance.

5.3.4 PROTON NUCLEAR MAGNETIC RESONANCE (\textsuperscript{1}H-NMR) SPECTROSCOPY

Peptides were mixed with ZnSO\textsubscript{4}.7H\textsubscript{2}O at a molar ratio of 1:10 in deuterium oxide and allowed to stand for 10 min at room before analysis. To study zinc-peptide interactions, \textsuperscript{1}H-NMR spectra of the peptides and their zinc complexes were recorded using Bruker Avance 300 spectrometer (Billerica, MA, USA) comprising of a 5 mm auto-tuning broadband probe with a Z-gradient, at an operating frequency of 300.1 Hz, temperature was stabilized at 25 °C, and a total of 64 scans was performed. The spectra were processed using Bruker TopSin version 1.3 and calibrated based on the residual non-deuterated solvent (δ\textsubscript{H} 4.80). Assignment of protons was done using homonuclear correlation spectroscopy (\textsuperscript{1}H-\textsuperscript{1}H COSY) data.

5.3.5 ADAM17 INTRINSIC FLUORESCENCE EMISSION

ADAM17 intrinsic fluorescence emission was measured in the absence and presence of the peptides using Nova spectrofluorometer (SPEX Industries, NJ, USA) at excitation and emission bandwidths of 5 nm. ADAM17 (final concentration, 0.02 ng/µL) and tripeptides (final
concentration, 0.005, 0.05, 0.5, 1.5, 2.5, and 5 µM) were mixed in a quartz cuvette and equilibrated at 37 °C for 2 min. ADAM17 was substituted with Tris-HCl buffer (25 mM, pH 8.0) in the blank experiment containing the peptides. Fluorescence intensity was recorded at the excitation and emission wavelengths of 280 and 300-400 nm, respectively.

5.3.6 DETERMINATION OF FLUORESCENCE QUENCHING CONSTANT

Fluorescence quenching constant was determined using the Stern-Volmer equation: \( \frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{SV} [Q] \), where \( F_0 \) and \( F \) are the maximum fluorescence intensities in the absence and presence of the peptides, respectively; \( K_{SV} \) is the Stern-Volmer quenching constant; and [Q] is the concentration of the quencher (peptides). The slope of the plot of \( \frac{F_0}{F} \) versus [Q] was taken to be \( K_{SV} \). To determine the quenching mechanism, the bimolecular quenching constant, \( K_q \), was calculated from the Stern-Volmer equation as: \( K_q = K_{SV} / \tau_0 \), where \( \tau_0 \) is the fluorescence lifetime in the absence of a quencher, and is usually \( 10^{-8} \) s for biological macromolecules\(^{18}\).

5.3.7 ADAM17 INHIBITION ASSAY

ADAM17 inhibitory activity was determined at 0.005-5 µM of the peptides prepared in 25 mM Tris buffer (pH 8.0). In a 96-well microplate, 25 µL of 0.4 ng/µL rh ADAM17 was mixed with equal volumes of the peptide solutions and 10 µM ZnSO\(_4\)·7H\(_2\)O. The positive control experiment contained all assay components except the peptides, which were substituted with an equal volume of buffer. The reaction mixtures were equilibrated at 37 °C for 5 min, followed by the addition of 25 µL of 40 µM ADAM17 fluorogenic peptide substrate, Mca-PLAQAV-Dpa-RSSSR-NH\(_2\). The fluorescence intensity was measured at excitation and emission wavelengths of 320 and 405 nm, respectively every 30 s for 5 min. ADAM17 enzymatic activity was determined as the rate of release (R) of the fluorescent reaction product. Percentage inhibitions were calculated as: \( (R_0 - R)/R_0 \times 100\)
\[ \frac{R}{R_0} \times 100, \quad \text{where } R_0 \text{ and } R \text{ are the reaction rates in the absence and presence of the peptides, respectively.} \]

**5.3.8 MOLECULAR DOCKING**

Molecular docking was performed using the Accelrys Discovery Studio software 2.5 (DS 2.5). The structures of the peptides were generated with DS 2.5, and energy was minimized with the CHARMM program. A crystal structure of the catalytic domain of ADAM17 (1bkc, Protein Data Bank) was used for docking. A binding site with a radius of 10 Å and coordinates, \( x: 4.263; y: -4.2; \) and \( z: 5.617 \), was created in the Subunit I of ADAM17. Automated molecular docking was performed using the partial flexibility CDOCKER tool of the DS 2.5 software in the presence of zinc cofactor. Evaluation of the molecular docking was done according to the scores and binding energy values in order to obtain the best peptide poses. DS 2.5 software was also used to identify hydrogen bonds as well as hydrophobic, hydrophilic and coordination interactions with amino acid residues in the enzyme active site.

**5.3.9 STATISTICAL ANALYSIS**

All experiments (except peptide production, NMR and docking) were done in triplicate, and results were expressed as mean ± standard deviation. Significant differences \((P < 0.05)\) between results were determined by one-way analysis of variance followed by a Holm–Sidak multiple comparison test. Statistical analyses were done using SigmaPlot 12.1 (Systat Software, San Jose, CA, USA).

**5.4 RESULTS AND DISCUSSION**

**5.4.1 RYE PROTEINS AS A SOURCE OF BIOACTIVE PEPTIDES**
Rye (*Secale cereale* L.) is a staple food in the human diet and accounts for 1% of the world’s total cereal production\(^\text{19}\). The major rye proteins are secalins including \(\gamma\)-40k, \(\gamma\)-75k, \(\omega\) and high molecular weight secalins, which are among the plant storage proteins known as prolamins\(^\text{20}\). Similar to prolamins in other cereal grains such as wheat and barley, secalins are regarded to be of poor nutritional value due to their low content of the essential amino acids lysine, tryptophan and threonine\(^\text{21}\). Moreover, prolamins are constituents of gluten, which can evoke autoimmune response in the intestine of people with celiac disease\(^\text{22}\). Valorization of prolamins can be achieved through enzymatic processing and fermentation to produce peptides that can be bioactive and potentially useful for human health promotion\(^\text{23,24}\). This process can also deactivate antigenic epitopes in the cereal proteins since the smallest currently known antigenic gluten peptides are nonapeptides\(^\text{25}\). Moreover, cereal grain proteins are thought to be sources of peptides (e.g. lunasin, VPP, IPP, LQP, LLP, etc.) that possess activities for combating chronic diseases such as cancer, diabetes, and cardiovascular diseases\(^\text{19,24,25,26,27}\).

Bioactive peptide discovery can be facilitated by the use of bioinformatics, which can aid the identification of sustainable food protein precursors of peptides, proteases for their release, and prediction of their biological activities\(^\text{28}\). For instance, ACE-inhibitory peptide sequences have been identified in rye secalins using this approach\(^\text{29}\). *In silico* hydrolysis of rye gluten proteins resulted in the release of tripeptides, QCA and CQV, by proteinase K from f343-345 and f324-326 of rye secalin, respectively. The protease has potential for use in food processing considering its broad cleavage specificity at wide pH and temperature ranges\(^\text{30}\), to ensure the deactivation of antigenic sequences. Analysis of secalin with *ZincExplorer* revealed several possible zinc-binding sites, each with scores of <0.4 (0 for no binding and 1 for strong binding), but only QCA and CQV were released as small peptides by proteinase K, with zinc-binding cysteine residue scores of
0.36559 and 0.15098, respectively. Moreover, glutamine can also participate in zinc chelation; therefore, the tripeptides were further evaluated for \textit{in vitro} zinc-chelating capacity at physiological pH and temperature. Food-derived bioactive di- and tripeptides are thought to have strong prospects for translation as active components of functional foods, compared to larger peptides, as they are more stable to gut proteolytic deactivation due to their lower number of scissile bonds, and can be transported across the intestine into the blood \textit{via} dedicated transporters\textsuperscript{31}.

\subsection*{5.4.2 ZINC-CHELATING CAPACITY OF THE TRIPEPTIDES}

The tested tripeptides exhibited a dose-dependent increase in their zinc-chelating capacity, with the highest activity observed at 0.5 µM (Fig. 1). However, the chelating capacity dropped by 63-75% at 5 µM. This is possibly due to increased proximity of the peptides in solution, which can facilitate intermolecular interactions leading to the depletion of the zinc-binding sites. The arrangement of amino acid residues in a peptide chain can influence their chemical behaviour and biological activities\textsuperscript{32}, including zinc-chelating capacity\textsuperscript{11}. For instance, the presence of a histidine residue at the N-terminal position enhanced the metal-chelating capacity of peptides\textsuperscript{33}. In our study, structural differences did not significantly alter the zinc-chelating capacity of the secalin tripeptides and analogues. Other di-, tri- and tetrapeptides derived from sesame and rapeseed proteins using trypsin and Alcalase, respectively, were reported to have 34-82% zinc-chelating capacity at higher concentrations, with tripeptides Asn-Cys-Ser (sesame) and Asn-Ser-Met (rapeseed) exhibiting the highest zinc chelation\textsuperscript{34,35}. Based on zinc chelation, the cysteine-containing tripeptides are expected to inhibit the catalytic activity of ADAM17 by interacting with its zinc cofactor.

\subsection*{5.4.3 NMR CHARACTERIZATION OF THE PEPTIDE AND THEIR COMPLEXES}
NMR spectra of the peptides and their complexes were obtained to confirm their binding and to gain insight on the peptidic ligands that participated in zinc chelation. $^1$H NMR spectra effectively showed that complexes were formed and that their proton signals overlapped with those of the unbound peptides. For instance, in the $^1$H NMR spectrum of QCA (Fig. 2A), protons of the methylene group of cysteine appeared as doublet doublets at $\delta$ 2.91 ppm (Cys-H$\beta$1, $J = 8.7, 14.4$ Hz) and 3.15 ppm (Cys-H$\beta$2, $J = 5.1$ and 14.4 Hz). In the Zn-QCA complex (Fig. 2B), the protons had similar chemical shifts but they appeared as multiplets, hence coupling constants could not be determined. The methine proton of cysteine in QCA appeared at $\delta$ 4.62 (Cys-H$\alpha$, dd, $J = 5.1$ and 8.7 Hz) compared to $\delta$ 4.63 ppm (Cys-H$\alpha$, m) in the free and Zn-QCA, respectively, which suggests a change in proton environment in the presence of zinc. However, ionic strength and pH of the chelating assay could not be used in the NMR study. The labile protons of $-\text{SH}, -\text{NH}$ and $-\text{COOH}$ were not detected due to their exchange with deuterium from the solvent, and it was therefore not possible to identify these zinc-binding sites.

**5.4.4 EFFECT OF THE TRIPEPTIDES ON ADAM17 FLUORESCENCE EMISSION**

Proteins intrinsically emit fluorescence due to their aromatic amino acid residues, tryptophan, tyrosine and phenylalanine$^{36}$. The fluorescence emission spectra of these residues provide information on their microenvironment, the conformational state of proteins, and binding interactions between proteins and their ligands. ADAM17 region starting from the catalytic to the cysteine-rich domains (Arg215-Asn671) contains three tryptophan and many tyrosine residues, which are the protein fluorophores that absorb at 280 nm$^{37,38}$. The fluorescence emission of these fluorophores, particularly tryptophan, is dependent on the polarity of their environment. The wavelength of maximum fluorescence emission ($\lambda_{\text{max}}$) of ADAM17 was observed at 310 nm (Fig. 3); a similar emission wavelength of 316 nm was previously reported for ADAM17 catalytic
domain, and indicates that the fluorophores are buried in hydrophobic cores. In the presence of the tripeptides, no substantial shift was observed in ADAM17 $\lambda_{\text{max}}$, which suggests that microenvironments of tryptophan and tyrosine residues were largely unperturbed, hence, conformational changes such as protein unfolding did not occur in the presence of the peptides. However, ADAM17 fluorescence emission was dose-dependently quenched by 9-40% in the presence of the peptides (Fig. 3), with CQV producing the highest effect as shown by the magnitude of the quenching constant, $K_{SV}$, (Table 1). The occurrence of quenching is evidence that the peptides were in direct contact with the fluorophore(s) or they were close enough to quench the fluorescence. Thus, CQV must have better interaction with ADAM17 despite the structural similarity of CQV and its analogues, QCV and QVC. Therefore, the relative position of amino acid residues in the peptides affected their interaction with ADAM17, since they are likely to be positioned differently on the enzyme structure. Fluorescence quenching occurs by two main mechanisms, which are dynamic (or collisional) and static quenching. Dynamic quenching occurs at the excited electronic state of the fluorophore, where it interacts with the quencher, and returns to the ground state without photon emission, thereby decreasing the fluorescence intensity. Conversely, static quenching results from complex formation between the quencher and the fluorophore at the ground electronic state, which consequently decreases the number of fluorophores, and hence the fluorescence intensity. The type of quenching occurring in a given system can be determined from Stern-Volmer equation by calculating the bimolecular quenching constant ($K_q$), which is usually less than $2 \times 10^{10}$ M$^{-1}$ s$^{-1}$ for collisional mechanism but greater for static quenching. Also, both mechanisms can occur concurrently. As shown in Fig. 4, linear Stern-Volmer plots were observed, indicating that the quenching of ADAM17 fluorescence emission by the tripeptides occurred by one mechanism, and that the enzyme fluorophores were
equally accessible to the quenchers. Moreover, the Kq values of the peptides ranged from 6.5-9.9 (×10^6 M⁻¹ s⁻¹) (Table 1), indicating that the peptides quenched ADAM17 fluorescence emission by the static mechanism. This further demonstrates that a specific binding interaction occurred between the peptides and the enzyme⁴², which can lead to inhibition of the enzymatic activity.

5.4.5 EFFECT OF THE TRIPEPTIDES ON ADAM17 ENZYMATIC ACTIVITY

ADAM17 enzymatic activity significantly decreased in the presence of the tripeptides; hence, the enzyme-peptide interaction observed in the fluorescence quenching study resulted in inhibition. As shown in Fig. 5, the peptides dose-dependently inhibited ADAM17, with the highest activity (70%) observed at 5 µM. The inhibitory capacity of the peptides at this concentration does not correlate with the zinc-chelating capacity, which suggests that, although zinc chelation can play a role in ADAM17 inhibition, other molecular interactions with the enzyme are likely involved. This is the first report of ADAM17 inhibitory activity of food protein-derived peptides. The tested peptides are small enough to access and occupy ADAM17 active site and have potential as desirable inhibitory molecules because their small sizes may make them bioavailable⁴³,⁴⁴. Some studies have demonstrated that enterally administered peptides are transported across the enterocytes into the blood stream via peptide transporter, PepT1⁴⁵, and the peptides can also exert anti-inflammatory activity in the gut in conditions such as inflammatory bowel disease.

5.4.6 STRUCTURE-FUNCTION RELATIONSHIP OF THE TRIPEPTIDES

Application of food-derived peptides as ADAM17 inhibitors would be facilitated by the understanding of their structure-function relationship. The mode(s) of interaction of the tripeptides with ADAM17 was elucidated by molecular docking. The peptides were stabilized in the active site of the enzyme by a combination of zinc coordination, hydrogen bonds, and hydrophobic
interactions, and mainly occupy the S1 and S1' subsites of the enzyme. These binding modes have been reported for synthetic ADAM17 inhibitors. The most preferred conformations of the tripeptides in the enzyme active site are shown in Fig. 6, and amino acid residues with at least one atom around the docked peptide are summarized in Table 2. As shown for synthetic ADAM17 inhibitors bearing carboxylate group as their zinc-binding ligand, the C-terminal carboxylate anions of QCA, QCV and QVC in our study served as bidentate ligands. Thus, zinc was in pentagonal coordination geometry with the three catalytic site histidine residues and the two oxygen atoms of the tripeptide carboxylate groups (Fig. 6). The spatial orientation of the C-terminal carboxylate anion of CQV was such that its interaction with zinc was prohibited; therefore, zinc coordination occurred through the carbonyl oxygen of the peptide bond between glutamine and valine residues. The distance between zinc and tripeptide donor atoms ranged from 2.145 to 2.339 Å, with CQV maintaining the closest interaction and having the least binding energy (Table 3). This indicated a more stable zinc coordinate complex with CQV and the proximity explained its pronounced ADAM17 fluorescence quenching effects. The tripeptide -SH moiety, the key zinc ligand, was observed to be located away from the zinc cofactor and therefore did not participate in zinc chelation. However, both the hydrogen and sulphur atom of the -SH in QCA, QCV, and QVC acted as either hydrogen bond donor or acceptor for binding with the S1' subsite residues, Gly346, Tyr436, Val440 and Leu348, with similar bond distances (Fig. 6, Table 4). Other hydrogen bond donors and acceptors in QCA, QCV, and QVC include the hydrogen atom of the N-terminal amino groups, the oxygen atom of the peptide bond, and the side chain -NH of glutamine amide group (Table 4). CQV had the highest number of hydrogen bond contacts but, unlike the other peptides, its –SH did not form hydrogen bonds with the enzyme. The hydrogen bonding between the peptides and Glu406 (Table 4), which serves as a base during substrate
catalysis\textsuperscript{47}, indicated a possible disruption of the highly conserved zinc structural motif and could explain the inhibitory activity. Peptides interactions with Tyr436 and Tyr433 as well as the residues in their vicinity \textit{viz.} Gly431, Lys432, Val434, Met435, Pro437, Ile438, Ala439 and Val440 can partly explain the fluorescence emission and static quenching effects on ADAM17. Interaction of ADAM17 inhibitors with the S1' hydrophobic pocket has been proposed to be key to achieving a selective inhibition and possibly eliminating physiological side effects\textsuperscript{48,49}. Therefore, data from this study provide important insight on peptide affinity and their possible selectivity for the enzyme.

5.5 CONCLUSION

Food protein-derived peptides can have regulatory functions in inflammation. In this study, rye secalin tripeptides and analogues chelated zinc and inhibited the activity of ADAM17, an enzyme that is involved in the activation of pro-inflammatory cytokine, TNF-\(\alpha\). Peptides inherently contain heterogeneous group of atoms that were found to interact with ADAM17 through zinc coordination, hydrogen bonds and hydrophobic interactions. This makes these tripeptides promising for clinical evaluation as anti-TNF-\(\alpha\) and anti-inflammatory agents.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors do not have any existing conflict of interest in this study.

5.6 REFERENCES


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**Fig. 5.1.** Zinc-chelating capacity of the tripeptides; bars in each chart of a group of peptides with different letters represent significantly different mean values with P<0.001.

**Fig. 5.2.** $^1$HNMR spectra of (A) QCA and (B) Zn-QCA.

**Fig. 5.3.** Fluorescence emission spectra of ADAM17 showing the quenching effect of (A) CQV, (B) QCV, (C) QVC, and (D) QCA at 0-5 µM.

**Fig. 5.4.** Stern-Volmer plots of ADAM17 with the tripeptides.

**Fig. 5.5.** ADAM17 inhibitory capacity of the tripeptides at 0.005-5 µM; bars in each chart of a group of peptides with different letters represent significantly different mean values with P<0.001.

**Fig. 5.6.** Molecular interactions between ADAM17 and the tripeptides after automated docking at ADAM17 active site; ADAM17 hydrophobic and hydrophilic residues are shown in red and green, respectively; hydrogen bonds are represented as green dotted line; the red ball is zinc atom; the four tripeptides are represented in stick models; image were obtained with Accelrys DS Visualizer software.
Table 5.1. Stern-Volmer fluorescence quenching constants and linear equations for the interaction of the tripeptides with ADAM17

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Linear equation</th>
<th>Linear coefficient $(R^2)$</th>
<th>$K_{SV}$ ($\times 10^3$ M$^{-1}$)</th>
<th>$K_q$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQV</td>
<td>$Y = 0.0997X + 1.1$</td>
<td>0.9704 ± 0.011</td>
<td>99.7 ± 0.0052$^a$</td>
<td>$9.97 \times 10^6$ $^a$</td>
</tr>
<tr>
<td>QCV</td>
<td>$Y = 0.0650X + 1.1$</td>
<td>0.9842 ± 0.011</td>
<td>65.0 ± 0.0078$^b$</td>
<td>$6.50 \times 10^6$ $^b$</td>
</tr>
<tr>
<td>QVC</td>
<td>$Y = 0.0698X + 1.1$</td>
<td>0.9680 ± 0.007</td>
<td>69.8 ± 0.0058$^b$</td>
<td>$6.98 \times 10^6$ $^b$</td>
</tr>
<tr>
<td>QCA</td>
<td>$Y = 0.0720X + 1.2$</td>
<td>0.9761 ± 0.004</td>
<td>72.0 ± 0.0005$^b$</td>
<td>$7.20 \times 10^6$ $^b$</td>
</tr>
</tbody>
</table>

Numbers in each column with different superscript letters represent significantly different mean values with $P<0.001$
Table 5.2. Residues of ADAM17 having at least one atom at a distance of 10 Å around the docked tripeptide.

<table>
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<tr>
<th>No.</th>
<th>ADAM17 residues</th>
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<th>QCV</th>
<th>QVC</th>
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Total 35 36 36 36

(✓) indicates the presence of the residue around the peptide
Table 5.3. Predicted binding energies and Zn$^{2+}$ coordination distances of the tripeptides with ADAM17.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Zn$^{2+}$ coordination</th>
<th>Atom</th>
<th>Distance (Å)</th>
<th>- CDocker energy</th>
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<tr>
<td>CQV</td>
<td></td>
<td>O2</td>
<td>2.145</td>
<td>47.1495</td>
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<td>QCA</td>
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<td>O21, 17</td>
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<td>QCV</td>
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<td>O18, 23</td>
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Table 5.4. Hydrogen bonds observed between the ADAM17 and docked top-ranked pose of the tripeptides.

<table>
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<tr>
<th>ADAM17 residues in H-bonding</th>
<th>Number of H-bonds and their corresponding distance (Å)</th>
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<td></td>
<td>CQV</td>
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<td>GLY346:O:H46</td>
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<td>GLY346:O:H40</td>
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<tr>
<td>LEU348:HN:S22</td>
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<td>LEU348:HN:O11</td>
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<td>GLY349:O:H41</td>
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<td>GLU398:O:H40</td>
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<tr>
<td>GLU406:OE2:H42</td>
<td>1(1.86224)</td>
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<td>GLU406:OE2:H36</td>
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<td>TYR436:O:H46</td>
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<td>TYR436:O:H38</td>
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<td>PRO437:O:H44</td>
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<td>ALA439:HN:O14</td>
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Fig. 5.1. Udechukwu, Tsopmo, He, Udenigwe
Fig. 5.2. Udechukwu, Tsopmo, He, Udenigwe
Fig. 5.3. Udechukwu, Tsopmo, He, Udenigwe
Figure 5.4. Udechukwu, Tsopmo, He, Udenigwe
Figure 5.5 Udechukwu, Tsopmo, He, Udenigwe
Figure 5.6. Udechukwu, Tsopmo, He, Udenigwe
CHAPTER 6 CONCLUSION

Food-derived zinc-chelating peptides have gained relevance in human nutrition as dietary zinc delivery agents. Peptides as dietary zinc carriers can respond to changes in the pH of the gastrointestinal tract due to the structural diversity of the chelating ligands, and this can affect their zinc complexes. Thus, one of the aims of this study was to gain insight on the behaviour of zinc-peptide complexes under gastrointestinal conditions. The findings demonstrate that the structural properties of properties can promote the gastric stability of their zinc complexes and limit zinc bioaccessibility. Although this information can serve as a lead for future application of these peptides, further evaluation in vivo would be needed. Studies on the identification of zinc-chelating peptides are intended for their use in enhancing zinc absorption and bioavailability; however, this study identified zinc-chelating peptides to have regulatory functions in inflammation as ADAM17 inhibitors. In fact, the zinc-binding capacity of these peptides was observed to be a mechanism of their inhibitory activity, and particularly for the peptides isolated from WPH-Esp. Indeed, peptides possess the structural features that can enable them to interact with different biological targets, and this can be exploited in the inactivation of aberrant physiological processes. Although, the secalin tripeptides were selected after in silico hydrolysis based on zinc-binding property, molecular docking shows that they not only interacted with the enzyme by zinc cofactor coordination, but also through hydrogen bonds and hydrophobic interactions. The latter interaction indicates the secalin tripeptides may have selectivity for ADAM17; however, this needs to be further investigated. The optimum commercialization of bioactive peptides as health-promoting agents is impeded by lack of biological effects in vivo. This is mostly due to further proteolysis when administered enterally, and molecular weight being an important factor influencing further peptide degradation. The secalin and whey peptides are likely to resist the proteolytic action of digestive
proteases, and absorbed into the blood stream where they can exert their effects, but the preservation of the ADAM17 inhibitory capacity of these peptides needs further evaluation \textit{in vivo}. However, the ability of the zinc-chelating whey and secalin tripeptides to inhibit ADAM17 suggests they can modulate TNF-\textit{\textalpha} levels in inflammatory diseases.
REFERENCES


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