# EFFECTS OF EXOPEPTIDASE TREATMENT ON ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY ACTIVITY, ANTIHYPERTENSIVE ACTIVITY AND TASTE OF WHEY PROTEIN HYDROLYSATES

by

Lennie Ka Yan Cheung

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#### Abstract

Hypertension is commonly treated with angiotensin I-converting enzyme (ACE) inhibitors. Synthetic ACE inhibitors are associated with adverse side effects while ACE inhibitors sourced from food protein hydrolysates are not. Protein hydrolysates have potential use as functional food ingredients, but often have bitter taste. Since terminal amino acids are implicated to cause peptide bitterness, hydrolysates can be debittered when treated with enzymes such as exopeptidases, which release N- or C-terminal amino acids (aminopeptidases or carboxypeptidases, respectively) from peptides to produce shorter peptides and free amino acids. However, exopeptidase treatment may influence peptide ACE-inhibitory activity as well. Thus, this study aimed to determine the effects of exopeptidase treatment on ACE-inhibitory activity, antihypertensive activity and taste of a whey protein hydrolysate (WPH).

A Thermoase PC10F-produced WPH (T3) with high ACE-inhibitory activity (IC<sub>50</sub> = 0.15 mg/mL) was treated with the carboxypeptidase Accelerzyme<sup>®</sup> CPG (T3-AC7), the aminopeptidase Peptidase R (T3-PR7), or the aminopeptidase and proteinase ProteAX (T3-PX7). The three exopeptidase-treated hydrolysates exhibited high ACE-inhibitory activities (IC<sub>50</sub> = 0.24–0.34 mg/mL). All size fractions of T3, obtained using size exclusion chromatography, exhibited relatively high ACE-inhibitory activity while the 200–1000 Da fractions of exopeptidase-treated hydrolysates exhibited the highest activity. All hydrolysates lowered systolic blood pressure (maximum reductions of 30–35 mm Hg) in spontaneously hypertensive rats (SHRs) for 24 h after a single dose of 100 mg/kg body weight.

In terms of taste, the exopeptidase-treated hydrolysates were found to have significantly lower bitterness than T3 when evaluated by a trained sensory panel (n = 12), with T3-AC7 and T3-PR7 having bitterness similar to a commercial WPH without ACE-inhibitory activity. The three exopeptidase-treated hydrolysates also had higher umami taste and saltiness than T3, which may be partly explained by the release of free amino acids. Bitterness correlated negatively with overall acceptability, emphasizing the importance of debittering hydrolysates prior to their use as food ingredients.

The WPH treated with either aminopeptidase or carboxypeptidase in this study was significantly debittered and still exhibited ACE-inhibitory activity *in vitro* and antihypertensive activity in SHRs. Exopeptidase treatment, therefore, has potential use for debittering antihypertensive hydrolysates prior to their incorporation into functional foods.

### Preface

This thesis contains original work conducted by myself, Lennie Ka Yan Cheung, under the supervision of Dr. Eunice Li-Chan, with additional guidance from my committee members Dr. Christine Scaman and Dr. Margaret Cliff. Under the supervision and ongoing guidance of Dr. Li-Chan, I was responsible for planning the experimental design, conducting the experiments, obtaining the data, analyzing and interpreting the data collected, and writing the thesis, which was reviewed by Dr. Li-Chan, Dr. Scaman and Dr. Cliff.

The animal study described in Chapter 2 was conducted in collaboration with Dr. Rotimi Aluko of the University of Manitoba. The experiments were performed at the Richardson Centre of Functional Foods and Nutraceuticals, following the guidelines of the Canadian Council on Animal Care Ethics and using a protocol approved by the University of Manitoba Animal Protocol and Management Review Committee (protocol number F11-015/1/2). I was responsible for producing the four experimental hydrolysates, and analyzing and interpreting the data.

The sensory analysis study described in Chapters 2 and 3 was conducted following the guidelines and approval of the Behavioural Research Ethics Board of the University of British Columbia. The corresponding ethics certificate number was H13-01909. The application was approved on August 1, 2013 for "Debittering whey protein hydrolysates" (Project title: Functional peptides for food applications; Sponsoring agency: Natural Sciences and Engineering Research Council of Canada (NSERC)). The statistical analysis of sensory data was conducted by myself under the guidance of Dr. Eunice Li-Chan and Dr. Margaret Cliff.

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# List of Abbreviations

- $\alpha$ -La =  $\alpha$ -lactalbumin ACE = angiotensin I-converting enzyme Ang I = angiotensin IAng II = angiotensin II AP = aminopeptidase  $\beta$ -Lg =  $\beta$ -lactoglobulin BCAAs = branched-chain amino acids BU = binding unit bw = body weight CI = confidence interval CP = carboxypeptidase  $ddH_2O = distilled$ -deionized water DBP = diastolic blood pressure DH = degree of hydrolysis EH = extent of hydrolysis hTAS2R = human TAS2 receptors IPP = Ile-Pro-ProLKP = Leu-Lys-Pro LKPNM = Leu-Lys-Pro-Asn-Met LSD = least significant difference QM/MM = quantum mechanics/molecular mechanics
- QSAR = quantitative structure-activity relationship

- RAS = renin-angiotensin system
- RSM = response surface methodology
- SBP = systolic blood pressure
- SHR = spontaneously hypertensive rat
- SU = stimulating unit
- T3 = Whey protein hydrolysate produced using Thermoase PC10F after a 3 h incubation
- T3-AC1 = T3 treated with Accelerzyme<sup>®</sup> CPG for 1 h
- T3-AC7 = T3 treated with Accelerzyme<sup>®</sup> CPG for 7 h
- T3-PR1 = T3 treated with Peptidase R for 1 h
- T3-PR7 = T3 treated with Peptidase R for 7 h
- T3-PX1 = T3 treated with ProteAX for 1 h
- T3-PX7 = T3 treated with ProteAX for 7 h
- VPP = Val-Pro-Pro
- WPH = whey protein hydrolysate
- WPI = whey protein isolate

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# Dedication

I dedicate this thesis to my loving parents, Jack and Catherine Cheung.

#### **Chapter 1: Introduction**

#### 1.1 Study context

Hypertension is a condition of chronic high blood pressure that, if untreated, can lead to a variety of diseases such as cardiovascular disease (Public Health Agency of Canada, 2010). In the body, blood pressure and sodium levels in the circulation are predominantly regulated by the renin-angiotensin system (RAS) (Kobori, Nangaku, Navar, & Nishiyama, 2007). Stimulation of this system leads to the angiotensin I-converting enzyme (ACE)-catalyzed production of angiotensin II, a potent vasoconstrictor (Kobori et al., 2007). Angiotensin II directly causes arterial constriction, and indirectly increases sodium retention and blood volume by increasing aldosterone release (Brown & Vaughan, 1998; Kobori et al., 2007).

ACE inhibitors are the class of synthetic drugs most prescribed for cardiovascular diseases in Canada (Public Health Agency of Canada, 2009). However, these drugs can induce side effects such as chronic coughing and life-threatening occurrences of angioedema (Weber & Messerli, 2008). Enzymatic hydrolysis of various food proteins, such as whey proteins, can yield natural ACE inhibitors in the form of peptides. Such peptides have lower potency than their synthetic counterparts, but have not been reported to cause the aforementioned side effects (Haque, Chand, & Kapila, 2008). Therefore, ACE-inhibiting peptides sourced from proteins could be useful ingredients in food products with non-traditional health benefits (i.e., functional foods).

Success of functional foods is largely influenced by their taste (Granato, Branco, Nazzaro, Cruz, & Faria, 2010). In the case of peptides, total hydrophobicity, hydrophobic amino acids at the C-terminal and bulky, aliphatic amino acids at the N-terminal were correlated with bitterness (Kim and Li-Chan, 2006). Hydrophobic amino acids, particularly at the C-terminal of short peptides, were also found to be important for peptide ACE-inhibitory activity (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980; He et al., 2012; Wang et al., 2011; Wu, Aluko & Nakai, 2006a). In fact, ACE-inhibitory activity and bitterness have been correlated in peptides using computer modelling (Pripp & Ardö, 2007; Tan et al., 2013), as well as in hydrolysates (Cheung & Li-Chan, 2010).

The fraction of a whey protein hydrolysate containing the most hydrophobic peptides (i.e., peptides eluting from styrene-based adsorption resins between washes with 45 and 75% alcohol) was the most bitter, but also exhibited the highest ACE-inhibitory activity and contained high amounts of essential amino acids (Cheison, Wang, & Xu, 2007b). Thus, debittering hydrolysates by the removal of bitter peptides may influence their ACE-inhibitory activity and amino acid profile. Treatment with exopeptidases, or enzymes that release amino acids from the N- or C-terminal of peptides (aminopeptidases or carboxypeptidases, respectively), can debitter hydrolysates without the removal of peptides or amino acids (Raksakulthai & Haard, 2003). However, the cleavage of terminal amino acids may influence peptide ACE-inhibitory activity. Currently, relatively few studies assessing hydrolysates for ACE-inhibitory activity also evaluate their overall taste. In addition, the overall taste of exopeptidase-treated hydrolysates by sensory

evaluation would provide valuable information on their sensory profiles and aid their incorporation into functional foods.

#### 1.2 Hypertension and the renin-angiotensin system

Hypertension, defined as chronic high arterial blood pressure, is diagnosed when a patient has systolic blood pressure (i.e., blood pressure when the heart is contracted) of  $\geq$  140 mm Hg or diastolic blood pressure (i.e., blood pressure when the heart is relaxed between contractions) is  $\geq$  90 mm Hg (Public Health Agency of Canada, 2010). This condition can lead to many chronic diseases if not controlled and is the leading risk factor for cardiovascular disease and death worldwide (Public Health Agency of Canada, 2009, 2010). Although systolic blood pressure was first thought to inevitably increase with aging and thus was not considered for diagnosing hypertension, it has since been reported to have stronger association with cardiovascular complications than diastolic blood pressure (Tin, Beevers, & Lip, 2002).

Hypertensive individuals typically require blood pressure medication, as well as lifestyle changes, to control their blood pressure (Sherwood & Kell, 2010). For example, hypertensive individuals are commonly advised to limit the ingestion of dietary salt, high intake of which increase arterial blood volume, blood pressure and risk for developing heart disease and stroke (Heart and Stroke Foundation, 2011). In the body, blood pressure changes in response to the amount of salt present in extracellular fluids (Sherwood & Kell, 2010). Specifically, increasing the amount of sodium in the plasma increases blood volume and pressure, whereas chloride anions passively follow the flow of sodium cations (Sherwood & Kell, 2010).

Salt content and blood pressure are predominantly regulated by the renin-angiotensin system (RAS), which is activated by three stimuli: low extracellular fluid volume, low arterial blood pressure, and low salt (NaCl) content (Sherwood & Kell, 2010). RAS-activation prompts the kidneys to release renin into the circulation (Sherwood & Kell, 2010). Renin (EC 3.4.23.15) is a protease that produces the deca-peptide angiotensin (Ang) I by cleaving angiotensinogen, a protein present in the blood at high concentrations (Kobori et al., 2007). Ang I is then converted into Ang II, an octa-peptide, by the angiotensin I-converting enzyme (ACE) (EC 3.4.15.1, peptidyl-dipeptidase A). This metalloenzyme, a dipeptidyl carboxypeptidase, is circulated in the blood and other body fluids and present at the surface of endothelial cells in the vasculature (Brown & Vaughan, 1998; Kobori et al., 2007). In addition to producing Ang II, ACE also degrades bradykinin, a peptide that promotes vasodilation and vascular permeability (Brown & Vaughan, 1998). Ang II causes vasoconstriction, contributes to local inflammatory responses, promotes the aggregation of adhesion molecules, and increases the formation of blood clots and atherosclerosis (McFarlane, Kumar, & Sowers, 2003). Ang II also increases sodium retention by regulating aldosterone levels (Kobori et al., 2007), and may increase sodium intake by decreasing salt sensitivity in angiotensin II type 1 receptor-expressing taste cells (Shigemura et al., 2013).

#### **1.3** ACE inhibitors for treatment of hypertension

While the RAS is a system of homeostasis in normotensive individuals, stimulation of the RAS, particularly in the kidneys, in the absence of the previously mentioned stimuli can result in hypertension (Kobori et al., 2007; Mann, 2012). A diet excessive in sodium (i.e.,  $\geq 6-7$  g

NaCl/day) can also place the body under chronic hypertension (Takahashi, Yoshika, Komiyama, & Nishimura, 2011). In 2011, 17.6% of Canadians over the age of 12 had hypertension (Statistics Canada, 2011). Furthermore, based on a study that conducted personal interviews with and collected physiological data such as blood pressure measurements from 3, 514 individuals aged 20–79, it was estimated that 17% of hypertensive Canadian adults were unaware of their condition (Wilkins et al., 2010). However, 95% of those who were aware received treatment for their hypertension (Wilkins et al., 2010). This study also estimated that 20% of Canadian adults were prehypertensive (Wilkins et al., 2010). Individuals with prehypertension have systolic and diastolic blood pressures of 120–139 and 80–89 mm Hg, respectively, and are encouraged to make lifestyle changes to prevent further increases in blood pressure (Sherwood & Kell, 2010).

ACE inhibitors, which are prescribed for hypertension and heart failure, were the most prescribed medication for cardiovascular diseases in Canada based on data collected in 2007 (Public Health Agency of Canada, 2009). In fact, ACE inhibitors are the initial choice of therapy for some physicians (Guthrie, 2010), and are recommended as the first-line treatment for hypertension in individuals aged  $\leq$  55 years by the National Institute for Health and Clinical Excellence in the United Kingdom (Krause, Lovibond, Caulfield, McCormack, & Williams, 2011). ACE inhibitors are commonly used because they protect against the development of cardiovascular (e.g., heart failure) and metabolic (e.g., diabetes) diseases, in addition to effectively managing hypertension (Guthrie, 2010). ACE inhibitors commonly prescribed to Canadians at the hospital are ramipril and perindopril (Lapointe-Shaw et al., 2012). Although there are several ACE inhibitors available on the market, they are generally similar in their effect (Mann, 2012).

#### **1.3.1** Synthetic ACE inhibitors

The first ACE inhibitors were peptides isolated from snake venom (Ondetti et al., 1971). Subsequent studies aimed to synthesize compounds that interacted specifically with the active site of ACE, and retained activity after oral ingestion (Cushman, Cheung, Sabo, & Ondetti, 1978). Using bovine carboxypeptidase A as a model for ACE, it was hypothesized that the active site of ACE had three components: a carboxyl-binding group, an affinity group (with positive charge) for the substrate C-terminal group (with negative charge), and a zinc ion that aids scission of the penultimate peptide bond (Cushman, Cheung, Sabo, & Ondetti, 1977). Succinyl amino acids (i.e., amino acids with a succinyl carboxy group) were originally hypothesized to be good inhibitors of ACE as they contained components that interacted with all three of the aforementioned groups (Cushman et al., 1977). The ACE-inhibitory activity of synthesized compounds was determined using a spectrophotometric assay that measured the production of hippuric acid by ACE from hippuryl-L-His-L-Leu, which is a tri-peptide that most tissue enzymes do not hydrolyze (Cushman and Cheung, 1971). Cushman et al. (1977) showed that succinyl-L-Pro (Fig. 1.1a) had the highest ACE inhibition (IC<sub>50</sub> = 330  $\mu$ M) of the succinyl amino acids analyzed, hypothesizing that the rigid ring structure of Pro oriented the carboxyl group to interact with the positively-charged group in the ACE active site. Replacing the succinyl carboxyl group of succinyl-L-Pro with a sulfhydryl group resulted in 3-mercaptopropanoyl-L-Pro (Fig. 1.1b), which had markedly higher ACE inhibition (IC<sub>50</sub> =  $0.20 \mu$ M) than the former (Cushman et al., 1977). The increase in activity was attributed to the sulfur in the sulfhydryl group having stronger coordination to zinc than oxygen (Cushman et al., 1977). Subsequent modification resulted in the development of D-3-mercapto-2-methylpropanoyl-L-Pro (Fig. 1.1c), which had potent ACE-inhibitory activity (IC<sub>50</sub> = 0.023  $\mu$ M) (Cushman et al., 1977). This peptide analogue was reported to effectively reduce blood pressure in animal and human trials and was marketed as the antihypertensive drug captopril (Cushman, Cheung, Sabo, & Ondetti, 1982). Although this compound was analogous to the peptide L-Ala-L-Pro (Fig. 1.1d), the latter had much lower potency as an ACE inhibitor (IC<sub>50</sub> = 230  $\mu$ M) (Cheung et al., 1980). Other ACE inhibitors have since been developed.



Fig. 1.1 – Comparison of structures: succinyl-L-Pro (a), 3-mercaptopropanoyl-L-Pro (b), D-3-mercapto-2-methylpropanoyl-L-Pro (c) and L-Ala-L-Pro (d).

#### **1.3.1.1** Adverse side effects of synthetic ACE inhibitors

Although effective at controlling hypertension, synthetic ACE inhibitors are associated with adverse side effects. A common adverse effect is chronic coughing, which affects 15-30% of patients (Lombardi et al., 2005). While up to a fifth of patients will cease ACE inhibitor therapy due to this cough, a side effect of greater medical concern is angioedema (Weber & Messerli, 2008). ACE inhibitor-induced angioedema is a swelling of the skin and mucosal tissues, usually manifesting at the face (86%) and tongue (39%), but may also occur on the skin (14%) and rarely in the intestines (< 5%) (Cicardi, Zingale, Bergamaschini, Agostini, 2004). In a study following primarily male, elderly, white veterans, about 1 in 500 patients starting treatment with ACE inhibitors developed angioedema (Miller et al., 2008). Depending on the study, 30-64% of angioedema presented at the emergency department of various hospitals were ACE inhibitorinduced, occurrences of which can be life-threatening (Banerji et al., 2008; Sondhi, Lippmann, & Murali, 2004; Weber & Messerli, 2008). Reviews of physician practices have shown that the occurrence of side effects is not well known (Lombardi et al., 2005), often misdiagnosed and mistreated (Vegter & de Jong-van den Berg, 2010). Other known side effects of ACE inhibitors are hypotension, hyperkalemia and bone marrow depression (Hoover, Lippmann, Grouzmann, Marceau, & Herscu, 2009).

Due to the aforementioned consequences, alternatives to synthetic ACE inhibitors with similar activity and less adverse side effects are of commercial and scientific interest. For example, angiotensin receptor blockers (ARBs) also target the RAS and are as effective as ACE inhibitors

(Mann, 2012). However, due to the high cost of ARBs, they are generally only prescribed to patients who have demonstrated incompatibility with ACE inhibitors (Mann, 2012).

#### **1.3.2** Bioactive peptides as natural ACE inhibitors

There has been much research in recent decades on the production of natural, food-sourced ACE inhibitors. In particular, food proteins have been extensively studied as a source of ACE-inhibiting peptides, which have potential to be used as ingredients for functional foods or taken as nutraceuticals. Side effects for ACE-inhibiting peptides have not been reported (Haque et al., 2008), which was thought to be due to their milder pharmacological effects relative to synthetic ACE inhibitors (Hata et al., 1996).

Over the years, there has been much research on the contribution of amino acid characteristics, as well as their location along the peptide chain, to ACE-inhibitory activity in peptides. In dipeptides, aromatic amino residues such as Trp (highest activity), Tyr and Phe, as well as Pro, at the C-terminal resulted in high ACE-inhibitory activity ( $IC_{50} = 30-630 \mu M$ ) while Asp resulted in low activity ( $IC_{50} = 9.2 mM$ ) (Cheung et al., 1980). The same study showed that the aliphatic branched-chain amino acids (BCAAs) Val and Ile at the N-terminal position also resulted in peptides with ACE-inhibitory activity ( $IC_{50} 1.1-1.2 mM$ ) (Cheung et al., 1980). In fact, the dipeptides containing the most favourable groups on each terminal resulted in the highest activities, with Val-Trp having the highest potency ( $IC_{50} = 1.6 \mu M$ ) (Cheung et al., 1980). Both aromatic and branched-chain amino acids are hydrophobic amino acids. Quantitative structure-activity relationship (QSAR) analysis showed that the amino acid at the C-terminal had more

influence on ACE-inhibitory activity than those at the N-terminal, and that amino acids with increased steric properties and hydrophobicity at the C-terminal would increase activity (He et al., 2012; Wang et al., 2011; Wu et al., 2006a).

The properties for ACE-inhibiting tri-peptides are similar to those described for di-peptides. All three positions of tri-peptides are reported to contribute to their ACE-inhibitory activity, in which hydrophobic aliphatic amino acids (e.g., the BCAAs Val, Leu, and Ile) at the N-terminal and aromatic and hydrophobic, bulky amino acids (e.g., Phe and Trp) at the C-terminal all increased ACE inhibition (Jing et al., 2014; Wu et al., 2006a). Similar to di-peptides, the C-terminal amino acid was found to be most influential of ACE inhibition in tri-peptides, followed by the N-terminal amino acid (Lin, Long, Bo, Wang, & Wu, 2008; Wang et al., 2011).

The structure-activity relationship becomes less defined as peptide length increases above three amino acid residues. While the entire length of peptides was thought to contribute to overall peptide activity, only the C-terminal tri-peptide was believed to directly interact with the active site of ACE (Cushman et al., 1982). However, using QSAR analysis, Wu, Aluko, and Nakai (2006b) showed that the ACE-inhibitory activity of tetra- to deca-peptides was largely influenced by the fourth amino acid residue from the C-terminal, which at times had more influence than the penultimate and antepenultimate amino acid residues. Specifically, ACE inhibition would be increased in longer peptides (i.e., tetra- to deca-peptides) if Tyr or Cys was present at the C-terminal (the first amino acid from the C-terminal, or the C-1 position), His, Trp, or Met, at the penultimate position (C-2 position), Ile, Leu, Val or Met at the antepenultimate position (C-3 position), and Trp at the preantepenultimate position (C-4 position) (Wu et al., 2006b). Sagardia,

Roa-Ureta, and Bald (2013) also reported that the C-1 and C-4 positions were most influential of ACE-inhibitory activity, particularly when small aliphatic amino acids (e.g., Gly) and bulky hydrophobic amino acids were present, respectively. In another QSAR study, the C-terminal amino acid was predicted to have significant influence on ACE-inhibitory activity in peptides up to eight amino acid residues long, although the N-terminal amino acid was not found to affect ACE-inhibitory activity of peptides 2–8 amino acid residues long in this study (Pripp, Isaksson, Stepaniak & Sørhaug, 2004). Therefore, QSAR modelling of peptides containing four or more amino acid residues suggested that peptide ACE-inhibitory activity was influenced by C-terminal amino acids. In fact, the C-terminal tetra-peptide was found to be more influential than the C-terminal tri-peptide.

Tan et al. (2013) found in their QSAR model that increasing peptide length above tetra-peptides had little effect on increasing ACE-inhibitory activity, explaining this as ACE only interacting with the C-terminal of peptides and that N-terminal elongation would not influence this interaction. This explanation was supported in a study using quantum mechanics/molecular mechanics (QM/MM) analysis, which is a method that simulates enzymatic reactions by modelling the enzyme active site in detail to assess interactions at the atomic level, and modelling the rest of the enzyme only structurally. Using this method to model the interaction between ACE and several hexa-peptides, Zhou, Yang, Ren, Wang and Tian (2013) found that the C-terminal di-peptide, and to a lesser extent the amino acids at the C-3 and C-4 positions, interact the most with ACE, whereas the C-5 and C-6 amino acids have little influence on peptide binding. The first and fourth amino acids from the N-terminal, however, were found in yet another QSAR study to be most influential of ACE inhibition in nona-peptides, with Pro

yielding high activity (Wang et al., 2011). The discrepancy among these studies may arise from the different descriptors used to develop these QSAR models. For example, Wu et al., (2006b) used either three or five descriptors to develop their QSAR model while Wang et al. (2011) used a novel set of eight descriptors. Nevertheless, hydrophobic amino acids at the peptide terminals were consistently associated with ACE-inhibitory activity.

Food protein-derived peptides with significant ACE-inhibitory activity generally contain 12 or less amino acid residues (Li, Le, Shi, & Shrestha, 2004). Due to the different molecular weights of amino acids, which range from 75 to 204 Da for Gly and Trp, respectively, the molecular weight fraction of hydrolysates that contain the highest ACE inhibition is less defined. The permeates of the lowest molecular weight cut-off are often reported to have higher ACEinhibitory activity than the higher molecular weight fractions or the unfractionated hydrolysate, although the lowest molecular weight cut-off varies among studies. Both 1 kDa (O'Loughlin et al., 2014; Pihlanto-Leppälä et al., 2000) and 3 kDa cut-offs have been commonly and successfully used to concentrate peptides with ACE-inhibitory activity from hydrolysates (Fernández-Musoles et al., 2013; Tavares et al., 2011; Vermeirssen, Van Camp, & Verstraete, 2005). Studies on different whey protein hydrolysates using both cut-offs have found the 1 kDa permeate to have higher (Estévez et al., 2012) and lower (Mullally, Meisel, & FitzGerald, 1997) ACE-inhibitory activity than the 3 kDa permeate, suggesting that the ACE-inhibitory activity of such size fractions depend not only on peptide size, but also on peptide composition. Cut-offs of 5 kDa (Ko, Lee, Byun, Lee, & Jeon, 2012; Lee et al., 2012) or 6 kDa (Pan, Cao, Guo, & Zhao, 2012) have also been used as the lowest molecular cut-off in studies assessing hydrolysate ACEinhibitory activity, but are relatively less common.

It should be noted, however, that the lowest molecular weight fraction of hydrolysates may not always have the highest ACE-inhibitory activity. For example, when three whey protein hydrolysates (WPHs) were filtered sequentially through 10 kDa and 3 kDa membranes, the < 3 kDa fraction of Alcalase- and Neutrase-produced WPHs had the highest ACE-inhibitory activity while the 10 kDa permeate of a thermolysin-produced WPH had the highest activity (Estévez et al., 2012). Similarly, the < 1 kDa fraction of two whey proteins,  $\alpha$ -lactalbumin ( $\alpha$ -La) and  $\beta$ lactoglobulin ( $\beta$ -Lg), hydrolyzed by various digestive enzymes did not always have the highest ACE-inhibitory activity when compared to the 1–30 kDa fraction or the unfractionated hydrolysates (Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000). In fact, the unfractionated hydrolysates of other food proteins, sourced from foods such as hemp seed (Girgih, Udenigwe, Li, Adebiyi, & Aluko, 2011) and tilapia (Raghavan & Kristinsson, 2009), were reported to have higher ACE-inhibitory activities than their molecular weight fractions, suggesting that peptides of various molecular weights synergistically inhibited ACE in these hydrolysates.

Although such discrepancies exist, the < 3 kDa fraction of various WPHs have been shown to have high ACE-inhibitory activity *in vitro* (Mullally et al., 1997; Vermeirssen et al., 2005), and reduce blood pressure in spontaneously hypertensive rats in both short (i.e., single dose) and long term (i.e., 24 days) studies through *in vivo* ACE inhibition (Fernández-Musoles, Manzanares, Burguete, Alborch, & Salom, 2013; Tavares, Sevilla, Montero, Carrón, & Malcata, 2012).

#### **1.3.2.1** Commercial peptide ACE inhibitors derived from natural sources

The majority of food-derived, ACE-inhibiting peptides available commercially are marketed as nutraceuticals and sold in the encapsulated form. An example of this is PeptACE<sup>TM</sup> from Natural Factors (USA), an encapsulated product containing nine peptides from bonito (Hartmann & Meisel, 2007). The peptide Leu-Lys-Pro-Asn-Met (LKPNM) is found in PeptACE<sup>TM</sup> and is classified as a prodrug type inhibitor, which refers to peptides that require further hydrolysis after consumption (e.g., by digestive enzymes or ACE) to yield shorter peptides with higher and long-lasting ACE-inhibitory activity (Fujita & Yoshikawa, 1999). In contrast, substrate type inhibitors are degraded upon contact with ACE and subsequently lose ACE-inhibitory activity, whereas true inhibitor type peptides exhibit ACE-inhibitory activity that is not degraded by ACE or other enzymes they may come in contact with after consumption (e.g., digestive enzymes) (Fujita & Yoshikawa, 1999). As a prodrug inhibitor, the ACE-inhibitory activity of LKPNM (IC<sub>50</sub> = 2.4  $\mu$ M) increases when it is shortened to LKP (IC<sub>50</sub> = 0.32  $\mu$ M) by ACE (Fujita, Yamagami, & Ohshima, 2001). LKPNM is also found in "Peptide Soup," a Japanese functional food produced by Nippon (Japan) (Hartmann & Meisel, 2007).

Two casein-derived tri-peptides with sequences Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) have also had success as nutraceuticals and ingredients of functional foods. These two peptides account for the hypotensive effect of a functional fermented milk product produced by Calpis Co., (Japan) and are added as functional ingredients to milk products under the Flora/Becel pro.active<sup>®</sup> (Unilever, Netherlands and United Kingdom) and Evolus<sup>®</sup> (Valio Ltd., Finland) brands (Haque et al., 2008). In 2008, a meta-analysis consisting of five Japanese and four Finnish studies (for a total of 12 trials and including 623 individuals) assessed the antihypertensive effect of IPP and VPP (2.6–5.6 mg/day) on prehypertensive and hypertensive subjects (Xu, Qin, Wang, Li, & Chang, 2008). The pooled systolic blood pressure reduction of 5.6 mm Hg (95% confidence interval (CI) 4.8–6.4) in hypertensive subjects was significantly larger than the reduction of 3.2 mm Hg (95% CI 1.9–4.5) in prehypertensive subjects (Xu et al., 2008). A reduction in systolic blood pressure by 6 mm Hg has been estimated to decrease the rate of ischemic heart disease- and cardiovascular disease-related mortality by 13% (Stamler, 1997). Prolonged administration of IPP and VPP from 2 to 8 weeks was observed to increase the antihypertensive effect, although no statistical significances among the time intervals were found (Xu et al., 2008). The authors also noted that the antihypertensive effects of IPP and VPP were similar between Japanese and Finnish studies (Xu et al., 2008).

A more recent meta-analysis, consisting of the nine previous studies and nine additional studies for a total of 1691 subjects, concluded that IPP and VPP ( $9.4 \pm 13.3 \text{ mg/day}$ ) had pooled reductions of 3.73 (95% CI 1.76–6.70) and 1.97 mm Hg (95% CI 0.64–3.85) for systolic and diastolic blood pressure, respectively (Cicero, Gerocarni, Laghi, & Borghi, 2011). However, this meta-analysis found that the tri-peptides had a larger antihypertensive effect in Asian subjects than in Caucasian subjects, with systolic blood pressure reducing by 6.93 mm Hg (95% CI 2.94– 10.95) in Asians and only 1.17 mm Hg (95% CI 0.72–2.82) in Caucasians (Cicero et al., 2011). This meta-analysis also concluded that IPP and VPP had antihypertensive effects irrespective of baseline blood pressure and treatment duration in individuals. Although the authors indicated that their results were based on individuals not receiving antihypertensive medication, one of the trials included did consist primarily of individuals (32 of 36 individuals) treated with antihypertensive medication (Cicero et al., 2011; Hata et al., 1996). The discrepancy between the two meta-analyses may be due to their differences in inclusion criteria (e.g., studies comprised of subjects from all blood pressure levels or only those with prehypertension or hypertension), as well as the actual studies included. For example, the meta-analysis by Cicero et al. (2011) included two large trials from the Netherlands that concluded IPP and VPP had no effect on blood pressure reduction, and the participants from these two trials accounted for 24% of the total number of participants included in the meta-analyses.

Biozate 1<sup>®</sup> is a WPH produced by Davisco Foods International, Inc., an American cheese and food ingredient company. This product was reported to reduce systolic and diastolic blood pressure in individuals (n = 30) by 11 and 7 mm Hg, respectively, relative to the unhydrolyzed whey protein control group after ingestion at 20 g/day for 8 weeks (Fitzgerald, Murray, & Walsh, 2004; Pins & Keenan, 2002). However, this product is said to have a "slightly bitter" taste (Davisco, 2012). Other WPH food ingredients have also been found to have ACE-inhibitory activity. Peptigen IF 3080 and Peptigen IF 3090 (Arla Food Ingredients, Denmark), which are commercial WPH products used in infant formula to reduce allergenic reactions (Arla Food Ingredients, 2011), were found to have ACE-inhibitory activity (IC<sub>50</sub> = 0.124 and 0.111 mg/mL, respectively) (Otte, Shalaby, Zakora, Pripp, & El-Shabrawy, 2007). Whether these two hydrolysates elicit hypotensive effects in infants at the amounts present in the final infant formula has not been reported.

#### 1.3.2.2 Whey protein as a source of ACE-inhibiting peptides

In addition to the few commercial products mentioned above, many WPHs with high ACEinhibitory activity have been produced experimentally using various proteases (Costa, Gontijo, & Netto, 2007; Estévez et al., 2012; Guo, Pan, & Tanokura, 2009; Morais et al., 2014; O'Loughlin et al., 2014; Otte et al., 2007; Tavares et al., 2012; van der Ven, Gruppen, de Bont, & Voragen, 2002; Wang, Mao, Cheng, Xiong, & Ren., 2010; Wang et al., 2012). While there is a vast amount of knowledge available on the ACE-inhibitory activity of WPHs, an exhaustive list is not presented as the objective of this research was not to find the conditions to create the WPH with highest activity, but to explore the conditions affecting hydrolysate activity. The following discussion describes some common hydrolysis conditions, such as the enzyme used, incubation temperature, pH and time of hydrolysis, that can affect the ACE-inhibitory activity of WPHs. Although discussed separately, it should be noted that their effects are interrelated during hydrolysis. For example, the incubation temperature and pH would influence enzyme activity.

Proteolysis refers to the hydrolysis of peptide bonds between amino acid residues of a protein, resulting in a mixture of shorter peptides and free amino acids, termed protein hydrolysates. These hydrolysates have been reported to contain peptides that possess functions and bioactivity that are improved from, or not inherently present in, their parent proteins (Madureira, Tavares, Gomes, Pintado, & Malcata, 2010). Protein hydrolysis can be achieved enzymatically or chemically by acid or alkali treatment, although the generation of hydrolysates for food use is typically conducted using enzymatic hydrolysis with endoproteases. One reason for this is the production of carcinogenic chloropropanols when hydrolysates are reacted with hydrochloric
acid in the presence of lipids (Collier, Cromie, & Davies, 1991). The milder conditions used, as well as the specificity of enzymatic reactions, thus make enzymatic hydrolysis more suitable than chemical hydrolysis for producing hydrolysates that are to be consumed.

The conditions employed during enzymatic hydrolysis can affect the ACE-inhibitory activity of the hydrolysates produced. One important factor is the enzyme used for hydrolysis and its substrate specificity (Morais et al., 2014; Mullally et al., 1997; Otte et al., 2007; Wang et al., 2010). Hydrolysis of whey proteins is necessary to release peptides with ACE-inhibitory activity from intact proteins (Morais et al., 2014; Mullally et al., 1997; Otte et al., 2007). Enzymes used for generating WPHs with ACE-inhibitory activity have varied from digestive enzymes such as trypsin, chymotrypsin and pepsin (Mullally et al., 1997; Pihlanto-Leppälä et al., 2000, Ferreira et al., 2007), to enzymes sourced from bacteria (Ortiz-Chao et al., 2009; Wang et al., 2010), fungi (Morais et al., 2014), and plants (Tavares et al., 2012). Fermentation of whey by various microflora can also yield ACE-inhibiting hydrolysates (Hamme, Sannier, Piot, Didelot, & Bordenave-Juchereau, 2009). In terms of individual whey proteins,  $\alpha$ -La,  $\beta$ -Lg, and lactoferrin have released ACE-inhibiting peptides (Fernández-Musoles et al., 2013; Pihlanto-Leppälä 2000; Tavares et al., 2011).

Due to varying hydrolysis conditions across studies (e.g., differences in enzyme concentrations or times of hydrolysis) and variances among enzymes themselves (e.g., differences in enzyme activity or substrate specificity), it is difficult to directly compare enzymes for their ability to produce WPHs with ACE-inhibitory activity. However, several enzymes have been repeatedly shown to yield WPHs with high ACE-inhibitory activity. For example, the digestive enzyme trypsin (Mullally et al., 1997; Philanto-Leppälä et al., 2000; Vermeirssen et al., 2005; Otte et al., 2007) and enzymes specifically targeting hydrophobic amino acid residues, such as Alcalase, have been successful at producing WPHs with ACE inhibition (Estévez et al., 2012; Costa et al., 2007; Morais et al., 2014; Wang et al., 2010). Thermolysin was also shown to yield WPHs with high ACE inhibition, which may be related to the fact that both enzymes are zinc metalloproteases with the same binding motif and specifically cleave at hydrophobic amino acid residues (Otte et al., 2007). In addition, thermolysin is known to be heat-stable (Contreras, Hernández-Ledesma, Amigo, Martín-Álvarez, & Reico, 2011) and can exhibit enzymatic activity at the high incubation temperatures applied to unfold whey proteins. To further promote hydrolysis, some studies have looked at hydrolysis with two enzymes, in which one is added after incubation with the first. Sequential hydrolysis has been shown to increase the degree of hydrolysis (Pihlanto-Leppälä et al, 2000) and ACE inhibition of WPHs, the final activity of which is affected by the enzymes used (Wang et al., 2010).

Enzyme activity can be influenced by hydrolysis conditions such as incubation temperature and pH. For example, Protease N had higher activity at its optimal temperature (55 °C) but lower stability, while temperatures below optimal temperature increased its stability but reduced activity (Cheison, Wang, & Xu, 2007a). Using response surface methodology (RSM), the optimal degree of hydrolysis of a WPH hydrolyzed by proteases from *Lactobacillus helveticus* LB13 was achieved under specific incubation temperature and pH (about 35 °C and pH 8, respectively), the levels of which were thought to reflect the influence of these attributes on enzyme activity (Guo et al., 2009).

Furthermore, the influence of incubation temperature and pH on enzyme activity may partly explain their effects on hydrolysate ACE-inhibitory activity. Stabilization of incubation pH, which spontaneously decreases as more hydronium ions  $(H_3O^+)$  are produced during protein hydrolysis, was found to have an enzyme-dependent effect on not only the degree of hydrolysis, but the ACE inhibition of WPHs (Wang et al., 2010). While Alcalase- and Neutrase-produced WPHs had higher ACE-inhibitory activity when produced under pH-controlled conditions, trypsin-produced WPHs had higher activity when pH was not controlled during incubation (Wang et al., 2010). In addition, specific incubation temperature and pH (45 °C and pH 8–9, respectively) yielded a Corolase PP-produced WPH with optimal ACE-inhibitory activity, as determined by RSM, while other hydrolysis conditions such as the time of hydrolysis or enzyme concentration did not appear to have maximum values (van der Ven et al., 2002). This may be attributed to their influence on enzyme activity, for which optimal activity occurs under specific temperature and pH conditions. The incubation temperature and pH used during the production of hydrolysates often reflect those for optimal enzyme activity, as indicated by the suppliers of the enzymes (Estévez et al., 2012; Morais et al., 2014).

In addition to affecting enzyme activity, incubation temperature and pH can influence hydrolysate production by inducing changes to the quaternary structure and conformation of proteins, thereby affecting the accessibility of the enzyme to its substrate. At higher temperatures (e.g., 55–70 °C),  $\beta$ -Lg, which accounts for about 50% of whey proteins, undergoes reversible denaturation and begins to unfold into its molten globular state, resulting in the loss of its tertiary structure and allowing for easier access and cleavage by enzymes (Cheison et al., 2007a).  $\beta$ -Lg completely denatures at temperatures above 70 °C, dissociates from dimers to monomers from

30-50 °C, and is highly stable in octamer form at temperatures < 30 °C (Cheison et al., 2007a). Similarly,  $\beta$ -Lg dissociates from dimers to monomers between pH 2.5 and 4 while denaturation occurs at pH > 9 (Taulier & Chalikian, 2001). Heating whey protein isolate (WPI) to 65 °C prior to enzymatic hydrolysis was found to yield WPHs with high ACE-inhibitory activity, which was attributed to the partial unfolding of whey proteins at 65 °C facilitating contact between enzymes and their substrate (Costa et al., 2007). In comparison, WPHs produced from native WPI (i.e., without pre-heating) or WPI pre-heated to 95 °C had lower ACE-inhibitory activity due to the whey proteins existing in more compact form or as large aggregates held together by hydrophobic attraction, respectively, both of which hinder the contact between enzymes and their substrate (Costa et al., 2007). Interestingly, the effect of pre-hydrolysis temperature on the ACE-inhibitory activity of a Corolase PP-produced WPH was found to be influenced by the enzyme concentration, in which ACE-inhibitory activity increased with increasing pre-treatment temperature, but only when the enzyme concentration was low (van der Ven et al., 2002).

Other hydrolysis conditions that may affect the ACE-inhibitory activity of WPHs include the time of hydrolysis and type of substrate used, both of which may also be partly influenced by the enzyme used. Increasing the time of hydrolysis has been associated with higher activity in studies using RSM to optimize the ACE-inhibitory activity of WPH produced using Corolase PP (van der Ven et al., 2002) or proteases extracted from *Cynara cardunculus*, a wild thistle (Tavares et al., 2011). In contrast, the commercial pancreatic proteinase PTN 3.0S released most of the ACE-inhibiting peptides from a whey protein concentrate during the first 20 min of hydrolysis (Mullally et al., 1997). Increasing the hydrolysis time from 3 to 24 h was also found to only slightly increase ACE-inhibitory activity in various WPHs produced by thermolysin,

proteinase K, trypsin, pepsin, and *Bacillus licheniformis* protease (Otte et al., 2007). Therefore, while increased time of hydrolysis may generally increase ACE-inhibitory activity, the magnitude of increasing activity may vary depending on the other production factors such as the enzyme used.

Whether a mixture of whey proteins (i.e., whey protein concentrate or isolate) or isolated proteins (e.g.,  $\beta$ -Lg or  $\alpha$ -La) is used as substrate can affect the ACE inhibition of resulting hydrolysates. For example, hydrolysates of  $\alpha$ -La and  $\alpha$ -La enriched whey protein isolate generally had higher ACE-inhibitory activities than hydrolysates of whey protein isolate or  $\beta$ -Lg when produced using the same enzymes (Otte et al., 2007). Although variants of  $\beta$ -Lg differing in certain amino acids exist, a study comparing these variants as substrate for hydrolysis found no differences in ACE-inhibitory activity among the final hydrolysates (Otte et al., 2007).

The extent of hydrolysis and the degree of hydrolysis are both measurements of hydrolysis progression during the production of hydrolysates. The extent of hydrolysis (EH) measures the amount of peptide bonds cleaved, whereas the degree of hydrolysis (DH) presents the EH as a percentage of all the peptide bonds present. Guo et al. (2009) associated low DH (< 20%) with increased ACE inhibition while Wang et al. (2010) and van der Ven et al. (2002) associated activity with more extensive hydrolysis (25–30% and 30%, respectively). This may be due to the inconsistency of classifying DH as high or low. Other studies have found no association between DH and ACE-inhibition (Mullally et al., 1997).

Although many studies have focused on *in vitro* ACE-inhibitory activity, it is important to recognize its distinction from antihypertensive activity *in vivo*. Peptides may undergo further degradation, such as gastrointestinal digestion, after oral consumption that could risk their inactivation. Thus, the peptides or hydrolysates with highest ACE inhibition *in vitro* may not necessarily translate to the highest antihypertensive activity *in vivo*. Costa et al. (2007) showed that while Alcalase-produced WPHs had lower activity than  $\alpha$ -chymotrypsin-produced WPHs *in vitro*, the antihypertensive activity of the former increased after introduction to spontaneously hypertensive rats (SHRs). This demonstrated not only the discrepancy between *in vitro* and *in vivo* testing, but also suggested that further hydrolysis by *in vivo* enzymes and release of shorter peptides may increase activity, as is the case for the previously discussed prodrug inhibitors such as LKPNM (Fujita & Yoshikawa, 1999).

Various WPHs have been reported to reduce blood pressure in SHRs (Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998; Fernández-Musoles et al., 2013; Wang et al., 2012). Although the hypotensive effects of some of these hydrolysates were attributed to *in vivo* ACE-inhibitory activity, other mechanisms of blood pressure reduction have also been reported (Fernández-Musoles et al., 2013; Wang et al., 2012). For example, peptides from the < 3 kDa fraction of a lactoferrin hydrolysate were shown to reduce vasoconstriction in rabbit arteries by inhibiting the endothelin-converting enzyme, which produces endothelin-1, a vasoconstrictor of the endothelin system (Fernández-Musoles et al., 2013). Other hypotensive mechanisms suggested for peptides are an indirect, vasodilatory effect through the activation of opioid receptors, and a protective effect against the oxidative damage associated with cardiovascular diseases (e.g., antioxidative activity) (Hernández-Ledesma, Contreras, & Reico, 2011). Therefore, the hypotensive effects of

peptides in addition to, or instead of, ACE inhibition can also contribute to the discrepancies observed between *in vitro* and *in vivo* studies.

## **1.4** Peptides for use in functional foods

Due to growing evidence that hydrolysates of milk proteins, and specifically whey proteins, can exhibit health-enhancing (e.g., antihypertensive) effects, these hydrolysates have potential and are appropriate for use as ingredients for functional foods (Haque et al., 2008; Sinha, Radha, Prakash, & Kaul, 2007; Urista, Fernández, Rodriguez, Cuenca, & Jurado, 2011). Despite the continuously expanding research on WPHs, and protein hydrolysates in general, with ACE-inhibitory activity and their theoretically ideal applications as functional ingredients, few products have successfully incorporated such hydrolysates. In fact, the examples named earlier in the chapter are among the few that are available on the market (Hartmann & Meisel, 2007). One reason for this is the bitter taste of peptides. This attribute has prevented the practical use of antihypertensive hydrolysates in foods and is not easily decreased in food systems (Hernández-Ledesma et al., 2011). Other limitations include the potentially high ash content of hydrolysates resulting from pH adjustment during production, and the possibility of losing peptide activity during food processing and storage (Hernández-Ledesma et al., 2011). The following discussion will focus on bitterness.

## **1.4.1** Perception of bitter peptides

Bitterness perception is thought to be of evolutionary importance for mammals as a protective mechanism against the ingestion of harmful substances, such as natural poisons and compounds generated during food spoilage (Meyerhof et al., 2010). In fact, the amount of naturally-existing bitter molecules humans encounter is estimated to be in the range of tens of thousands and encompass a wide range of compounds including peptides (Meyerhof et al., 2010). Despite its importance, the actual mechanism of detecting bitter compounds, let alone bitter peptides, has only been discovered within the past two decades (Adler et al., 2000; Chandrashekar et al., 2000; Maehashi et al., 2008). Humans detect bitter compounds using approximately 25 receptors, termed human TAS2 receptors or hTAS2Rs, which vary in their specificity for bitter compounds (Meyerhof et al., 2010). Human TAS2 receptors are a class of G protein-coupled receptors that, when activated by the binding of a bitter compound, attaches to a nearby G protein such as  $\alpha$ gustducin to initiate a signaling cascade (Maehashi & Huang, 2009). Human TAS2 receptors have seven transmembrane segments, a short, extracellular N-terminal, and mostly differ in their extracellular regions to presumably recognize a wide range of compounds (Adler et al., 2000). In particular, hTAS2R1, hTAS2R4, hTAS2R14, and hTAS2R16 have been shown to recognize bitter peptides, with hTAS2R1 showing specificity for bitter di-and tri-peptides (Maehashi et al., 2008; Upadhyaya, Pydi, Singh, Aluko, & Chelikani, 2010).

# 1.4.2 Bitterness in peptides and protein hydrolysates

To predict peptide bitterness based on the hydrophobicity of their amino acid composition, Ney used Equation 1 to solve for a Q value, which was the free energy required to transfer the amino acid side chains from ethanol to water ( $\Delta f_{tR}$ ), averaged over the number of amino acid residues in the peptide (*n*) (Guigoz & Solms, 1976).

$$Q = \sum \Delta f_{tR} / n \tag{1}$$

Ney proposed that peptides with Q values > 1400 cal/mol have bitter taste while peptides with Q values <1300 cal/mol were not bitter, and that the peptide sequence had no effect on bitter taste (Guigoz & Solms, 1976). A majority of the bitter peptides sourced from hydrolysates of casein, ovalbumin, gliadin, and proteins from soy and corn, as well as synthetic peptides, reviewed in Guigoz and Solms (1976) conformed to Ney's hypotheses. However, as the authors noted, the review only focused on bitter peptides while peptides with other taste attributes and varying average hydrophobicities were not included (Guigoz & Solms, 1976). Although subsequent studies have uncovered limitations to Ney's hypotheses, such as the importance of spatial orientation of amino acid residues for eliciting bitter taste (Kim et al., 2008; Otagiri et al., 1985), hydrophobicity remains as an important factor for bitter taste. Hydrophobicity is thought to induce bitterness by partitioning peptides from the saliva and into the hydrophobic cavity in bitter receptors (Asao et al., 1987), and by aiding the binding of peptides to the active site of bitter taste receptors (Maehashi & Huang, 2009).

Following the connection between hydrophobicity and bitter taste, there was much interest in determining the roles of hydrophobic amino acids in causing peptide bitterness. These studies

were initially conducted by synthesizing and tasting amino acids and short peptides. Through these studies, amino acid stereochemistry was found to greatly affect the taste perceived. For example, L-Phe was bitter while D-Phe was 2.5 times sweeter than sucrose (Ishibashi et al., 1987b). Another study showed that N-acetyl L-amino acids were bitter while their D enantiomers were sour or neutral, whereas sweet-tasting L-amino acids were bitter when in their D configuration (Asao et al., 1987). In addition to configuration, amino acids may contain inherent flavour attributes that compete with their ability to cause other taste modalities. Such is the case of Cys and Met, both of which could not be assessed for bitterness accurately due to an overwhelming sulfurous odour and taste (Asao et al., 1987). Hydrophobic amino acids such as the BCAAs Ile, Leu and Val, the aromatic ring-containing Phe, Trp and Tyr, as well as the basic amino acid Arg were reported to have bitter taste in their natural L-conformation (Ishibashi et al., 1987a, 1987b; Ishibashi et al., 1988b; Lemieux & Simard, 1992; Li-Chan & Cheung, 2010). Some amino acids were also shown to have higher bitterness when repeated in peptide form. For example, Arg-Arg and Phe-Phe were three and fifteen times as bitter as Arg and Phe, respectively, while Phe-Phe-Phe was about 100 times as bitter as Phe (Otagiri et al., 1985).

Contrary to Ney's hypotheses, the position of the amino acids along the peptide chain also greatly influences peptide bitterness. For example, in the assessment of di-peptides, Gly-Pro was extremely bitter while Pro-Gly was sweet, and Arg-Gly was much more bitter than Gly-Arg (Otagiri et al., 1985). In general, the hydrophobic amino acids Leu, Val, Phe and Tyr were found to increase peptide bitterness when present at the C-terminal (Ishibashi et al., 1987a, 1987b; Ishibashi et al., 1988b) and basic amino acids at the N-terminal generally attributed to bitter taste in di- and tri-peptides (Otagiri et al., 1985). Position alone, however, did not solely dictate

bitterness. Although both Pro-Val and Pro-Gly had proline at the N-terminal, the former elicited a citrus sensation and the latter a fruity sensation (Asao et al., 1987). Similarly, the presence of Phe at the C-terminal did not guarantee bitterness (Ishibashi et al., 1987b). Furthermore, peptides were suggested to exhibit bitterness when they had amino acids with side chains containing 3 or more carbons, which is a trait related to hydrophobicity and include amino acids such as Val, Leu, Phe and Tyr (Ishibashi et al., 1988b).

Peptide length has also been shown to affect peptide bitterness. For example, elongation of the peptide sequence Arg-Pro-Phe (i.e., when additional Arg, Pro, or Phe units were added while keeping the sequence of Arg-Pro-Phe) significantly increased bitterness, with the octa-peptide Arg-Arg-Pro-Pro-Pro-Phe-Phe having bitterness (threshold value 0.002 mM) within an order of magnitude of brucine (threshold value 0.0008 mM), the most bitter substance known (Otagiri et al., 1985). Tamura et al. (1990a) found that elongation of peptides with seven or less amino acids markedly increased bitterness, while major differences in bitterness of peptides with eight or more amino acids were not observed. Kim and Li-Chan (2006) made similar conclusions based on their QSAR model, from which peptide bitterness was predicted to increase as the molecular weight of peptides increased up to 1000 Da, or about 8–10 residues, while certain peptides as long as 14 amino acid residues also had intense bitterness. Tan et al. (2013) also found in their QSAR model that bitterness increased as the peptide length was elongated from two to six amino acids, explaining this observation to be due the larger number of residues having increased interactions with bitter receptors.

The association between bitterness and peptides of shorter length (i.e., < 8 amino acid residues) was thought to be due to the size of the binding "pocket" on the bitter receptor (Maehashi & Huang, 2009). Ishibashi, Kouge, Shinoda, Kanehisa, and Okai (1988a) suggested that the bitter receptor contained two sites essential for bitterness detection: a "binding unit" (BU) that binds with the stimulant and at least one "stimulating unit" (SU) in close proximity. Subsequent research suggested that the BU had an affinity for hydrophobic groups, the SU for bulky basic groups and the distance between the two to be 0.41 nm (Ishibashi et al., 1988a). These proposed requirements for peptide-receptor interaction may explain the lower threshold value for Arg-Pro (0.8 mM) than either Arg or Pro alone (25 and 13 mM, respectively) since the hydrophobic imino ring of Pro and positively-charged side chain of Arg were oriented within 0.3–0.5 nm of each other (Otagiri et al., 1985). Using QSAR modelling, bulky hydrophobic groups and bulky basic groups at the C- and N-terminal of oligopeptides, respectively, were also positively correlated with bitterness (Kim & Li-Chan, 2006). These requirements, however, did not explain how different intensities of bitterness were perceived. Tamura et al. (1990a) suggested that the actual site that determines bitterness intensity, referred to as the "hydrophobicity recognition zone," existed on the wall of the binding pocket while the BU and SU existed at the base. Since the bitterness of peptides larger than 1.5 nm (or longer than seven amino acid residues) did not differ, it was estimated that the pocket size was about 1.5 nm (Tamura et al., 1990a).

Due to the importance of hydrophobicity and small size for peptide bitterness, it is not surprising that protein hydrolysates typically have bitter taste. While hydrophobic amino acids are hidden in the core of intact proteins, these amino acids may become exposed as shorter peptides are liberated during enzymatic hydrolysis. Indeed, the WPH fraction containing the most hydrophobic peptides (i.e., peptides eluting from styrene-based adsorption resins using 75% alcohol after a wash with 45% alcohol) was the most bitter and consisted predominantly (71%) of peptides  $\leq 0.6$  kDa (Cheison et al., 2007b). In contrast, the least hydrophobic fraction containing peptides that eluted with 20% alcohol had minimal bitterness, and consisted of both short peptides (41% of peptides  $\leq 0.6$  kDa) and medium-length peptides (52% of peptides 0.6–5 kDa) (Cheison et al., 2007b). WPH bitterness has also been attributed to peptides of lower molecular weights. For example, when 22 commercial WPHs were assessed for bitterness, high bitterness was correlated to low concentration of peptides > 2000 Da and high concentration of peptides < 1000 Da (Leksrisompong, Miracle & Drake, 2010). In addition, the < 3 kDa fraction of a commercial WPH was more bitter than the > 3 kDa fraction and the unfractionated WPH (Liu, Jiang, & Peterson, 2013). Interestingly, the bitterness of this commercial WPH was contributed by predominantly four peptides, one of which was sourced from  $\beta$ -casein (Liu et al., 2013). In shrimp byproduct hydrolysates, the < 3 kDa fraction obtained using size exclusion chromatography had the most bitter taste, although it was less bitter than the unfractionated hydrolysate (Cheung & Li-Chan, 2010).

The specificity of the enzymes included in the protease preparation used for hydrolysis can also be an important contributing factor of hydrolysate bitterness. For example, when three proteinase preparations from the *Bacillus* sp. were used to prepare WPH, the Alcalase-produced WPH had significantly higher bitterness than the Corloase 7089- and Prolyve 1000-produced WPHs (Spellman, O'Cuinn, & Fitzgerald, 2009). This was attributed to presence of a glutamyl endopeptidase in Alcalase, which, when purified from the commercial enzyme preparation and included during hydrolysate production with the other two proteinase preparations, increased the bitterness of the Corloase 7089- and Prolyve 1000-produced WPHs (Spellman et al., 2009).

In addition to bitterness, WPHs have been described as malty, burnt or scorched, potato or brothy, and having high or low animal flavour, all of which were detected at concentrations 10 times below that of bitterness (Leksrisompong et al., 2010). Therefore, as with many other food ingredients, the incorporation of WPHs into food products may change the overall flavour profile and impart off-flavours regardless of bitter intensity. Furthermore, the bitterness perceived in the final food product may be influenced by additional food processing techniques. For example, the bitterness of rye-flour hydrolysates was perceived to be higher when tasted as aqueous suspensions (40 g rye flour in 60 g water) than as crackers produced after the suspensions were baked (Heiniö et al., 2012).

## 1.4.3 Correlating ACE-inhibitory activity and bitter taste in peptides

While synthesis of peptides for tasting and assaying can provide information on the bitterness and ACE-inhibitory activity of specific peptide sequences, it would be impractical to synthesize and assay all peptides, which would have 400 combinations for di-peptides alone. As an alternative, QSAR modelling based on amino acid properties has been used to correlate the bitterness and ACE-inhibitory activity in peptides, which have led to conflicting conclusions (Pripp & Ardö, 2007; Tan et al., 2013; Wu & Aluko, 2007). Wu and Aluko (2007) found no significant correlation ( $p \ge 0.05$ ) between bitterness and ACE-inhibitory activity using QSARpredicted activities for 53 di-peptides and 55 tri-peptides. On the contrary, Pripp and Ardö (2007) used experimental and QSAR-predicted values for bitterness and ACE inhibition and reported strong correlations. The relationship between ACE-inhibitory activity and bitterness were thought to stem from the importance of hydrophobicity for both attributes (Pripp & Ardö, 2007). However, the correlation was less strong in oligopeptides 3–6 amino acids long, suggesting the possibility for finding longer peptides with potent ACE-inhibitory activity and low bitterness (Pripp & Ardö, 2007). Tan et al. (2013) reported similar findings, wherein the attributes were correlated for di-peptides, but not for tri- to hexa-peptides, and postulated that peptides with four or more amino acid residues had potential for having high antihypertensive activity and low bitterness. Kawakami, Kayahara, and Tadasa (1995), however, showed that peptides as short as three amino acid residues can have high ACE inhibition and low bitterness. Although still bitter, the tri-peptides IIe-Lys-Phe, Leu-Lys-Phe, and Val-Lys-Phe had reduced bitterness and increased ACE-inhibition when the C-terminal Phe was replaced with Pro (Kawakami et al., 1995).

Despite the disagreement from studies based on QSAR modelling, an association between bitterness and ACE-inhibitory activity has also been observed in WPH (Cheison et al., 2007b). Several peptides from whey proteins have also been reported in separate studies to have bitter taste and antihypertensive activity. For example, Tyr-Gly-Leu-Phe of  $\alpha$ -La and Leu-Leu-Phe from serum albumin were found to have bitter taste (Liu et al., 2013), while Tyr-Gly-Leu-Phe of  $\alpha$ -La and Leu-Leu-Phe of  $\beta$ -Lg were reported to reduce systolic blood pressure in SHRs (Hernández-Ledesma et al., 2011). Furthermore, a bitter-tasting WPH produced by Davisco Foods International, Inc. was shown to reduce the systolic and diastolic blood pressure of prehypertensive and hypertensive adults in a clinical trial (Pins & Keenan, 2006). In other commodities, ACE inhibition has been positively correlated to bitterness in hydrolysates of shrimp processing byproducts (Cheung & Li-Chan, 2010), while the hydrolysates of wakame, a type of brown seaweed, were reported to have ACE-inhibitory activity and varying degrees of bitter taste (Sato et al., 2002).

Recently, a  $\beta$ -Lg hydrolysate produced using an integrative process incorporating adsorption and microfiltration components was reported to have high ACE-inhibitory activity (IC<sub>50</sub> = 0.128 mg/mL) but did not significantly change the taste of partially-skimmed milk when added at 200–400 µg/mL (Welderufael, Gibson, Methven, & Jauregi, 2012). Hydrolysates of corn gluten and egg white have also been reported to have high ACE-inhibitory activity and low bitterness, although the methods used for bitterness assessment were not described in these studies (Chiang, Lee, Guo, & Tsai, 2006; Suh, Whang, Kim, Bae, & Noh, 2003).

#### **1.4.4 Debittering of hydrolysates**

Various methods have been assessed for their efficacy in debittering hydrolysates prior to their application into foods. Physical removal of bitter peptides using activated carbon, which form hydrophobic interactions with bitter peptides, has been shown to be an effective debittering method effective for hydrolysates such as those of corn gluten (Suh et al., 2000) and skim milk, the latter of which had bitterness completely eliminated with the use of 100% carbon (Helbig, Ho, Christy, & Nakai, 1980). A similar method utilizing hydrophobic interaction for physical removal of bitter peptides was described by Cheison et al. (2007b), as briefly described earlier. However, while the most hydrophobic fraction was the most bitter, it also had the highest content

of essential amino acids, emulsifying properties, and ACE-inhibitory activity (Cheison et al., 2007b). Thus, while physical removal of bitter peptides through these or other (e.g., chromatographic separation) methods is effective at reducing bitter taste, these methods would not only result in reduced yields, but may lead to undesirable changes to the nutritional profile, functional properties, and bioactivity of the debittered hydrolysates.

An alternative to removing bitter peptides is to introduce ingredients that will alter the perception of bitterness present. Mixture suppression is a term explaining the antagonism of taste sensations when taste stimuli are present simultaneously (Keast, 2008), resulting in a masking of the inherent bitter taste. This method has been investigated for its effectiveness for debittering hydrolysates. Leksrisomprong, Gerard, Lopetcharat, and Drake (2012) recently assessed the bitter-masking ability of various food flavourants on WPHs. In this study, bitter taste inhibition was presented as a percentage that was derived by subtracting the treatment score (i.e., the bitterness value indicated by panelists when samples contained a WPH and a masking agent) from the reference score (i.e., the bitterness value of the WPH), divided by the reference score (Leksrisompong et al., 2012). The addition of sweeteners significantly suppressed bitter taste, the most effective sweetener of which was sucralose (44% bitterness inhibition) (Leksrisompong et al., 2012). Salts such as sodium gluconate (33% bitterness inhibition) and sodium acetate (36% bitterness inhibition) as well as the nucleotide adenosine 5'monophosphate (38% bitterness inhibition) were as effective as fructose and sucrose in suppressing bitterness (Leksrisompong et al., 2012). Monosodium glutamate, an additive inducing the umami taste modality, inhibited bitterness by 37% (Leksrisompong et al., 2012). While the amino acids L-Arg and L-Lys

inhibited bitterness of the alkaloid quinine by 37 and 40%, respectively, they were ineffective at reducing bitterness of WPHs (Leksrisompong et al., 2012).

The perceived bitterness of WPHs was similar with or without blockage of the orthonasal passage, and common flavour-enhancing volatiles (e.g., vanillin and maltol) inhibited bitter taste poorly (<12%), suggesting that aroma did not affect the bitterness perceived (Leksrisompong et al., 2012). Volatiles released from WPHs also did not correlate with bitterness, although they may affect the overall flavour profile (Leksrisompong et al., 2010).

One limitation of flavour masking is the additional flavour profile changes induced when the WPH and flavour additive mixture are introduced to a food system. Although intensities of taste attributes may be controlled, the taste quality is less easy to control and may result in undesirable changes in taste (Leksrisompong et al., 2012). In addition, the addition of salt, monosodium glutamate and sugars to food products may be undesirable due to their associated nutritional concerns. Other compounds shown to be ineffective at debittering bitter peptides include starch at room temperature, fatty substances (e.g., margarine and vegetable oil), and  $\alpha$ -cyclodextrin (Tamura et al., 1990b).

A relatively newer method of debittering is encapsulation, which is achieved by surrounding the bitter compounds, termed the core material, with an outer layer of material, termed the wall material. WPH encapsulated with a mixture of maltodextrin and  $\beta$ -cyclodextrin through spray drying significantly reduced bitterness intensity by eight times relative to the WPH control (Yang et al., 2012). When the dilution of the WPH content by the addition of the wall material

(30:70 w/w core to wall material) was taken into account, encapsulation reduced bitterness by 2– 4 times (Yang et al., 2012). Encapsulation was thought to decrease bitterness by reducing the amount of exposed hydrophobic amino acid residues (Yang et al., 2012). In addition, encapsulation of bioactive peptides may have other advantages, such as increasing shelf-stability and controlling the release of its core materials (Hernández-Ledesma et al., 2011).

Enzymatic debittering reduces peptide bitterness by releasing terminal amino acids. This method of debittering is the most promising of debittering methods as it does not remove any of the nutritional benefits or require the addition of taste-active compounds (FitzGerald & O'Cuinn, 2006; Saha & Hayashi, 2001). Exopeptidases are enzymes that release free amino acids, dipeptides (dipeptidyl-peptidases) or tri-peptides (tripeptidyl-peptidases) from either peptide terminal. Exopeptidase activity is influenced by the polypeptide terminus and adjacent amino acid residues, as well as the polypeptide conformation and length (Raksakulthai & Haard, 2003). Since exopeptidases release amino acids from positions that are linked to bitter taste (i.e., the N- and C-terminal of peptides), they have been used as a debittering method for hydrolysates of various food proteins sourced from dairy products, soy, corn, fish and squid (Raksakulthai & Haard, 2003).

There are two types of exopeptidases: aminopeptidases (APs) and carboxypeptidases (CPs). APs act on the N-terminal of peptides while CPs release substrates from the C-terminal. Both APs and CPs may be classified by the compounds or functional groups they require for activity, the main classes of which are metallo, cysteine and serine exopeptidases (Raksakulthai & Haard, 2003). Metallo exopeptidases require a bound zinc ion to act as a cofactor for their hydrolytic

activity (Raksakulthai & Haard, 2003). More APs and CPs classify as metallo exopeptidases than other classes (Raksakulthai & Haard, 2003). Present in the active site of serine-exopeptidases are the catalytic trio of Ser, Asp, and His (Raksakulthai & Haard, 2003), while cysteine exopeptidases have a reactive thiol group (Barrett & Rawlings, 1996).

AP and CP that have been assessed for debittering activity include both crude and purified enzymes from various sources including fungi, bacteria, plant and animal. For example, a CP sourced from the fungus *Actinomucor elegans* was shown to decrease bitterness of soy hydrolysates by favoring the release of hydrophobic amino acids (Fu, Li, & Yang, 2011) while a wheat-sourced CP was also found to have the same effect on casein hydrolysates (Umetsu, Matsuoka, & Ichishima, 1983). In another study, the debittering and deodorizing activity of a crude preparation of fungal-sourced aspergillopeptidase A on soybean protein hydrolysates were attributed to its CP and endopeptidase, respectively (Arai, Nogushi, Kurosawa, Kato, & Fujimaki, 1970). De-odorization of soy hydrolysates by the endopeptidase was explained by the release of *n*-hexanal and *n*-hexanol from the substrate (Arai et al., 1970).

Although CP are theoretically more effective at debittering peptides (due to the importance of Cterminal amino acids for eliciting bitter taste), APs have been reported in experimental studies to have promising debittering activities and in fact most commercially available exopeptidases are APs (Raksakulthai & Haard, 2003). APs from fungal and bacterial sources have been shown to debitter soy and casein hydrolysates, which was attributed to the release of hydrophobic amino acids from the N-terminal of bitter peptides (Izawa, Tokuyasu, & Hayashi, 1997; Minagawa, Kaminogawa, Tsukasaki, & Yamauchi, 1989; Nishiwaki, Yoshimizu, Furuta & Hayashi, 2002). In studies assessing both soy and casein hydrolysates, larger decreases in bitterness were observed in the former hydrolysates. The bitterness of soy hydrolysates decreased as AP treatment was prolonged and reached threshold concentrations after > 20 h (Izawa et al., 1997; Nishiwaki et al., 2002). On the other hand, prolonging AP treatment from 6–7 h to > 20 h had minimal additional debittering effect on casein hydrolysates, despite continued release of free amino acids, and the resulting hydrolysates did not have bitterness as low as the casein hydrolysates (Izawa et al., 1997; Nishiwaki et al., 2002). Furthermore, even casein hydrolysates prepared by different enzymes had varying decreases in bitterness after treatment by the same CP and AP mixture (Ge & Zhang, 1996). Therefore, investigation of exopeptidase treatments, differing for example in hydrolysis conditions (e.g., treatment duration), the type of exopeptidase used (e.g., AP or CP), and the specificity of the exopeptidases, would be necessary to achieve an optimal debittering effect for a particular hydrolysate.

While exopeptidase treatment seems ideal for debittering hydrolysates, this debittering method may risk the release of terminal amino acids that influence ACE-inhibitory activity, or any bioactivity. The validity of this concern remains to be addressed.

## **1.4.5** Taste and functional foods

It may be assumed that undesirable changes to the taste of foods after the addition of functional ingredients would be accepted by consumers on the basis that they have health-enhancing benefits, since health-enhancing medications are readily consumed despite their typically undesirable taste. However, taste is a more influential factor than health-enhancing attributes on

functional food use (Barrios, Bayarri, Carbonell, Izquierdo, & Costell, 2008). Consumer surveys revealed that despite an increase over time (2001 to 2004) in valuing foods for health, consumers were less willing to tolerate poor taste for health benefits (Verbeke, 2006). Therefore, the taste of the functional ingredients influences the success of the final functional food product. Consequently, a pre-treatment (i.e., debittering method) of bitter-tasting functional food ingredients such as ACE-inhibiting protein hydrolysates is required if their successful incorporation into functional foods is to be realized. This suggests that, as opposed to strictly focusing on maximizing the bioactivity of hydrolysates, focus should also be given on the taste of bioactive hydrolysates.

# **1.4.6** Taste assessment

Human panelists remain the gold standard for assessing taste. Despite the development of promising electronic taste-sensing equipment, it was concluded that such equipment could never replicate in a single analysis the simultaneous processing of sensory, physiological, and psychological responses that occur in humans (Woertz, Tissen, Kleinebudde, & Breitkreutz, 2011). However, taste assessments by sensory analysis with human panelists are not without their shortcomings. Panelists can be influenced by both environmental disturbances and cues inherent to the sensory evaluation (Meilgaard, Ceville, & Carr, 2007). To address these, the testing facilities must be carefully oriented to minimize disturbances, whether from outside the facility or created by other panelists (Meilgaard et al., 2007). In addition, the presentation of samples must be carefully arranged to minimize any biases or expectations the panelists may

have (Meilgaard et al., 2007). All of these factors require time to address and are costly to implement.

The panelists themselves also have their shortcomings. Although training of panelists can improve their use of line scales and lead to higher discriminatory ability (Labbe, Rytz, & Hugi, 2004), panelists are prone to assess samples differently (Romano, Brockhoff, Hersleth, Tomic, Næs, 2008). While some of these inconsistencies are the result of physiological differences among panelists, a majority of these differences stem from the different uses of line scales by panelists (Romano et al., 2008). Differences in scale use are predominantly of three types: level effect, range or scaling effect, and variability effect (Næs, 1990; Romano et al., 2008). Level effect occurs when panelists rank samples similarly and with the same distance between samples, but the absolute values are different (e.g., on the low or high end of the scale) (Næs, 1990). Range or scaling effect occurs when panelists rank samples similarly, but with varying distances between samples (Næs, 1990; Romano et al., 2008). Variability effect occurs when panelists differ in their rating of replicates of the same sample (Romano et al., 2008). Combinations of these effects can also occur (Næs, 1990). These differences can result in a significant panelist effect, which is common in descriptive analysis (Chapman, Matthews & Guinard, 2004; Cliff, Stanich, Edwards, & Saucier, 2012; Moon & Li-Chan, 2007; Schlosser, Reynolds, King, & Cliff, 2005; Song et al., 2010).

Sample differences can still be discerned in the presence of panel inconsistencies (i.e., significant interaction effects) by treating panelists as random effects (i.e., representative of the entire consumer population) and recalculating treatment *F*-values as described, for example, in Goniak

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and Noble (1987). However, whether panelists are treated as random or fixed effects should reflect the intended outcomes of the research and will dictate the type of conclusions that can be made (O'Mahony, 1995; O'Mahony 1998). O'Mahony and Goldstein (1987) classified sensory testing into two categories: Sensory Evaluation I and Sensory Evaluation II. In Sensory Evaluation I, the goal was to detect or quantify the attributes of samples, the panelists were a fixed effect, and the conclusions made could not be generalized to a wider population (O'Mahony, 1995). Significant panelist effects should ideally not occur, as panelists should be as consistent and repeatable as analytical instruments. In contrast, Sensory Evaluation II had the goal of determining how consumers (e.g., the general public or a specific subset of the public) would respond to samples, the panelists were a random effect, and the conclusions made could be generalized (O'Mahony, 1995). Thus, the recalculation of *F*-values to overcome panel interactions would be inappropriate for Sensory Evaluation I.

To overcome significant panelist and interaction effects, Reid and Durance (1992) minimized the aforementioned panel differences in line scale use by performing *Z*-score transformation of sensory data scores prior to statistical analysis. The equation for standardizing the data was as follows:

$$Z = (x - X)/SD \tag{2}$$

where Z is the standardized score, x is the original score, X is the mean for one attribute from one panelist, and SD is the standard deviation of X for the same panelist. Few studies have followed this approach to minimize panel effects (Cheung & Li-Chan, 2014; Moon & Li-Chan, 2007; Mui, Durance, & Scaman, 2002). Romano et al. (2008) compared this method (i.e., scaling by standardization) with two other methods (i.e., scaling by minimizing differences between pairs of

panelists, and scaling by modelling all individual differences) for reducing the differences in line scale use. Although all three methods removed individual differences, scaling by standardization was relatively more susceptible to bias, such as when the random error of a panelist was not proportional to their differences in line scale use.

## 1.5 Thesis objectives and hypothesis

As uncovered in the previous section, protein hydrolysates with ACE-inhibitory activity have the potential to be incorporated into functional food products aimed to help control hypertension, but only if they do not impart adverse changes to taste in the final product. Hydrolysates, particularly those with ACE-inhibitory activity, are typically bitter because they contain peptides with structures similar to bitter peptides (i.e., peptides with hydrophobic amino acid residues, especially when situated at the peptide terminals). In addition, hydrolysate fractions containing low molecular weight peptides (e.g.,  $\leq 3$  kDa) have been associated with both bitter taste and ACE-inhibitory activity. Exopeptidase with either aminopeptidases treatment or carboxypeptidases has been shown to be efficient as debittering methods for peptides that retain the nutritional value and yield of the resulting hydrolysates. However, this debittering method may also influence peptide bioactivity. Therefore, the objective of this study was to assess exopeptidase treatment as a potential method for debittering whey protein hydrolysates (WPHs) with ACE-inhibitory activity. The four hypotheses of this study were as follows:

H<sub>1</sub>: WPHs with high ACE inhibitory activity will have bitter taste

- H<sub>2</sub>: WPH treated with an aminopeptidase will reduce in bitter taste and still have ACE-inhibitory activity
- **H<sub>3</sub>:** WPH treated with a carboxypeptidase will reduce in bitter taste and still have ACEinhibitory activity
- **H**<sub>4</sub>: Peptides in a fraction between the 0.5–3.0 kDa size range will retain the most ACEinhibitory activity after exopeptidase treatment

These hypotheses were tested using the following objectives:

- O1: Prepare WPHs with ACE-inhibitory activity and determine bitter intensity
- O2: Determine the effect of aminopeptidase treatment on ACE-inhibitory activity and taste of WPH
- O3: Determine the effect of carboxypeptidase treatment on ACE-inhibitory activity and taste of WPH
- O<sub>4</sub>: Compare the ACE-inhibitory activity of fractions collected from WPH after aminopeptidase treatment, after carboxypeptidase treatment, and without exopeptidase treatment

# 1.5.1 Overview of experimental approach



Fig. 1.2 – Experimental design of thesis research

# Chapter 2: Effects of exopeptidase treatment on ACE-inhibitory activity, antihypertensive activity and bitterness of whey protein hydrolysates

# 2.1 Synopsis

Whey protein hydrolysates (WPHs) produced from whey protein isolate by endoproteases and various exopeptidase preparations were assessed for angiotensin I-converting enzyme inhibitory activity, antihypertensive activity and bitterness. Whey protein isolate was hydrolyzed for 3 h by one of seven commercial endoproteases, of which Thermoase PC10F yielded a WPH with the highest ACE-inhibitory activity (IC<sub>50</sub> = 0.15 mg/mL) and bitter taste. This hydrolysate, T3, was significantly debittered after a 7 h treatment with the carboxypeptidase Accelerzyme® CPG (T3-AC7), the aminopeptidase Peptidase R (T3-PR7), or the aminopeptidase and proteinase preparation ProteAX (T3-PX7). In fact, T3-AC7 and T3-PR7 evoked the same amount of bitterness as a commercial WPH product. All three exopeptidase-treated hydrolysates exhibited ACE-inhibitory activity (IC<sub>50</sub> = 0.24-0.34 mg/mL). T3 and the exopeptidase-treated WPHs also reduced systolic blood pressure (SBP) in spontaneously hypertensive rats after a single dose of 100 mg/kg body weight, with effects lasting for at least 24 h. T3 and exopeptidase-treated hydrolysates resulted in SBP reductions of up to 35 mm Hg and 30-31 mm Hg, respectively. Size exclusion chromatography revealed that the  $\sim 200-1000$  Da fractions in all exopeptidasetreated hydrolysates were associated with the highest ACE-inhibitory activity. The results of this study suggest that aminopeptidase or carboxypeptidase treatment is a viable method for debittering antihypertensive protein hydrolysates prior to their addition into functional foods.

# 2.2 Introduction

Hypertension, the main risk factor for cardiovascular disease, accounts for 45% and 51% of heart disease- and stroke-related deaths worldwide, respectively, and causes more than nine million deaths annually (World Health Organization, 2013). In Canada, synthetic angiotensin Iconverting enzyme (ACE) inhibitors are the class of drugs most commonly prescribed for cardiovascular diseases (Public Health Agency of Canada, 2009), with more than 17 million prescriptions filled in 2006 (Jackevicius et al., 2009). These drugs inhibit ACE, an enzyme that promotes sodium retention and vasoconstriction, to effectively control hypertension (Ritter, 2011). However, synthetic ACE inhibitors may cause undesirable side effects such as chronic dry cough or angioedema, the latter of which occurs less frequently but is of greater medical concern (Weber & Messerli, 2008). ACE inhibitor-induced angioedema affects 0.2% of patients and can lead to serious respiratory distress requiring ventilatory support or, in rare cases, death (Banerji et al., 2008; Miller et al., 2008; Weber & Messerli, 2008). Therefore, there is growing interest for natural ACE inhibitors in the form of peptides from food protein hydrolysates, which have not been associated with the aforementioned side effects, for use in functional foods aimed to help control hypertension.

Protein hydrolysates often have a bitter taste that can be detectable even after their addition into foods. For example, the bitterness of cheese, which is typically regarded as a flavour defect, has been attributed to bitter peptides released during the ripening stage (Lemieux & Simard, 1992; Singh, Young, Drake, & Cadwallader, 2005). Since bitterness can decrease acceptance, and even cause rejection, of foods (Temussi, 2011), the use of bitter-tasting hydrolysates as ingredients

can risk decreased consumer acceptance of the target food product. Although studies employing computer models to assess correlations between bitterness and ACE-inhibitory activity in peptides have led to divergent conclusions (Pripp & Ardö, 2007; Tan et al., 2013; Wu & Aluko, 2007), a positive correlation between these two attributes has been reported in experimental protein hydrolysates (Cheung & Li-Chan, 2010). In addition, peptides sourced from whey proteins that reduce systolic blood pressure in spontaneously hypertensive rats have also been reported to have bitter taste (Hernández-Ledesma et al., 2011; Liu et al., 2013). This relationship is thought to arise because both ACE-inhibitory activity and bitterness have been associated with low molecular weight peptides composed of hydrophobic amino acid residues. ACE-inhibitory activity is often attributed to peptides bearing hydrophobic residues at the C-terminal, as well as at the N-terminal of di- and tri-peptides (Cheung et al., 1980; Sagardia et al., 2013; Wang et al., 2011; Wu et al., 2006a, 2006b). Fractionation of hydrolysates based on peptide size can be used to concentrate ACE-inhibitory peptides, with fractions < 3 kDa (Estévez et al., 2012; Tavares et al., 2012) and < 1 kDa (O'Loughlin et al., 2014; Pihlanto-Leppälä et al., 2000; Pihlanto-Leppälä, Rokka & Korhonen, 1998) often having higher activity than higher molecular weight fractions. Similarly, hydrophobic amino acid residues, particularly at the C-terminal, and molecular weights < 1000 Da were correlated with peptide bitterness using computer modelling (Kim & Li-Chan, 2006).

Aminopeptidases and carboxypeptidases are two types of exopeptidases, enzymes that release amino acid residues from the N- and C-terminal, respectively. Exopeptidase treatment can decrease hydrolysate bitterness by releasing hydrophobic amino acids from the N-terminal (Izawa et al., 1997; Minagawa et al., 1989; Nishiwaki et al., 2002), the C-terminal (Fu et al., 2011), or both terminals (Ge & Zhang, 1996). Although effective for debittering, hydrolysis with exopeptidases may risk cleaving off amino acid residues pertinent to ACE-inhibitory activity. To date, there is limited research on the effects of exopeptidase treatment on ACE-inhibitory activity, or any other bioactivity, of protein hydrolysates.

The objective of this study was to assess whether exopeptidase treatment is an effective method for debittering antihypertensive hydrolysates prior to their application into functional foods. Hydrolysates of whey protein have been widely studied for ACE-inhibitory activity (Madureira et al., 2010; Morais et al., 2014; O'Loughlin et al., 2014; Wang et al., 2012) and have been reported to have bitter taste (Leksrisomprong et al., 2010, 2012; Liu et al., 2013; Pins & Keenan, 2006). Therefore, whey protein was selected as the substrate for producing hydrolysates in the current study. The effects of exopeptidase treatments on *in vitro* ACE-inhibitory activity, *in vivo* antihypertensive activity, bitterness and molecular size distribution of the resulting whey protein hydrolysates were investigated.

# 2.3 Materials and methods

## 2.3.1 Materials

NZMP<sup>TM</sup> whey protein isolate (WPI) 895, donated by Fonterra Co-operative Group (Rosemont, IL), was used as the protein source for hydrolysate production. Protease M "Amano" SD, Protease P "Amano" 6 SD, Thermoase PC10F, Protin SD-AY10, Protin SD-NY10, Peptidase R, and ProteAX were donated by Amano Enzyme U.S.A, Ltd. (Elgin, IL). Validase<sup>®</sup> Papain Liquid, Maxazyme<sup>®</sup> NNP DS, and Accelerzyme<sup>®</sup> CPG were donated by DSM Food Specialties B.V. (Delft, The Netherlands). WPH 4003 was donated by PGP International (Eagan, MN), Hilmar<sup>TM</sup> 8350 and Hilmar<sup>TM</sup> 8390 were donated by Hilmar Ingredients (Hilmar, CA), and NZMP<sup>TM</sup> WPH 917 (Fonterra Co-operative Group; Rosemont, IL) was donated by Caldic Canada Inc. (Delta, BC). Trichloroacetic acid (TCA) and Food Chemical Codex grade hydrochloric acid (HCl), sodium hydroxide (NaOH) and caffeine were from Fisher Scientific (Fairlawn, NJ) while 2,4,6trinitrobenzenesulphonic acid (TNBS) was from Thermo Scientific (Rockford, IL). Hippuryl-His-Leu-OH (HHL) was from New Bachem (Torrance, CA). Rabbit lung angiotensin Iconverting enzyme, spectrophotometric-grade ethyl acetate, blue dextran, vitamin  $B_{12}$ , Lcarnosine, and Leu were from Sigma-Aldrich (St. Louis, MO). An antifreeze protein was donated by A/F Protein Canada Inc. (St. Johns, NF). Trp and Ala were from Bio Basic Inc. (Markham, ON). All other laboratory chemicals were of reagent grade.

# 2.3.2 Hydrolysate production

Hydrolysates were produced in the University of British Columbia Food Science program pilot plant (Vancouver, BC) following food safe procedures. WPI was dissolved in distilled-deionized water (ddH<sub>2</sub>O) at 3 g WPI/100 mL and pre-heated with constant stirring in a temperaturecontrolled water bath (Blue M Electric Company, Blue Island IL). The initial pH of the solution was  $6.86 \pm 0.09$ . When incubation temperature was reached and all WPI had dissolved, endoproteases were added to the mixture at 3 g/100 g WPI. The incubation temperatures for each of the endoproteases were as follows: Protease M "Amano" SD (50 °C), Protease P "Amano" 6 SD (40 °C), Thermoase PC10F (65 °C), Protin SD-AY10 (50 °C), Protin SD-NY10 (50 °C), Validase<sup>®</sup> Papain Liquid (65 °C) and Maxazyme<sup>®</sup> NNP DS (45 °C). Incubation temperatures were selected for optimal enzyme activity, as specified on the manufacturer's specification sheets (Amano Enzyme, 2012b, 2012c, 2012e; Daiwa Kasei, n.d.-a, n.d.-b; DSM Food Specialties, 2011a, 2011b). Additional information on the endoproteases used is shown in Table 2.1. After 3 h of incubation, aliquots (0.5 mL) were obtained for extent of hydrolysis analysis, and the hydrolysis was terminated by heating to 90 °C for 15 min. The mixture was then centrifuged (10 min at 13,200 x g) using a Du Pont Sorvall Centrifuge RC 5B (Mandel Scientific Co. Ltd., Guelph, ON) and the supernatant collected, frozen, lyophilized and stored at -20 °C until use.

Name	Source	Description (activity <sup>b</sup> )	Enzyme Class	Optimal pH	Manufacturer
Protease M "Amano" SD-K	Aspergillus oryzae	Acid and neutral protease, has peptidase activity, applicable to seasonings and short peptides $(\geq 40,000 \text{ u/g})$	3.4.23.18 (Aspergillo- pepsin I)	6.0	Amano Enzyme Inc., Japan
Protease P "Amano" 6SD-K	Aspergillus melleus	Semi alkaline protease, applicable to various seasonings (≥ 600,000 u/g)	3.4.21.63 (Oryzin)	7.0-8.0	Amano Enzyme Inc., Japan.
Thermoase PC10F	Bacillus stearothermophilus	Neutral metalloprotease (≥ 90,000 u/g)	3.4.24.27 (thermolysin)	7.0-8.5	Amano Enzyme Inc., Japan
Protin SD-AY10	Bacillus licheniformis	Alkaline serineproteinase (≥ 90,000 PU/g)	3.4.21.62 (subtilisin)	10.0–11.0	Amano Enzyme Inc., Japan
Protin SD-NY10	Bacillus subtilis	Neutral metalloproteinase, application in meat extracts (≥ 90,000 PU/g)	3.4.24.28 (bacillolysin)	7.0	Amano Enzyme Inc., Japan
Validase <sup>®</sup> Papain Liquid	<i>Carica papaya</i> fruit	Protease with broad specificity and extensive hydrolysis, applied to (animal) processing co-products (190 – 230 TU/mg)	3.4.22.2 (papain)	4.8-6.2	DSM Food Specialties B.V., The Netherlands
Maxazyme <sup>®</sup> NNP DS	Bacillus amyloliquefaciens (subtilis)	Neutral protease, for proteins/ amino acids extraction in alcohol and spirits production $(\geq 180,000 \text{ PC/g})$	NS	5.2–5.8	DSM Food Specialties B.V., The Netherlands

Table 2.1 – Characteristics<sup>a</sup> of the proteases used for the hydrolysis of whey protein isolate.

<sup>a</sup> Data from supplier sheets (Amano Enzyme, 2012b, 2012c, 2012e; Daiwa Kasei, n.d.-a, n.d.-b; DSM Food Specialties, 2011a, 2011b) <sup>b</sup> Enzyme activity of Amano enzymes was determined using the Folin method as unit (u) or PU (Protease Unit). The assay and units used for determining enzyme activity of DSM enzymes were not specified (NS).

# 2.3.3 Exopeptidase treatment

The hydrolysate produced after a 3 h hydrolysis with Thermoase PC10F (T3), as described in Section 2.3.2, was reconstituted to 10 g hydrolysate/100 mL in ddH<sub>2</sub>O and used as the starting material for exopeptidase treatments. This sample was pre-heated under constant stirring in a temperature-controlled water bath until reaching incubation temperature, after which it was treated with Accelerzyme<sup>®</sup> CPG (AC), Peptidase R (PR), or ProteAX (PX) at 4 g/100 g for 1 or 7 h. The incubation temperature, pH and hydrolysis time used (Table 2.2) were based on those for optimal enzyme activity, as provided by the suppliers (Amano Enzyme 2012a, 2012d; DSM Food Specialties, n.d.). Hydrolysis was terminated by heating to 90 °C for 15 min and the exopeptidase-treated hydrolysates were then lyophilized before storing at -20 °C until use. The hydrolysate treated with AC, PR, or PX were abbreviated as T3-AC, T3-PR or and T3-PX, respectively. Sample codes were followed by a 1 or 7 to denote 1 or 7 h of exopeptidase treatment. AC-treated hydrolysates were adjusted to pH 6 using 3 M NaOH prior to lyophilization. At least two batches of T3, T3-AC7, T3-PR7 and T3-PX7 were produced to assess reproducibility (Appendix A)
# Table 2.2 – Characteristics<sup>a</sup> of the exopeptidase preparations used for debittering

Product name (microbial source)	Supplier description (amino acid specificity; activity)	Enzyme class	Hydrolysis conditions
Peptidase R ( <i>Rhizopus oryzae</i> )	High peptidase activity for debittering bitter protein hydrolysates (Arg > Ala, Lys, Phe, Leu; $\geq 420 \text{ u/g}^{b}$ )	3.4.11.1 leucyl amino- peptidase	52 °С pH б
ProteAX (Aspergillus oryzae)	High peptidase and proteinase activity (Gln, Ser, Thr, Met > Arg > Ala, Lys, Phe, Leu; $\geq 1400 \text{ u/g}^{c}$ )	3.4.11.1 leucyl amino- peptidase	60 °C pH 6
Accelerzyme <sup>®</sup> CPG ( <i>Aspergillus niger</i> strain ISO-528)	Accelerates ripening and promotes flavour in cheese production (broad activity with highest specificity for Phe, Ile, Leu, Met, Val; $\geq 900 \text{ CPGU/g}^d$ )	serine carboxy- peptidase	37 °C pH 4

# treatment of a whey protein hydrolysate.

<sup>a</sup> Data from supplier resources (Amano Enzyme, 2010; Amano Enzyme 2012a, 2012d; DSM Food Specialties, n.d.)
<sup>b</sup> Activity based on the L-leucyl-glycyl-glycine method
<sup>c</sup> Activity based on the leucyl-naphthylamide method.
<sup>d</sup> Activity based on hydrolysis of furyl acryloyl-L-Phe-L-Ala.

# 2.3.4 Extent of hydrolysis

The  $\alpha$ -amino content of protein hydrolysates, determined in triplicate using the TNBS method as described in Liceaga-Gesualdo & Li-Chan (1999), was used to indicate the extent of hydrolysis (EH). Aliquots (0.5 mL) of hydrolysates obtained prior to the heat inactivation step during hydrolysis production or exopeptidase treatment (i.e., WPH at 3 g/100 mL and exopeptidasetreated WPH at 10 g/100 mL) and of reconstituted T3 and commercial WPH (10 g/100 mL) were added to 24% TCA at a 1:1 ratio. Samples were then centrifuged at 5,000 rpm (~2000 x g) for 15 min using a bench-top centrifuge (VWR Galaxy 16, VWR International; Radnor, PA). The supernatant of these samples were diluted 10-100 times in ddH<sub>2</sub>O. Hydrolysates required a 50 times dilution, while the exopeptidase-treated hydrolysates, reconstituted T3, and all commercial WPHs except WPH 4003 required a 100 times dilution. WPH 4003 required a 10 times dilution. Diluted solutions (0.1 mL) were mixed with 1 mL of 0.05 M sodium tetraborate buffer (pH 9.2) and 0.50 mL of 4.0 mM TNBS, vortexed, and incubated in the dark at room temperature. After 30 min, 0.5 mL of 2.0 M sodium phosphate containing 18 mM Na<sub>2</sub>SO<sub>3</sub> was added to each tube; the mixture was vortexed and absorbance at 420 nm determined using a Shimadzu UV-160 UV-Visible Recording Spectrophotometer (Mandel Scientific Company Inc.). Absorbance values were compared to a Leu standard curve (0-3.0 mM Leu) constructed each day the assay was conducted. The EH was reported as milliequivalents of Leu (meq Leu)/g sample.

# 2.3.5 ACE-inhibitory activity

The protocol for determining ACE-inhibitory activity was based on that described in Cheung & Li-Chan (2010). Samples were reconstituted in 0.050 M sodium tetraborate buffer with 0.3 M NaCl (pH 8.3) to 0.1–0.3 mg/mL final assay concentrations. Aliquots (30  $\mu$ L) were pre-incubated in a water bath at 37 °C for 15 min with 30  $\mu$ L ACE (2.5 mU in buffer). After pre-incubation, 150  $\mu$ L of 6.5 mM HHL dissolved in pre-warmed (37 °C) buffer was added to all samples and incubated for 1 h at 37 °C. Hippuric acid was produced by the ACE-catalyzed hydrolysis of HHL. The reaction was terminated after 1 h by adding 250  $\mu$ L of 1N HCl and vortexing. Ethyl acetate (1 mL) was added to each sample and the mixture vortexed for 30 seconds (to aid ethyl acetate extraction of hippuric acid) before centrifugation at 2000 x *g* for 5 min using a bench-top centrifuge (to separate ethyl acetate from the aqueous phase). The top layer (700  $\mu$ L) (ethyl acetate with extracted hippuric acid) was pipetted into a glass test tube and set in a heating block at 120 °C until all solvents were evaporated. The dried material was solubilized in 1.3 mL ddH<sub>2</sub>O and read at 228 nm. ACE-inhibitory activity in percentage was calculated from the average absorbance values obtained from triplicate analyses as follows:

% ACE activity = 
$$[Abs_{sample} - Abs_{HHL control}] \times 100\%$$
 (3)  
[Abs\_positive control - Abs\_{HHL control}]

% ACE-inhibitory activity = 
$$100\% - \%$$
 ACE activity (4)

where the HHL control (i.e., with  $ddH_2O$  in lieu of ACE and WPH) acts as a blank for all readings, and the positive control (i.e., with  $ddH_2O$  in lieu of WPH) represents un-inhibited ACE activity. The IC<sub>50</sub> value of hydrolysates was obtained by cubic polynomial regression analysis of ACE-inhibitory activities (%) at five or more sample concentrations (0.5–5 mg hydrolysate/mL).

# 2.3.6 Sensory evaluation

Sensory evaluation using a tasting procedure based on that described in Cheung & Li-Chan (2010) was conducted with the approval of the Behavioural Research Ethics Board of the University of British Columbia (Vancouver, BC). As this chapter discusses the debittering effect of exopeptidase treatment, only the methods pertaining to the assessment of bitterness is discussed. Additional detail on panelist recruitment, panelist training and sample assessment can be found in Chapter 3 (Section 3.3.2).

Preliminary screening of WPI and 18 WPH samples by three researchers was used as the basis for selecting six WPH samples for evaluation by the trained sensory panel. Panelists were recruited from the Food Nutrition and Health program at the University of British Columbia (Vancouver, BC). The trained sensory panel consisted of eight females and four males aged 20– 35 years. One participant was Caucasian and the remaining were Asian. The sensory panel assessed in duplicate the bitterness of 10% (10 g hydrolysate/100 mL ddH<sub>2</sub>O) solutions of T3, T3-AC7, T3-PR7, T3-PX7, and the commercial products WPH 4003 and Hilmar<sup>TM</sup> 8350. Panelists participated in two training sessions, during which they were asked to differentiate aqueous solutions of bitter standards (5, 10 and 15 mM caffeine in ddH<sub>2</sub>O) in two sets of triangle tests, and rank bitter standards consisting of various caffeine concentrations (2.5–15 mM caffeine dissolved in WPH 4003) in two sets of ranking tests. The commercial product WPH 4003 was selected as the base for the reference standard due to its low intensities of taste attributes. Samples (0.3 mL) were deposited directly on the back of the tongue using a 1 mL syringe (BD; Franklin Lakes, NJ). Bitterness intensity was evaluated on 15-cm line scales with anchors at 1 and 14 cm labelled low and high, respectively. After group discussion, the panelists concluded that 10 mM caffeine in WPH 4003 had the bitterness intensity most similar to the experimental hydrolysates, and therefore selected it to be the reference standard for bitterness. This reference was positioned on the 15-cm line scale at 11.0 cm to calibrate panelists.

The sensory evaluation of hydrolysates was conducted over two sessions. Panelists tasted samples (10 g/100 mL ddH<sub>2</sub>O) in assigned random order under red lighting to minimize any possible visual cues, such as differences in colour, among samples. The reference standard was provided to panelists for their use as necessary. Panelists were able to provide written comments during the evaluation. Water, low sodium club soda (45 mg Na/355 mL) (Canada Dry Mott's Inc.; Mississauga, ON), and unsalted crackers were provided as palate cleansers and used at least once between samples.

# 2.3.7 Size exclusion chromatography

T3 and the 7 h exopeptidase-treated hydrolysates were reconstituted to 20 mg/mL in 0.05 M tetraborate buffer (pH 8.3) and filtered through a 0.45  $\mu$ m filter (MILLEX®HV filter unit, Millipore, Cork, Ireland). The sample (500  $\mu$ L) was loaded into a fast protein liquid

chromatography system (FPLC) (ÄKTA purifier 100/10; GE Healthcare Life Sciences, Baie d'Urfe, QC) and eluted through the Superdex<sup>TM</sup> Peptide 10/300 GL column (24 mL, GE Healthcare, Uppsala, Sweden) at a flow rate of 0.2 mL/min using tetraborate buffer as the eluent solvent. The fractionation range of this column was 100–7000 Da. Elution profiles were monitored at 215 nm (mV) and 280 nm (Abs), with the eluate collected in 0.4 mL volumes (i.e., every 2 minutes). These collected samples were pooled into fractions based on peaks obtained in the elution profile. These fractions were lyophilized and stored at -20 °C until use. After each elution, the column was washed with ddH<sub>2</sub>O and equilibrated with buffer. The molecular weight ranges of the fractions were estimated based on the elution volumes of the following molecular weight standards: antifreeze protein (3240 Da), vitamin B<sub>12</sub> (1355.47 Da), L-carnosine (226.24 Da), and a mixture of Trp (204.2 Da) and Ala (89.09 Da). The resulting estimations of molecular weight size range were < 200 Da, 200–1000 Da, and > 1000 Da. Blue dextran (2000 kDa) was used to determine the column void volume.

# 2.3.8 Antihypertensive activity in spontaneously hypertensive rats

Hydrolysates (i.e., T3, T3-AC7, T3-PR7 and T3-PX7) produced as described in Sections 2.3.2 and 2.3.3 were assessed for antihypertensive activity. Spontaneously hypertensive rats (SHRs) housed at the Animal Housing Facility at the Richardson Centre of Functional Foods and Nutraceuticals (University of Manitoba, Winnipeg, MB) were used to determine the *in vivo* antihypertensive effect of hydrolysates based on the protocol described in Girgih et al. (2011). Experiments were carried out following the Canadian Council on Animal Care ethics guidelines and using a protocol approved by the University of Manitoba Animal Protocol and Management Review Committee. Twenty-four male rats about 20 weeks old and weighing 350–415 g were randomly assigned into six groups (n = 4): (1) T3, (2)–(4) the three 7 h exopeptidase-treated hydrolysates (100 mg protein/kg body weight), (5) captopril (10 mg/kg body weight, positive control), or (6) saline (negative control). All samples were dissolved in phosphate buffered saline (pH 7.4) and 1 mL was administered to the rats by oral gavage. At 2, 4, 6, 8 and 24 h after initial administration, the systolic blood pressure (SBP) of SHRs was measured in triplicate by tail-cuff plethysmography. The change in SBP was determined as the difference between SBP at the different time points and baseline SBP (186  $\pm$  1 mm Hg), and was expressed as the mean and standard deviation of the averaged triplicate SBP readings from four rats per group.

### 2.3.9 Statistical analysis

The EH and ACE-inhibitory activity of hydrolysates were analyzed by a one-factor analysis of variance (ANOVA). Significant differences ( $p \le 0.05$ ) were determined using Tukey's method and Fisher's least significant differences (LSD) test. Correlation between EH and ACE-inhibitory activity for the WPHs (n = 7) produced in Section 2.3.2 was determined by calculating the Pearson correlation coefficient (R) and its p-value on Microsoft Excel using the function adapted from that described by Miles and Banyard (n.d., 2007): TDIST((R\*SQRT(n-2)/SQRT(1-(R\*R))), n-2, 2).

The effect of captopril, T3, and the three 7 h exopeptidase-treated hydrolysates on the SBP reduction of SHRs was analyzed by a two-factor ANOVA with repeated measures on one factor, with sample and time (repeated factor) as main effects. Significant differences ( $p \le 0.05$ ) of the 5

samples at 5 time intervals were determined using Tukey's method. The effect of sample on SBP reduction at each time interval was further assessed using a one-factor ANOVA. Significant differences ( $p \le 0.05$ ) among samples (n = 5) were determined using Fisher's LSD test. Saline, the negative control, was not included in the statistical analysis for significant differences.

The sensory scores were quantified in cm on the 15-cm line scale. A two-factor ANOVA was performed on the data from each panelist, using sample and replicate as main effects, to assess panelist repeatability. One panelist had a significant replication effect ( $p \le 0.05$ ), suggesting a lack of repeatability, and was removed from the data set. Additional information on panel assessment is described in Chapter 3 (Section 3.3.5.1). The remaining sensory data was analyzed by a three-factor ANOVA followed by determination of significant differences ( $p \le 0.05$ ) using Fisher's LSD test.

All analyses except correlation analyses were conducted using the Minitab software (version 16, Minitab Inc., State College, PA).

# 2.4 Results and discussion

# 2.4.1 Screening of WPHs for ACE-inhibitory activity and bitterness

Hydrolysis times of 1–6 h were assessed in preliminary studies for their effect on ACE-inhibitory activity, with 3 h of hydrolysis being found to be sufficient for releasing ACE-inhibiting peptides (data not shown). Therefore, WPI, which does not have ACE-inhibitory activity, was hydrolyzed by one of seven endoproteases and the resulting WPHs assessed for EH and ACE-inhibitory activity. Since Tukey's method was more conservative and had lower risk of committing Type I error than Fisher's LSD test (O'Mahony, 1986), only the results analyzed using Tukey's method are discussed (Table 2.3). The results analyzed by Fisher's LSD test can be found in Table B.1 in Appendix B.

The seven hydrolysates varied in their EH from 0.35-3.74 meq Leu/g and, with the exception of the Maxazyme<sup>®</sup> NNP DS-produced hydrolysate, all displayed greater than 50% ACE-inhibitory activity when tested at 0.3 mg/mL (final assay concentration) (Table 2.3). Thermoase PC10F and Protin SD NY10 yielded hydrolysates with the highest ACE-inhibitory activities. A correlation between EH and ACE-inhibitory activity was not observed in the WPHs (R = 0.3893, n = 7, p = 0.3880). High ACE-inhibitory activity in WPHs has been associated with both high (> 25%) and low (< 20%) degree of hydrolysis in other studies (Guo et al., 2009; van der Ven et al., 2002; Wang et al., 2010), the discrepancies of which may be partly explained by the different hydrolysis conditions employed (e.g., the enzyme used for hydrolysis).

The unhydrolyzed WPI was not bitter, but all seven WPI hydrolysates exhibited bitterness albeit with varying intensities. The most bitter hydrolysate was that produced after hydrolysis with Thermoase PC10F for 3 h (hereinafter referred to as T3), which, interestingly, also had the highest ACE-inhibitory activity. While bitterness has been correlated to ACE-inhibitory activity in hydrolysates (Cheung & Li-Chan, 2010), a trend between these two characteristics was not observed in the current samples. Hydrolysates produced by Protin SD NY10, Protease P "Amano" 6, and Validase<sup>®</sup> Papain Liquid had high ACE-inhibitory activity and lower bitterness than T3, but developed other undesirable taste and texture attributes. Protin SD NY10-produced hydrolysate elicited an undesirable film-like sensation while Protease P "Amano" 6 and Validase<sup>®</sup> Papain Liquid-produced hydrolysates exhibited noticeably higher intensities of cardboard and sour tastes. Sour taste and cardboard flavour have been used to describe WPHs in other studies, with the former being attributed to lipid oxidation products and the latter being attributed to peptides (Leksrisompong et al., 2010). T3 had the highest ACE-inhibitory activity, strongest bitter taste and least amount of other undesirable taste sensations of the hydrolysates assessed. Therefore, T3 was selected for exopeptidase treatments with Accelerzyme® CPG, Peptidase R, or ProteAX.

	Extent of	ACE	Taste attributes and sensation	
Protease used	hydrolysis (meq Leu/g) <sup>a</sup>	inhibitory activity (%) <sup>b</sup>	Bitterness	Other
Thermoase PC10F	1.57c	75a	++++	Initially sour
Protin SD NY10	0.54e	69ab	+	Film-like coating
Protease P "Amano" 6	3.74a	67b	+	Cardboard taste,
				sour/sweet
Validase <sup>®</sup> Papain Liquid	0.88d	67b	+++	"Odd", sour and
				cardboard taste
Protease M "Amano" SD	2.18b	59c	++	Sour/sweet
Protin SD AY10	0.84d	54c	+	Sour, slight
				cardboard taste
Maxazvme <sup>®</sup> NNP DS	0.35e	27d	++	Mild taste
None (WPI)	0 03f	-88e	_	Metallic flat
	0.031	000		metanic, nat

 Table 2.3 – Extent of hydrolysis, ACE-inhibitory activity and taste of whey protein isolate

 and seven whey protein hydrolysates.

<sup>a</sup> Expressed as  $\alpha$ -amino groups in milliequivalents of L-Leu per gram of protein (meq Leu/g). Samples not sharing common lowercase letters are significantly different ( $p \le 0.05$ ) as determined using Tukey's method.

<sup>b</sup> At final assay concentration of 0.3 mg/mL. Samples not sharing common lowercase letters are significantly different ( $p \le 0.05$ ) as determined using Tukey's method.

<sup>c</sup> Samples (10% w/v) were tasted by 3 researchers in preliminary studies. Intensities of bitterness were coded as follows: no bitterness (-), detectable bitterness (+), mildly bitter (++), moderately bitter (+++), and highly bitter (+++).

# 2.4.2 Effect of exopeptidase treatment on ACE-inhibitory activity and bitterness of the Thermoase PC10F-produced WPH

The ACE-inhibitory activity and EH of T3 before and after exopeptidase treatments were analyzed using Tukey's method (results of the analysis using Fisher's LSD test can be found in Table B.2 in Appendix B). As shown in Table 2.4, all exopeptidase-treated hydrolysates displayed ACE-inhibitory activity, albeit their activities were generally lower than T3. EH significantly increased when the hydrolysis time of exopeptidase treatments increased from 1 to 7 h, but only resulted in significantly lower ACE-inhibitory activity in the case ProteAX-treated hydrolysates. The ProteAX enzyme preparation contains proteinase in addition to aminopeptidase activity, unlike Accelerzyme<sup>®</sup> CPG and Peptidase R, which only exhibit carboxypeptidase and aminopeptidase activity, respectively. The high EH of ProteAX-treated hydrolysates suggests that the proteinase was active under the hydrolysis conditions used, potentially fragmenting active peptides and resulting in lower ACE-inhibitory activity.

Preliminary taste assessment found that prolonging the exopeptidase treatment from 1 to 7 h increased the debittering effect (Table 2.4). The hydrolysates resulting from the exopeptidase treatments of 1 h were described as bitter and having mild, milky, and cardboardy taste sensations similar to T3. In contrast, the exopeptidase treatments of 7 h resulted in a larger debittering effect as well as induced changes to other taste attributes, such as saltiness and umami taste. Four commercial WPH products were also assessed for benchmarking the bitterness of experimental hydrolysates in preliminary studies. As food ingredients, these commercial hydrolysates act as nutrient (i.e., amino acid) sources and have "minimal off-flavors

and bitterness" (WPH 4003), "bland" taste (NZMP<sup>TM</sup> WPH 917 and Hilmar<sup>TM</sup> 8350), or "flavor enhancement benefits" (Hilmar<sup>TM</sup> 8390), as described by the manufacturers product information sheets (PGP International, 2009; Fonterra, 2007; Hilmar Ingredients, 2012). Hilmar<sup>TM</sup> 8390 was found to have higher bitterness than T3. Interestingly, this product was also the only commercial WPH exhibiting ACE-inhibitory activity at a level comparable to exopeptidase-treated hydrolysates. WPH 4003 and NZMP<sup>TM</sup> WPH 917 had lower bitterness than the exopeptidasetreated hydrolysates, but did not display ACE-inhibitory activity. NZMP<sup>TM</sup> WPH 917 also had a noticeably thicker consistency than the other hydrolysates tasted. The low bitterness of WPH 4003 and NZMP<sup>TM</sup> WPH 917 may be due to their low EH, since degree of hydrolysis has been reported to positively correlate with bitterness (Lekrsisompong et al., 2010) and the starting WPI was found to develop bitterness only after hydrolysis with various proteases (Table 2.3).

To quantify and compare the bitterness among WPHs, the experimental and commercial hydrolysates were tasted by a trained sensory panel consisting of 11 Asians and 1 Caucasian. Asians have been reported to have more sensitivity than Caucasians for bitter tastants such as 6-*n*-propylthiouracil, sensitivity for which is associated with the rejection of other bitter tastants and decreased acceptance of various bitter-tasting food products (Drewnowski, Kristal, & Cohen, 2001). Thus, the panel used in the current study may be expected to have high acuity for bitter taste. Since the hydrolysates treated with exopeptidases for 7 h had lower bitterness and similar ACE-inhibitory activity to hydrolysates treated with exopeptidases for 1 h, only the 7 h exopeptidase-treated hydrolysates, as well as T3, were selected for evaluation by the sensory panel. Hilmar<sup>TM</sup> 8390 and NZMP<sup>TM</sup> WPH 917 were also excluded from the sensory panel due to their extreme bitter taste and thicker consistency, respectively.

Sample differences were determined using Fisher's LSD test, a pairwise comparison test commonly used in sensory research (O'Mahony, 1986), the results of which are shown in Fig 2.1 The trained panel found T3 to be the most bitter sample, exhibiting bitterness slightly less than the reference standard (10 mM caffeine in WPH 4003) (Fig. 2.1). The exopeptidase-treated hydrolysates were significantly less bitter than T3. Among the three exopeptidase-treated hydrolysates, T3-PX7 had the highest bitterness. This may be due to its proteinase activity generating high contents of low molecular weight peptides, which have been reported to be correlated to bitterness in hydrolysates (Leksrisompong et al., 2010). Both T3-AC7 and T3-PR7 had similar bitterness to Hilmar<sup>™</sup> 8350, suggesting that exopeptidase-treatments with either aminopeptidase or carboxypeptidase can potentially decrease the bitterness of bioactive hydrolysates to levels comparable to commercially available hydrolysates.

# Table 2.4 – Extent of hydrolysis, ACE-inhibitory activity, and taste of a whey protein

	Extent of	ACE-	Taste attributes and sensations <sup>e</sup>	
Sample <sup>a</sup>	hydrolysis (meq Leu/g) <sup>b,c</sup>	inhibitory activity (%) <sup>c,d</sup>	Bitterness	Other
Т3	1.12f	76a	++++	Mild taste, milky, cardboardy
Carboxypeptidase-tr	reated			
T3-AC1	1.99e	69b	+++	Mild
T3-AC7	2.46cd	67b	+	Mild, good flavour, complex
Aminopeptidase-treated				
T3-PR1	2.25d	65bc	+++	Odd, cardboardy
T3-PR7	3.92b	60cd	++	Salty, umami, acceptable
T3-PX1	2.66c	70ab	+++	Milky, cardboardy
T3-PX7	4.55a	48e	++	Sour, good, not good
Commercial products				
WPH 4003	0.01i	-42g	-	Very mild, sweet, milky
Hilmar <sup>TM</sup> 8350	0.71g	16f	++++	Cracker-like, milky
Hilmar <sup>TM</sup> 8390	2.38d	57d	+++++	Like rotten cheese
NZMP <sup>TM</sup> WPH 917	0.33h	21f	+	Thick, milky

ing along suce (10), chopep hause headed 10, and rout commercial produces	ydrolysate (	T3), exope	otidase-treated	l T3, and	four	commercial	products.
---------------------------------------------------------------------------	--------------	------------	-----------------	-----------	------	------------	-----------

<sup>a</sup> A whey protein hydrolysate produced after hydrolysis with Thermoase PC10F for 3 h (T3) was treated with Accelerzyme<sup>®</sup> CPG (T3-AC), Peptidase R (T3-PR) or ProteAX (T3-PX7). Sample codes were followed by 1 or 7 to represent the exopeptidase treatment of 1 or 7 hours, respectively.

<sup>b</sup> Expressed as α-amino groups in milliequivalents of L-Leu (meq Leu)/g hydrolysate.

<sup>c</sup> Samples not sharing common lowercase letters are significantly different ( $p \le 0.05$ ) as determined using Tukey's method.

<sup>d</sup> At final assay concentration of 0.3 mg/mL.

<sup>e</sup> Samples (10% w/v) were tasted by 3 researchers. Intensities of bitterness were coded as follows: no detectable bitterness (-), detectable bitterness (+), mildly bitter (+++), moderately bitter (++++), highly bitter (++++), and extremely bitter (++++).



Fig. 2.1 – Mean bitterness scores (n = 22) for four experimental whey protein hydrolysates and the commercial products Hilmar<sup>TM</sup> 8350 and WPH 4003.

Duplicate samples of a whey protein hydrolysate produced after hydrolysis with Thermoase PC10F for 3 h (T3), and of T3 treated for 7 h with Accelerzyme<sup>®</sup> CPG (T3-AC7), Peptidase R (T3-PR7), or ProteAX (T3-PX7) were tasted at 10% w/v by a trained sensory panel of 11 panelists. Samples not sharing common lowercase letters are significantly different ( $p \le 0.05$ ). The bitter score (11.0 cm) of the reference standard consisting of 10 mM caffeine in WPH 4003 is indicated as a dotted line.

# 2.4.3 Size fractions of WPHs and their ACE-inhibitory activity

Size exclusion chromatography was used to obtain the different size fractions of T3, T3-AC7, T3-PR7, and T3-PX7 based on their elution profiles at 215 and 280 nm (Fig. 2.2). Distinct peaks were observed below about 1000 Da while peptides above this size range appeared to co-elute. Therefore, peptides with molecular weights > 1000 Da were pooled together in the same fraction (Fraction 1), while peptides  $\leq 1000$  Da were fractionated according to the peaks observed in their elution profiles. T3 was found to consist predominantly of peptides with molecular weights >1000 Da (Fraction 1), with a smaller proportion of peptides between 200–1000 Da (Fractions 2) and 3) and virtually no free amino acids (Fig. 2.2a). Exopeptidase treatments increased the content of peptides and free amino acids in lower molecular weights fractions (approximately < 200 Da), as evident in Fractions 4–6 of T3-AC7 (Fig. 2.2b), and Fractions 5–6 of T3-PR7 (Fig. 2.2c) and T3-PX7 (Fig. 2.2d). The elution profiles of exopeptidase-treated hydrolysates monitored at 280 nm suggested that a high amount of aromatic amino acids originally embedded in peptides in T3 was released as free amino acids in Fraction 6 of all three exopeptidase-treated hydrolysates (Fig. 2.2b, c, d). This may partly explain the decrease of ACE-inhibitory activity in the unfractionated exopeptidase-treated hydrolysates compared to T3 (IC<sub>50</sub> = 0.15 mg/mL, 76% at 0.3 mg/mL). Aromatic amino acids, particularly at the C-terminal, have been correlated to ACE-inhibitory activity in peptides (Cheung et al., 1980; Jing et al., 2014; Wu et al., 2006a), but display no activity as free amino acids. Since T3-PR7 appeared to have the most aromatic amino acids released, as suggested by the absorbance at 280 nm of Fraction 6, it may be expected to have the lowest ACE-inhibitory activity. Similarly, T3-AC7, which was the only sample to release C-terminal aromatic amino acids, may be expected to have low potency since C-terminal

amino acids were correlated to ACE-inhibitory activity. However, both T3-PR7 (IC<sub>50</sub> = 0.27 mg/mL, 60% at 0.3 mg/mL) and T3-AC7 (IC<sub>50</sub> = 0.24 mg/mL, 67% at 0.3 mg/mL) had higher inhibitory activity than T3-PX7 (IC<sub>50</sub> = 0.34 mg/mL, 48% at 0.3 mg/mL), suggesting that other factors may have contributed to the decrease in ACE-inhibitory activity of exopeptidase-treated hydrolysates

All five fractions collected from T3 had ACE-inhibitory activity, with Fractions 1–3 having higher activities ( $\geq$  60% at a final assay concentration of 0.1 mg/mL) than Fractions 4 and 5 (Fig. 2.2a). In comparison, only one fraction in T3-AC7 (Fig. 2.2b) and T3-PX7 (Fig. 2.2d) and two fractions in T3-PR7 (Fig. 2.2c) exhibited activities similar to or higher than 60%. These fractions corresponded to peptides with approximate molecular weights between 200–1000 Da in T3-AC7 and T3-PX7 (Fraction 2), and around 1000 Da (Fraction 2) and less than 200 Da (Fraction 4) in T3-PR7. These size fractions (i.e., < 1000 Da) are similar to those reported to exhibit the highest ACE-inhibitory activity in other protein hydrolysates (Estévez et al., 2012; O'Loughlin et al., 2014; Pihlanto-Leppälä et al., 2000; Pihlanto-Leppälä, et al., 1998).

The peptides eluting after the 200–1000 Da fractions, which are presumably di- and possibly tripeptides, and collected in Fractions 4 and 5 of T3-AC7, Fraction 5 of T3-PR7, and Fractions 3–5 of T3-PX7 had relatively low activity. This may be explained by the specificity of the exopeptidases for hydrophobic amino acids, which are associated with ACE-inhibitory activity in di- and tri-peptides when present at either peptide terminal (Wu et al., 2006a). Accelerzyme<sup>®</sup> CPG, which has high specificity for five hydrophobic amino acids (Table 2.2), would presumably degrade di- and tri-peptides containing hydrophobic amino acids at the C-terminal into free amino acids. Therefore, some, if not most, of the remaining di- and tri-peptides present in Fractions 4 and 5 may not contain hydrophobic amino acids at the C-terminal and therefore are less likely to exhibit ACE-inhibitory activity. Likewise, the removal of hydrophobic amino acids (e.g., Ala, Phe, Lys, and Met) from the N-terminal by Peptidase R and ProteAX may explain the low activities observed in Fraction 5 of T3-PR7 and Fractions 3–5 in T3-PX7.



# Fig. 2.2 – Elution profiles at 215 or 280 nm and collected fractions of T3 (a), T3-AC7 (b), T3-PR7 (c), and T3-PX7 (d) obtained using size exclusion chromatography, and the ACE-inhibitory activity of the corresponding fractions.

A whey protein hydrolysate produced after hydrolysis with Thermoase PC10F for 3 h (T3) was treated for 7 h with Accelerzyme<sup>®</sup> CPG (T3-AC7), Peptidase R (T3-PR7) or ProteAX (T3-PX7). Fractions collected from each hydrolysate are shown as shaded bars on each elution profile, and numbered above the elution profiles and below the bar graph depicting ACE-inhibitory activity (0.1 mg/mL final assay concentration). The arrows above elution profiles denote the elution volume of four molecular weight standards: antifreeze protein (AFP, 3240 Da), vitamin  $B_{12}$  (VB, 1355.47 Da), L-carnosine (LC, 226.24 Da), and a mixture of Trp (204.2 Da) and Ala (89.09 Da).

# 2.4.4 Antihypertensive activity of WPHs in spontaneously hypertensive rats

The SHR data was analyzed by a two-factor ANVOA with repeated measures on one factor (time), the summary of mean square, F-values and p-values of which can be found in Table 2.5. Time was a highly significant main effect, with the magnitude of the change (i.e., reduction) in SBP decreasing over time (Fig. C.1 in Appendix C). This was expected as the antihypertensive activity of peptides and ACE inhibitors were not expected to be permanent. Synthetic ACE inhibitors are eliminated from the body by the kidneys (Brown & Vaughan, 1998) while peptides in the plasma may be rapidly degraded by proteinases and peptidases (Walsh et al., 2004).

Sample was also a significant effect, suggesting that the samples varied in the SBP reduction. Furthermore, the significant sample × time interaction suggested that the antihypertensive effect of T3, the 7 h exopeptidase-treated hydrolysates and captopril differed over the 24 h sampling time. This may be seen in Fig. C.2 of Appendix C, in which captopril was shown to have more sustained activity over 24 h while T3 had higher initial effect (i.e., larger reduction in SBP at 2 h), but a steeper decrease in effect (i.e., faster return to initial SBP). Captopril has one of the shortest elimination half-lives of synthetic ACE inhibitors at 1.7 h (Brown & Vaughan, 1998), suggesting that only about 6% of its original activity remains 7 h after ingestion. However, various studies, including the present study, have observed that the effects of captopril on SBP reduction in SHRs lasted for at least 24 h (Girgih et al., 2011; Ruiz-Giménez et al., 2012). This may be partly due to their continued ACE-inhibitory activity on the tissues of various organs even after ACE-inhibitory activity in the plasma had diminished (Cushman et al., 1989). Since the number of means for multiple comparison was high (n = 25 for 5 samples at 5 times) and the possibility of committing Type I errors increases with the number of comparisons made when using Fisher's LSD test (O'Mahony, 1986), Tukey's method for multiple comparison was used to determine sample differences. The results of this analysis are shown in Table 2.6. Some sample differences were observed, such as the exopeptidase-treated hydrolysates having significantly less effect than captopril after 24 h. To further compare sample differences, a one-way ANOVA was conducted on the data using sample as the only main effect. Due to the decreased number of sample comparisons (n = 5), Fisher's LSD test was used to determine sample differences.

All three exopeptidase-treated hydrolysates and T3 lowered SBP in SHRs as early as 2 h after a single oral administration of 100 mg/kg body weight (Fig. 2.3). Of all the samples and controls tested, T3 lowered SBP the most at 2 and 4 h after administration and had a maximum effect of  $-35 \pm 4$  mm Hg SBP after 2 h. This reduction in SBP was significantly higher than that displayed by the positive control captopril at 10 mg/kg body weight (bw), which had a maximum effect of  $-28 \pm 2$  mm Hg after 4 h. The effect of T3 on SBP reduction remained at a level comparable to captopril at 6 and 8 h after administration, before having significantly less effect than captopril after 24 h. The exopeptidase-treated hydrolysates decreased SBP the most during the first 4 h after administration, with T3-AC7, T3-PR7, and T3-PX7 having maximum effects of  $-31 \pm 2$ ,  $-31 \pm 1$ , and  $-30 \pm 2$  mm Hg, respectively. The exopeptidase-treated hydrolysates also had similar effects as captopril on SBP reduction at 2, 4 and 6 h after administration. Interestingly, T3-PX7, which had relatively lower ACE-inhibitory activity *in vitro* than T3-AC7 or T3-PX7, displayed antihypertensive effects similar to those of the other two exopeptidase-treated hydrolysates. A

possible explanation for this observation may be the presence of pro-drug inhibitors in T3-PX7, whereby shorter peptides with increased antihypertensive activity were released from longer peptides with lower activity upon interaction with gastrointestinal enzymes or ACE.

The reduction of SBP by T3 and exopeptidase-treated hydrolysates was greater than those reported for similar or higher doses of protein hydrolysates of sweet potato (Ishiguro et al., 2012), hemp seed (Girgih et al., 2011) and pea (Li et al., 2011). The WPHs in this study lowered SBP at a magnitude similar to an Alcalase-hydrolyzed WPH (80-1200 mg/kg bw) (Wang et al., 2012), but less than that of a Proteinase K-hydrolyzed WPH (8 mg/kg) (Abukabar et al., 1998). However, the hydrolysates in the current study were active within 2 h of administration and had longer-lasting effects, while the aforementioned hydrolysates from other studies were active only after 6 h of administration and had no effect after 24 h (Wang et al., 2012; Abukabar et al., 1998). Similarly, although tri-peptides sourced from  $\alpha$ -casein were shown to achieve greater reductions in SBP at lower concentrations (e.g., Ile-Val-Phe and Ile-Phe-Phe decreased SBP by 47 and 34 mm Hg in SHRs, respectively, at 1.5 mg/kg bw), these peptides had highest effects after 3 h of administration and minimal activity 5 h after administration (Jing et al., 2014). In comparison, reduced SBP was still observed in SHRs 24 h after a single administration of the current hydrolysates. The effect of prolonged WPH administration on SBP reduction was not determined in this study, although long-term (ca. 20 days) daily administration of WPHs to SHRs has been reported in other studies to decrease heart rate, plasma ACE activity, serum angiotensin II and plasma aldosterone levels (Fernández-Musoles et al., 2013; Wang et al., 2012).

Table 2.5 – Summary of mean squares, F-values and p-values for sample, subject, time and subject  $\times$  time interaction from a two-factor ANOVA with repeated measures on one factor using the change in systolic blood pressure of spontaneously hypertensive rats.

	Change in systolic blood pressure			
Source of variation	Mean Squares	<i>F</i> -value	<i>p</i> -value <sup>a</sup>	
Sample (S)	52.71	4.96	0.010	
Subject	11.04	1.67	0.082	
Time (T)	832.01	125.92	0.000	
$\mathbf{S}  imes \mathbf{T}$	27.84	4.21	0.000	

<sup>a</sup> Significant effects ( $p \le 0.05$ ) are shown in bold font.

# Table 2.6 – Mean systolic blood pressure reduction in spontaneously hypertensive rats over

	Time after single administration <sup>b</sup> (h)				
Sample <sup>a</sup>	2	4	6	8	24
Saline	5	2	3	3	2
Captopril	-27defgh	-28efghi	-24cdefg	-23cde	-20bcd
T3	-35i	-34hi	-25cdefg	-23cde	-16ab
T3-AC7	-29efghi	-31ghi	-28efgh	-24cdef	-12a
T3-PR7 <sup>c</sup>	-27defg	-31ghi	-27efgh	-19abc	-12a
T3-PX7	-30fghi	-27defgh	-24cdef	-18abc	-12a

24 h after a single administration of saline, captopril, T3, T3-AC7, T3-PR7 or T3-PX7.

<sup>a</sup> A whey protein hydrolysate produced after hydrolysis with Thermoase PC10F for 3 h (T3) was treated for 7 h with Accelerzyme<sup>®</sup> CPG (T3-AC7), Peptidase R (T3-PR7) or ProteAX (T3-PX7).

<sup>b</sup> Means (n = 4) not sharing common lowercase letters are significantly different ( $p \le 0.05$ ).

<sup>c</sup> Significant subject effect (p = 0.017).



Fig. 2.3 – Change in systolic blood pressure of spontaneously hypertensive rats (mean  $\pm$  standard deviation of 4 rats) over 24 h after a single administration of saline, captopril (10 mg/kg bw), T3 (100 mg/kg bw) or one of three exopeptidase-treated T3 (100 mg/kg bw). A whey protein hydrolysate produced after hydrolysis with Thermoase PC10F for 3 h (T3) was treated for 7 h with Accelerzyme<sup>®</sup> CPG (T3-AC7), Peptidase R (T3-PR7) or ProteAX (T3-PX7). Samples not sharing common lowercase letters at each time interval are significantly different ( $p \leq 0.05$ ).

# 2.5 Conclusion

Exopeptidase treatment of a Thermoase PC10F-produced whey protein hydrolysate (T3) resulted in hydrolysates that were significantly lower in bitterness but still exhibited ACE-inhibitory activity. Fractionation of the exopeptidase-treated hydrolysates by size exclusion chromatography showed highest ACE-inhibitory activity in the fractions consisting of peptides with molecular weights of approximately 200-1000 Da. The fractions eluting after this range, presumably containing di- and tri-peptides, had relatively lower ACE-inhibitory activity that may be explained by the specificity of exopeptidases for hydrophobic amino acids. The three 7 h exopeptidase-treated hydrolysates (100 mg/kg bw) resulted in similar reductions of SBP in spontaneously hypertensive rats, effects of which were comparable to that achieved by captopril (10 mg/kg bw) for the first 8 h and lasted 24 h after administration. Two of the exopeptidasetreated hydrolysates, namely T3 treated with Accelerzyme<sup>®</sup> CPG or Peptidase R, had similar bitterness to a commercial WPH with bland flavour. Interestingly, changes in the other taste attributes were observed in exopeptidase-treatment. These newly-developed taste profiles of exopeptidase-treated hydrolysates could influence their compatibility with different food commodities. Therefore, understanding the taste profile of exopeptidase-treated protein hydrolysates would help their application into foods. This research suggested that exopeptidase treatment with either aminopeptidases or carboxypeptidases has potential use for debittering antihypertensive hydrolysates prior to their incorporation into functional foods.

# Chapter 3: Taste attributes of antihypertensive whey protein hydrolysates produced with or without exopeptidase treatment

# 3.1 Synopsis

An antihypertensive whey protein hydrolysate (WPH) was assessed for changes in taste after treatment with various exopeptidases. WPH produced using an endoprotease preparation (Thermoase PC10F) was treated with the carboxypeptidase Accelerzyme<sup>®</sup> CPG, the aminopeptidase Peptidase R or the aminopeptidase and proteinase mixture ProteAX, and evaluated for five taste attributes and overall acceptability by a sensory panel (n = 12) using descriptive analysis. A three-factor ANOVA was used to quantify panel performance. Significant panelist and interaction effects were eliminated and mean square error values were reduced after standardization of the sensory data by Z-score transformation. The exopeptidase treatments improved the taste of WPHs by decreasing bitterness to levels comparable to a commercial WPH product that displayed minimal ACE-inhibitory activity in vitro. In addition, umami taste and saltiness were significantly higher in exopeptidase-treated hydrolysates. The changes in taste of WPHs after exopeptidase treatments may be partly explained by the release of free amino acids. Specifically, the release of hydrophobic amino acids from the terminals of bitter peptides may explain the decreased bitter taste of exopeptidase-treated hydrolysates while the release of Glu may have increased their umami taste. A significant negative correlation was found between overall acceptability and bitterness of WPHs, suggesting the need for debittering antihypertensive hydrolysates prior to their incorporation into functional foods.

# 3.2 Introduction

Functional foods are food products that exhibit health benefits in addition to their inherent nutritional functions. Although some functional foods are already available to consumers, particularly dairy products such as fortified milks or probiotic yogurts, growth and success of the functional food sector depends on consumer acceptance (Granato et al., 2010). However, consumers are reluctant to compromise poor taste for health benefits in functional foods (Verbeke, 2006). In addition, the continued consumption of a functional food product was dependent on its flavour and texture, as opposed to its health benefits (Barrios et al., 2008). Acceptable or negligible taste of functional ingredients, therefore, is a prerequisite for their successful integration into functional foods.

Whey protein hydrolysates are food ingredients commonly used in the food industry, notably in sports nutrition and infant formula as a source of bioavailable amino acids. Many studies have isolated bioactive peptides that exhibit physiological effects, including antihypertensive activity, from whey protein hydrolysates (Madureira et al., 2010). However, whey protein hydrolysates, and protein hydrolysates in general, are typically bitter (Leksrisompong et al., 2010, 2012). Bitter taste generally leads to food rejection, as opposed to sweet and umami taste, which are generally associated with food acceptance (Temussi, 2011). As a result, hydrolysates have had limited use in functional foods for their bioactive properties.

The bitterness of peptides is attributed to the presence of hydrophobic amino acids, particularly when situated at the C-terminal (Kim & Li-Chan, 2006). Various methods have been explored

for debittering protein hydrolysates, such as the removal of hydrophobic peptides, with exopeptidase treatment providing efficient debittering effects without decreasing nutritional quality or yield (Rakaksulthai & Haard, 2003; Saha & Hayashi, 2001). Exopeptidases are enzymes that release the terminal amino acid residues of peptides, with aminopeptidases and carboxypeptidases acting at the N-terminal and C-terminal, respectively. Chapter 2 described the significant debittering effect of three commercial exopeptidases on a Thermoase PC10Fproduced whey protein hydrolysate. The exopeptidase-treated hydrolysates exhibited not only ACE-inhibitory activity *in vitro*, but antihypertensive activity in spontaneously hypertensive rats as well. The exopeptidase treatments, interestingly, were also observed to change the taste attributes of hydrolysates. Although exopeptidase treatment may be an effective debittering method for bioactive hydrolysates, their effect on the full taste profile of hydrolysates is rarely addressed and should be studied to better assess their appropriateness for producing food ingredients.

The use of human panelists remains the gold standard for assessing flavour, despite emerging methods of instrumental assessment using taste-sensing equipment (Woertz et al., 2011). The training of panelists can improve their use of line scales and lead to higher discriminatory ability (Labbe & Hugi, 2004). Despite panelist training, significant panelist and panel interaction effects are not only common in sensory research but generally recognized, and are attributed to the physiological differences of panelists, as well as their differences and inconsistency in scale use (Chapman et al., 2004; Cliff et al., 2012; Moon & Li-Chan, 2007; Schlosser et al., 2005; Song et al., 2010). In these studies, panel inconsistencies are often overcome by recalculating the treatment F-values. This is achieved by treating panelists as random effects (i.e., representative

of the entire consumer population), resulting in the calculation of a mixed-model, as opposed to a fixed-model, ANOVA. The mean square for the panelist  $\times$  treatment effect is used in lieu of the mean square error for the calculation for treatment *F*-values, leading to a more conservative estimate of treatment effects. Although commonly used, this method is not universally accepted in sensory evaluation (Lawless, 1998).

While such procedures are acceptable for estimating consumer perception, this loss of sample discrimination is not particularly satisfactory for analytical sensory testing, wherein panelists are regarded as analytical instruments as opposed to a segment of a general population (O'Mahony, 1998). Ideally, significant panelist effect should not occur, analogous to how replicate readings from one instrument or readings of a sample from multiple similar instruments are not expected to differ significantly. Reid & Durance (1992) proposed that standardization of sensory data by the computation of *Z*-scores minimized the differences of line scale usage by panelists, resulting in the elimination of a significant panelist effect. Although beneficial for their data analysis, this method of data standardization has not been widely adopted in sensory evaluation and the benefits of *Z*-score transformation of sensory data remain to be assessed.

The first objective of this study was to characterize and compare the bitterness, umami taste, sweetness, saltiness, sourness, and acceptability of the aforementioned WPH and exopeptidase-treated hydrolysates (i.e., WPH produced by Thermoase PC10F and further treated by Accelerzyme<sup>®</sup> CPG, Peptidase R, or ProteAX for 7 h), and two commercial WPHs. The second objective of this study was to apply *Z*-score transformation on the sensory data and assess the benefits of this data standardization method in sensory research.

#### **3.3 Materials and methods**

# 3.3.1 Materials

The whey protein hydrolysate produced after a 3 h hydrolysis with Thermoase PC10F (T3) and three exopeptidase-treated hydrolysates, namely T3 treated for 7 h with Accerlerzyme<sup>®</sup> CPG (T3-AC7), Peptidase R (T3-PR7) and ProteAX (T3-PX7), were prepared as described in Sections 2.3.2 and 2.3.3, respectively. The "exopeptidase-treated hydrolysates" refer to T3-AC7, T3-PR7 and T3-PX7 while the "experimental hydrolysates" refer to the exopeptidase-treated hydrolysates and T3. Two commercial whey protein hydrolysates, Hilmar<sup>TM</sup> 8350 and WPH 4003, were obtained as described in Section 2.3.1. Monosodium glutamate was from Ajinomoto North America, Inc. (Teaneck, NJ). Food grade hydrochloric acid (HCl), sodium hydroxide (NaOH) and caffeine meeting Food Chemical Codex requirements, citric acid anhydrous meeting United States Pharmacopeia requirements were from Fisher Scientific (Fairlawn, NJ). Iodized table salt (Windsor<sup>®</sup>; Anjou, QC) and sugar (Rogers Sugar Inc.; Vancouver, BC) were purchased from the grocery store. All other reagents and chemicals were as described in Section 2.3.1.

# 3.3.2 Sensory evaluation

Sensory evaluation was conducted with the approval of the Behavioural Research Ethics Board of the University of British Columbia.

# 3.3.2.1 Panelist recruitment

Panelists of the sensory panel were recruited from the Food Nutrition and Health program at the University of British Columbia (Vancouver, BC). Individuals who have food allergies, a family history of food allergies, or have never eaten dairy foods in the past (and thus may be unaware of an allergy) were excluded from the study. Since the WPH may contain small amounts of lactose carried over from the starting WPI, individuals with severe lactose intolerance were also excluded. The panel consisted of eight females and four males aged 20–35 years. One participant was Caucasian and the remaining were Asian. All participants were asked to refrain from using strong, odourous materials such as perfumes before participation in sensory evaluations and to refrain from eating, drinking, or smoking within 30 minutes of entering the sensory laboratory.

# 3.3.2.2 Sensory training

#### **3.3.2.2.1** First training session

The objective of the first training session was to have panelists practice identifying taste attributes and discriminating different levels of bitterness, and be familiarized with the samples and potential reference standards. All samples (0.2 mL) were tasted by directly depositing on the back of the tongue as described in Section 3.3.2.3. Panelists were trained to identify umami taste, sweetness, saltiness, and sourness with aqueous solutions of the following reference standards: 1% monosodium glutamate, 2% sucrose, 1% sodium chloride, and 0.5% citric acid in ddH<sub>2</sub>O. Panelists were asked to discriminate different levels of bitterness in two sets of triangle tests: 5

and 15 mM caffeine in ddH<sub>2</sub>O, and 10 and 15 mM caffeine in ddH<sub>2</sub>O. Product-specific reference standards were also prepared by dissolving the aforementioned reference standards and 15 mM caffeine in WPH 4003 (10 g/100 mL ddH<sub>2</sub>O). This commercial product was selected as the base for reference standards because it had the overall taste profile of the experimental WPHs, but otherwise low intensities of taste attributes. The inclusion of product-specific reference standards in the sensory evaluation served to calibrate panelists in their taste assessments. During this session, panelists requested a decrease in sourness for the sour standard and the development of a standard for "milky."

# 3.3.2.2.2 Second training session

The objective of the second training session, conducted one day after the first training session, was to have panelists practice discriminating different levels of bitterness in WPHs, and for panelists to agree on which reference standards to include in the tasting sessions and where these reference standards should be anchored on their respective line scales. At the beginning of this session, the amount of sample tasted was increased from 0.2 mL to 0.3 mL, as agreed upon by the entire panel. Discrimination of bitterness at varying intensities was assessed over two ranking tests: five samples (2.5–15 mM caffeine in WPH 4003) were presented in the first test and two samples (5 and 10 mM caffeine in WPH 4003) in the second test. In the first test, three panelists ranked all five samples in the correct order, four panelists ranked three samples in the correct order and four panelists ranked one sample correctly. One panelist did not rank any of the samples correctly, although all samples were placed within two positions of the correct ranking. Ten panelists correctly recognized that the sample containing 2.5 mM caffeine had the lowest

bitterness, while seven panelists correctly ranked the sample containing 15 mM caffeine as the highest bitterness. The number of panelists placing samples containing 5, 7.5 and 10 mM caffeine in the correct rank position were 5, 3 and 6, respectively. By the second ranking test, 11 of 12 panelists correctly ranked samples. The results from the first and second ranking tests suggested that panelists could discriminate among 2.5, 5 and 10 mM of caffeine in WPH 4003.

Panelists were presented with three standards for "milky" taste consisting of 50% diluted skim milk, skim milk, and 2% milk. Upon further discussion, the panelists agreed that "milky" was not a representative descriptor for the samples and unanimously decided to eliminate it from this study. A decreased concentration of citric acid (0.4%) in WPH 4003 was deemed appropriate for use as the reference standard for sourness. During discussion, the sample containing 10 mM caffeine in WPH 4003 was deemed more appropriate (i.e., more representative of the bitterness intensity in the experimental hydrolysates) than the standard used in the first training session (15 mM caffeine in WPH 4003) and therefore was selected as the reference standard for bitterness.

A reference standard for each of the taste attributes was marked on their respective 15-cm line scales at a location agreed upon by the panelists. The reference standards used and their distance on the line scale were as follows: 10 mM caffeine for bitterness (11.0 cm), 1% monosodium glutamate (MSG) for umami taste (12.0 cm), 2% sucrose for sweetness (10.5 cm), 1% sodium chloride (NaCl) for saltiness (10.0 cm), and 0.4% citric acid for sourness (7.5 cm).

# 3.3.2.3 Sample assessment

Sensory evaluation was conducted using the procedure briefly described in Chapter 2 (Section 2.3.6) and was based on that described in Cheung & Li-Chan (2010). The T3 and exopeptidasetreated hydrolysates as well as two of the commercial products, WPH 4003 and Hilmar<sup>TM</sup> 8350, were prepared as 10% solutions (10 g/100 mL ddH<sub>2</sub>O) and assessed for bitterness, umami taste, sweetness, saltiness, sourness, and overall acceptability by 12 panelists in duplicate. This concentration (i.e., 10%) has been used in other studies assessing the taste of WPHs (Leksrisompong et al., 2010, 2012; Liu et al., 2013). Samples were labelled with random threedigit codes and assigned in random order to panelists. Panelists tasted samples by directly depositing 0.3 mL on the back of the tongue using a 1 mL syringe (BD; Franklin Lakes, NJ) and were instructed to wait 5 s before swallowing or discarding samples and 2 min between samples. An example of the scorecard used is shown in Fig. D.1 in Appendix D. Tastings were conducted under red lighting to minimize any possible visual cues. Between samples, panelists were instructed to use water, low sodium club soda (45 mg Na/355 mL) (Canada Dry Mott's Inc.; Mississauga, ON), and unsalted crackers as palate cleansers at least once. Panelists also rated overall acceptability of the samples. All five taste attributes and overall acceptability were evaluated on 15-cm line scales with anchors at 1 and 14 cm labelled low and high, respectively. Reference standards for the five taste attributes, as described in the previous section, were positioned on their respective 15-cm line scale. Panelists were able to provide comments on their scorecards.
### 3.3.3 Salt content

Salt content of hydrolysates was measured using a MeterLab<sup>®</sup> CDM210 conductivity meter (Radiometer Analytical SAS, Lyon, France). The electrode was submerged into samples (ca. 15 mL of 10% aqueous solutions), and the conductivity reading (mS/cm) recorded when the reading was stabilized. A standard curve (0.0125–1.0000% NaCl) was constructed and used to determine the salt content as % NaCl equivalents.

### 3.3.4 Amino acid analysis

Total and free amino acid contents of hydrolysates were analyzed at the Advanced Protein Technology Centre at the Hospital for Sick Children (Toronto, ON) by reversed-phase high performance liquid chromatography with detection at 254 nm following pre-column derivatization of hydrolysates with phenyl isothiocyanate. Free amino content was assessed without prior hydrolysis while assessment for total amino acid content required a 24 h hydrolysis with 6 N HCl and 1% phenol at 110 °C prior to pre-column derivatization. The content of bound amino acid was estimated from the difference between total amino acid content and free amino acid content.

### 3.3.5 Statistical analysis

### **3.3.5.1** Assessment of panel performance on taste attributes

The sensory data was analyzed based on the method based on that described by Cheung & Li-Chan (2014). The sensory scores were first analyzed by a three-factor ANOVA general linear model (GLM) with sample, panelist and replication as main effects as described in Section 3.3.5.3. Plots depicting selected significant main and interaction effects can be found in Appendix D (Figs. D.2–D.9). Panelists were then screened for (1) their individual repeatability and (2) their correlation with other panelists. The repeatability of each panelist for each taste attribute and overall acceptability was assessed by conducting a two-factor ANOVA general linear model (GLM) on sensory scores (n = 12) with sample and replication as main effects. Panelists with a significant replication effect ( $p \le 0.05$ ) had their sensory scores removed from the overall data set. This resulted in the elimination of Panelist 6 for bitterness, Panelist 11 for umami taste, and Panelists 9, 11 and 13 for saltiness (Table D.1 in Appendix D). A three-factor ANOVA GLM was used to track the improvements in panel performance, which showed that bitterness no longer had significant interaction effects and therefore did not require additional assessment of panel performance.

The correlation of individual panelists with the panel was determined as described in Section 2.3.9 for taste attributes still exhibiting significant interaction effects, namely umami taste, sweetness, saltiness and sourness. Panelists were removed when their six sample means did not significantly correlate ( $p \ge 0.05$ ) with sample means obtained by averaging the scores of the

other panelists (Table D.2 in Appendix D). This resulted in the removal of Panelists 3, 6, 7, and 9 for umami taste and Panelists 6 and 7 from saltiness. Furthermore, this assessment revealed that the panel lacked correlation in their assessment for the sweetness and sourness taste attributes. Thus, these two taste attributes were not included in subsequent analyses.

### **3.3.5.2** Data standardization

The sensory scores for bitterness (n = 22), umami taste (n = 14) and saltiness (n = 14) were normalized into Z-scores using Equation 2 as described in Mui, Durance, & Scaman (2002) and Reid & Durance (1992):

$$Z = (x - X)/SD \tag{2}$$

where Z is the transformed score, x is the original score, X is the mean of all scores for one attribute from one panelist, and SD is the standard deviation of X for the same panelist. This method of data standardization removed individual differences in line scale use by creating a scale with a mean of zero for each panelist. Samples with Z-scores above and below the mean have positive and negative values, respectively.

### **3.3.5.3** Data analysis

The original sensory scores and Z-scores for bitterness, umami taste and saltiness, and the original scores for overall acceptability were analyzed using a three-factor ANOVA GLM with panelists (P), sample (S), and replication (R) as fixed effects and all two-factor interactions (P  $\times$ 

S,  $P \times R$ ,  $S \times R$ ) (Minitab 16; Minitab Inc., State College, PA). Significant differences among samples were determined by Fisher's least significant difference (LSD) test. Correlations among attributes and overall acceptability were determined as described in Section 2.3.9. Correlation analyses were also performed between sensory scores and results of the compositional analyses: saltiness and umami taste with salt content (% NaCl equivalent), and umami taste with free Glu content. All statistical significances were determined at  $p \le 0.05$ .

### 3.4 Results and discussion

### 3.4.1 Analysis of variance and standardization of data

The experimental (T3, T3-AC7, T3-PR7 and T3-PX7) and commercial WPH (Hilmar<sup>TM</sup> 8350 and WPH 4003) samples were evaluated for bitterness, umami taste and saltiness, and the results analyzed by a three-factor ANOVA. Significant sample effects were found for all three taste attributes, suggesting that the hydrolysates had distinguishable intensities of bitterness, umami taste and saltiness (Table 3.1). However, umami taste and saltiness also had significant panelist × sample interactions, which increase the likelihood of committing type I and type II errors and forming false conclusions of sample differences (Lundhal & McDaniel, 1991). In addition, the panelist × replication interaction was significant for saltiness, suggesting that the saltiness of samples was perceived differently by panelists between the two tasting sessions.

While the calculation of an adjusted *F*-value using the mean square of interaction effects can overcome significant interactions and allow for sample discrimination, the three taste attributes also had significant panelist effect and large mean square error values. Significant panelist effects are common in sensory research and are attributed to physiological differences among panelists, as well as their differences and inconsistences of line scale usage (Chapman et al., 2004; Cliff et al., 2012; Moon & Li-Chan, 2007; Schlosser et al., 2005; Song et al., 2010). Indeed, closer inspection of panelist sensory scorecards showed that some panelists were different and inconsistent in their use of line scales, despite panelist training, inclusion of reference standards for each attribute, and screening for panel performance based on panelist

repeatability and correlation with the panel. For example, some panelists tended to use the entire line scale, while some others preferred to use a narrow range of the scale. In addition, panelists differed in their evaluation of sample differences such that some panelists found large differences among samples, while others found only small differences. These observations can be visualized, for example, in the panelist  $\times$  sample interaction plot for saltiness (Fig. D.7). While Panelists 10 and 12 tended to use a wider range of the scale and found larger differences among samples, Panelists 2, 4, and 8 tended to use a narrow range on scale and found only small differences among samples. These differences in scale use may explain the significant panelist effect observed in all taste attributes (Table 3.1).

Reid and Durance (1992) reduced the error resulting from different and inconsistent use of line scales by forcing all scores onto the same scale, effectively eliminating the significant panelist effect. This was achieved by *Z*-score transformation of sensory scores prior to statistical analysis (Mui et al., 2002). When applied to the current sensory scores, data standardization eliminated significant panelist effects and significant interactions (Table 3.2). In addition, the mean square error values were decreased in all three taste attributes, with error terms for bitterness decreasing more than 10 fold.

The mean sensory and Z-scores of experimental and commercial hydrolysates are shown in Table 3.3. The aforementioned reduction in the mean square error values led to increased sample discrimination, as observed for the scores for bitterness. For example, T3-PR7 was found to be similar to both T3-AC7 and T3-PX7 without data standardization. However, the use of Z-scores showed that T3-PR7 was more similar to T3-AC7, both of which were significantly less bitter

than T3-PX7. While these differences may be minor when the hydrolysates are incorporated as ingredients into food products, the analysis suggested that the use of *Z*-scores led to more sensitive detection of statistical differences among samples.

Sample differences were not determined for umami taste and saltiness using sensory scores as both taste attributes had significant interaction effects (Table 3.1). However, the sample effects of umami taste and saltiness (F = 63.51 and 50.19, respectively) were larger than their panelist and interaction effects (F = 7.39 and 2.02, and F = 26.18, 2.67, and 2.57, respectively), suggesting that the differences among the samples were larger than, and could be detected despite, differences and interactions among panelists. The large sample effect may be attributed to both the training of the panelists, as well as the pre-screening analyses for panel performances.

The three-factor ANOVA on Z-scores for umami taste and saltiness did not have significant panelist and interaction effects (Table 3.2), allowing sample differences to be determined (Table 3.3). However, presenting sample means as Z-scores has its disadvantages. For example, differences in taste intensities are only relative to the samples assessed because sample differences are indicated by their positive or negative distances around a mean of zero. Sensory scores, on the other hand, provide information on the taste intensity of a sample (e.g., on a scale from 0–15) and its relative distance from a known reference standard. Nevertheless, Z-score transformation allowed for the analysis of significant sample differences with minimal panel inconsistency and, therefore, decreased the likelihood of committing Type I or Type II errors. Standardization by Z-score transformation, therefore, has potential to be a valuable tool for

analytical sensory testing, allowing statistical sample differences to be discerned despite shortcomings in panel performance.

While panelists chose to include sweetness and sourness as descriptors for the hydrolysates, the assessment for panel consistency revealed that there was minimal agreement among panelists in their evaluation of these two attributes (Table D.2 in Appendix D). This may be due to the inherently low sweetness and sourness of the experimental and commercial hydrolysates, which had mean (n = 144) sensory scores of  $3.4 \pm 2.2$  and  $2.1 \pm 1.3$ , respectively. In addition, the perceived intensities of some taste attributes, such as bitterness and sourness, when all are present simultaneously (Keast, 2008). These hypotheses are supported by the comments provided by the panelists. For example, one panelist commented that "no … difference in sourness [was detected]… [sourness] may not be the characteristic taste of your samples," while another commented "the bitter taste outpowers a lot, including sweetness." Therefore, these two taste attributes may not be relevant descriptors of the experimental and commercial hydrolysates.

Table 3.1 – Summary of *p*-values, *F*-values and mean square error terms for panelist,

sample, replication, and their interactions from a three-factor ANOVA using sensory

	<i>p</i> -value <sup>a</sup>				
Source of variation	Bitterness	Umami taste	Saltiness	Overall acceptability	
Panelist (P)	0.000	0.000	0.000	0.255	
Sample (S)	0.000	0.000	0.000	0.000	
Replication (R)	0.755	0.631	0.210	0.912	
$\mathbf{P} \times \mathbf{S}$	0.055	0.030	0.004	0.000	
$\mathbf{P} \times \mathbf{R}$	0.958	0.641	0.326	0.806	
$\mathbf{S}  imes \mathbf{R}$	0.998	0.457	0.048	0.900	
Sample <i>F</i> -value	34.24	63.51 2.692	50.19	18.82 (6.39) <sup>b</sup>	
Wear square critic	T.U/I	2.072	1.702	5.710	

scores.

<sup>a</sup> Significant effects ( $p \le 0.05$ ) are shown in bold font. Bitterness, umami taste, saltiness and overall acceptability of six whey protein hydrolysate samples were assessed in duplicate by 11, 7, 7 and 12 panelists, respectively.

<sup>b</sup> An adjusted *F*-value of 6.38 was calculated using the mean square of the panelist × sample interaction effect (15.952) as the denominator. The sample effect was significant ( $p \le 0.001$ ).

Table 3.2 – Summary of *p*-values, *F*-values and mean square error terms for panelist,

		<i>p</i> -value"		
Source of variation	Bitterness	Umami taste	Saltiness	
Panelist (P)	1.000	1.000	1.000	
Sample (S)	0.000	0.000	0.000	
Replication (R)	0.772	0.469	0.115	
$\mathbf{P} \times \mathbf{S}$	0.067	0.485	0.838	
$\mathbf{P} \times \mathbf{R}$	0.963	0.587	0.512	
$S \times R$	0.958	0.297	0.059	
Sample <i>F</i> -value	41.20	50.85	36.96	
Mean square error	0.3588	0.2360	0.3011	

sample, replication, and their interactions from a three-factor ANOVA using Z-scores.

<sup>a</sup> Significant effects ( $p \le 0.05$ ) are shown in bold font. Bitterness, umami taste and saltiness of six whey protein hydrolysate samples were assessed in duplicate by 11, 7 and 7 panelists, respectively.

Table 3.3 – Mean sensory and Z-scores for bitterness, umami taste, saltiness and overall acceptability of T3, exopeptidase-treated T3, and two commercial products Hilmar<sup>TM</sup> 8350 and WPH 4003.

			Taste attri	bute <sup>b</sup>			
	Bitterness Umami taste Saltiness				Overall acceptability <sup>b</sup>		
Sample <sup>a</sup>	sensory <sup>c</sup>	$Z^{d}$	sensory <sup>e</sup>	$Z^{d}$	sensory <sup>e</sup>	$Z^{d}$	-
Т3	10.4a	1.1a	4.2	-0.5b	3.5	-0.3c	3.8d
T3-AC7	6.5c	-0.1c	9.9	1.0a	7.4	1.2a	6.7bc
T3-PR7	7.1bc	0.1c	9.0	0.8a	5.0	0.3b	6.4bc
T3-PX7	8.2b	0.5b	8.5	0.7a	6.0	0.6b	5.7cd
Hilmar <sup>TM</sup> 8350	5.8c	-0.2c	2.8	-0.9bc	2.2	-0.8d	8.3ab
WPH 4003	2.3d	-1.4d	1.9	-1.1c	1.4	-1.1d	9.7a

<sup>a</sup> A whey protein hydrolysate produced after hydrolysis with Thermoase PC10F for 3 h (T3) and treated for 7 h with Accelerzyme<sup>®</sup> CPG (T3-AC7), Peptidase R (T3-PR7) or ProteAX (T3-PX7) were tasted as 10% solutions in duplicate. Two commercial products Hilmar<sup>TM</sup> 8350 and WPH 4003 were also tasted.

<sup>b</sup> Means in the same column not sharing common lowercase letters are significantly different ( $p \le 0.05$ ). Bitterness, umami taste, saltiness and overall acceptability were assessed in duplicate by 11, 7, 7 and 12 panelists, respectively.

<sup>c</sup> Data from Chapter 2.

 $^{d}Z$ -scores that are above and below the mean have positive and negative values, respectively.

<sup>e</sup> Significant sample differences were not calculated due to significant panelist and interaction effects when the data was analyzed by a three-factor ANOVA using sample, panelist, and replication as main effects.

### 3.4.2 Exopeptidase treatment effects on taste and overall acceptability

T3 had the highest bitterness of all hydrolysates tasted and was significantly more bitter than the exopeptidase-treated hydrolysates (Table 3.3). Of the exopeptidase-treated hydrolysates, T3-PX7 was the least debittered. As suggested in Chapter 2, this may be due to the proteinase activity of ProteAX resulting in more extensive hydrolysis of T3 (Table 2.4) as high DH has been positively correlated to bitterness in another study assessing WPHs (Leksrisompong et al., 2010). The two hydrolysates treated with only exopeptidases (i.e., T3-AC7 and T3-PR7) had bitterness similar to Hilmar<sup>TM</sup> 8350, a commercial product described as having "bland" taste (Hilmar Ingredients, 2012). This suggested that the exopeptidase treatments not only significantly debittered T3, but that the bitterness was decreased to a level comparable to those of currently-used WPH food ingredients.

Exopeptidase treatment also influenced the other taste attributes of the exopeptidase-treated hydrolysates. Umami taste, while minimally present in T3 and the two commercial products, was significantly increased in exopeptidase-treated hydrolysates (Table 3.3). Umami taste describes a savoury, satisfying taste and is often associated with the taste improvement ability of monosodium glutamate (Beauchamp, 2009). Therefore, exopeptidase treatment of T3 appeared to enhance the savoury taste in addition to debittering the hydrolysates. It is possible that the increase in umami taste contributed to the debittering effect of exopeptidase treatment, since the umami taste-enhancing compound monosodium glutamate (100 mM) has been reported to decrease the bitterness of a 10% WPH (Leksrisompong et al., 2012). The saltiness was also significantly higher in exopeptidase-treated hydrolysates (Table 3.3), with T3-AC7 having the

highest saltiness of the samples assessed. This was consistent with the results of compositional analyses, where T3-AC7 had the highest NaCl equivalent of 0.56%. The other WPHs had NaCl equivalents as follows: T3-PX7 (0.39%) and T3-PR7 (0.35%), T3 (0.27%), Hilmar<sup>TM</sup> 8350 (0.20%) and WPH 4003 (0.10%). In fact, saltiness was significantly correlated to the salt content of experimental hydrolysates (R = 0.9871, n = 6, p = 0.0002).

In addition to these three taste attributes, the experimental and commercial hydrolysates were assessed for their overall acceptability. As shown in Table 3.1, this characteristic had a significant sample effect without a significant panelist effect. To overcome the significant panelist  $\times$  sample interaction, the *F*-value for the sample effect was recalculated using the mean square of the interaction effect as described in Goniak & Noble (1987) (MS<sub>p×s</sub> = 15.952,  $F_{adj}$  = 6.39,  $p \le 0.001$ ) (Table 3.1). This method treats panelist as a random effect, which is appropriate when assessing overall acceptability since it is a consumer sensory characteristic, as opposed to analytical sensory characteristics such as the aforementioned taste attributes (O'Mahony, 1998). T3-AC7 and T3-PX7 had significantly higher overall acceptability than T3 and were comparable to Hilmar<sup>TM</sup> 8350 (Table 3.3). Therefore, the treatments with aminopeptidase and carboxypeptidase were equally effective at debittering and improving the overall acceptability of T3. In contrast, T3-PX7 was similar to both T3 and the other exopeptidase-treated hydrolysates. The generally higher overall acceptability of exopeptidase-treated hydrolysates compared to T3 was consistent with their sensory profiles of individual taste attributes such that desirable taste attributes (i.e., umami taste and saltiness) were increased while undesirable bitterness was decreased (Beauchamp, 2009; Temussi, 2011). The lack of significant differences in overall acceptability among exopeptidase-treated hydrolysates may be attributed to the small panel size

(n = 12). While this size serves to estimate the general direction of overall acceptability, it is not representative of the general consumer population and may not be sufficient to establish smaller sample differences.

### 3.4.3 Amino acid analysis

Total and free amino acid contents of the experimental hydrolysates were determined to gain further insight on the changes in taste profile after exopeptidase treatment. These results are shown in Fig. 3.1. The original hydrolysate, T3, had high contents of Glu, Leu, Asp, and Lys, the four of which accounted for 50% of the total amino acids present. These values were similar to those of the starting WPI product used for hydrolysate production (Fonterra, 2007), as well as to WPHs reported elsewhere (Sindayikengera & Xia, 2006; Sinha et al., 2007). Very low amounts of free amino acids (5.61 µg/mg sample), consisting mostly of Trp and Val, were present in T3. These observations were consistent with the results described in Chapter 2, in which T3 was reported to have a low extent of hydrolysis and contain predominantly high molecular weight peptides.

The two aminopeptidase-treated hydrolysates, T3-PR7 and T3-PX7, contained higher contents of free amino acids (35 and 40% of total amino acids, respectively) than the carboxypeptidase-treated hydrolysate T3-AC7 (23% of total amino acids). Treatment with ProteAX, and to a lesser extent Peptidase R, released the majority of Ile, Leu, Val, Phe and Arg as free amino acids from their peptide-bound form (Fig. 3.1). Hydrophobic amino acids such as the branched-chain Ile, Leu and Val, and the aromatic ring containing Phe, Trp, and Tyr (Ishibashi et al., 1987a, 1987b;

Ishibashi et al., 1988b; Lemieux & Simard, 1992), as well as the basic amino acid Arg (Li-Chan & Cheung, 2010) are bitter in their natural L-conformation. While these may impart bitterness in hydrolysates, the exopeptidase-treated hydrolysates were less bitter than T3. This may be due to hydrophobic amino acids having higher bitterness when present in peptide-bound form than as free amino acids (Otagiri et al., 1985). In addition, Arg was more bitter when repeated in di- and tri-peptide form (i.e., Arg-Arg and Arg-Arg-Arg, respectively) than as a free amino acid (Matoba & Hata, 1972). Therefore, hydrolysates can be debittered despite the release of free, bitter-tasting amino acids after exopeptidase treatment. Although hydrophobic C-terminal amino acids were predicted to be correlated to bitterness in peptides (Kim & Li-Chan, 2006) and thus their removal may be expected to result in larger decreases of bitterness than the removal of N-terminal hydrophobic amino acids, no differences in debittering effect were observed between aminopeptidase- and carboxypeptidase-treated hydrolysates in this study.

While the exopeptidase-catalyzed release of bitter amino acids may have an overall debittering effect, the release of free glutamate may be associated with the increased umami taste of hydrolysates after exopeptidase treatment. All of the experimental hydrolysates had high total Glu contents. However, the three exopeptidase-treated hydrolysates had significantly higher umami taste than T3. This may be because the exopeptidase-treated hydrolysates contained higher contents of free Glu, which exists in its umami-tasting salt form glutamate at neutral pH. High contents of Glu, as opposed to peptides with umami taste, were postulated to contribute more to the umami taste of protein hydrolysates (Temussi, 2011). Indeed, the free Glu content was significantly correlated to umami taste with *R* of 0.9642 (n = 4; p = 0.0358). The increase in

free Glu has been reported to increase the umami taste in other hydrolysates (Schlichtherle-Cerny & Amadò, 2002).



# Fig. 3.1 – Free and bound contents of nonpolar (a) and polar amino acids (b) in the hydrolysate produced by Thermoase PC10F (T3), and T3 treated with Accelerzyme® CPG (T3-AC7), Peptidase R (T3-PR7), or ProteAX (T3-PX7).

Amino acid contents are shown as stacked bars with dark and light gray colour denoting free (bottom bars) and bound (top bars) amino acid contents, respectively, the sum of which account for the total amino acid content. Four samples were assessed (from left to right): the hydrolysate produced by Thermoase PC10F (T3, solid pattern), and T3 treated with Accelerzyme® CPG (T3-AC7, diagonal striped pattern), Peptidase R (T3-PR7, vertical striped pattern), or ProteAX (T3-PX7, checkered pattern). The free amino acid content of Asx is the sum of Asp (lowest bars with dark gray colour) and Asn (centre bars with gray colour), while the bound amino acid content of Asx (top bars with light gray colour) is the sum of bound Asp and Asn. The free amino acid content of Glx is the sum of Glu (lowest bars with dark gray colour) and Gln (centre bars with gray colour), while the bound amino acid content of Glx has with light gray colour) is the sum of Glx (highest bars with light gray colour) is the sum of bound Glu and Gln. Cys and Trp contents were underestimated due to their sensitivity to acid hydrolysis.

### **3.4.4** Correlations of taste attributes and acceptability

Potential correlations among the three taste attributes and overall acceptability were determined to better understand their inter-relationships (Table 3.4). Bitterness was the only taste attribute significantly correlated to overall acceptability, to which it correlated negatively (R = -0.9697, n = 6, p = 0.0014). This demonstrated that the low overall acceptability of T3 was largely due to its bitter taste and suggested that overall acceptability can be increased if the bitterness was decreased. Since consumer acceptance is required for functional food products to be successful (Barrios et al., 2008; Verbeke, 2006), debittering of hydrolysates prior to incorporation into functional foods would be highly beneficial.

The positive correlation observed between umami taste and saltiness (R = 0.9503, n = 6, p = 0.0036) may reflect the fact that the two attributes were not readily distinguishable (Table 3.4), as discussed by panelists during the training sessions. Panelists may be prone to associate umami and salty tastes due to the likelihood of tasting both attributes simultaneously during meals (Ikeda, 2002). Furthermore, salty and umami-tasting compounds, such as sodium chloride and monosodium glutamate, respectively, have been reported to interact synergistically in savoury foods, and result in enhanced umami taste and decreased desire to consume additional salty foods (Halpern, 2000). In fact, the hydrolysate salt concentrations (i.e., % NaCl equivalents) were significantly correlated with umami taste (R = 0.9164, n = 6, p = 0.0102), which may suggest that the salt present in the hydrolysates interacted with the free glutamate produced after exopeptidase treatment and led to an enhancement of umami taste and saltiness that was difficult

to differentiate. Of the experimental hydrolysates, T3-AC7 had the highest umami taste, saltiness, and salt content (Table 3.3).

Sourness, although not a key taste attribute, was correlated to bitterness in whey protein hydrolysates in another study (Leksrisompong et al., 2010). As the hydrolysate sourness in the current study was too low to evaluate consistently, this attribute was not assessed for correlations with other taste attributes.

Table 3.4 – Correlation matrix displaying the Pearson correlation coefficient and *p*-value

		Pearson corre	Pearson correlation coefficient ( <i>p</i> -value) <sup>a</sup>					
Attribute	Bitterness	Umami	Saltiness	Overall acceptability				
Bitterness	1.0000	0.3664	0.4431	-0.9697				
	(0.0000)	(0.4750)	(0.3788)	(0.0014)				
Umami		1.0000	0.9503	-0.4121				
		(0.0000)	(0.0036)	(0.4168)				
Saltiness			1.0000	-0.4953				
			(0.0000)	(0.3178)				
Acceptability				1.0000				
				(0.0000)				

among three taste attributes and overall acceptability	among	three	taste	attributes	and	overall	acce	ptability	y.
--------------------------------------------------------	-------	-------	-------	------------	-----	---------	------	-----------	----

<sup>a</sup> Significant correlations ( $p \le 0.05$ ) are shown in bold (n = 6, df = 4).

### 3.5 Conclusion

This study showed that exopeptidase treatment not only changed the bitterness of hydrolysates, but the other taste attributes as well, and can also influence overall acceptability. A bitter-tasting, antihypertensive whey protein hydrolysate (WPH) was treated with one of three exopeptidases and the resulting hydrolysates, as well as two commercial WPHs, were assessed for five taste attributes and overall acceptability. Since the three-factor ANOVA on sensory scores revealed significant panelist and interaction effects, it was desirable to standardize sensory scores prior to assessing sample differences. Z-score transformation of the sensory data eliminated the significant panelist and interaction effects and reduced the mean square error terms. The analysis of Z-scores showed that exopeptidase treatment significantly reduced the bitterness of the antihypertensive WPH to commercially-accepted levels and increased its umami and salty tastes. Amino acid analysis suggested that exopeptidase treatment debittered hydrolysates by releasing hydrophobic amino acids from bitter peptides. In addition, the release of free Glu may explain the significantly higher umami taste of exopeptidase-treated hydrolysates. Bitterness was negatively correlated to overall acceptability, demonstrating that an effective debittering method for protein hydrolysates is essential for their successful incorporation into functional foods.

# **Chapter 4: Conclusion**

### 4.1 Main findings of this research study

Until now, it was known that (1) the removal of terminal amino acids in peptides via exopeptidase treatment is an effective debittering method for protein hydrolysates, which require debittering if they are to be successfully incorporated into foods and (2) the ACE-inhibitory activity of peptides is highly influenced by the terminal amino acid residues. However, this raised the question of whether exopeptidase treatment can be used to debitter antihypertensive hydrolysates, considering the importance of terminal amino acids for both bitterness and ACE-inhibitory activity.

This study sought to determine whether exopeptidase treatment could be used to debitter antihypertensive protein hydrolysates prior to their incorporation into functional foods. To achieve this, seven whey protein hydrolysates were produced and screened for ACE-inhibitory activity and bitterness. It was hypothesized that the WPHs with high ACE-inhibitory activity will also have bitter taste. As shown in Table 2.3, all WPHs with high ACE-inhibitory activity did indeed have bitter taste, allowing the acceptance of the first hypothesis in this study. It should be noted, however, that the intensity of the bitterness varied amongst the WPHs with high ACEinhibitory activity. For example, the WPH produced by Protin SD NY10 inhibited 69% of ACEinhibitory activity (0.3 mg/mL final assay concentration) and yet was designated a low bitterness rating (Table 2.3). Nonetheless, bitterness was detected in all WPHs regardless of ACE- inhibitory activity, emphasizing the importance of an effective debittering method for protein hydrolysates in general.

From these seven hydrolysates, the Thermoase PC10F-produced hydrolysate (T3) was selected for treatment with the carboxypeptidase Accelerzyme<sup>®</sup> CPG (T3-AC7), the aminopeptidase Peptidase R (T3-PR7) or the aminopeptidase and proteinase mixture ProteAX (T3-PX7). The antihypertensive activity and taste of these hydrolysates are briefly summarized in Table 4.1. Prior to exopeptidase treatment, T3 had the highest ACE-inhibitory activity and resulted in the greatest reduction of systolic blood pressure (SBP) in spontaneously hypertensive rats (SHRs), but was also the most bitter and had low overall acceptability. Although exopeptidase treatment decreased the ACE-inhibitory activity of T3, the exopeptidase-treated hydrolysates still exhibited high ACE-inhibitory activity (IC<sub>50</sub> = 0.24-0.34 mg/mL). All three exopeptidase-treated hydrolysates also reduced the SBP of SHRs, decreasing SBP by a maximum of 30-31 mm Hg after a single dose (100 mg/kg bw). In fact, the magnitude of SBP reduction resulting from exopeptidase-treated hydrolysates was similar to the positive control captopril (10 mg/kg bw). The large reduction in the SBP of SHRs by T3 and the exopeptidase-treated hydrolysates may be due to their ACE-inhibitory activity, other hypotensive mechanisms they may exhibit (e.g., opiate- and antioxidant-like activities), as well as their slow elimination (Tavares et al., 2012). These mechanisms may also partly explain the sustained (i.e., 24 h) reduction of SBP after a single administration. Synthetic ACE inhibitors have been shown to reduce the SBP of SHRs for 24 h, the effect of which was attributed to the retention of inhibitors in various tissues (e.g., in the heart and kidneys) leading to tissue-specific ACE inhibition even after the inhibitors were eliminated from the plasma (Fabris, Chen, Pupic, Perich, & Johnston, 1990).

In terms of taste, exopeptidase treatments decreased the bitterness of T3, possibly by releasing hydrophobic amino acids from the terminals of bitter peptides. The exopeptidase-treated hydrolysates also had higher overall acceptability than T3. This could be explained by both the reduction of bitterness, as bitterness was significantly correlated to overall acceptability (R = -0.9697, p = 0.0014), as well as the increase in other desirable taste attributes such as umami taste. An increase in free Glu content may explain the increase in umami taste, the effects of which may have influenced the perception of salty taste. It was hypothesized that WPH treated with either aminopeptidase or carboxypeptidase treatment would have reduced bitter taste and still have ACE-inhibitory activity. Since all three exopeptidase-treated hydrolysates exhibited ACE-inhibitory activity, and had lower bitterness than T3, both the second and third hypotheses were accepted.

The fourth hypothesis in this study was that peptides in a fraction between the 0.5–3.0 kDa size range would have the highest ACE-inhibitory activity after exopeptidase treatment. The upper limit was selected to be 3 kDa as this cut-off has been commonly and successfully used to concentrate peptides with ACE-inhibitory activity from WPHs in other studies (Fernández-Musoles et al., 2013; Tavares et al., 2011; Vermeirssen et al., 2005). For the lower range, QSAR modelling had predicted high correlation between terminal amino acids and ACE-inhibitory activity in di- and tri-peptides (Wu et al., 2006a). Therefore, exopeptidase treatment and consequent removal of terminal amino acids was hypothesized to eliminate the ACE-inhibitory activity of many di- and tri-peptides. Since di- and tri-peptides typically have molecular weights of 200–500 Da, and free amino acids have not been found to exhibit ACE-inhibitory activity, the fraction < 500 Da was hypothesized to have minimal ACE-inhibitory activity.

Size exclusion chromatography revealed that the 200–1000 Da fraction had the highest ACEinhibitory activity (Fig. 2.2). While the upper limit of 1000 Da fell within the range of 0.5–3 kDa hypothesized, the lower limit of 200 Da fell below the hypothesized range, leading to the rejection of the fourth hypothesis. The high ACE-inhibitory activity of fractions containing peptides 200–1000 Da suggested that at least some of the di- and tri-peptides present after exopeptidase-treatment exhibited ACE-inhibitory activity. In addition, Fractions 4 and 5 of exopeptidase-treated hydrolysates collected after the 200–1000 Da fraction also presumably contained di- and tri-peptides and exhibited ACE-inhibitory activity, albeit with lower potency than the 200–1000 Da fractions. These results suggest that exopeptidase treatment did not eliminate the ACE-inhibitory activity of all di- and tri-peptides. Additional purification and peptide sequencing would be necessary to determine which peptides accounted for the ACEinhibitory activity observed in the <200 and 200–1000 Da fractions of exopeptidase-treated hydrolysates, and whether these peptides were the same as, or shorter versions of, the ACEinhibiting peptides in T3.

The presence of ACE-inhibiting di- and tri-peptides in aminopeptidase-treated hydrolysates may be explained as N-terminal amino acids having less influence on ACE-inhibitory activity than Cterminal amino acids in tri-peptides (Lin et al., 2008) and their removal are thus less detrimental to the bioactivity of the peptide. On the other hand, the removal of C-terminal amino acids from di- and tri-peptides may be expected to have more consequence on their ACE-inhibitory activity. However, not all amino acids associated with ACE-inhibitory activity at the C-terminal were removed by Accelerzyme<sup>®</sup> CPG. For example, Accelerzyme<sup>®</sup> CPG released very low amounts of Pro, which contributed to high ACE-inhibitory activity when situated at the C-terminal of dipeptides (Cheung et al., 1980).

The results of this study showed that exopeptidase-treatment was a viable debittering method for antihypertensive hydrolysates. In addition to having lower bitterness and higher overall acceptability than the original whey protein hydrolysate, the exopeptidase-treated hydrolysates also exhibited ACE-inhibitory activity *in vitro* and antihypertensive activity in SHRs. Therefore, the production of exopeptidase-treated hydrolysates with high activity and low bitterness appears to be possible. These findings have implications for the development of functional foods, for which additional research and improvements in exopeptidase treatments may balance hydrolysate bioactivity and taste, and finally allow for the health-enhancing effects of bioactive peptides to be utilized in the food system at a larger scale.

## Table 4.1 – Summary of ACE-inhibitory activity, systolic blood pressure reduction,

bitterness and overall acceptability of experimental and commercial whey protein

	IC <sub>50</sub> for ACE-	SBP reduction		
	inhibitory activity	at 2 h		Overall
Sample <sup>a</sup>	(mg/mL) <sup>b</sup>	(mm Hg) <sup>c,d</sup>	Bitterness <sup>c,e</sup>	acceptability <sup>c,f</sup>
T3	0.15	-35b	1.1a	3.8d
T3-AC7	0.24	-29a	-0.1c	6.7bc
T3-PR7	0.27	-27a	0.1c	6.4bc
T3-PX7	0.34	-30ab	0.5b	5.7cd
Hilmar <sup>TM</sup> 8350	NA	ND	-0.2c	8.3ab
WPH 4003	NA	ND	-1.4d	9.7a

hydrolysates.

<sup>a</sup> A whey protein hydrolysate produced after hydrolysis with Thermoase PC10F for 3 h (T3) was treated for 7 h with Accelerzyme<sup>®</sup> CPG (T3-AC7), Peptidase R (T3-PR7) or ProteAX (T3-PX7). Two commercial WPH were included for comparison, namely Hilmar<sup>TM</sup> 8350 and WPH 4003. <sup>b</sup> NA = activity too low to be determined; ND = not determined

<sup>c</sup> Means in the same column not sharing common lowercase letters are significantly different ( $p \le 0.05$ ).

<sup>d</sup> Change in systolic blood pressure (SBP) in spontaneously hypertensive rats (mean values of 4 rats per treatment) 2 h after a single dose of hydrolysate (100 mg/kg body weight); ND = not determined.

<sup>e</sup> Mean of Z-scores (n = 22). Duplicate samples were assessed by 11 panelists as 10% solutions. <sup>f</sup> Mean of sensory scores (n = 24). Duplicate samples were assessed by 12 panelists as 10% solutions.

### 4.2 Future areas of research

The objective of the current study was to assess whether a whey protein hydrolysate treated with various exopeptidase preparations would still exhibit ACE-inhibitory activity and decrease in bitterness. However, neither the ACE-inhibitory activity nor decrease in bitterness of exopeptidase-treated hydrolysates was optimized. Further research employing different hydrolysis conditions to optimize these attributes would be required to produce hydrolysates with optimal ACE-inhibitory activity and debittered taste. A common method for optimizing particular attributes is the use of response surface methodology (RSM), which assesses various production factors (e.g., hydrolysis time, incubation temperature, and enzyme concentration) at several levels to determine which combination of levels would yield hydrolysates with the optimal values of the desired attribute. This method has been used for optimizing ACE-inhibitory activity in WPHs (Contreras et al., 2011; Guo et al., 2009; Tavares et al., 2011) but less often for taste. A recent study, however, employed RSM to determine the optimal conditions for producing Alcalase-hydrolyzed soybean with the highest antioxidant activity and lowest bitterness (debittered by the addition of  $\beta$ -cyclodextrins) (Hou, Wang, & Zhang, 2013), suggesting that RSM is a viable method to assess optimal conditions for exopeptidase treatments.

The use of RSM to optimize hydrolysis conditions for the best sensory characteristic (i.e., lowest bitterness) would require the sensory evaluation of a large number of samples and may not always be feasible since sensory panels require many resources to implement (Meilgaard et al., 2007). Therefore, alternative methods of bitterness quantification may be required to pre-screen samples. Electronic taste-sensing equipment has been used in recent years to assess the taste of various compounds in food and pharmaceutical science. Recent studies have found strong 119

correlations between sensor values and sensory scores of food protein hydrolysates for some taste attributes, suggesting that taste-sensing equipment is a potential method for pre-screening hydrolysate bitterness *in vitro* (Cheung & Li-Chan, 2014; Goudarzi & Madadlou, 2013). However, this method is largely dependent on the materials comprising the sensor membrane, which are currently not representative of human taste buds (Woertz et al., 2011).

An emerging method of *in vitro* bitter assessment is the use of cell-based (i.e., TAS2Rsexpressing cells) assays. First used to identify receptor-ligand specificity, TAS2R-expressing cell assays have since been suggested for use in quantifying bitterness (Misaka, 2013; Narukawa et al., 2011). These assays quantify receptor response by measuring the influx of extracellular calcium after TAS2Rs are activated by their respective ligands (Misaka, 2013). As several hTAS2Rs have been shown to respond specifically to bitter peptides, notably hTAS2R1 (Maehashi et al., 2008; Upadhyaya et al., 2010), cells expressing hTAS2Rs may be used to quantify peptide bitterness. Maehashi et al. (2008) showed that hTAS2R1, hTAS2R4, hTAS2R14, and hTAS2R16 all responded to a bitter peptide fraction from a trypsin-produced casein hydrolysate. Furthermore, the authors found that the bitter peptides Gly-Leu and Gly-Phe activated hTAS2R1 strongly while the tasteless peptide Gly-Gly did not activate any hTAS2Rs (Maehashi et al., 2008). In addition, hTAS2R4 responded strongly to the bitter casein hydrolysate fraction but not as strongly to Gly-Leu and Gly-Phe, suggesting that other bitter peptides present in the hydrolysate fraction activated hTAS2R4 (Maehashi et al., 2008). These results suggested that despite the presence of both bitter and non-bitter peptides in hydrolysate fractions, the receptors responded specifically to bitter peptides. Although relatively new, this method is already being improved. For example, Toda, Okada, and Misaka (2011) increased the

specificity of this method by replacing the commonly-used calcium-sensitive fluorescent dyes, which react with all fluorescent compounds including polyphenols, with luminescing photoproteins for response detection. Therefore, the use of cell-based assays specific to bitter peptides is another viable method for *in vitro* pre-screening of hydrolysate bitterness prior to final taste assessment by a sensory panel.

In addition to having low bitterness and high ACE-inhibitory activity, exopeptidase-treated hydrolysates need to demonstrate antihypertensive activity in vivo at doses appropriate for food use in order to be applicable in functional foods. Spontaneously hypertensive rats have similar vascular responses as humans and are often used to test the *in vivo* antihypertensive activity of peptides and hydrolysates, with studies showing that hydrolysates displaying blood pressurereducing activity in SHRs also depressed blood pressure in humans (Fujita et al., 2001; Iwaniak, Minkiewicz, & Darewicz, 2014; Li et al., 2011). However, controlled trials using human subjects could further establish the health-enhancing effects of bioactive hydrolysates and may facilitate their usage as food ingredients. Currently, the antihypertensive effect of exopeptidase-treated WPHs in humans is unknown. In fact, there are very few studies assessing the antihypertensive activity of WPHs in humans, which have drawn varying conclusions on the effectiveness of WPHs at reducing blood pressure (Fekete, Givens, & Lovegrove, 2013; Fluegel et al., 2010; Goudarzi & Madadlou, 2013; Pins & Keenan, 2006). As shown in Table 4.2, however, these differences may also be partly attributed to the differences in the experimental designs and conditions used. In these studies, the treatments usually consist of 20-28 g of a WPH dissolved in flavoured beverages. In comparison, SLIMFAST<sup>®</sup> protein meal shakes by Unilever contains 19.44 g protein in a 295 mL bottle (USDA, n.d.). Therefore, the concentration of WPHs shown

in experimental studies to have antihypertensive activity in humans are slightly higher than concentrations typically found in commercial protein shakes, but below the recommended dietary allowance of proteins for adults (46–56 g protein/day) (Health Canada, 2010).

In conclusion, currently available research suggests that WPHs have potential for reducing blood pressure in humans, although the antihypertensive effect in humans of WPHs debittered by exopeptidase treatment still needs to be determined. Further research is also required to optimize the antihypertensive activity and taste of exopeptidase-treated WPHs before they can be used as functional ingredients. Once WPHs with high antihypertensive activity are successfully debittered, these ingredients can be applied to a wide range of functional food products, such as whey-based beverages, and provide a natural supplement for prehypertensive and hypertensive individuals.

# Table 4.2 – Comparison of experimental designs in studies assessing the antihypertensive activity of whey protein hydrolysates

## in human subjects

Whey protein bydrolysate (WPH) used	Study design	Study population <sup>a</sup>	Effect (mm Hg)	Significance	Reference
Flavourzyme-produced WPH	24 g/d WPH or unmodified whey protein isolate (control) in water; 200 mL before breakfast and 200 mL before lunch every day for 1 week	Males ( <i>n</i> = 10) 26.4 ± 3.4 years SBP: 130 ± 5.2 DBP: 84.6 ± 3.9	Treatment group:   SBP: $-5.4 \pm 2.1$ DBP: $-2.5 \pm 1.5$ Control group:   SBP: $-1.8 \pm 1.0$ DBP: $-1.4 \pm 1.2$	No significant differences in SBP or DBP between treatment and control groups	Goudarzi & Madadlou, 2013
Validase <sup>®</sup> BNP L- produced WPH	28 g/day WPH or unmodified whey protein concentrate (WPC) (control) in 295 mL fruit flavoured whey drink; once/day for 6 weeks	Adults ( $n = 71$ ) <b>Treatment group:</b> 20.4 ± 1.7 years SBP: 123.8 ± 9.4 DBP: 74.4 ± 7.8 <b>Control group:</b> 20.7 ± 1.9 years SBP: 122.4 ± 10.0 DBP: 75.4 ± 6.5	BP after 6 weeks: <b>Treatment group:</b> SBP: $121.4 \pm 9.0$ DBP: $73.6 \pm 6.5$ <b>Control group:</b> SBP: $123.7 \pm 9.4$ DBP: $74.4 \pm 7.8$	Significant SBP and DBP reduction in hypertensive and prehypertensive individuals (WPC and WPH data combined)	Fluegel et al., 2010
Trypsin-produced WPH, produced by Davisco Foods International, Inc. (Eden Praire, MN)	1 packet/day containing 20 g WPH or unmodified whey protein (control), 1 g cocoa powder, and 0.5 g aspartame for 6 weeks	Adults ( $n = 30$ ) 45.6 ± 13.1 years <b>Treatment group:</b> SBP: 137 ± 2.8 DBP: 84 ± 1.8 <b>Control group:</b> SBP: 135 ± 2.3 DBP: 82 ± 1.5	Treatment group: SBP: $126 \pm 1.6$ DBP: $77 \pm 1.4$ Control group: SBP: $133 \pm 1.9$ DBP: $80 \pm 1.5$	Significantly lower SBP and DBP in treatment group	Pins & Keenan, 2006

<sup>a</sup> SBP = systolic blood pressure; DBP = diastolic blood pressure

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# Appendices

Appendix A Reproducibility of T3 and the exopeptidase-treated hydrolysates

Table A.1 – Comparison of extent of hydrolysis and ACE-inhibitory activity between two

batches of T3 and the exopeptidase-treated hydrolysates.	

	Extent of hydrolysi	$s (EH)^{b} (meq Leu/g)$	ACE-inhibitor	ry activity <sup>c</sup> (%)
Sample <sup>a</sup>	Batch 1	Batch 2	Batch 1	Batch 2
T3	$1.12\pm0.16$	$1.07\pm0.02$	$73 \pm 2$	$76 \pm 1$
T3-AC7	$2.46\pm0.02$	$2.02\pm0.04$	$70 \pm 1$	$67 \pm 2$
T3-PR7	$3.92 \pm 0.11$	$3.26\pm0.00$	$64 \pm 1$	$60 \pm 3$
T3-PX7	$4.55\pm0.08$	$4.04\pm0.01$	$42 \pm 2$	$48 \pm 2$

<sup>a</sup> A whey protein hydrolysate produced after hydrolysis with Thermoase PC10F for 3 h (T3) was treated for 7 h with Accelerzyme<sup>®</sup> CPG (T3-AC7), Peptidase R (T3-PR7) or ProteAX (T3-PX7). <sup>b</sup> Expressed as  $\alpha$ -amino groups in milliequivalents of L-Leu per gram of hydrolysate (meq Leu/g).

<sup>c</sup> At final assay concentration of 0.3 mg/mL.

Appendix B Sample differences as determined using Fisher's LSD test

### **B.1** Screening of whey protein hydrolysates for ACE-inhibitory activity and bitterness

Table B.1 – Extent of hydrolysis, ACE-inhibitory activity and taste of whey protein isolate and seven whey protein hydrolysates.

	Extent of	ACE	Taste attributes	and sensations <sup>c</sup>
Protease used	hydrolysis (meq Leu/g) <sup>a</sup>	inhibitory activity (%) <sup>b</sup>	Bitterness	Other
Thermoase PC10F	1.57c	75a	++++	Initially sour
Protin SD NY10	0.54e	69b	+	Film-like coating
Protease P "Amano" 6	3.74a	67b	+	Cardboard taste, sour/sweet
Validase <sup>®</sup> Papain Liquid	0.88d	67b	+++	"Odd", sour and cardboard taste
Protease M "Amano" SD	2.18b	59c	++	Sour/sweet
Protin SD AY10	0.84d	54d	+	Sour, slight cardboard taste
Maxazyme <sup>®</sup> NNP DS	0.35f	27e	++	Mild taste
None (WPI)	0.03g	-88f	-	Metallic, flat

<sup>a</sup> Expressed as  $\alpha$ -amino groups in milliequivalents of L-Leu per gram of protein (meq Leu/g). Samples not sharing common lowercase letters are significantly different ( $p \le 0.05$ ) using Fisher's least significant difference test.

<sup>b</sup> At final assay concentration of 0.3 mg/mL. Samples not sharing common lowercase letters are significantly different ( $p \le 0.05$ ) using Fisher's least significant difference test

<sup>c</sup> Samples (10% w/v) were tasted by 3 researchers in preliminary studies. Intensities of bitterness were coded as follows: no bitterness (-), detectable bitterness (+), mildly bitter (++), moderately bitter (+++), and highly bitter (+++).

### **B.2** Effect of exopeptidase treatment on ACE-inhibitory activity and bitterness

### Table B.2 – Extent of hydrolysis, ACE-inhibitory activity, and taste of a whey protein

	Extent of	ACE-	Taste attribu	ites and sensations <sup>e</sup>
	hydrolysis	inhibitory		
Sample <sup>a</sup>	$(\text{meq Leu/g})^{b,c}$	activity (%) <sup>c,d</sup>	Bitterness	Other
T3	1.12g	76a	++++	Mild taste, milky, cardboardy
Carboxypeptidase-tr	reated			
T3-AC1	1.99f	69bc	+++	Mild
T3-AC7	2.46d	67bc	+	Mild, good flavour, complex
Aminopeptidase-trea	ited			
T3-PR1	2.25e	65c	+++	Odd, cardboardy
T3-PR7	3.92b	60d	++	Salty, umami, acceptable
T3-PX1	2.66c	70b	+++	Milky, cardboardy
T3-PX7	4.55a	48e	++	Sour, good, not good
Commercial product	S			
WPH 4003	0.01j	-42h	-	Very mild, sweet, milky
Hilmar <sup>TM</sup> 8350	0.71h	16g	++++	Cracker-like, milky
Hilmar <sup>TM</sup> 8390	2.38d	57d	+++++	Like rotten cheese
NZMP <sup>TM</sup> WPH 917	0.33i	21f	+	Thick, milky

hydrolysate (T3)	, exopeptidase-treated	T3, and four	commercial	products.
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<sup>a</sup> A whey protein hydrolysate produced after hydrolysis with Thermoase PC10F for 3 h (T3) was treated with Accelerzyme<sup>®</sup> CPG (T3-AC), Peptidase R (T3-PR) or ProteAX (T3-PX7). Sample codes were followed by 1 or 7 to represent the exopeptidase treatment of 1 or 7 hours, respectively.

<sup>b</sup> Expressed as  $\alpha$ -amino groups in milliequivalents of L-Leu (meq Leu)/g hydrolysate.

<sup>c</sup> Samples not sharing common lowercase letters are significantly different ( $p \le 0.05$ ) using Fisher's least significant difference test.

<sup>d</sup> At final assay concentration of 0.3 mg/mL.

<sup>e</sup> Samples (10% w/v) were tasted by 3 researchers. Intensities of bitterness were coded as follows: no detectable bitterness (-), detectable bitterness (+), mildly bitter (++), moderately bitter (++++), highly bitter (++++), and extremely bitter (++++).



Appendix C Two-factor ANOVA with repeated measures on one factor on SBP



The change in systolic blood pressure in spontaneously hypertensive rats at each time interval was the mean of 20 rats in 5 treatment groups (4 rats per treatment group). The horizontal line represents the mean change in systolic blood pressure of 20 rats at 5 time intervals (n = 100).



Fig. C.2– Plot depicting the mean change in systolic blood pressure in spontaneously hypertensive rats of four experimental whey protein hydrolysates and captopril over 24 h, as graphed using Minitab 16.

# Appendix D Sensory Evaluation

# D.1 Sensory scorecard

PANELIST NUMBER: <u>1</u>							
	s	ENSORY EVALUA	TION OF WHEY P	ROTEIN HYDRO	OLYSATE PRODUC	TS	
Please rate the ine with the o	e taste attr orrespondi	ibutes for each sa ng sample code.	ample by placing a	a vertical line on	each of the scale	s below and label	each
NSTRUCTION: before swallow	S: Place 0.3 ving or disc	anl of each samp carding. Between	le solution in its er samples, rinse pa	ntirety onto the late with club so	back of your tong da and water. Uns	ue and wait 5 secor alted crackers may	nds / be
consumed if fla asting sample	avours pers es. Referen	ce standards for e	ach taste attribut	es are provided a	lease wait at leas and can be referre	t two minutes betw d to as needed.	veen
Please evaluat	e samples	in the following o	order:		1		
	182	505	783	354	476	648	
	_		BITTE	RNESS		_	
_	Low				R	High	
	I				I	Ι	
	_		UMAM	TASTE	_		
_					<u>к</u>		
	I		SWEE	TNESS	I	Ι	
	Low		30011	111233	R	High	
-							
	I		SΔIT	NESS	I	I	
	Low		GALI	R	1	High	
-	<u> </u>						
	,		SOUR	NESS		I	
	Low		F	ł		High	
_	<u> </u>					_ <b>_</b>	

Fig. D.1a – Example scorecard used for sensory evaluation (page 1).



Fig. D.1b – Example scorecard used for sensory evaluation (page 2).

### **D.2** Panelist screening





Whey protein hydrolysates (WPHs) were produced by Thermoase PC10F after a 3 h incubation (T3) and treated with Accelerzyme<sup>®</sup> CPG (AC), Peptidase R (PR), or ProteAX (PX) for 7 h. These are coded T3-AC7, T3-PR7, and T3-PX7, respectively. Two commercial WPHs were included for comparison, namely Hilmar<sup>TM</sup> 8350 and WPH 4003. Samples were assessed in duplicate. The horizontal line represents the mean umami taste (n = 144) of all six WPHs.



Fig. D.3 – Plot depicting the panelist × sample interactions of 12 panelists and four experimental and two commercial whey protein hydrolysates for umami taste based on sensory scores and graphed using Minitab 16.

Whey protein hydrolysates (WPHs) were produced by Thermoase PC10F after a 3 h incubation (T3) and treated with Accelerzyme<sup>®</sup> CPG (AC), Peptidase R (PR), or ProteAX (PX) for 7 h. These are coded T3-AC7, T3-PR7, and T3-PX7, respectively. Two commercial WPHs were included for comparison, namely Hilmar<sup>TM</sup> 8350 and WPH 4003. Samples were assessed in duplicate.



Fig. D.4 – Plot depicting the mean sweetness of four experimental and two commercial whey protein hydrolysates based on the sensory scores of 12 panelists and graphed using Minitab 16.

Whey protein hydrolysates (WPHs) were produced by Thermoase PC10F after a 3 h incubation (T3) and treated with Accelerzyme<sup>®</sup> CPG (AC), Peptidase R (PR), or ProteAX (PX) for 7 h. These are coded T3-AC7, T3-PR7, and T3-PX7, respectively. Two commercial WPHs were included for comparison, namely Hilmar<sup>TM</sup> 8350 and WPH 4003. Samples were assessed in duplicate. The horizontal line represents the mean sweetness (n = 144) of all six WPHs.



Fig. D.5 – Plot depicting the panelist × sample interactions of 12 panelists and four experimental and two commercial whey protein hydrolysates for sweetness based on sensory scores and graphed using Minitab 16.

Whey protein hydrolysates (WPHs) were produced by Thermoase PC10F after a 3 h incubation (T3) and treated with Accelerzyme<sup>®</sup> CPG (AC), Peptidase R (PR), or ProteAX (PX) for 7 h. These are coded T3-AC7, T3-PR7, and T3-PX7, respectively. Two commercial WPHs were included for comparison, namely Hilmar<sup>TM</sup> 8350 and WPH 4003. Samples were assessed in duplicate.



Fig. D.6 – Plot depicting the mean saltiness of four experimental and two commercial whey protein hydrolysates based on the sensory scores of 12 panelists and graphed using Minitab 16.

Whey protein hydrolysates (WPHs) were produced by Thermoase PC10F after a 3 h incubation (T3) and treated with Accelerzyme<sup>®</sup> CPG (AC), Peptidase R (PR), or ProteAX (PX) for 7 h. These are coded T3-AC7, T3-PR7, and T3-PX7, respectively. Two commercial WPHs were included for comparison, namely Hilmar<sup>TM</sup> 8350 and WPH 4003. Samples were assessed in duplicate. The horizontal line represents the mean saltiness (n = 144) of all six WPHs.



Fig. D.7 – Plot depicting the panelist × sample interactions of 12 panelists and four experimental and two commercial whey protein hydrolysates for saltiness based on sensory scores and graphed using Minitab 16.

Whey protein hydrolysates (WPHs) were produced by Thermoase PC10F after a 3 h incubation (T3) and treated with Accelerzyme<sup>®</sup> CPG (AC), Peptidase R (PR), or ProteAX (PX) for 7 h. These are coded T3-AC7, T3-PR7, and T3-PX7, respectively. Two commercial WPHs were included for comparison, namely Hilmar<sup>TM</sup> 8350 and WPH 4003. Samples were assessed in duplicate.


Fig. D.8 – Plot depicting the mean sourness of two replications (Rep) of four experimental and two commercial whey protein hydrolysates based on the sensory scores of 12 panelists and graphed using Minitab 16.

The horizontal line represents the mean sourness (n = 144) of all six whey protein hydrolysates.



Figure D.9 – Plot depicting the panelist × replication (Rep) interactions of 12 panelists and two replicate assessments of four experimental and two commercial whey protein hydrolysates for sourness based on sensory scores and graphed using Minitab 16.

	<i>p</i> -values <sup>a</sup>								
						Overall			
Panelist	Bitterness	Umami	Sweetness	Saltiness	Sourness	acceptability			
1	0.676	0.683	0.667	0.201	0.660	0.289			
2	0.356	0.528	0.246	0.748	0.562	0.226			
3	0.942	0.216	0.263	0.887	0.069	0.520			
4	0.688	0.371	0.289	0.126	0.764	0.697			
6	0.031	0.882	0.122	0.418	0.138	0.458			
7	0.870	0.534	0.621	0.170	0.377	0.949			
8	0.733	0.421	0.486	0.383	0.316	0.059			
9	0.505	0.277	0.449	0.009	0.135	0.951			
10	0.561	0.856	0.182	0.249	0.798	0.352			
11	0.523	0.005	0.620	0.019	0.668	0.399			
12	0.426	0.507	0.793	0.075	0.104	0.289			
13	0.846	0.339	0.435	0.030	0.384	0.854			

 Table D.1 – Summary of *p*-values for panelist repeatability based on sensory scores.

<sup>a</sup> Significant replication effects ( $p \le 0.05$ ) are shown in bold font.

	_	Taste attribute <sup>a</sup>							
Panelist		Bitterness	Umami	Sweetness	Saltiness	Sourness			
1	R	0.8992	0.9918	0.0403	0.9648	0.3176			
	р	0.0147	0.0001	0.9396	0.0018	0.5396			
2	R	0.8911	0.9921	0.1158	0.9083	0.3275			
	р	0.0171	0.0001	0.8271	0.0122	0.5263			
3	R	0.9011	0.1945	0.2146	0.8726	-0.0072			
	р	0.0142	0.7120	0.6831	0.0233	0.9892			
4	R	0.8864	0.9546	0.9161	0.9520	-0.0269			
	р	0.0186	0.0030	0.0103	0.0166	0.9596			
6	R	-	0.7338	0.1858	0.3998	0.5127			
	р	-	0.0968	0.7245	0.4322	0.2984			
7	R	0.8148	0.7781	0.6420	0.5692	0.3328			
	р	0.0483	0.0684	0.1693	0.2384	0.5192			
8	R	0.7389	0.8677	0.4308	0.9028	0.5300			
	р	0.0934	0.0251	0.3938	0.0137	0.2795			
9	R	0.6885	0.7799	0.2497	-	0.5233			
	р	0.1304	0.0673	0.6332	-	0.2867			
10	R	0.5959	0.9353	0.0185	0.9136	0.8894			
	р	0.2119	0.0061	0.9722	0.0109	0.0177			
11	R	0.8428	_	0.1700	-	0.3730			
	р	0.0351	-	0.7474	-	0.4665			
12	R	0.8640	0.8424	0.6763	0.9542	0.5771			
	р	0.0265	0.0353	0.1402	0.0031	0.2304			
13	R	0.9310	0.8982	0.8020	-	-0.0600			
	р	0.0070	0.0150	0.0549	-	0.9102			

Table D.2 – Summary of Pearson correlation coefficients (R) and p-values of individual panelists with the remaining panelists for all attributes.

<sup>a</sup> Non-significant correlations ( $p \ge 0.05$ ) are shown in bold font and represent panelists whose scores did not correlate with the other panelists in the panel.