# ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY PEPTIDES FROM THE HYDROLYSIS OF PACIFIC HAKE FILLETS BY COMMERCIAL PROTEASE

by

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# Abstract

In addition to modifications of diet and lifestyle, angiotensin I-converting enzyme (ACE) inhibitory prescription drugs have been conventionally used to control high blood pressure. Recently, protein hydrolysates, mainly those from milk and fish sources, have been shown to exhibit ACE-inhibitory activity, the most potent of which are now being marketed as nutraceutical alternatives for treatment of hypertension. In this study, Pacific hake (*Merluccius productus*) fillet was investigated as a source of ACE-inhibitory peptides. Specifically, the objectives were to assess the effects of (1) hydrolysis conditions, (2) raw material variability, (3) ultrafiltration separation, (4) simulated gastrointestinal digestion, and (5) peptide-ACE pre-incubation on hydrolysate ACE-inhibitory activity *in vitro* to establish if further study of antihypertensive efficacy *in vivo* is warranted. Using Protamex® commercial protease according to a Response Surface Methodology (RSM) Central Composite Design, hydrolysis time and enzyme-to-substrate ratio factors were found to be the most significant predictors of ACE-inhibitory activity ($P<0.001$), with 125 minutes and 3.0%, respectively, producing hydrolysate with an $IC_{50}$ of 165 ± 9 μg peptides/mL; higher time-enzyme combinations showed no significant improvement in ACE-inhibitory activity ($P>0.05$). ACE-inhibitory activity of hydrolysates produced from fish of different catch months and *Kudoa* infections did not differ significantly ($P>0.05$). Ultrafiltration of hydrolysate to <10 kDa resulted in an $IC_{50}$ value of 44 ± 7 μg peptides/mL, making it over 2.5 times more potent than the commercial product PeptACE® ($IC_{50}$ 114 ± 8 μg peptides/mL). However, after simulated gastrointestinal digestion, unfractionated hydrolysate and the <10 kDa fraction had similar activity at $IC_{50}$ 90 ± 9 μg peptides/mL and 89 ± 4 μg peptides/mL, repectively, and PeptACE® was approximately 30% more active at $IC_{50}$ 64 ± 6 μg peptides/mL. Therefore, as a 'pro-drug type' inhibitor released by gastrointestinal digestion, further enrichment processing of unfractionated Pacific hake hydrolysate is unnecessary. Further, the ACE-inhibitory activity of digested unfractionated and <10 kDa hydrolysates were not significantly affected by pre-incubation with ACE ($P>0.05$) and the latter was found to exhibit a competitive inhibitory mode. Together, these results suggest Pacific hake fillet hydrolysates are a commercially viable source of ACE-inhibitory peptides that necessitate further *in vivo* study.

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| | |
|---|---|
| AA | amino acid |
| ACE | angiotensin-converting enzyme |
| EH | extent of hydrolysis as mequivalents free α-amino groups / L |
| DH | Degree of Hydrolysis |
| %E/S | enzyme mass to protein substrate mass ratio expressed as percent |
| FAPGG | 2-furanacryloyl-1-phenylalanylglycylglycine |
| FOSHU | Food for Special Health Use (Japan) |
| GI | gastrointestinal |
| HA | hippuric acid |
| HHL | hippuryl-L-histidyl-L-leucine |
| $IC_{50}$ | Inhibitory Concentration 50 (concentration of inhibitory agent required to inhibit ACE by 50%) |
| $K_{app}$ | substrate concentration required to achieve ½ maximum reaction velocity in the presence of inhibitor |
| $K_i$ | dissociation constant for competitive inhibitor binding |
| $K_m$ | substrate concentration required to achieve ½ maximum reaction velocity |
| MALDI-ToF | Matrix-Assisted Laser Desorption/Ionization-Time of Flight |
| MW | molecular weight |
| PHF | Pacific hake fillet |
| RAS | renin-angiotensin system |
| RSM | Response Surface Methodology |
| [S] | substrate concentration |
| SBP | systolic blood pressure |
| SHR | spontaneously hypertensive rat |
| TNBS | trinitrobenzenesulfonic acid |
| UF | ultrafiltration separation |
| $V_{max}$ | maximum velocity |
| $V_o$ | initial velocity |

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For Dr. Susan Arntfield,

whose belief in me I will always treasure

as a gift of inspiration

# CHAPTER I Introduction

## 1.1 Study Context

Cardiovascular disease (CVD) claims the lives of over 16 million people worldwide every year. It is the leading cause of death in people over 60 years of age and is second only to HIV/AIDS in people aged 18-59 years (Mackay and Mensah 2006). Considered a 'slow killer', the global burden of this disease is expected to rise from the 47 million 'healthy life years lost' in 1990 to 82 million in 2020 (Mackay and Mensah 2006).

High blood pressure is one of the leading risk factors for the onset of CVD in developed countries. Medications, including angiotensin I-converting enzyme (ACE) inhibitors, are conventionally used for its control. High blood pressure can however be remediated by modifications in diet and lifestyle such as maintaining a healthy weight (body mass index of 18.5-24.9 $kg/m^2$, Groff and Gropper 2000), reducing sodium intake, eliminating tobacco use, and participating in regular physical activity (Heart and Stroke Foundation 2006). In addition, a FOSHU-approved nutraceutical, ACE-inhibitory peptides from dried bonito fish, has now been introduced in Japan as a new alternative for lowering potential and existing mild and borderline hypertension (Fujita and others 1995; Fujita and Yoshikawa 1999; Fujita and others 2001; Nippon Supplement 2006).

A variety of other fish protein hydrolysates, including those from tuna (Kohama and others 1988), sardine (Matsui and others 1993), and pollack (Je and others 2004) have been shown to exhibit ACE-inhibitory activity *in vitro*, while those from fermented fish sauce (Ichimura and others 2003), salmon (Ono and others 2003), and sole (Jung and others 2006), have exhibited antihypertensive efficacy in the spontenously hypertensive rat (SHR) model, suggesting that this potential is not necessarily species specific. Pacific hake, an abundant species off the west coast of Canada and United States undervalued for its poor fillet texture, should therefore be investigated as another possible source of ACE-inhibitory hydrolysates. Using ACE-inhibitory hydrolysates from Pacific hake as an ingredient in functional foods would not only be beneficial in lowering and preventing hypertension, but would also generate a value-added niche market for this otherwise under-utilized natural resource.

## 1.2 Hydrolysis by Commercial Proteases

### 1.2.1 Hydrolysis for value-added peptide functionality

Protein hydrolysis, the cleavage of peptide bonds to degrade protein molecules into smaller peptides, is a process catalyzed by acid, base, or enzymes that can be applied to improve the functional properties of food proteins (Adler-Nissen 1986). Casein hydrolysates, selectively processed to remove aromatic amino acids, have long been used in formulas for infants with phenylketonuria or tyrosinosis (Mahmoud and Cordle 2000). Protein hydrolysates are also used as a more bioavailable nitrogen source for individuals with impaired gastrointestinal function (Mahmoud and Cordle 2000). Besides nutritional applications, hydrolysis can be used to convert intact proteins into value-added ingredients. For example, fish protein hydrolysates with improved solubility, water holding, emulsifying, and foaming properties have been generated (Kristinsson and Rasco 2000a, 2000b).

Yet most recently, bioactive functionality has become the focus of many protein hydrolysate studies. For instance, peptides with immunomodulatory and opioid activities have been derived from rice and soybean, and wheat and milk sources, respectively (Meisel 2001; Kitts and Weiler 2003). As well, ACE-inhibitory peptides have been produced from the hydrolysis of milk, chicken, bovine, wheat, corn, and fish proteins (Yamamoto 1997; Li and others 2004; Meisel and others 2006). In the case of bovine skin gelatin, bovine blood plasma, and fish frame protein, traditionally food processing by-products with little value, the production of bioactive hydrolysates may expand their use (Kim and others 2001; Wanasundara and others 2002; Je and others 2004, Jung and others 2006)

Pacific hake (*Merluccius productus*) is an ideal protein source for investigation of value-added uses of hydrolysates. Despite its abundance off the west coast of Canada and United States, the value of Pacific hake has historically been limited to its use in surimi processing and plagued by the overly soft and mushy texture of its cooked fillets (Morrissey and others 1995). Patashnik and others (1982) and Kudo and others (1987) proposed that proteolytic enzymes released by the spores of *Kudoa paniformis* and *Kudoa thyrsites*, prevalent myxosporean parasites found in the Pacific hake population, induce the myoliquifaction of the fillet. Myoliquifaction by endogenous proteases triggered by parasitic infection has also been

postulated (An and others 1994). Regardless of the cause, *Kudoa* infection of wild Pacific hake cannot be controlled. In order to increase the value of this commodity, non-traditional processing, such as the production of hydrolysates, should be investigated.

### 1.2.2 Enzymatic hydrolysis variables

For any given enzymatic reaction, rate of catalysis is dependent on process conditions. Temperature (T), pH, enzyme-to-substrate ratio (%E/S), and substrate concentration (S), are all factors which affect the final extent of hydrolysis achieved (Adler-Nissen 1986; Kristinsson and Rasco 2000b). Degree of hydrolysis (DH), as defined by Adler-Nissen (1986) as the percent ratio of the number of peptide bonds broken to the total number of peptide bonds in a given type of protein, can then be quantified by a variety of different techniques. These include monitoring the reaction of free primary amino groups with trinitrobenzenesulfonic acid (TNBS) or *o*-phthaldialdehyde (OPA) (Adler-Nissen 1979; Nielsen and others 2001), or monitoring the amount of base consumed to keep the hydrolysis reaction at a constant pH (Adler-Nissen 1986). Alternatively, Hoyle and Merritt (1994) have expressed DH as the percent ratio of 10% trichloroacetic acid soluble nitrogen after hydrolysis to the total nitrogen in the sample as determined by Kjeldahl analysis. While the temperature, pH, and %E/S ratio variables are dictated by the specification ranges of the protease to be used, a mince to water ratio of 1:2 (mass:volume) for the hydrolysis slurry is conventionally used for fish protein hydrolysis studies. At this level, the slurry is concentrated enough to allow protease access to the protein substrate, yet dilute enough to prevent early product inhibition of the protease (Diniz and Martin 1996; Benjakul and Morrissey 1997; Kristinsson and Rasco 2000c).

Protein source and protease type used to perform the hydrolysis are also factors influencing the final hydrolysate product. These factors influence hydrolysate functionality by dictating the shape, size, amino acid composition and sequence, net charge and distribution of charges, hydrophobicity-hydrophilicity ratio, and molecular rigidity of the peptides produced. Various kinds of protease preparations are available commercially, each having a unique set of product specifications and cleaving site preferences. Firstly, the broader the cleaving specificity of the enzyme(s), the more diverse the peptide sequences in the mixture become (Kristinsson and Rasco 2000b). Secondly, mode of proteolysis dictates peptide length distribution. In

theory, proteolytic reactions can either proceed as a 'one-by-one' reaction in which an enzyme acts on a particular protein until it is fully hydrolyzed to its end products, or conversely, can proceed as a zipper reaction in which native protein molecules are rapidly converted to an intermediary form (peptide) which is then more slowly converted to end products (Adler-Nissen 1986). In reality hydrolysis occurs as a combination of these activities, thereby resulting in a heterogeneous hydrolysate mixture. Thirdly, the use of exopeptidases in conjunction with endoproteases can improve the flavour profile of peptides. It has been observed that free amino acids are much less bitter than when in peptide form and that bitterness of peptides is highest when hydrophobic residues are non-terminal, therefore carboxypeptidases or aminopeptidases are useful in de-bittering hydrolysate mixtures (Adler-Nissen 1986). As an example of protease diversity, Table 1.1 outlines the process conditions, standardized activity levels and cleaving specificities of three popular commercial protease preparations.

### 1.2.3 Use of Response Surface Methodology in optimizing functionality

Response Surface Methodology (RSM) experimental designs have been successfully applied to simultaneously investigate the individual and interactive effects of hydrolysis process factors on the final composition and functionality of hydrolysate mixtures. RSM models are very useful as they can be used to predict hydrolysate functionality based on process conditions, and in turn be used to select the optimal conditions for the hydrolysate characteristics desired (Myers and Montgomery 2002; Minitab 2006). A 1996 study by Diniz and Martin used RSM to determine, for the hydrolysis of dogfish muscle by Alcalase 2.4L®, the optimal conditions for achieving the highest DH possible (i.e. most efficient proteolytic activity) at 2 hours of hydrolysis. At a pH of 8.3, a hydrolysis temperature of 53.6 °C, and an E/S of 3.6% w/w, DH was maximized. In a subsequent study, Diniz and Martin (1997) used these conditions to then monitor changes of hydrolysate functional properties like emulsification and foaming capacities as a function of DH over time. They found that the 6.50% DH sample had significantly improved values for both functional properties compared to those of the unhydrolysed control. However, in the case of the 13.0% DH and 18.8% DH samples, functionality was significantly decreased compared to the 6.50% DH sample. This suggests that peptide functionality can be

created and then subsequently degraded, further confirming that a given functional property can be maximized by an optimal set of hydrolysis conditions.

More recently, van der Ven and others (2002) conducted an RSM study to optimize the ACE-inhibitory activity of whey protein hydrolysates. As an improvement to the approach of the Diniz and Martin (1996, 1997) studies, ACE-inhibitory activity of the hydrolysates was directly monitored as a response of the RSM. Using Corolase PP, the hydrolysates with the highest ACE-inhibitory activity were produced at pH 8 and 45 °C. In terms of %E/S and hydrolysis time, no optimum ACE-inhibitory activity was found since '%E/S'$^2$ and 'time'$^2$ were not significant factors in the model of the experimental area. Rather, increases in %E/S and hydrolysis time within the experimental area only resulted in further increase in ACE-inhibitory activity. Instead of testing a new RSM with higher upper limits for %E/S and time, van der Ven and others (2002) conducted confirmation trials at the optimal pH and temperature conditions and the maximum %E/S condition (2.19%), then monitored the ACE-inhibitory activity over time. Prolonged hydrolysis time resulted in only a slight improvement in ACE-inhibitory activity, with subsequent decreases in ACE-inhibitory activity, implying again that functionality of the hydrolysate mixture is continuously in flux. Response surface methodology is therefore instrumental in establishing process conditions.

## 1.3 Angiotensin-I Converting Enzyme and its Inhibition

### 1.3.1 Overview of the renin-angiotensin system

Angiotensin-I converting enzyme (ACE, EC 3.4.15.1) is a dipeptide-liberating carboxypeptidase found on the cell surface of vascular endothelial cells of the circulatory system as well as organ specific sites in the heart, brain, kidneys, placenta, bone marrow, pancreas, and testis (Ehlers and Riordan 1989, Meisel and others 2006). It is a key component of the endocrine renin-angiotensin system (RAS), a control system for salt conservation, blood volume, and blood pressure preservation in the body (Eriksson and others 2002; Montani and van Vliet 2004). It is also thought to have functions in local tissue RAS systems with links to reproduction and immunity (Ehlers and Riordan 1989).

In the classical view of the endocrine RAS, as illustrated in Figure 1.2, a decrease in blood pressure or blood volume stimulates the release of the proteolytic enzyme renin (EC 3.4.23.15) from the kidney. While in circulation, renin cleaves angiotensinogen, a protein produced by the liver, to produce angiotensin I. Angiotensin-I, a decapeptide (DRVYIHPFHL), is then cleaved by angiotensin-I converting enzyme removing the C-terminal HL to yield angiotensin-II, an octapeptide (Meisel and others 2006). Angiotensin-II acts as a strong vasoconstrictor by binding to specific cell surface receptors that trigger the production of contractile proteins and activation of calcium channels, which contract vascular tissue and promote cell proliferation, respectively (Opie 1992; Matsui and others 2005). Angiotensin-II further regulates sodium levels by promoting sodium and water reabsorption by the kidney (Montani and van Vliet 2004). ACE also inactivates bradykinin, a vasodilator (Opie 1992). All of these activities result in increased blood pressure.

More recently, a homologue of ACE, ACE2, has been shown to function in generating previously unknown RAS peptides. This includes the hydrolysis of angiotensin-I to the inactive angiotensin$_{(1\text{-}9)}$ peptide which is then cleaved by ACE to the vasodilator metabolite angiotensin$_{(1\text{-}7)}$ (Eriksson and others 2002). In effect, angiotensin$_{(1\text{-}7)}$ is an antagonist of angiotensin-II. While this seems contrary to the role of ACE as a catalyst in the production of vasoconstrictor angiotensin-II, it also highlights the importance of these enzymes in maintaining homeostatic mechanisms. It is hypothesized that the emergence of genetic polymorphisms

responsible for the under-production of angiotensin$_{(1-7)}$ forming and/or angiotensin-II degrading enzymes causes dysregulation of this control mechanism which has, in turn, led to an evolution of a population with increased incidence of intrinsic hypertension (Ferrario and Chappell 2004).

While its activity is essential for blood flow and renal function, over-activity of ACE and of the RAS as a whole can result in conditions such as hypertension (a systolic blood pressure greater than 140 mm Hg and/or a diastolic pressure greater than 90 mm Hg) and cardiovascular hypertrophy (chronic stiffening of the heart and vessel tissue), and can promote vascular inflammation and atherosclerosis (Montani and van Vliet 2004). Amongst hypertension management drugs, ACE-inhibitors and angiotensin receptor antagonists have become the preferred choice for prescription (Ibrahim 2006). Compared to others, including diuretics, beta-blockers, and calcium antagonists, ACE-inhibitors have no advantage in reducing overall cardiovascular morbidity and mortality, yet provide better organ protection including regression of left ventricular hypertrophy and reduction of diabetes in hypertensive individuals (Ibrahim 2006). Diuretics, whose actions decrease blood pressure by increasing salt and water excretion by the kidney, cause excessive urination and excretion of potassium (Opie 1992). Beta-blockers and calcium antagonists act by intercepting adrenal simulation of the heart and blocking calcium channel-induced contraction of vessels, respectively, yet commonly cause fatigue and edema (swelling of the feet) (Waeber and Brunner 1995). On the other hand, besides rare cases of hypotension and impaired renal function, use of ACE inhibitors may cause a side-effect of persistent dry cough. This is thought to be associated with the altered activity of bradykinin (Opie 1992; Waeber and Brunner 1995).

### 1.3.2 Development of ACE inhibitors from food protein sources

ACE inhibitors, first developed in the 1970s, intercept the vasoconstrictive effects of the RAS, thereby allowing control of high blood pressure. Using a hypothetical model of the ACE active site, Ondetti and Cushman (1977) designed carboxyalkanoyl and mercaptoalkanoyl derivatives of proline that inhibited the constriction response of muscle tissue to angiotensin-I, interpreted as inhibition of ACE. Interestingly, the use of proline had been inspired by the C-terminal sequences AP or PP previously discovered as common to ACE-inhibitory peptides found in snake venom (Ondetti and others 1971). Captopril® (SQ 14,225, D-3-mercapto-2-

methylpropanoyl-L-proline, MW 217 g/mole), one of the most commonly used ACE inhibitors today, has a very low $IC_{50}$ value of 0.023 μg/L when used on ACE from rabbit lung, where $IC_{50}$ is the concentration of inhibitor required to reduce ACE activity by 50% (Cushman and others 1980; Rubin and Antonaccio 1980).

Researchers are currently investigating natural sources of ACE inhibitors, namely in the form of peptides derived from food protein, as a preventative rather than pharmaceutical approach to borderline hypertension (120-139/80-89 mm Hg, Heart and Stroke Foundation 2006). Based on the work by Fujita and Yoshikawa (1999) and Fujita and others (2001) in Japan, Nippon Supplement, Inc. has marketed the ACE-inhibitory oligopeptide LKPNM, derived from the hydrolysis of dried bonito, under the Food for Specific Health Use (FOSHU) status (Nippon Supplement, 2006). Yet this is only one of many fish-source peptides demonstrated as having antihypertensive potential. Table 1.2 provides examples of the varied sequences and potencies of ACE-inhibitory peptides isolated from fish hydrolysates. PeptACE®, a product of Natural Factors Inc. (Coquitlam, BC), which is claimed to contain nine ACE-inhibitory peptides also from fish muscle has now been introduced to the North American market; however, under this trademark name, its efficacy has not yet been reported in peer-reviewed literature. Of other food protein sources, ACE-inhibitory peptides from dairy and fermented dairy resources have been extensively researched and marketed. For example, AmealPeptide®, containing the tripeptides IPP and VPP, has been added to a yogurt drink under the Flora/Becel pro.activ® brand in the United Kingdom (Calpis 2006; Flora 2006). See Section 1.4.3 and Table 1.5 below for details of the clinical trials supporting these products.

The structure-activity relationship of ACE-inhibitory peptides has not yet been fully established, but some trends in their composition and sequence have been proposed. For example, many dipeptides and tripeptides with higher potency have an aromatic or proline residue at their C-terminus and a branched aliphatic residue at the N-terminus; similarly, active oligopeptides often have a sequence of 3 hydrophobic residues at the C-terminus (Li and others 2004; Meisel and others 2006). Peptides with positively-charged residues penultimate to the C-terminus have also been shown to exhibit ACE-inhibitory activity. As contrary as this seems, these results correspond with new insights into the structure of ACE: the existence of active sites on each of the N and C domains, each with distinct substrate specificities (Sturrock and others 2004). The interaction of the ACE-inhibitory drug Linopril®, a tripeptide analogue of

FKP, with the C-domain active site as documented by X-ray crystallography illustrates this specificity (Acharya and others 2003). The C-domain active site is covered by a lid-like structure which is highly dependent on chloride ion activation, but once open the phenyl ring interacts with the $S_1$ sub-site, the lysine with $S_1'$ and proline with $S_2'$, which keeps the inhibitor in the active site without cleavage. On the other hand, the N-domain site is 50 times more active towards the haemoregulatory peptde N-acetyl-SDKP meaning that the N-domain active site hydrolyses, rather than is inhibited by, peptides with a positively-charged residue at the penultimate C-terminal position (Acharya and others 2003). Captopril®, an N-thioalkyl derivative of the dipeptide AP with a hydrophobic residue in the penultimate C-terminal position is, however, an inhibitor of the N-domain. Difference in hydrophobicity and charge in the lid-like structure of the N-domain active site compared to the C-domain are attributed to the difference in substrate specificity and inihibitor binding (Acharya and others 2003). Just as Linopril® and Captopril® are more selective towards inhibiting the C and N domains, respectively, the wide variety of the three C-terminus residues amongst ACE-inhibitory peptides may reflect active site preference, with positively charged residue-containing peptides occupying that of the C-domain and more hydrophobic residue-containing peptides occupying the N-domain active site. Alternatively, peptides may be acting in regions other than the active sites by non-competitive inhibition.

Quantitative structure-activity relationship modeling has also provided a great deal of insight into trends of ACE-inhibitory peptide composition and sequence. Using a database containing the $IC_{50}$ values and sequences of 140 ACE-inhibitory tripeptides from the literature, Wu and others (2006a) constructed a model to predict $IC_{50}$ using partial least squares regression based on hydrophobicity, bulkiness, and charge scores of 20 amino acids. Confirming the trends already noted, the most favorable residues for the C-terminus and the N-terminus were determined as aromatic and hydrophobic residues, respectively. When positively charged residues occupied the middle position of these tripeptides, $IC_{50}$ was further reduced. In a similar modelling study by Pripp and others (2004), less bulky residues in the middle position of tripeptides were also noted as favorable. In terms of predictive power, the Wu model was successfully validated, as experimental $IC_{50}$ results obtained upon synthesis and assay of selected peptides matched the predicted $IC_{50}$ values. When used to identify highly favorable

sequences in parent proteins, computational modeling can be considered a tool for pre-screening food proteins as potential sources of ACE-inhibitory peptides.

### 1.3.3 Use of ultrafiltration in ACE-inhibitory hydrolysate development

In the course of ACE-inhibitory hydrolysate development, it has been argued that isolation of single peptides is not necessary for hydrolysates to deliver an antihypertensive effect (Fujita and others 2001; van der Ven 2002). Given the diversity of the peptides that have demonstrated ACE-inhibitory activity, one could presume that extensive chromatographic separation would actually result in the loss of part of the active peptides from the hydrolysate mixture (van der Ven 2002). The $IC_{50}$ value of a hydrolysate mixture is higher than its isolated peptides, but could still contain doses of the active peptides at levels high enough to produce a significant ACE-inhibitory effect. There are multiple benefits to this holistic approach. It is economically advantageous from a production point of view since multiple peptide fractionations would be very costly. Secondly, a milder antihypertensive effect could reduce the chances of developing the undesirable side-effects associated with the current ACE-inhibitory drugs. Thirdly, hydrolysate mixtures could be used as ingredients in functional foods at the gram or milligram level, making product formulation less sensitive than at the microgram or nanogram level.

The use of ultrafiltration (UF), however, has proven to significantly decrease the $IC_{50}$ value of hydrolysate mixtures from many difference sources (Jeon and others 1999; Hyun and Shin 2000; Pihlanto-Leppala and others 2000; Je and others 2004; Kim and others 2004; Vermeirssen and others 2005b). Table 1.3 provides a summary of the effects of UF on hydrolysate $IC_{50}$ values from these numerous studies. Overall, hydrolysates in the molecular weight range of less than 1 kDa have lower $IC_{50}$ values than those of exclusively higher molecular weight or those containing mixtures of low and high molecular weight peptides. This outcome is logical since many of the most active ACE-inhibitory peptides isolated so far contain 5 residues or less (Ariyoshi 1993; Li and others 2004; Meisel and others 2006). Addition of an UF unit to a production scheme would therefore be beneficial in increasing the ACE-inhibitory potency of the hydrolysate product, however, recovery yields are greatly reduced. In fact, Hyun and Shin (2000) found that upon filtration to a cut-off of 1 kDa, the

percent yield of hydrolysate was reduced to less than 9% of the original, unfractionated hydrolysate yield. Ultrafiltration processes coupled with bioreactors could possibly be employed to increase yield of small molecular weight peptides through continuous removal and further hydrolysis of reaction products, although solving issues of membrane fouling is still being researched (Korhonen and Pihlanto 2003)

### 1.3.4 *In vitro* assays for ACE-inhibitory activity

Numerous studies over the past two decades have used *in vitro* assays as a means of pre-screening the ACE-inhibitory potential of food protein hydrolysates and gastric digests as well as their individual peptide constituents (Kohama and others 1988; Yokoyama and others 1992; Matsui and others 1993; Yano and others 1996; Mullally and others 1997; Hyun and Shin 2000; Fujita and others 2000; Kim and others 2001; van der Ven and others 2002, Wanasundara and others 2002; Wu and Ding 2002; Vermeirssen and others 2003; Hernandez-Ledesma and others 2004a, 2004b; Megias and others 2004; Je and others 2004; Ruiz and others 2004; Lo and Li-Chan 2005; Hasan and others 2006; Miguel and others 2006; Ono and others 2006; Wu and others 2006b). This pre-screening is essential as *in vivo* studies are very costly and lengthy. However, there is currently no standard method or substrate for measuring ACE activity *in vitro*. This makes absolute comparison of hydrolysate $IC_{50}$ values between studies difficult due to differences in the $K_m$ of the synthetic substrate used. Even between studies employing the same substrate, the kinetics of the ACE reaction may be altered by different enzyme/substrate ratios, buffer conditions, and inhibitor concentrations. Nonetheless, $IC_{50}$ values, regardless of the assay employed, still give valuable information regarding the relative inhibitory activity of the sample compared to internal study standards such as ACE-inhibitory nutraceutical products (PeptACE®) or ACE-inhibitory drugs (Captopril®), as well as allow for comparison of the relative effects of fractionation processes.

The precedent-setting publication by Cushman and Cheung (1971), outlining a spectrophotometric assay and the properties of ACE from rabbit lung, still provides the foundation for most of the ACE-inhibitory activity assays used today. This method, in its classical form, quantifies ACE activity by monitoring the liberation of hippuric acid (HA) from the synthetic substrate hippuryl-histidyl-leucine (HHL) by its absorbance at 228 nm. As such,

ACE-inhibitory activity of an added sample can be determined by the reduction in absorbance compared to a control. However, since both HA and HHL absorb at 228 nm, a step in which HA is selectively extracted into ethyl acetate must also be carried out. In order to eliminate this step, Hayakari and others (1978) and Serra and others (2005) developed methods in which HA in the original reaction solution is further reacted with 2,4,6-trichloro-*s*-triazine or TNBS, and absorbance read at 382 nm or 415 nm, respectively. However, due to possible elevated absorbance readings resulting from reaction of the colourimetric reagents with amino groups, these methods are not suitable for peptide-based ACE-inhibitory samples. A recent method by Li and others (2005) employs the colourimetric reaction of HA with benzene sulfonyl chloride in the presence of quinoline to quantify ACE activity/inhibition at 492 nm. With no apparent reaction of HHL or peptide sample controls, this method may prove a simple and rapid alternative to the original. Other methods using the HHL substrate take further care to separate and quantify the HA product from the unhydrolysed HHL and HL products by HPLC coupled with electrospray-mass spectrometry to avoid any confounding HHL that may also be extracted in the ethyl acetate layer (Xiao and others 2006).

Assays employing a colourimetric substrate 2-furanacryloyl-1-phenylalanyl-glycylglycine (FAPGG) and an internally quenched fluorescent substrate *o*-aminobenzoylglycyl-*p*-nitrophenylalanylproline have also been developed to facilitate rapid screening of ACE-inhibitory peptides (Bunning and others 1983; van Elswijk and others 2003; Sentandreu and Toldra 2006). In comparing assays of different substrates, Shalaby and others (2006) found that the $IC_{50}$ of a tryptic whey protein isolate digest, when assayed using FAPGG as ACE substrate, was one order of magnitude higher than that found when using HHL as ACE substrate. As such, FAPGG results are more conservative in terms of estimating ACE-inhibitory potency. Despite this, modifications of the Cushman and Cheung (1971) method still remain the most widely used, possibly because cleavage of HL from HHL has been made to mimic the dipeptidase activity of ACE *in vivo* (Meisel and others 2006), and possibly, simply to facilitate results comparison.

## 1.4 *In Vivo* Efficacy Considerations

### 1.4.1 Gastrointestinal digestion and absorption

As with all bioactive compounds to be ingested orally, in order to have effect *in vivo* bioactive peptides from food sources must be able to reach target sites in the body in a form that still retains the intended activity. This entails the peptides either being resistant to gastrointestinal (GI) enzyme degradation or, upon hydrolysis, the products exhibiting bioactivity as well. These peptides must then be able to be absorbed intact through the GI wall into the blood stream.

During the digestion process, after pepsin hydrolysis in the stomach, proteins move to the small intestine where they are further hydrolysed by pancreatic proteases, namely trypsin and chymotrypsin (Kutchai 1996). Figure 1.2 shows the brush border of mucosal cells in the small intestine along with associated peptidases. In the traditional view of GI digestion, it is at this point that brush border peptidases cleave oligopeptides into tripeptides, dipeptides, and free amino acids, with these products then being absorbed into the mucosal cell via transporter proteins. Within the mucosal cell, the remaining peptides are degraded to free amino acids and released into the blood stream, thus completing the transcellular absorption process (Kutchai 1996). However, small peptides that are detected in the blood, including those that have demonstrated bioactivity *in vivo,* are thought to have bypassed exhaustive hydrolysis in the mucosal cells by being absorbed paracellularly (Figure 1.2) (Shimizu 1999; Vermeirssen 2004).

Upon investigating the effect of digestive enzymes on the ACE-inhibitory activity of peptides, some studies have found evidence of GI-resistance. For example, Hernandez-Ledesma and others (2004a, 2004b) found that *in vitro* digestion simulation of fermented milk and hydrolysed infant formula did not significantly change the moderate ACE-inhibitory activity of the peptide mixtures. Similarly, Wu and Ding (2002) showed that ACE-inhibitory peptides prepared from the Alcalase 2.4L® hydrolysis of soy protein retained activity though treatment with pepsin and pancreatin. Both of these results suggest that these ACE-inhibitory peptides are either resistant to degradation by digestive enzymes, or that ACE-inhibitory peptides are degraded into a mixture of smaller peptides, each with lower, higher, or the same ACE-

inhibitory activity as the parent peptides. As such, the total amount of ACE-inhibitory activity in the hydrolysate as a whole may remain the same.

However, apparent resistance to the GI tract is not always the case as proven in studies by Vermeirssen and others (2003) and Lo and others (2006). Firstly, using a dynamic model system of the upper GI tract, Lo and others (2006) found that the ACE-inhibitory activity of soy protein isolate hydrolysates after 30 minute hydrolysis in the stomach chamber was half that of the hydrolysates that had been exposed to an 30 additional minutes in the duodenum chamber. Yet, when the incubation times were increased to 90 minutes, the stomach + duodenum hydrolysates were less active than those generated by the stomach alone. This indicates that the peptides of pepsin hydrolysis, whose length and composition are time dependent, in turn affect the products able to be generated by pancreatin. Further, Vermeirssen and others (2003) modeled the effects of time in stomach and time in small intestine phase on ACE-inhibitory activity of pea protein hydrolysates by RSM. Within the ranges of the model, maximum time in the stomach (180 minutes) and maximum time in the small intestine (240 minutes) yielded hydrolysates with the lowest $IC_{50}$ values, indicating that during the digestion of this particular protein source, the generation of bioactive peptides was continuous and the ACE-inhibitory activity was continually in flux. The effects of digestion time on other proteins and/or hydrolysates are yet to be determined, however, it is clear that movement of bioactive peptides through the GI tract may affect survival of the intact peptide to the blood stream.

In terms of isolated peptides, the effect of GI enzymatic hydrolysis is more pronounced. For example, ACE-inhibitory oligopeptides, namely IWHHT and IVGRPRHQG, have been shown to increase in activity upon incubation with chymotrypsin and trypsin, respectively (Fujita and others 2000). These are classed as 'pro-drug type' bioactive peptides because they contain amino acid sequences that must first become exposed by GI hydrolysis before they can exhibit any significant ACE-inhibitory activity. On the contrary, Miguel and others (2006) found that the peptides YAEERYPIL and RADHPFL isolated from ovalbumin hydrolysate were degraded by simulated pepsin-pancreatin digestion to yield products with approximately 10-fold higher *in vitro* $IC_{50}$ values. Together, these findings reveal the variable activity of the GI tract on bioactive peptides and emphasize the need for simulated GI digestion in the pre-screening process of potential bioactive peptide sources.

After a bioactive peptide is either generated by or resists GI enzymatic hydrolysis, it must then pass the intestinal wall in order to enter the blood stream and reach its target site. Both cell culture and *in vivo* studies have been employed in assessing the absorption potential of ACE-inhibitory peptides. Using a Caco-2 cell monolayer, the ACE-inhibitory activity of whey and pea protein GI-digests were found to be resistant to brush border peptidases, but little or no activity was detected even in concentrated basolateral samples after 1 hr, indicating that the peptides had not been absorbed in sufficient quantities (Vermeirssen and others 2005a). Slightly more promising results were found by Satake and others (2002) where 2% of the VPP, an ACE-inhibitory peptide, was transported across the monolayer. In addition, this transport was not significantly inhibited by a competitive substrate for the transcellular absorption transporter PepT1, thereby supporting the theory that transport of bioactive peptides is paracellular. Yet Caco-2 cell monolayers are known to be tighter than *in vivo* human intestinal cells which may explain these low transport yields (Vermeirssen and others 2005a). In clinical trials, however, results have been much more promising, where peptides as long as 12 residues matching segments of κ-casein and β-casein have been detected in the human blood stream after digestion of milk (Chabance and others 1998). In terms of ACE-inhibitory peptides, a more recent study by Matsui and others (2002) demonstrated a dose-dependent increase in plasma VY levels 2 hours after administration of a VY drink. Yet again, absorption ratios were very low (<0.014%), so it is thought that paracellular transport was induced only by saturation of the transporter protein (Matsui and others 2002) and that peptide hydrophobicity, a preferential characteristic for transcellular transport, determined the dominant transport route (Shimizu and others 1997; Groff and Gropper 2000). This was counterbalanced, however, by the observation that the blood ACE-inhibitory activity of the absorbed VY was 300 times more potent than the $IC_{50}$ of the peptide *in vitro* (Matsui and others 2002). As such, amino acid composition and dose will be very important in functional food formulation.

It is possible as well that microencapsulation or lipophilization technologies could be used to help deliver intact bioactive peptides to the blood stream by protecting peptides against gastric enzyme degradation or increasing their interaction with intestinal cell junctions (Ko and others 2006). Paracellular permeability, in turn, is controlled by tight junction protein structure, which has been shown can be opened by interaction with certain sweet pepper and horseradish

capsinanosides (Shimizu 1999). Incorporation of these nanotechnologies and compounds into functional foods may prove a novel way to increase overall bioavailability.

### 1.4.2 Inhibitory activity kinetics

Once in the blood stream, ACE-inhibitory peptides must still reach their target site, that is, an ACE molecule. But given that ACE is a peptidase itself, there is the possibility that the peptide will be cleaved by ACE before, or instead of, it acting as an inhibitor. Not surprisingly, research by Fujita and others (2000) found that not all peptides deemed strong ACE inhibitors by *in vitro* assay are as effective *in vivo*. To investigate, Fujita and others pre-incubated isolated peptides with ACE before *in vitro* measurement of ACE-inhibitory activity. For some peptides, mainly dipeptides and tripeptides, pre-incubation with ACE did not affect the ability of the peptide to inhibit ACE hydrolysis of HHL. Other peptides, such as LKPNM, were hydrolyzed by ACE to a shorter peptide, LKP, which was more inhibitory towards ACE than the parent peptide (Table 1.2). Finally, contact with ACE degraded some peptides into peptides of little or no ACE-inhibitory activity. Following the example of this study, pre-incubation with ACE is now being used to classify ACE-inhibitory peptides according to their relative activity upon contact with ACE (Ruiz and others 2003; Hasan and others 2006). These classifications include: 'prodrug-type', meaning that the antihypertensive effect *in vivo* must be first activated by hydrolysis with GI proteases and/or ACE, 'true-drug type' or 'inhibitor type', where antihypertensive activity is not affected by digestion or ACE, and 'substrate type', where antihypertensive activity is reduced by contact with ACE. Strictly speaking, both 'substrate type' and 'pro-drug type' peptides are not ACE inhibitors, but rather are ACE substrates exerting an apparent ACE activity-lowering effect *in vitro* due to their transient occupation of the active site. The products of 'pro-drug type' peptides are, however, true inhibitors. It is, therefore, imperative during *in vitro* screening, as is simulated GI digestion, to pre-incubate hydrolysates with ACE before postulating *in vivo* efficacy.

The concept of ACE-inhibitory peptides as ACE substrates also provides possible explanations for seemingly anomalous results in other ACE inhibition studies. For example, in a study by Mullally and others (1997), the 3 kDa filtrate of whey protein GI digests showed stronger ACE-inhibitory activity than the 1 kDa filtrate. Yet, it is more common that the most

potent ACE-inhibitory fraction is less than 1 kDa in molecular weight (Section 1.3.3). It is possible in this case that the peptides in the 1-3 kDa range are acting as ACE substrates, and when hydrolysed to < 1 kDa, are no longer as active. Conversely, peptide products of a simulated GI digestion of ovalbumin exhibited low *in vitro* ACE-inhibitory activity yet exerted significant blood pressure lowering effects in the SHR model (Miguel and others 2006). Since the peptides administered had already undergone GI hydrolysis, it is possible that ACE *in vivo* had acted on primarily 'pro-drug type' peptides to generate 'true-drug' type products.

Inhibitory peptide classification can then be further divided by 'inhibitor mode' into 'competitive' and 'non-competitive' inhibitors. Inhibitor mode is determined by monitoring the effect of substrate concentration ([S]) on the intital velocity ($V_0$) of the enzymatic reaction in the presence or absence of an inhibitor (Stauffer 1989; Whitaker 1994). By plotting the reciprocals of [S] and $V_0$, a Lineweaver-Burk plot is generated, from which the key terms in the determination of inhibitor type, maximum velocity ($V_{max}$) and $K_m$, are calculated. Characteristic plots of competitive inhibition and non-competitive inhibition are shown in Figure 1.3. $K_m$, the concentration of substrate needed to achieve one-half the maximum reaction velocity in the absence of an inhibitor, and $K_{app}$, the concentration of substrate needed to reach one-half the maximum reaction velocity in the presence of an inhibitor, can then be used to determine $K_i$, the dissociation constant for competitive inhibitor binding, by the following equation in the units of inhibitor concentration: $K_i$ = Inhibitor concentration / $[(K_{app}/K_m) - 1]$ (Whitaker 1994). The lower the $K_i$ value of a competitive inhibitor, the higher the affinity of the inhibitor for the enzyme active site, and the greater the displacement of substrate from the active site. This then manifests during *in vitro* experiments as a lower $IC_{50}$ value.

Recently, several studies have investigated the inhibitory kinetics of ACE-inhibitory peptides with varying results. For example, using the Cushman and Cheung (1979) method for ACE activity, Hernandez-Ledesma and others (2003) carried out an experiment using yogurt (fermented) whey peptides as inhibitors in which the concentration of ACE was kept constant at 26 mU/mL and the concentration of HHL substrate was varied from 5 to 40 mM. Even at very high concentrations of HHL, substrate was not able to overcome the inhibition. Characteristic of a non-competitive inhibitor, the whey peptides would have bound to an area other than the active site and hindered binding to HHL by deforming the ACE conformation. On the other

hand, the ACE-inhibitory activity of the synthesized peptide LRW and the peptide analogue drug Captopril® have been found to be competitive (Hernandez-Ledesma and others 2003; Wu and others 2006b). In an effort to elucidate relationships between sequence and inhibition mode, Ono and others (2006) synthesized six tryptophan-containing ACE-inhibitory dipeptides AW, VW, MW, IW, LW, and their corresponding reversed sequences. Of these peptides, all those with tryptophan as the C-terminal residue exhibited non-competitive inhibition while all those with reversed sequences exhibited competitive inhibition, except WL. These results indicate that inhibition mechanism can be affected by amino acid sequence; however, a definitive mechanism for tryptophan C-terminal peptides cannot be elucidated as the latter results do not correspond with the competitive inhibition mechanism of LRW (Wu and others 2006b).

Yet overall, these categories of 'inhibition type' and 'inhibition mode' not only reflect the diverse possibilities of ACE-inhibitory peptide composition, sequences, and lengths, they more importantly explain discrepancies in the *in vitro* versus *in vivo* effects of some individual peptides. Since *in vivo* studies are not practical for pre-screening purposes, determination of a hydrolysate mixture's dominant 'inhibition type' *in vitro* may be a preliminary way of estimating antihypertensive efficacy *in vivo*, where 'pro-drug' and 'true-drug' types would likely be more effective than 'substrate' type hydrolysates. In addition, non-competitive inhibitors may provide a more consistent acute antihypertensive effect as their action would not be mediated by the concentration of ACE substrates in the blood.

### 1.4.3 Animal model and clinical trials of antihypertensive efficacy

Interestingly, even though synthetic ACE-inhibitory drugs like Captopril® have demonstrated higher activity potency *in vitro*, the effects of some ACE-inhibitory peptides and Captopril® *in vivo* are comparable (Table 1.4). Among fish protein hydrolysates, those from dried bonito, fermented fish sauce, salmon, fermented mackerel, and yellowfin sole hydrolysates have already been successfully tested in the SHR model (Fujita and Yoshkawa 1999; Ichimura and others 2003; Ono and others 2003; Itou and Akahane 2004; Jung and others 2006). As shown in Table 1.4, both isolated peptides and unfractionated hydrolysate exert maximal antihypertensive effect at approximately 4 to 6 hours after administration. Further,

antihypertensive effect appears to be dose-dependent only at lower concentrations, as Itou and Akahane (2004) demonstrated that there was a maximal decrease in systolic blood pressure (SBP) achieved after which increases in ACE-inhibitory peptide dose did not exert further activity. In terms of blood pressure depression duration, unfractionated hydrolysate or longer pro-drug type peptides, such as LKPNM and MFGAGGPEL, seem to have a more prolonged effect *in vivo* than shorter true-drug peptides. This is plausible since they may firstly be decreasing ACE activity as substrates, then secondly as true inhibitors, before they are eliminated from the body (Fujita and Yoshikawa 1999).

Very few clinical trials have been carried out on ACE-inhibitory peptides, although those few have had promising results. As summarized in Table 1.5, at an average daily dose of approximately 1.5 mg of isolated peptides, or 1500 mg of ultrafiltered hydrolysate, mildly hypertensive subjects from the three studies experienced a maximum decrease in SBP of approximately 12 mm Hg. A significantly lower SBP than the placebo group was sustained for a minimum of 2 weeks post-administration. These studies confirm that certain ACE-inhibitory peptides are bioavailable and effective in humans, which therefore justifies further screening of potential ACE-inhibitory peptide sources and the future development of functional foods and nutraceuticals for the prevention of hypertension.

## 1.5 Thesis Objectives and Hypotheses

To investigate Pacific hake (*Merluccius productus)* fillet hydrolysates as a source of ACE-inhibitory peptides, and to establish if further study of antihypertensive efficacy *in vivo* is warranted, the objectives of this thesis study are:

(1) To assess the effects of hydrolysis conditions (pH, %E/S, hydrolysis time), starting material variability (*K. thyrsites* spore count, catch date), ultrafiltration separation, simulated gastrointestinal (pepsin-pancreatin) digestion, and peptide-ACE pre-incubation on Pacific hake fillet hydrolysate ACE-inhibitory activity *in vitro*.

(2) To determine the inhibitory kinetics of hydrolysate samples with the lowest $IC_{50}$ values after pepsin-pancreatin digestion and peptide-ACE pre-incubation.

Figure 1.4 provides a flow-diagram overview of the experimental stages and analyses of this thesis study. As such, it is hypothesized that:

(1) Pacific hake fillet hydrolysate with *in vitro* ACE-inhibitory activity, comparable to or higher than that of other ACE-inhibitory hydrolysate sources, can be produced by an optimal set of hydrolysis conditions within the ranges tested regardless of starting material variability.

(2) Ultrafiltration will serve to separate and concentrate peptides of higher ACE-inhibitory activity from this unfractionated hydrolysate.

(3) In turn, molecular weight range of unfractionated hydrolysate and ultrafiltrates will have an impact on the effects imparted by pepsin-pancreatin digestion and peptide-ACE pre-incubation on ACE-inhibitory activity.

(4) Finally, inhibitory kinetics will also be influenced by extent of ultrafiltration.

# 1.6 Tables

**Table 1.1 Product specifications, standardized activity levels, and cleavage specificities of various commercial proteases** (Novo Nordisk 1998a, 1998b; Kristinsson and Rasco 2000c; Gilmartin and Jervis 2002; Novozymes 2004)

| | **Alcalase 2.4L®** | **Protamex®** | **Flavourzyme 1000L®** |
|---|---|---|---|
| **Source** | *Bacillus licheniformis* | multiple | *Aspergillus oryzae* |
| **Optimal pH** | 6.5-8.5 | 5.5-7.5 | 5.0-7.0 |
| **Temperature range, °C** | 55-70 | 35-60 | 50 |
| **Protease type** | endoprotease | protease complex | endoprotease/exopeptidase |
| **Azocoll activity units/g** [1] | 668,896 | not tested | 59,630 |
| **Activity on synthetic substrate**[2] | | | |
| Suc-Leu-Leu-Val-Tyr-AMC | 4370 | 942.9 | 326.8 |
| Suc-Ala-Ala-Ala-pNA | 259.5 | 179.9 | 40.06 |
| BZ-Pro-Phe-pNA | 1.29 | 0.59 | 1.78 |
| BZ-Arg-AMC | 1.79 | 0 | 1.1 |
| Gly-Pro-AMC | 0 | 0 | 7.05 |
| Leu-AMC | 69.44 | 6.94 | 9268 |
| Arg-AMC | 4.69 | 2.36 | 326.8 |
| Ala-AMC | 1.85 | 0 | 13.51 |
| Gly-AMC | 0 | 0 | 1.17 |
| Pro-AMC | 0 | 0 | 8.64 |

[1]Azocoll activity units per gram of enzyme determined by proteolysis of Azocoll synthetic substrate by the given enzyme at pH 7.5, 40 °C, for 15 min., in 0.1 M sodium phosphate buffer, where 1 AzU is the amount of enzyme producing an absorption of 0.1 at 520 nm.

[2]Activity on synthetic substrates expressed as nmol of 7-amino-4-methylcoumarin (AMC) or μmol p-nitroalinine (pNA) released per min per mg of protein in 0.1 Tris, pH 7.5, at 40 °C.

**Table 1.2 Examples of sequences and potencies of ACE-inhibitory peptides prepared and isolated from the hydrolysis of various fish protein sources**

| Peptides | Source | Hydrolysis method | $IC_{50}$ (μM) | Reference |
|---|---|---|---|---|
| PTHIKWGD | tuna muscle | acid extraction | 2 | Kohama and others 1988 |
| IKPLNY, IKP, LYP | dried bonito | pepsin, chymotrypsin, trypsin, & thermolysin | 43, 1.7, 6.6 | Yokoyama and others 1992 |
| LKPNM, LKP | dried bonito | thermolysin | 2.4, 0.32 | Fujita and Yoshikawa 1999 |
| IY, IW | bonito muscle | thermolysin | 2.1, 5.1 | Fujita and others 2000 |
| FGASTRGA | Alaska pollock | pepsin | 14.7 | Je and others 2004 |
| FL, VW | salmon muscle | thermolysin | 13.6, 2.5 | Ono and others 2003 |
| MIFPGAGGPEL | yellowfin sole | chymotrypsin | 28.7 | Jung and others 2006 |

**Table 1.3 Comparison of $IC_{50}$ values of protein hydrolysates of different molecular weight ranges as obtained by ultrafiltration**

| Protein source | Enzyme(s) used | MW range/cut-off (kDa) | $IC_{50}$ (μg/mL) | Reference |
|---|---|---|---|---|
| cod frame | protease | unfractionated | 320 | Jeon and others 1999 |
| | from tuna | <30 | 380 | |
| | pyloric caeca | <10 | 350 | |
| | | <5 | 180 | |
| | | <1 | 80 | |
| lactalbumin | trypsin | unfractionated | 345 | Pihlanto-Leppala and others 2000 |
| | | 1-30 | 1134 | |
| | | <1 | 109 | |
| | pepsin, trypsin, chymotrypsin | unfractionated | 509 | |
| | | 1-30 | 485 | |
| | | <1 | 314 | |
| bovine blood plasma | Alcalase® | unfractionated | 560 | Hyun and Shin 2000 |
| | | <10 | 470 | |
| | | <3 | 300 | |
| | | <1 | 120 | |
| corn gluten | Flavourzyme® | unfractionated | 180 | Kim and others 2004 |
| | | <5 | 50 | |
| whey | pepsin, chymotrypsin | >3 | 41 | Vermeirssen and others 2005 |
| | | <3 | 14 | |

**Table 1.4 Decrease in systolic blood pressure (SBP) following single oral dose of ACE-inhibitory peptides (or Captopril® control) in the spontaneously hypertensive rat (SHR) model**

| Peptide(s) | Source | Dose (mg/kg body wt) | Maxmum change in SBP (mm Hg) [1] | Significant Duration (hrs) [2] | Reference |
|---|---|---|---|---|---|
| unfractionated hydrolysate | Porcine myosin | 30 | -25 at 6 hrs | >20 | Nakashima and others 2002 |
| LKPNM | Dried bonito | 15 | -14 at 4 hrs | 4 | Fujita and Yoshikawa 1999 |
| LKP | | 4.5 | -16 at 2 hrs | 2 | |
| Captopril® | -- | 5 | -14 at 4 hrs | 4 | |
| KP | Fermented fish sauce | 50 | -10 at 6 hrs | 6 | Ichimura and others 2003 |
| unfractionated hydrolysate | Salmon muscle | 500 | -30 at 4 hrs | <4 | Ono and others 2003 |
| unfractionated acid extract | Fermented mackerel | 50 | -40 at 4 hrs | 4 | Itou and Akahane 2004 |
| | | 10 | -40 at 4 hrs | 4 | |
| | | 5 | -15 at 4 hrs | <4 | |
| MFGAGGPEL | Yellowfin sole frame | 10 | -35 at 6 hrs | >8 | Jung and others 2006 |
| Captopril® | -- | 10 | -35 at 6 hrs | >8 | |

[1] Hours after administration

[2] Time SBP response of treatment group was significantly different ($P<0.05$) than placebo group after time of first significant difference.

**Table 1.5 Effect of short-term oral administration of ACE-inhibitory peptides on systolic blood pressure (SBP) in human subjects**

| Source / Peptide(s) | Dose (mg/kg body wt/day) [1] | Duration of administration | Max. decrease SBP (mm Hg) [2] | Duration post-administration | Reference |
|---|---|---|---|---|---|
| sour milk/ VPP, IPP | ~0.033, 0.025 | 8 weeks | -12 at 8 weeks | >4 weeks | Hata and others 1996 |
| Sardine muscle/ VY | ~0.09 | 4 weeks | -10 at 1, 2, 3, and 4 weeks | 3 weeks | Kawasaki and others 2000 |
| Dried bonito/ <3 kDa thermolysin digest | ~23 | 5 weeks | -12 at 5 weeks | 2 weeks | Fujita and others 2001 |

[1] Given a mean body weight of 65 kg for all subjects in all studies

[2] Time after start of administration period

## 1.7 Figures

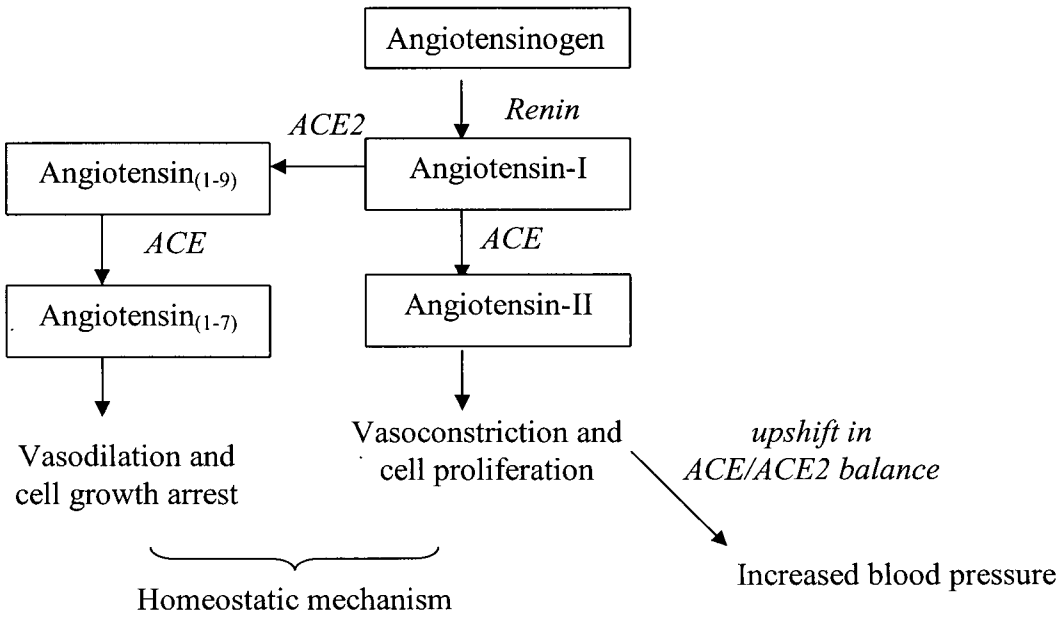



**Figure 1.1** **Schematic of the endocrine renin-angiotensin system as it affects the kidneys, heart, vascular smooth muscle cells, and adrenal glands**
(Eriksson and others 2002)

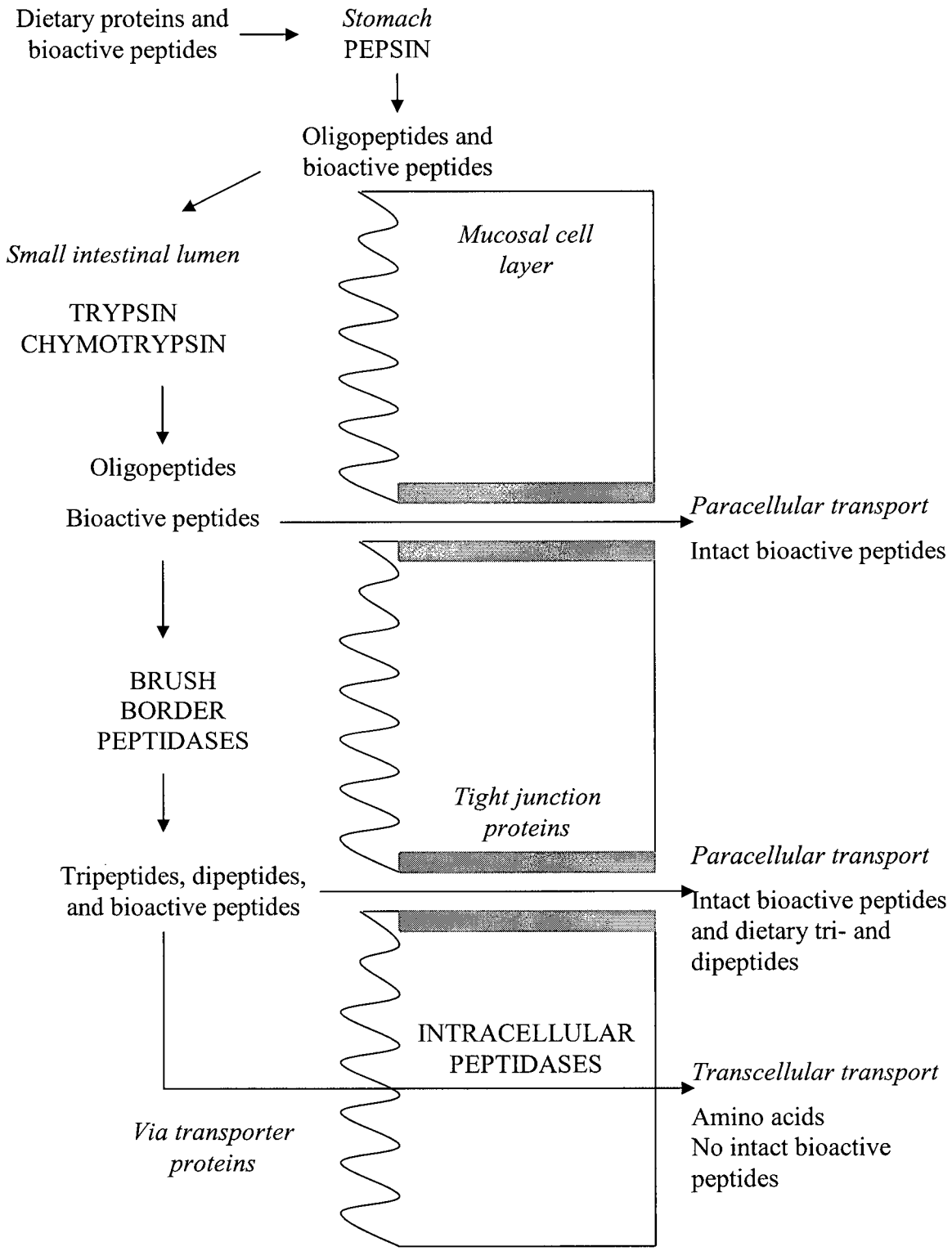
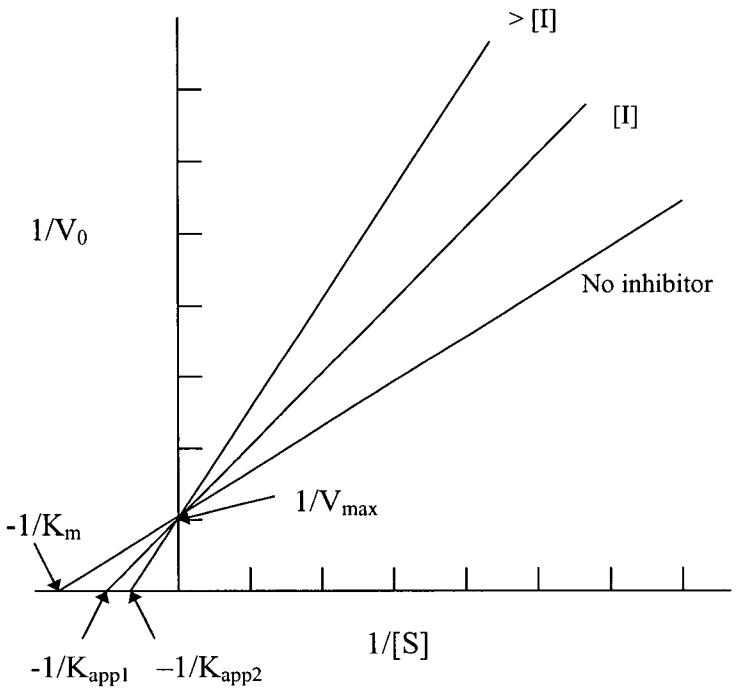



**Figure 1.2** **Digestion and modes of absorption of dietary proteins and bioactive peptides in the gastrointestinal tract** (Shimizu 1999; Vermeirssen and others 2004)

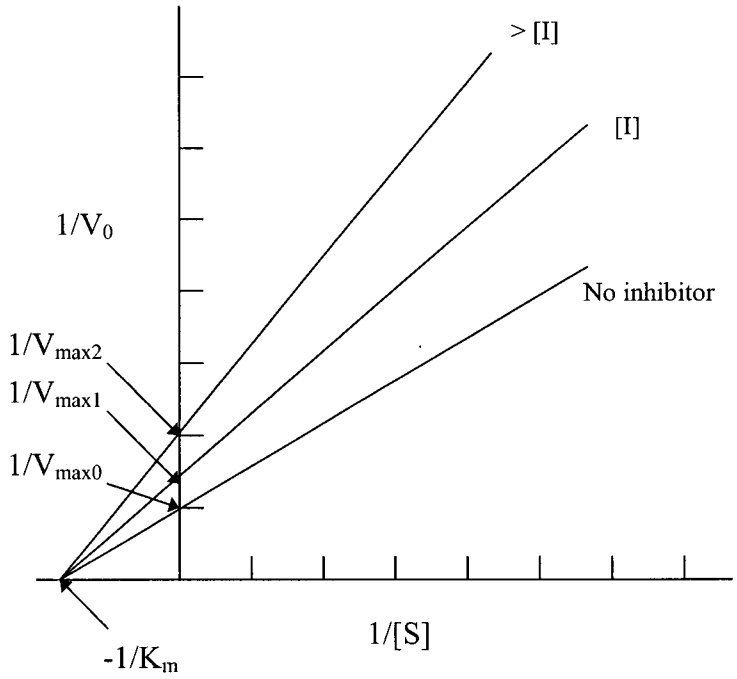



**Competitive Inhibition –**

Presence of inhibitor does not affect maximum velocity ($V_{max}$) of reaction

Increased concentration of inhibitor increases amount of substrate needed to reach ½ $V_{max}$ ($K_m < K_{app1} < K_{app2}$)

Inhibition can be overcome by increasing concentration of substrate




**Non-Competitive Inhibition –**

Increased concentration of inhibitor decreases maximum velocity ($V_{max}$) of reaction

Presence of inhibitor does not affect amount of substrate needed to reach ½ of respective$V_{max}$ ($K_m = K_{app}$)

Inhibition cannot be overcome by increasing concentration of substrate

**Figure 1.3 Lineweaver-Burk plots for the determination of inhibitor type**

(adapted from Whitaker 1994)

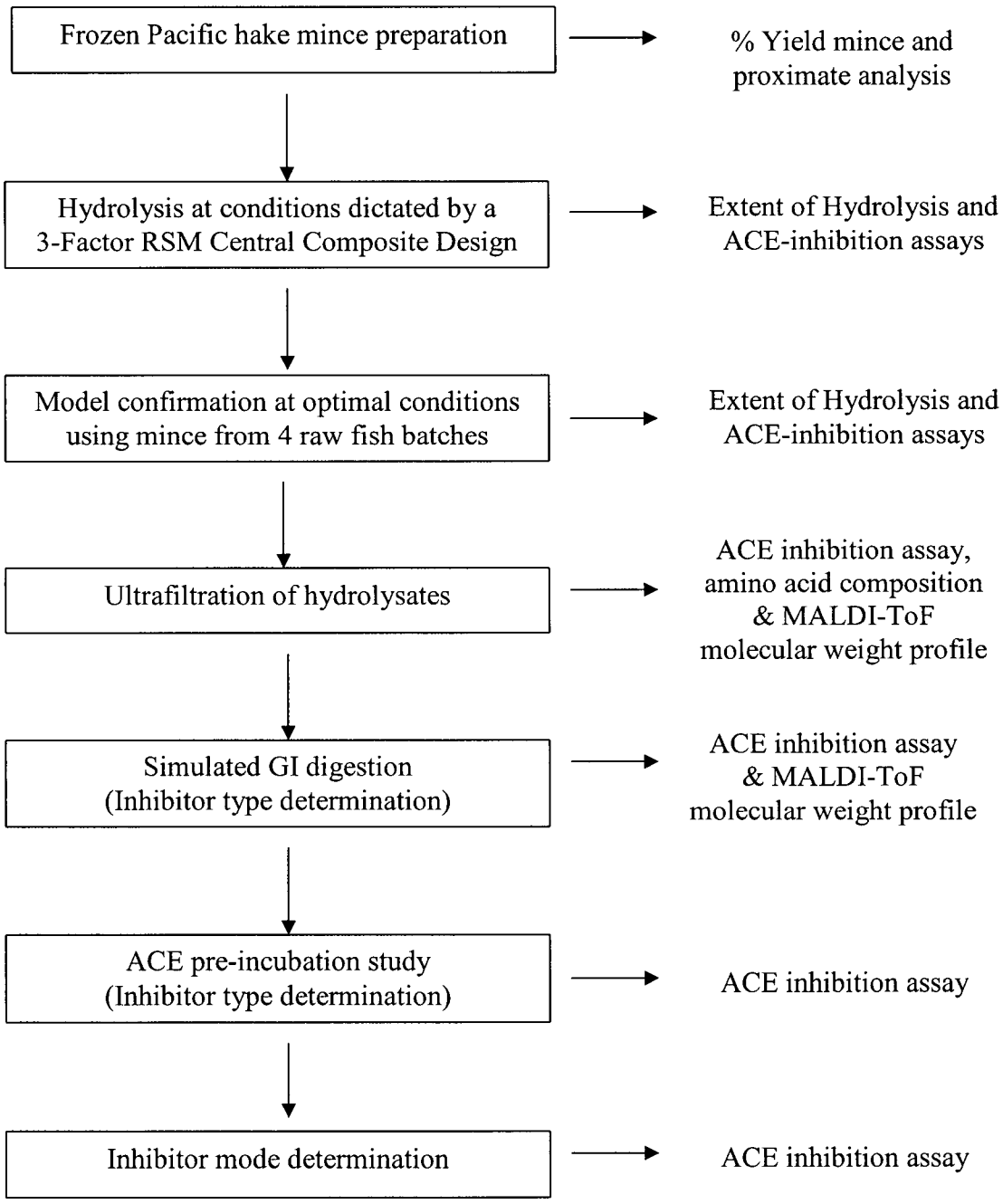



**Figure 1.4 Flow-diagram overview of thesis study experimental stages and analyses**

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# CHAPTER II Use of RSM and Ultrafiltration in Optimizing the Angiotensin I-Converting Enzyme Inhibitory Activity of Pacific Hake Fillet Hydrolysate[1]

## 2.1 Introduction

In addition to modifications of diet and lifestyle, prescription drugs classed as angiotensin I-converting enzyme (ACE) inhibitors have been conventionally used to control high blood pressure by reducing the production of angiotensin II, a potent vasoconstrictor, and lowering the inactivation of bradykinin, a vasodilator (Opie 1992; Meisel and others 2006). Altered activity of bradykinin however, has been attributed to the onset of a persistent dry cough side-effect (Opie 1992). Starting in the late 1980s, hydrolysates from food protein sources have been shown to exhibit ACE-inhibitory activity as well, including those from milk, soy, chicken, wheat, corn, and fish (Li and others 2004; Meisel and others 2006). More importantly, clinical trials have reported that borderline and mildly hypertensive subjects displayed significantly lower ($P<0.05$) systolic blood pressure upon oral administration of 1500 mg hydrolysate per day from dried bonito, and had experienced no adverse side-effects (Fujita and others 2001). These results suggest that hydrolysates from food protein sources may provide a milder, yet effective alternative for blood pressure management.

A variety of other fish protein hydrolysates, including those from tuna (Kohama and others 1988), sardine (Matsui and others 1993), and pollack (Je and others 2004) have been shown to exhibit ACE-inhibitory activity *in vitro*, while those from fermented fish sauce (Ichimura and others 2003), salmon (Ono and others 2003), and sole (Jung and others 2006), have exhibited antihypertensive efficacy in the spontaneously hypertensive rat (SHR) model, suggesting that this potential is not necessarily species specific. In this study, Pacific hake (*Merluccius productus*), an abundant species off the west coast of Canada and the United States, was investigated as a source of ACE-inhibitory peptides.

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[1] A version of this chapter will be submitted for publication. Cinq-Mars, C.D. and Li-Chan, E.C.Y. Use of RSM and ultrafiltration in optimizing the angiotensin I-converting enzyme inhibitory activity of Pacific hake fillet hydrolysate. J. Food Sci.

Undervalued for its poor fillet texture attributed to the occurrence of *Kudoa* spore infection (Morrissey and others 1995), use of Pacific hake for the production of ACE-inhibitory peptides may provide a potential value-added niche market for this otherwise underutilized renewable resource. However, *Kudoa* spore infection influences intrinsic hydrolysis levels in the muscle (Samaranayaka and others 2006), which may in turn influence the substrate and cleavage sites of commercial hydrolysis processes. Similarly, catch date, which reflects possible differences in diet due to fish migration and the predominant feed available, may affect muscle composition. As such, ACE-inhibitory activity must be assessed in response to starting material variability.

In protein hydrolysis processes, variables such as protease used, pH, temperature, hydrolysis time, substrate concentration, and enzyme-to-substrate ratio (%E/S), must be considered and controlled as they all influence the extent of hydrolysis and therefore the functionality of the end product (Kristinsson and Rasco 2000). Response Surface Methodology (RSM) is a useful statistical tool to study such multi-variable processes as it reduces the number of trials required compared to full-factorial design, and allows study of process variable interactions. RSM provides an experimental design of orthogonal process variable combinations, to be used as trials, whose outcomes of hydrolysate functionality can be plotted as a predictive response surface (Myers and Montgomery 2002; Minitab 2006a). Statistically sound inferences between data points can then be made, and process conditions resulting in optimized end product functionality can be predicted by means of the model equation. Studies by Diniz and Martin (1997) and van der Ven and others (2002) have successfully employed RSM in the study of hydrolysates to optimize emusification and foaming capacities, and ACE-inhibitory activity, respectively. RSM predictions should however be verified by conducting trials at select process variable combinations, especially for models with a significant lack-of-fit term, or a low $r^2$ value (Myers and Montgomery 2002).

While isolating single ACE-inhibitory peptides by extensive chromatographic fractionation is important for elucidation of the structure-function relationships of ACE-inhibitory peptides with ACE, this is not practical from a commercial production point of view as it would be very costly, diminish product yields, and result in the loss of other potentially active peptides. A less extensive method to separate hydrolysates into fractions of a higher given activity is ultrafiltration (UF) (Korhonen and Pihlanto 2003). For ACE-inhibitory

activity, the most potent peptides have been estimated to be in the dipeptide to pentapeptide range (<1 kDa), yet this comprises only a small portion of the total hydrolysate mass (Matsui and others 1993; Fujita and others 1999). However, at a 10 kDa cutoff, UF may be an industrially advantageous way to 'enrich' the ACE-inhibitory potency of Pacific hake fillet (PHF) hydrolysate without compromising yield.

Therefore, the objectives of this study were to assess the effects of (1) hydrolysis conditions (using RSM), (2) starting material variability, and (3) UF separation, on the ACE-inhibitory activity of PHF hydrolysates *in vitro*. Further, ACE-inhibitory activity of the samples was compared to that of the commercial product PeptACE® (Natural Factors Inc., Coquitlam, BC) in order to establish if further study of PHF as a commercially competitive source of ACE-inhibitory peptides was warranted.

## 2.2 Materials and Methods

### 2.2.1 Materials

Whole, raw, Pacific hake was provided by Steveston Seafood Direct Ltd. (Richmond, BC) during the summer catch seasons of 2004 and 2005 from the approximate area of 48° N latitude and 124° to 125° W longitude off the west coast of Vancouver, BC. Fish were transported on ice to the University of British Columbia Food Science Laboratory within 2 days of capture. *Kudoa paniformis* infection levels had been determined by the method of Dawson Coates and others (2003) as modified by Samaranayaka and others (2006) prior to storage at –25 °C. Catch date, fish length, fish mass, and *Kudoa paniformis* infection level ranges used to create experimental batches to assess the effect of starting material variability on ACE-inhibitory activity are found in Table 2.1.

Sodium hydroxide (product ACS-816) and disodium tetraborate decahydrate (product ACS-861) were purchased from BDH Inc. (Toronto, ON). Sodium dihydrogen orthophosphate dihydrate (monobasic, product 3819-05) was purchased from JT Baker (Phillipsburg, NJ). Trinitrobenzenesulfonic acid (TNBS, product 8746) was purchased from Eastman Kodak Co. (Rochester, NY). Sodium sulfite (product S430-500), sodium chloride (product S271-3), hydrochloric acid (product A144-S212), and sodium azide (as sodium trinitride, product S227I-500) were purchased from Fisher Scientific Co (Ottawa, ON). Materials purchased from Sigma-Aldrich Canada (Oakville, ON) include L-leucine (product L-8912), ethyl acetate (99.5% ACS, spectrophotometric grade, product 154857), trichloroacetic acid (99% flakes, product T6399), hippuryl-L-histidyl-L-leucine (HHL as tetrahydrate, product H1635), and angiotensin-converting enzyme (ACE, from rabbit lung, product A6778). ACE was dissolved and portioned in 0.025 M sodium tetraborate buffer containing 0.3 M NaCl (pH 8.3) to the concentration of 2.5 mU/30 µL aliquot and stored at –25 °C.

Alcalase 2.4L® (protease from *Bacillus licheniformis*, 2.4 AU/g, product P4860) and Flavourzyme 500L® (protease from *Aspergillus oryzae,* 500 LAPU/g, product P6110) were purchased from Sigma-Aldrich Canada (Oakville, ON). Protamex® (1.5 AU/g) was a gift from Brenntag Canada Inc. (Langley, BC). All enzymes were produced by Novozymes (Bagsvaerd,

Denmark). PeptACE® is a product of Natural Factors Nutritional Products Ltd. (Coquitlam, BC) and was purchased from Finlandia Pharmacy (Vancouver, BC).

### 2.2.2 Mince preparation and proximate analysis

Whole fish, grouped into batches as described in Table 2.1, were first thawed overnight at 4 °C, then filleted, ground using a BEEM Gigant commercial meat grinder with a 4 mm screen (Butcher and Packer Supply Company, Detroit, MI) and homogenized using a 15 quart Duratax mixer (John Holt Bolton Limited, USA). Mince was portioned into 150 g amounts, sealed in plastic 5 mil stock vacuum bags (West Coast Foodpak Systems Limited, Vancouver, BC) using a Multivac AG5 vacuum sealer (Sepp Haggenmuller KG, Wolfertschwenden, Germany), and stored at –25 °C until use in hydrolysis trials. A 10 g portion of the homogenized mince from the preliminary batch was also kept for proximate analysis. Moisture content and ash were determined by AOAC Official Methods 930.15 and 942.05, respectively (AOAC 1995). The pH was determined by the method in the Handbook of Meat Analysis (Koniecko 1985) and lipid was determined by the Bligh and Dyer method (1959). The dried sample obtained from measuring moisture content was used for determination of nitrogen content by combustion method AOAC 992.15 (AOAC 1995), using a LECO instrument (LECO Instruments Ltd, Mississauga, ON). Crude protein content was calculated from nitrogen using a conversion factor of 6.25. All assays were performed in triplicate.

### 2.2.3 Preparation of hydrolysates

First, a 1:2 mince to water slurry was made by mixing 150 g of prepared PHF mince with 300 mL of distilled water in a Waring blender (Model 700G, Waring Commercial, Torrington, CT) and decanting into a 500 mL glass beaker. The slurry was then pre-heated to boiling in a microwave (Kenmore Model 87470, Sears Canada Incorporated, Toronto, ON) at maximum power (800 W) for 2 minutes, stirred manually, covered with foil, then heated at 95 °C in boiling water for 10 minutes to inactivate endogenous enzymes. After cooling at room temperature back to the desired temperature (55 °C for Alcalase 2.4L® trials, 50 °C for Flavourzyme 500L® trials, 40 °C for Protamex® trials), the covered beaker was incubated in a MagniWhirl constant temperature water bath (Blue Electric Company, Blue Island, IL) to

maintain process temperature. The pH was adjusted to that desired using 6 N HCl or 6 N NaOH. Commercial protease was then added at the desired %E/S ratio. Sodium azide was also added at a ratio of 0.05% w/v to prevent microbial growth in preliminary trials. The slurry was constantly stirred at 400 rpm with a 5 cm diameter propeller overhead stirrer (Model 5VA, Eastern Industries, Hamden, CT), and the pH and temperature monitored using an Accumet® portable pH meter (AP61 pH meter with 13-690-AP50 probe, Fisher Scientific Co., Ottawa, ON). pH was kept constant by addition of 6 N NaOH as hydrolysis progressed. After the desired process time, hydrolysis was terminated by submersion in boiling water for 15 minutes. The slurry was then centrifuged using a GSA rotor at 12,000 x g (Sorvall RC 5B Plus, DuPont Canada Inc., Mississauga, ON) at room temperature for 15 minutes. The supernatant was decanted through Whatman 4 filter paper into a graduated cylinder, the volume recorded, and the pH neutralized using 1 N NaOH or HCl. The conductivity (mS/cm) of the neutralized supernatant was measured (Meterlab CDM210, Radiometer Analytical, Lyon, France) and compared to a 0.01-0.2 M NaCl conductivity standard curve to monitor the salt concentration of the hydrolysate ($y= 97.796 x + 0.572$, $r^2 = 0.999$, where y is conductivity (mS/cm) and x is molar concentration of NaCl). The slurry supernatant solutions were freeze-dried (Model 4451F, Labconco Corporation, Kansas City, MO) to yield hydrolysate powder, and stored at -25 °C. The % yield was calculated by:

[(Mass dried powder – mass salt) / original mass of mince solids] X 100

[Equation 1]

Note that the mass of the salt, when calculated from the NaCl standard conductivity curve was less than 0.5% of the hydrolysate mass.

### 2.2.4 Extent of hydrolysis

Measurement of the extent of hydrolysis (EH) was carried out according to the method of Adler-Nissen (1979) with modifications by Liceaga-Gesualdo and Li-Chan (1999). Immediately prior to termination of hydrolysis, a 4 mL aliquot of the mince-water slurry was mixed with equal parts 24% trichloroacetic acid solution and centrifuged using a SS34 rotor at 12,100 x g (Sorvall RC 5B Plus, DuPont Canada Inc., Mississauga, ON) for 5 minutes. An

aliquot of the supernatant was then diluted 100X with distilled-deionized water to fit within the absorbance range of the leucine standard curve. Next, 0.2 mL of the diluted supernatant was added to 2.0 mL of 0.05 M sodium tetraborate buffer (pH 8.2). Once 1.0 mL of freshly made 4.0 mM TNBS was added, the mixture was immediately incubated in the dark at room temperature for 30 minutes. After incubation, 1.0 mL of 2.0M $NaH_2PO_4$ containing 18 mM $Na_2SO_3$ was added and the absorbance was measured at 420 nm using a spectrophotometer (Unicam UV/Vis Spectrometer UV2, ATI Unicam). The assay was carried out in triplicate and distilled water replaced the slurry sample as an assay blank.

In this method, quantitative estimation of the peptide bonds cleaved during hydrolysis is achieved by reaction of the peptide primary amino groups with TNBS at pH 8.2 to form chromophores with maximum absorbance at 420 nm. Extent of hydrolysis can then be expressed as milliequivalents free α-amino groups / L slurry, determined by comparison to an L-leucine standard curve (0.2-3.0 mM). It was not possible to express EH as a percentage, or degree of hydrolysis (DH) in this study, where $\%DH = h / h_{total} \times 100$, since $h_{total}$ (total number of peptide bonds in a given protein) is unknown in a hydrolysate solution.

### 2.2.5 ACE-inhibitory activity

ACE-inhibitory activity of hydrolysate samples was determined in triplicate by a method adapted from Lo and Li-Chan (2005) based on the ACE activity assay of Cushman and Cheung (1971). In this assay, ACE activity is quantified by spectrophotometric absorbance at 228 nm produced by the liberation of hippuric acid (HA) from synthetic substrate hippuryl-L-histidyl-L-leucine (HHL). In the presence of an ACE inhibitor at a given concentration, ACE activity and HA production are depressed; therefore % ACE inhibition can be calculated as:

$$1 - \left( \frac{(\text{absorbance of inhibitor-containing sample} - \text{absorbance of negative control})}{(\text{absorbance of positive control} - \text{absorbance of negative control})} \right) * 100$$

[Equation 2]

where the positive control is taken as 100% ACE activity, with inhibitor having been replaced with buffer, and the negative control is taken as 0% ACE activity, with ACE being added only after reaction termination. The $IC_{50}$ value, the concentration of peptide sample in the assay

required to inhibit the activity of ACE by 50%, is commonly used to quantitatively express and compare potency of ACE-inhibitory activity between samples. In this study, $IC_{50}$ values have been derived in triplicate from regression curves through the % ACE inhibition values of 5 peptide concentrations, as shown in Figure 2.1. All controls were also performed in triplicate to monitor the reproducibility of the assay.

To carry out the assay, 30 μL of peptide solution was first mixed with 30 μL (2.5 mU) of ACE and incubated at 37° C for 1 hour, after which 150 μL of 7.8 mM HHL was added and the solution was further incubated at 37° C for 1 hour. All assay components were dissolved in 0.025 M sodium tetraborate buffer containing 0.3 M NaCl (pH 8.3) for a total assay volume of 210 μL. HCl (250 μL, 1N) was used to terminate the reaction. In order to extract HA, 1.0 mL of ethyl acetate was added. The solutions were then vortexed for 30 seconds and microcentrifuged at 2000 x g (Mikro 20, Hettich Zentrifugen, Tuttlingen, Germany) for 5 minutes. After centrifugation, 0.7 mL of the ethyl acetate layer was removed into a 10 mm diameter clear glass tube and was evaporated by heating at 120°C (Analog Dry Block Heater, VWR International Ltd., Delta, BC) for 30 minutes. The remaining HA residue was redissolved with 1.3 mL of distilled water and the absorbance read at 228 nm in a quartz cuvette.

### 2.2.6 Ultrafiltration

Ultrafiltration was carried out using an Amicon® Model 8400 stirred cell (Millipore Corporation, Billerica, MA). Hydrolysate solution, the supernatant of the hydrolyzed mince-water slurry, was sequentially passed through Millipore membranes YM10, YM3, YM1, and YC05 (molecular weight cut-offs 10 kDa, 3 kDa, 1 kDa, and 500 Da, respectively) under 40 psi nitrogen gas at room temperature and aliquots of filtrate were collected at each filtration step. Filtrates were freeze-dried to powder form and stored at –25 °C until further use. Percent yield of hydrolysate upon ultrafiltration at 10 kDa was calculated as:

$$\left( \frac{\text{mass retentate powder } \textit{or} \text{ mass filtrate powder}}{\text{total mass of hydrolysates produced}} \right) * 100 \qquad \text{[Equation 3]}$$

### 2.2.7 Amino acid composition and hydrolysate ash analysis

Hydrolysate samples were sent for amino acid (AA) analysis to the Advanced Protein Technology Centre at the Hospital for Sick Children (Toronto, ON). Using the Waters Pico-Tag System, samples were subjected to vapour phase hydrolysis by 6N HCl with 1% phenol at 110°C for 24 hours, followed by pre-column derivatization of the hydrolyzates using phenylisothiocyanate (PITC) and quantification by reverse phase HPLC using PITC-labelled amino acids as standards (Hospital for Sick Children 2006). Picomoles of individual amino acids detected were converted to mass quantities using their respective residue weights. Conversion of hydrolysate solids concentration basis to peptide concentration basis for expression of $IC_{50}$ was therefore as follows, where percent mass amino acids was derived by the sum of individual amino acid masses detected by AA analysis compared to total solids mass analysed:

$$\mu g \text{ peptides / mL assay} = (\%\text{mass AA}) * (\mu g \text{ solids / mL assay}) \qquad \text{[Equation 4]}$$

Ash content of the hydrolysate solids was determined by AOAC Official Methods 942.05 (AOAC 1995).

### 2.2.8 Statistical analysis

MINITAB® version 12.21 (Minitab Inc., State College, PA) was used to create the Response Surface Methodology Central Composite Design for the preparation of hydrolysates, and to generate the response surface plots. Microsoft® Excel 2000 was used for the determination of $IC_{50}$ value by means of regression curve equations (Figure 2.1). For RSM model confirmation, predicted values were required to fall within the range of mean ± standard deviation of measured values. Statistical significance of differences between means was evaluated by analysis of variance (ANOVA) using a General Linear Model with pairwise comparisons by Tukey's method ($P<0.05$).

## 2.3 Results and Discussion

### 2.3.1 Proximate analysis and response surface model factor level selection

Following proximate analysis, the purpose of these preliminary studies was to determine if ACE-inhibitory hydrolysate, of *in vitro* potency comparable to literature values for other unfractionated hydrolysates ($IC_{50}$ <1.00 mg hydrolysate/mL; Matsui and others 1993; Jeon and others 1999; Hyun and Shin 2000; Pihlanto-Leppala and others 2000; Kim and others 2001; Kim and others 2004; Vermeirssen and others 2005), could be generated from PHF. Secondly, commercial protease and hydrolysis variable ranges to use for further study by RSM were to be selected based on yield and ACE-inhibitory activity of hydrolysates generated. Results of the proximate analysis of Pacific hake used in this study, shown in Table 2.2, are in accordance with the reference values reported in the Nutrient Data Laboratory database (USDA 2006). Lipid and protein contents are approximately 0.4% and 2.7% lower than the reference, however, this mass is substituted by higher moisture and ash content. Given that Pacific hake is a migratory species, possible differences in diet composition may have lead to differences in the proximate composition of the flesh.

As shown in Table 2.3, $IC_{50}$ values of <1.00 mg hydrolysate/mL and a hydrolysate yield of >80% (w/w solids basis) were achievable for unfractionated hydrolysate from Pacific hake mince using Alcalase® or Protamex®. However, Protamex® was chosen for further study because, compared to the other enzymes, it generated the most potent peptides in the least amount of time, it required the least amount of energy to reach optimal temperature, and it required the least amount of pH adjustment. Therefore, for the RSM study, the pH range was set at 5.8-7.2 to reflect Protamex® product specifications for activity of >60% at 40 °C (Novozymes 2004). The %E/S ratio range for was set at 1.00-3.20% with axial points of 0.25% and 3.95% to probe for ACE-inhibitory peptides generated at high levels of enzyme but also at more economical levels of enzyme. The hydrolysis time range was set at 35-125 minutes (± axial points) to include the decrease in ACE-inhibitory activity post 1 hour hydrolysis as shown in Table 2.3. In following the RSM design, a total of 20 hydrolysis trials were conducted, including 6 replicates at the centre point conditions of pH 6.5, 2.10% E/S, and hydrolysis time of 80 minutes (Table 2.4).

### 2.3.2 Response Surface Methodology - Extent of hydrolysis and ACE-inhibitory activity models

In keeping with the objective of assessing the effects of pH, %E/S, and hydrolysis time factors on the EH and ACE-inhibitory activity of resulting hydrolysate, as shown in Table 2.4, two response surface models were generated (Figures 2.2 and 2.3). By stepwise elimination, factors that had a significant effect on EH were 'time', '%E/S', 'time'$^2$, and '%E/S'$^2$ (Table 2.5). Given a 'square' model ($P<0.01$), the equation of the response surface is:

meq free amino/L =
(1.32 * 'time') + (47.2 * '%E/S') + (-0.00482 * 'time'$^2$) + (-6.36 * '%E/S'$^2$) - 17.2

[Equation 5]

By the same stepwise elimination process, factors which had a significant effect on the ACE-inhibitory activity of hydrolysate produced within the ranges of this RSM model included 'pH', 'time', '%E/S', 'time'$^2$, and '%E/S'$^2$ (Table 2.6). For the purposes of plotting Figure 2.3, pH was held at the centre value of 6.5 since, compared to the other variables of time and %E/S, it influenced ACE-inhibitory activity to the least extent. The equation of the response surface using a 'square' model ($P<0.01$) is:

% ACE-inhibitory activity (in the presence of 200 μg hydrolysate solids/mL assay) =
(-6.08 * 'pH') + (0.388 * 'time') + (17.9 * '%E/S') + (-0.00145 * 'time'$^2$)
+ (-2.56 * '%E/S'$^2$) + 24.7

[Equation 6]

The significant lack-of-fit term in this model, as shown in Table 2.6, indicates that the equation of the response surface may not accurately predict the true ACE-inhibitory activity in some regions of the model (Myers and Montgomery 2002; Minitab 2006b). This can be caused by a low standard deviation of the centre samples tested compared to the true variance of samples produced at more extreme conditions. It is also possible that a higher order model is required to simultaneously fit all responses in all regions. The response of the hydrolysate produced at the axial point conditions of pH 6.5, time 4 minutes, and %E/S 2.10 was deemed an outlier by a standardized residual analysis value of –3.2, indicating that response predictions in

this region of the model are not reliable. However, the power to predict true responses that lie on the quadratic response surface, that is, in all other regions of the model, is very good given the $r^2$ value of 88.4%.

Looking at the response surface model equations, the coefficients for hydrolysis time and %E/S are positive in the prediction of EH, meaning that increasing hydrolysis time and amount of enzyme added would result in a greater number of peptide bonds cleaved, as is expected. Similarly, %ACE-inhibitory activity is predicted to increase with higher levels of %E/S and longer hydrolysis times, and in addition, by more acidic pH hydrolysis condtions within the range tested. However, it is important to note as well that both models show a plateau in the 3.20-3.80% E/S and 120-150 minute ranges indicated by the significance of the square terms, where both EH and ACE-inhibitory activity of the hydrolysate produced are predicted to be maximized. Beyond these conditions, ACE-inhibitory peptides are possibly being generated then destroyed due to extended hydrolysis time, or protease at high concentration cleaves at sites that do not facilitate ACE-inhibitory activity. Similar results were found by Kim and others (2001) and Kim and others (2004) in which prolonged hydrolysis of bovine blood plasma beyond 6 hours with Alcalase®, Neutrase®, or Pronase E®, and corn gluten beyond 8 hours with Flavourzyme®, respectively, resulted in a decrease in ACE-inhibitory activity. It is therefore likely unnecessary to employ hydrolysis times beyond those of the RSM model plateau for production of ACE-inhibitory hydrolysate from PHF when using Protamex®. In a study by van der Ven and others (2002), however, the RSM model included '$pH'^2$ as significantly affecting ACE-inhibitory activity of whey protein hydrolysates produced using Corolase PP® (a proteolytic enzyme prepartion from pig pancreas), rather than '$\%E/S'^2$. It is possible that Protamex®, as a commercial mixture of exopeptidases and endoproteases from multiple microbial sources, has a more robust activity at non-physiolgical pH conditions than Corolase PP®. Rather than a maximum ACE-inhibitory activity, increases in pH within the ranges of this study have resulted in a continued increase in ACE-inhibitory activity of the hydrolysates produced.

### 2.3.3 Model confirmation and assessment of starting material variability

In order to confirm the models' predictions, five confirmation conditions, as shown in Figure 2.3, were chosen to verify the centre point (pH 6.5, 2.10% E/S, 80 minutes), the optimal region (pH 6.5, 3.00% E/S, 125 minutes and pH 6.5, 3.50% E/S, 140 minutes) and the outer regions (pH 6.5, 1.00% E/S, 25 minutes and pH 6.5, 3.95% E/S, 155 minutes). Two points in the optimal region were chosen specifically to verify that increased enzyme and time within this range would not result in a significant change in ACE-inhibitory activity. At the same time, effect of starting material variability on ACE-inhibitory activity of end-product was studied by using mince from four batches differing in either *Kudoa paniformis* spore count or catch date (Table 2.1).

As shown in Table 2.7, no significant difference ($P>0.05$) in ACE-inhibitory activity was found between batches at any of the five hydrolysis conditions. Predictions of extent of hydrolysis and ACE-inhibitory activity were confirmed for 14 of the 20 samples, and 18 of the 20 samples, respectively. Given that the samples which do not confirm the ACE-inhibitory activity model occur for hydrolysis conditions in the outer regions of the response surface (1.00% E/S, 25 minutes and 3.50% E/S, 140 minutes), and that other samples in that group are also higher and lower than predicted, respectively, it is possible that it is the prediction itself that is either slightly underestimated or overestimated due to the significant lack-of-fit term discussed above. Still, the samples produced at pH 6.5, %E/S 1.00, and 25 minutes, all measured an extent of hydrolysis lower than predicted. In this region, the model fails to accurately predict the effects of very low enzyme and hydrolysis time on extent of hydrolysis. This is not surprising as hydrolysis progresses at a falling logarithmic rate, therefore, an RSM focusing in on lower ranges of process variables would be useful in more accurately predicting extent of hydrolysis in this region of steeply changing responses.

Nonetheless, since means of hydrolysate ACE-inhibitory activity for all three plateau points were not significantly different ($P>0.05$), increases in %E/S and hydrolysis time beyond 3.0% and 125 minutes, respectively, were confirmed to not significantly improve hydrolysate ACE-inhibitory activity. Hydrolysates from all three hydrolysis conditions, however, were still considered for further study to determine possible differences in their activity after UF separation.

### 2.3.4 Effect of ultrafiltration on $IC_{50}$ value and amino acid profile

As shown in Figure 2.4, UF to <10 kDa significantly improved the ACE-inhibitory activity of hydrolysates compared to both unfractionated hake hydrolysate and PeptACE® ($P<0.05$) by approximately 70% and 58%, respectively. Comparatively, the decrease in $IC_{50}$ after filtration to <10 kDa found for cod frame hydrolysate and bovine albumin hydrolysate were only 10% and 20%, respectively (Jeon and others 1999; Hyun and Shin 2000). As such, UF to 10 kDa is particularly advantageous to PHF hydrolysates produced at the conditions of this study. Further, a significant trend of decreasing $IC_{50}$ values (increasing ACE-inhibitory potency) is seen as longer peptides are removed (Figure 2.4), indicating that the smaller molecular weight peptides are likely responsible for the ACE-inhibitory activity in each filtrate. However, given that the trend is quite weak, either all filtrates contain mainly low molecular weight peptides, or ACE-inhibitory peptides exist in all the molecular weight ranges so their removal does not result in a drastic overall change in activity. The former is in accordance with the results of ACE-inhibitory peptide isolation studies, which have found that the most active peptides contain 5 peptides or less (Li and others 2004; Meisel and others 2006), but the reason cannot be elucidated fully until the molecular weight profiles of these hydrolysates are determined.

Further UF treatment beyond 10 kDa would not be warranted in a commercial process for PHF hydrolysates since only an approximate 10% reduction in the $IC_{50}$ value is achieved with each successive filtration. Not only would this be very costly, filtration to <1 kDa has been found to reduce yield to less than 9% (w/w dry basis) of unfractionated hydrolysate mass in the case of bovine albumin hydrolysates produced using Alcalase® (Hyun and Shin 2000). At the optimized hydrolysis conditions employed in this study, ultrafiltration at a 10 kDa cut-off resulted in a maximum hydrolysate yield loss of only 5.4%, indicating that commercial UF treatment at 10 kDa is a reasonable option since ACE-inhibitory activity is also significantly enriched (Table 2.8).

Also shown in Figure 2.4, there was no significant difference in ACE-inhibitory activity found within filtrate groups, except for the <500 Da filtrate of hydrolysate produced at the high enzyme/long time condition. At this condition hydrolysis may have continued to an extent where small active peptides were getting destroyed. Therefore, within the ranges tested, there is no advantage, even if the hydrolysate is going to be further processed by UF, to employ

hydrolysis conditions greater than 3.00% E/S and 125 minutes in order to maximize ACE-inhibitory activity.

Table 2.9 summarizes the amino acid profiles of unfractionated hake hydrolysate, its UF fractions, the commercial product PeptACE®, and unhydrolysed fillet. PHF hydrolysate has an AA profile comparable to PeptACE® and all samples are most abundant in the acidic or amide-containing residues (D/N, E/Q), followed by A, L, G, K, and V respectively. These profiles are also comparable to ACE-inhibitory peptides cited in reviews in which, among other residues, K is common as penultimate to the C-terminus, L and V are common at the N-terminus of di- and tri-peptides, and hydrophobic residues are common at each of the 3 C-terminal positions of oligopeptides (Li and others 2004; Meisel and others 2006). Less bulky residues in the middle position of tripeptides, such as A and G, have also been noted as favorable by a quantitative structure-activity modeling study Pripp and others (2004). Further, separation by UF did not grossly alter the AA profile of the hydrolysates meaning that either peptides in all molecular weight ranges contain similar AA profiles, or that the majority of the peptides fall below the 1 kDa molecular weight range. The latter is more likely since, as discussed above, subsequent UF treatment did not result in a strong improvement in ACE-inhibitory activity either. In addition, peptides that increased in proportion in the <1 kDa filtrate include K, A, L, and F, which are all common residues of ACE-inhibitory peptides. The proportion of residues K, A, L, G, and F are also notably higher in the <1 kDa filtrate than was determined for the unhydrolysed hake fillet (in bold, Table 2.9), and than is reported for Pacific hake (whiting) muscle (Benjakul and Morrisey 1997), meaning that there is a 'concentration' of ACE-inhibitory activity-associated residues in processed hydrolysates. It is impossible however, to quantitatively correlate this AA profile to ACE-inhibitory activity since the individual ACE-inhibitory peptide sequences found in PHF hydrolysate are not yet known.

Ultrafiltration also resulted in an increase in non-peptide materials compared to peptide mass. Specifically, as shown in Table 2.9, proportion of ash increased and peptide decreased per unit solids as hydrolysate was filtered to lower molecular weight cutoffs. However, since the fractions were collected as aliquots of filtrate and not as retentates, all filtrates contained the same concentration of salt. Therefore, % ash was increasing only in proportion to a decrease in peptide yield, rather than due to a concentration effect on the salt by UF, as reflected in the consistent sum of approximately 65% per unit hydrolysate mass. A comparison of ash contents

in hydrolysate solids from fish fillet sources has not been otherwise reported in the literature. As a result it is essential to consider % peptide content when expressing $IC_{50}$ on a peptide versus solids basis, since on a peptide basis (i.e. the active portion) the $IC_{50}$ concentration will be comparatively lower.

In summary, hydrolysis time, %E/S, and pH, are all significant factors determining the ACE-inhibitory activity of Pacific hake hydrolysates produced with Protamex® commercial protease. Condition combinations beyond pH 6.5, 3.00% E/S, and 125 minutes did not significantly improve hydrolysate activity meaning these conditions are the most economical while still maximizing ACE-inhibitory activity. Consistent end-product quality is achievable as hydrolysate ACE-inhibitory activity was not significantly affected by starting material variability in terms of catch date or *K. paniformis* level. Finally, UF to <10 kDa significantly decreased the $IC_{50}$ value to 44 ± 7 μg peptides/mL, meaning Pacific hake has potential as a commercially competitive source of ACE-inhibitory peptides.

## 2.4 Tables

**Table 2.1 Ranges of catch dates, lengths, masses, and *Kudoa paniformis* spore counts of Pacific hake used to create experimental batches.**

| Experimental batch | Catch dates | Number of fish | Length range (cm) | Body weight range (g) | *K. paniformis* spore count [1] |
|---|---|---|---|---|---|
| Preliminary | 2004-05-15 | 30 | 46-56 | 661-992 | $10^5$-$10^7$ |
| 1 | 2004-07-03 | 6 | 45-56 | 518-966 | $10^6$ |
| | 2004-07-05 | 6 | | | |
| | 2004-07-07 | 4 | | | |
| | 2004-07-26 | 7 | | | |
| | 2004-08-21 | 5 | | | |
| | 2004-08-28 | 3 | | | |
| | 2004-08-30 | 5 | | | |
| | 2004-09-04 | 5 | | | |
| | 2004-09-11 | 3 | | | |
| | 2004-09-18 | 4 | | | |
| | 2004-09-22 | 4 | | | |
| 2 | 2004-06-12 | 8 | 45-56 | 550-997 | $10^6$ |
| | 2004-06-16 | 4 | | | |
| | 2004-06-19 | 3 | | | |
| | 2004-06-21 | 8 | | | |
| | 2004-06-23 | 15 | | | |
| | 2004-06-26 | 15 | | | |
| | 2004-06-28 | 8 | | | |
| 3 | 2005-05-06 | 2 | 48-52 | 630-821 | $10^6$ |
| | 2005-05-11 | 2 | | | |
| | 2005-05-17 | 1 | | | |
| | 2005-05-18 | 6 | | | |
| 4 | 2005-05-09 | 1 | 47-54 | 555-803 | $<10^4$ |
| | 2005-05-11 | 1 | | | |
| | 2005-05-14 | 3 | | | |
| | 2005-05-17 | 3 | | | |
| | 2005-05-23 | 2 | | | |

[1] *Kudoa paniformis* spore count (spores / g fish tissue) across all fish of all catch dates within an experimental batch

**Table 2.2** **Proximate analysis of raw Pacific hake (whiting, *Merluccius productus*) fillet[1] received from Steveston Seafood Direct Ltd. (Richmond, BC) as compared to values for 'Whiting, mixed species, raw' as found in the Nutrient Data Laboratory database** (USDA 2006)

| | **Measured [2]** | **Database values** |
|---|---|---|
| pH | 6.86 ± 0.02 | not given |
| Moisture | 82.50 ± 0.14% | 80.27% |
| Ash | 1.47 ± 0.25% | 1.30% |
| Lipid | 0.91 ± 0.02% | 1.31% |
| Crude protein | 15.6 ± 0.3% | 18.31% |
| Carbohydrate | n/a | 0.00% |
| Sum | 100.5% | 101.19% |

[1] From preliminary batch fillet as described in Table 2.1.

[2] All analyses performed in triplicate. Data presented as mean ± standard deviation.

**Table 2.3 Summary of hydrolysate yield, extent of hydrolysis, and ACE-inhibitory activity of hydrolysate generated by commercial proteases in a 1:2 Pacific hake mince-water slurry [1]**

| Sample | Hydrolysis time (minutes) | % Yield [2] | Extent of hydrolysis [3] (meq free amino groups/L) | $IC_{50}$ [4] (mg solids/mL) |
|---|---|---|---|---|
| **Alcalase 2.4L® 2% E/S** | 0 | 35.3 | 6.4 ± 0.1 | No inhibition effect |
| (55 °C, pH 7.5) | 10 | 66.8 | 107 ± 4 | 1.96 |
| | 60 | 77.0 | 182 ± 12 | 1.47 |
| | 240 | 86.0 | 260 ± 8 | 1.00 |
| | 1440 | 99.7 | 380 ± 35 | 0.70 |
| **Alcalase 2.4L® 4% E/S** | 0 | 29.4 | 13 ± 1 | No inhibition effect |
| (55 °C, pH 7.5) | 10 | 73.9 | 136 ± 13 | 1.76 |
| | 60 | 83.1 | 178 ± 11 | 1.45 |
| | 240 | 91.0 | 232 ± 0 | 1.21 |
| | 1440 | 100. | 302 ± 29 | 1.17 |
| **Flavourzyme 500L® 2% E/S** | 0 | 27.0 | 13 ± 0.1 | No inhibition effect |
| (50 °C, pH 7.0) | 10 | 43.2 | 44.1 ± 1.4 | >4.00 |
| | 60 | 51.2 | 72.5 ± 2.6 | >4.00 |
| | 240 | 62.5 | 125 ± 4 | 3.62 |
| | 1440 | 75.8 | 336 ± 3 | >4.00 |
| **Flavourzyme 500L® 4% E/S** | 0 | 26.1 | 13 ± 0.2 | No inhibition effect |
| (50 °C, pH 7.0) | 10 | 47.6 | 65.9 ± 0.3 | >4.00 |
| | 60 | 59.1 | 120 ± 2 | 3.36 |
| | 240 | 68.6 | 170 ± 2 | 3.19 |
| | 1440 | 79.8 | 331 ± 2 | >4.00 |
| **Protamex® 2% E/S** | 0 | 22.0 | 11 ± 1 | No inhibition effect |
| (40 °C, pH 6.2) | 10 | 53.7 | 66.9 ± 9.2 | 1.23 |
| | 60 | 62.3 | 104 ± 6 | 1.84 |
| | 240 | 72.9 | 160 ± 3 | 2.23 |
| | 1440 | 87.0 | 331 ± 11 | 2.33 |
| **Protamex® 4% E/S** | 0 | 21.4 | 13 ± 0.6 | No inhibition effect |
| (40 °C, pH 6.2) | 10 | 59.8 | 78.7 ± 5.7 | 1.60 |
| | 60 | 81.3 | 115 ± 11 | <1.00 |
| | 240 | 82.2 | 171 ± 9 | 2.22 |
| | 1440 | 100. | 246 ± 3 | 2.06 |

[1] Hydrolysates produced using preliminary batch fillet as described in Table 2.1

[2] Hydrolysate yield calculated based on mince solids basis

[3] meq free amino groups measured in slurry supernatant; results expressed as mean ± standard deviation of assay triplicates.

[4] $IC_{50}$ values calculated from a regression line though assays carried out at 4 peptide concentrations; Alcalase 2.4L® 2% and 4% based on a range of 0.1-2.0 mg hydrolysate solids/mL, Flavourzyme 500L® 2%, 4%, and Protamex® 2% on a range of 0.1-4.0 mg solids/mL, and Protamex® 4% on a range of 1- 4 mg hydrolysate solids/mL added to the assay.

**Table 2.4** **Response surface methodology central composite design factor levels for 20 hydrolysis trials and their corresponding responses for extent of hydrolysis and %ACE-inhibitory activity [1]**

| Standard Order | Run Order | pH | Time | %E/S | Extent of Hydrolysis (meq free amino groups/L slurry) [2] | %ACE-inhibitory activity [2] |
|---|---|---|---|---|---|---|
| **Corner Points** | | | | | | |
| 1 | 12 | 5.8 | 35 | 1.00 | 79.0 ± 1.6 | 20.9 ± 5.4 |
| 2 | 9 | 7.2 | 35 | 1.00 | 74.3 ± 0.9 | 14.7 ± 1.3 |
| 3 | 16 | 5.8 | 125 | 1.00 | 117 ± 2 | 29.1 ± 2.0 |
| 4 | 11 | 7.2 | 125 | 1.00 | 107 ± 3 | 18.2 ± 7.1 |
| 5 | 15 | 5.8 | 35 | 3.20 | 111 ± 3 | 37.1 ± 1.8 |
| 6 | 3 | 7.2 | 35 | 3.20 | 107 ± 1 | 24.5 ± 2.4 |
| 7 | 19 | 5.8 | 125 | 3.20 | 154 ± 4 | 43.3 ± 1.9 |
| 8 | 8 | 7.2 | 125 | 3.20 | 159 ± 2 | 38.6 ± 4.1 |
| **Axial Points** | | | | | | |
| 9 | 20 | 5.3 | 80 | 2.10 | 114 ± 3 | 37.0 ± 2.7 |
| 10 | 5 | 7.7 | 80 | 2.10 | 116 ± 6 | 22.9 ± 4.5 |
| 11 | 2 | 6.5 | 4 | 2.10 | 46.6 ± 0.3 | 4.51 ± 3.01 [3] |
| 12 | 14 | 6.5 | 156 | 2.10 | 146 ± 10 | 42.2 ± 3.2 |
| 13 | 13 | 6.5 | 80 | 0.25 | 56.8 ± 0.6 | 9.04 ± 1.67 |
| 14 | 17 | 6.5 | 80 | 3.95 | 148 ± 5 | 36.8 ± 1.1 |
| **Centre Point Replicates** | | | | | | |
| 15 | 1 | 6.5 | 80 | 2.10 | 128 ± 0.6 | 33.7 ± 4.7 |
| 16 | 4 | 6.5 | 80 | 2.10 | 146 ± 2 | 33.4 ± 4.8 |
| 17 | 6 | 6.5 | 80 | 2.10 | 121 ± 1 | 31.1 ± 3.2 |
| 18 | 7 | 6.5 | 80 | 2.10 | 129 ± 5 | 33.6 ± 3.5 |
| 19 | 10 | 6.5 | 80 | 2.10 | 136 ± 1 | 36.2 ± 3.8 |
| 20 | 18 | 6.5 | 80 | 2.10 | 129 ± 4 | 35.4 ± 2.5 |

[1] Hydrolysates produced from Batch 1 fillet as described in Table 2.1

[2] Results are the means ± standard deviation of triplicate assays.

[3] Outlier data point in model (standardized residual > ± 3)

**Table 2.5** **Regression coefficients, p-values, and other significance statistics of the model for the prediction of extent of hydrolysis of hydrolysates from Pacific hake fillet**

| | Coefficient | *p*-value |
|---|---|---|
| **Hydrolysis factor** | | |
| intercept | -17.2 | 0.207 |
| 'time' | 1.32 | 0.000 |
| '%E/S' | 47.2 | 0.000 |
| 'time' $^2$ | -0.00482 | 0.002 |
| '%E/S' $^2$ | -6.36 | 0.011 |
| **Statistics of model signifcance** | | |
| $r^2$ | 0.918 | -- |
| 'linear' regression | -- | 0.000 |
| 'square' regression | -- | 0.002 |
| lack-of-fit | -- | 0.140 |

**Table 2.6** **Regression coefficients, p-values, and other significance statistics of the model for the prediction of %ACE-inhibitory activity of hydrolysates from Pacific hake fillet**

| | Coefficient | *p*-value |
|---|---|---|
| **Hydrolysis factor** | | |
| intercept | 24.7 | 0.000 |
| 'pH' | -6.08 | 0.003 |
| 'time' | 0.388 | 0.000 |
| '%E/S' | 17.9 | 0.000 |
| 'time' $^2$ | -0.00145 | 0.022 |
| '%E/S' $^2$ | -2.56 | 0.016 |
| **Statistics of model significance** | | |
| $r^2$ | 0.884 | -- |
| 'linear' regression | -- | 0.000 |
| 'square' regression | -- | 0.010 |
| lack-of-fit | -- | 0.014 |

**Table 2.7 Comparison of extent of hydrolysis and %ACE-inhibitory activity of Pacific hake hydrolysates from different mince batches to model predictions at selected hydrolysis conditions within the RSM models**

| Hydrolysis Conditions | | | | meq /L slurry | | %ACE-inhibitory activity[1] | |
|---|---|---|---|---|---|---|---|
| pH | %E/S | Time (min.) | Batch[2] | Experimental[3] | Predicted | Experimental[3] | Predicted |
| 6.5 | 1.00 | 25 | 1 | 48.0 ± 0.7[a] | 53.5 | 11.7 ± 5.0 | 9.3 |
| | | | 2 | 50.1 ± 1.3[a] | | 11.1 ± 1.5 | |
| | | | 3 | 47.5 ± 3.2[a] | | 15.3 ± 2.5[a] | |
| | | | 4 | 49.9 ± 1.0[a] | | 12.9 ± 6.2 | |
| 6.5 | 2.10 | 80 | 1 | 126 ± 5 | 128 | 32.9 ± 2.9 | 33.2 |
| | | | 2 | 131 ± 4 | | 33.9 ± 1.2 | |
| | | | 3 | 129 ± 11 | | 32.7 ± 4.8 | |
| | | | 4 | 124 ± 5 | | 29.3 ± 8.8 | |
| 6.5 | 3.00 | 125 | 1 | 160 ± 4 | 156 | 39.9 ± 1.1 | 41.6 |
| | | | 2 | 156 ± 6 | | 41.8 ± 4.0 | |
| | | | 3 | 157 ± 4 | | 40.3 ± 3.8 | |
| | | | 4 | 153 ± 4 | | 44.0 ± 7.3 | |
| 6.5 | 3.50 | 140 | 1 | 155 ± 6 | 159 | 42.2 ± 3.6 | 42.3 |
| | | | 2 | 161 ± 6 | | 39.6 ± 7.5 | |
| | | | 3 | 161 ± 6 | | 38.8 ± 4.1 | |
| | | | 4 | 160 ± 7 | | 35.9 ± 7.9[a] | |
| 6.5 | 3.95 | 155 | 1 | 164 ± 4[a] | 158 | 39.8 ± 5.1 | 41.2 |
| | | | 2 | 163 ± 3[a] | | 40.8 ± 1.6 | |
| | | | 3 | 155 ± 5 | | 42.2 ± 2.5 | |
| | | | 4 | 159 ± 5 | | 39.9 ± 6.5 | |

[1] %ACE-inhibitory activity determined at 200 μg hydrolysate solids/mL assay

[2] Pacific hake mince batches as described in Table 2.1

[3] Results expressed as mean ± standard deviation of triplicate assays; values denoted by 'a' do not confirm the model prediction.

**Table 2.8** **Summary of Pacific hake hydrolysate % yield as a function of ultrafiltration with molecular weight cut-off at 10 kDa** [1]

| Hydrolysis Conditions | | | % Yield | |
|---|---|---|---|---|
| pH | time (min.) | %E/S | >10 kDa | <10 kDa |
| 6.5 | 125 | 3.00 | 5.6 | 94.4 |
| 6.5 | 140 | 3.50 | 4.6 | 95.4 |
| 6.5 | 155 | 3.95 | 0.1 | 99.9 |

[1] Hydrolysates produced from pooled fillet batches 1, 2, 3, and 4 (Table 2.1)

**Table 2.9** **Sample of amino acid profiles, % mass of amino acids to hydrolysate solids, and % ash of commercial product PeptACE®, unfractionated Pacific hake hydrolysates and its ultrafiltrates (produced at pH 6.5, 3.00% E/S, time 125 minutes), and unhydrolysed Pacific hake fillet [1]**

| | PeptACE® | Unfractionated Hydrolysate | <10 kDa filtrate | <1 kDa filtrate | Unhydrolysed Pacific hake fillet |
|---|---|---|---|---|---|
| Amino acid recovered | % abundancy [2] | % abundancy | % abundancy | % abundancy | % abundancy |
| **Hydrophobic** | | | | | |
| Gly (G) | 8.9 | 8.7 | 8.3 | 9.1 | 7.8 |
| Ala (A) | 10.7 | 10.8 | 10.6 | 12.7 | 9.4 |
| Val (V) | 6.4 | 6.0 | 6.0 | 6.0 | 6.3 |
| Leu (L) | 8.2 | 9.3 | 9.6 | 10.6 | 8.2 |
| Ile (I) | 4.4 | 4.3 | 4.5 | 4.5 | 4.9 |
| Phe (F) | 2.1 | 2.4 | 2.7 | 3.3 | 2.9 |
| **Polar** | | | | | |
| Ser (S) | 5.2 | 5.7 | 5.5 | 6.2 | 5.6 |
| Thr (T) | 4.5 | 4.4 | 4.5 | 5.7 | 5.2 |
| Pro (P) | 4.4 | 4.0 | 3.7 | 2.3 | 4.1 |
| Tyr (Y) | 2.4 | 2.2 | 2.3 | 2.0 | 2.9 |
| Met (M) | 2.6 | 2.8 | 2.9 | 3.0 | 1.7 |
| **Acidic/Amidic** | | | | | |
| Asx (D) + (N) | 11.0 | 10.3 | 10.1 | 7.8 | 11.2 |
| Glx (E) + (Q) | 15.2 | 14.3 | 14.2 | 12.3 | 15.8 |
| $NH_3$ | 1.3 | 0.6 | 0.7 | 0.9 | 0.6 |
| **Basic** | | | | | |
| Arg R | 5.2 | 6.2 | 6.1 | 4.3 | 5.7 |
| Lys (K) | 5.3 | 6.7 | 7.2 | 8.3 | 6.3 |
| His (H) | 3.5 | 1.9 | 1.9 | 1.9 | 2.0 |
| **% amino acids to hydrolysate solids (g/100g)** [3] | 61.2 | 54.7 | 53.7 | 38.6 | N/A |
| **% ash to hydrolysate solids (g/100g)** | 4.2 | 11.6 | 12.8 | 24.3 | N/A |

[1] Hydrolysates produced from pooled fillet batches 1, 2, 3, and 4 (Table 2.1)

[2] Calculated as % moles of total moles amino acids detected

[3] Without Trp or Cys

## 2.5 Figures

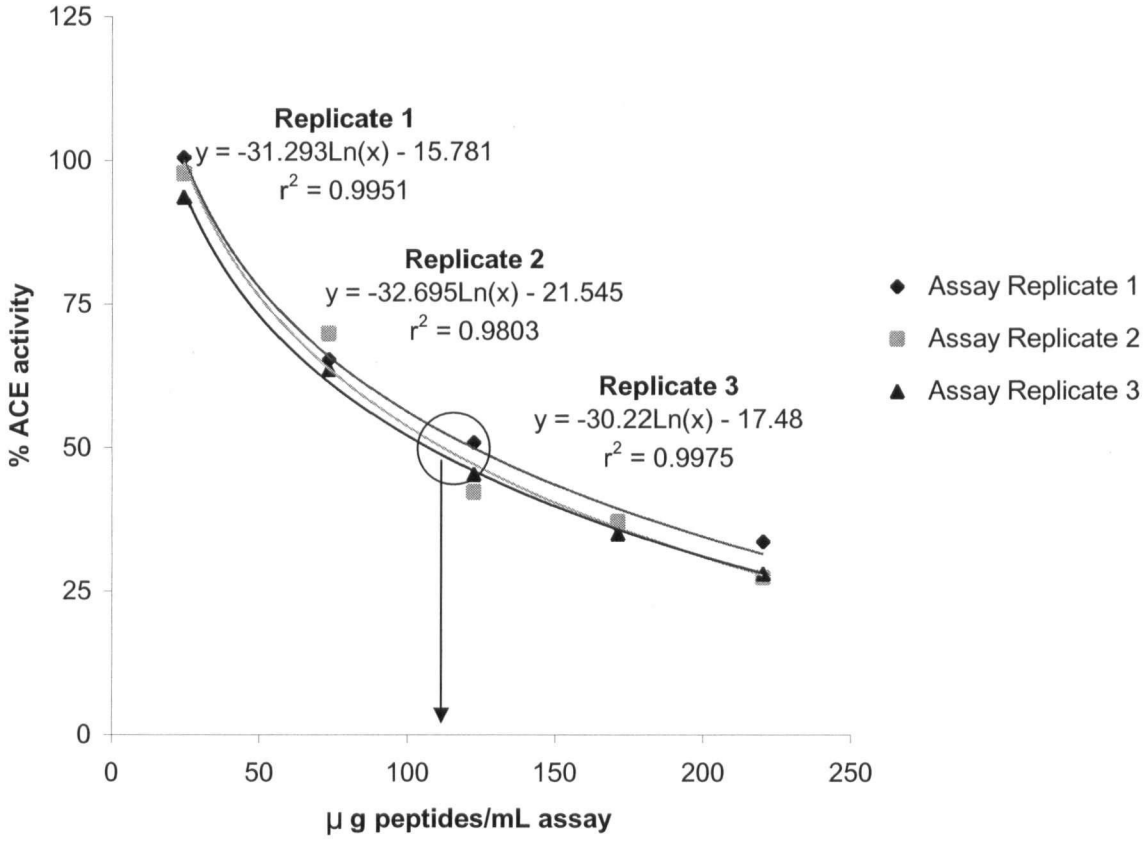


**Figure 2.1 - Example of $IC_{50}$ determination[1] by regression analysis**

[1] Results for PeptACE® commercial product

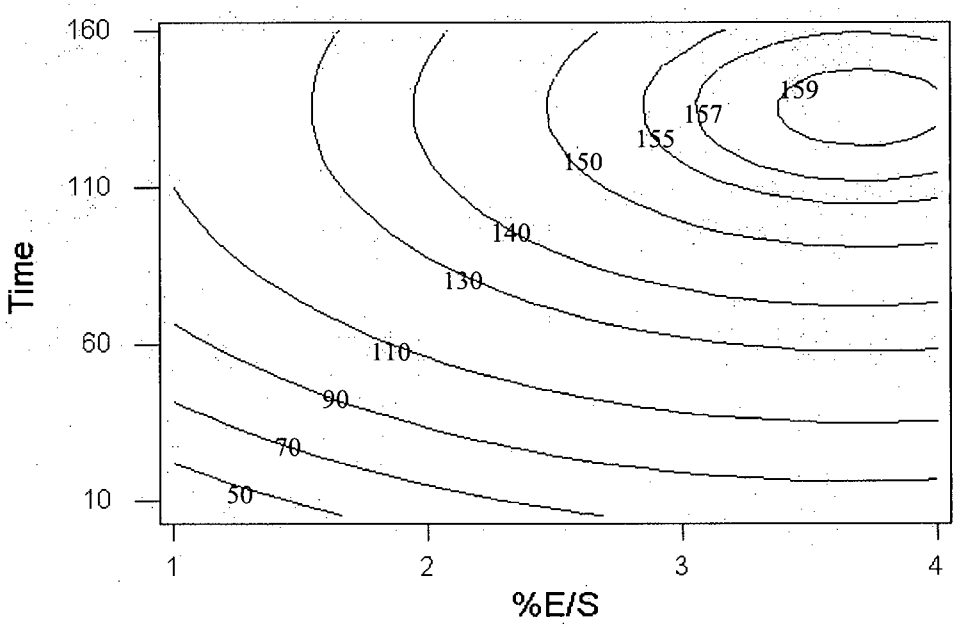


**Figure 2.2** **Response surface contour plot of extent of hydrolysis of Pacific hake hydrolysates[1] (expressed as milliequivalents free amino groups/L slurry) as a function of hydrolysis time (minutes) and enzyme-to-substrate ratio (%)[2]**

[1] Hydrolysates produced from Batch 1 fillet as detailed in Table 2.1

[2] pH held at 6.5

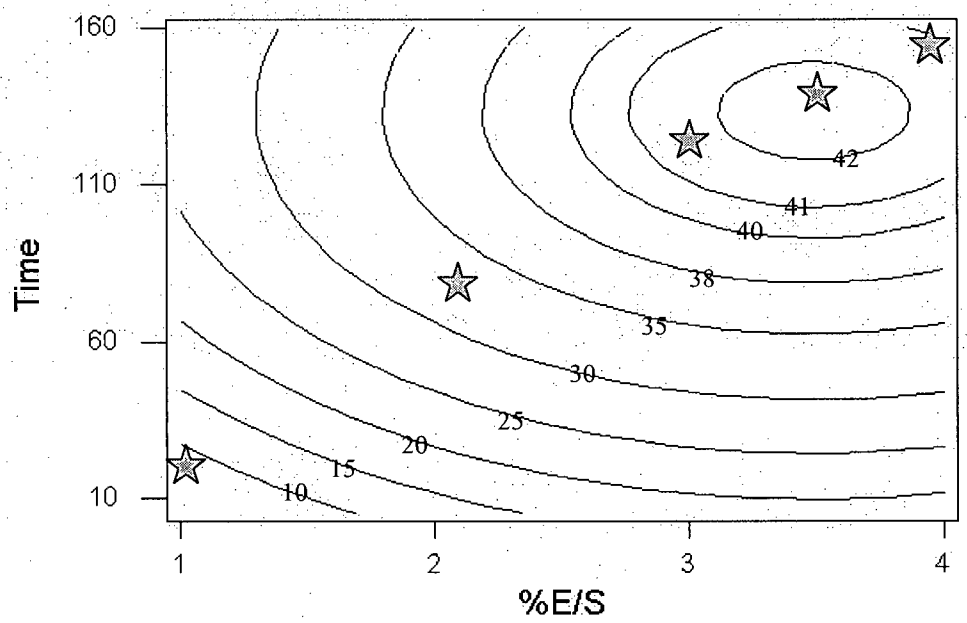


**Figure 2.3** **Response surface contour plot of ACE-inhibitory activity of Pacific hake hydrolysates[1] (expressed as % ACE inhibition at 200 µg hydrolysate solids/mL) as a function of hydrolysis time (minutes) and enzyme-to-substrate ratio (%)[2]**

[1] Hydrolysates produced from Batch 1 fillet as detailed in Table 2.1

[2] pH held at 6.5; stars indicate condition combinations chosen for model verification

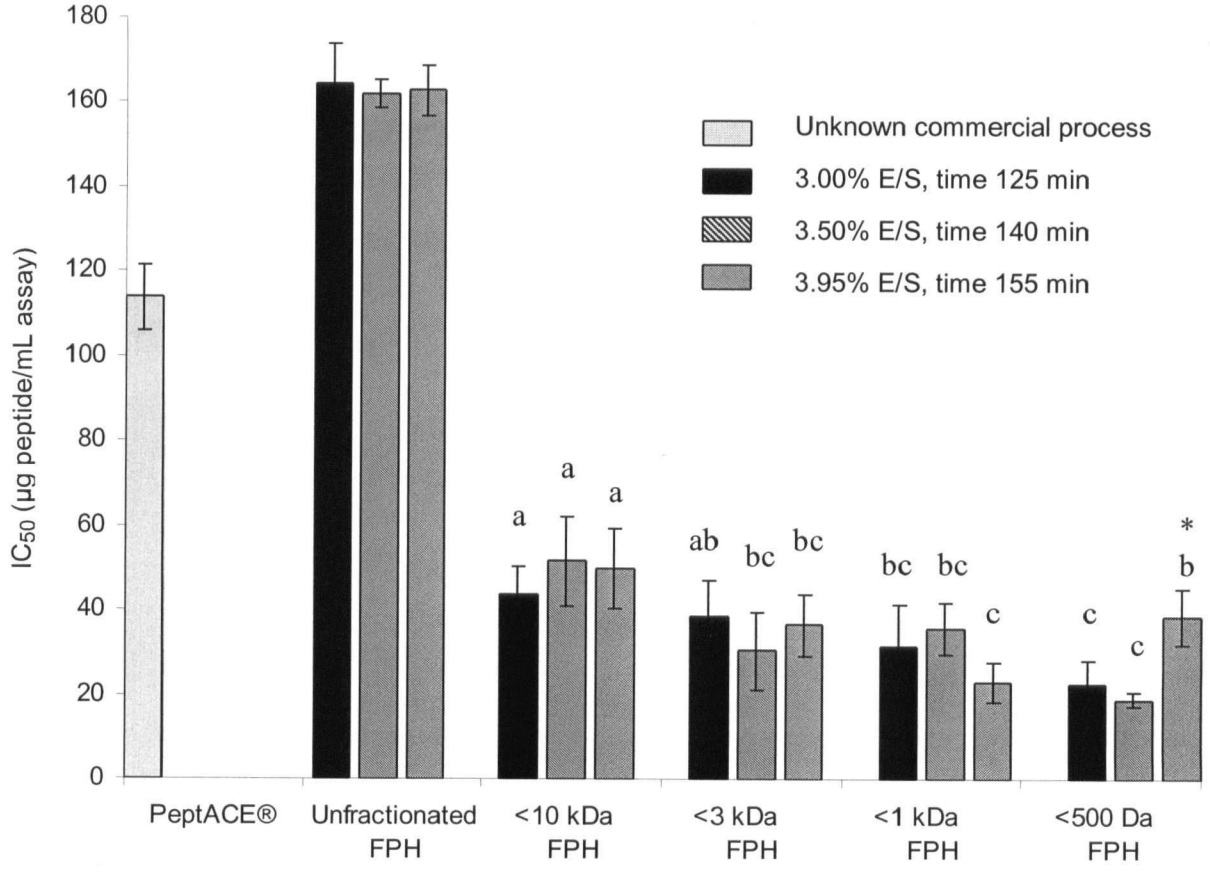


**Figure 2.4** **Comparison of ACE-inhibitory activity of PeptACE®, Pacific hake hydrolysate, and Pacific hake hydrolysate ultrafiltrates, produced at 3 optimal hydrolysis conditions** [1,2]

[1] Each bar represents the mean ± standard deviation of triplicate $IC_{50}$ values; bars with different letters within a hydrolysis condition group are significantly different ($P<0.05$), while the bar denoted by * is significantly different from others within the UF filtrate group

[2] Hydrolysates produced from pooled fillet batches 1, 2, 3, and 4 (Table 2.1)

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# CHAPTER III Investigations into Angiotensin I-Converting Enzyme Inhibitor Type and Mode of Pacific Hake Fillet Hydrolysate [2]

## 3.1 Introduction

One of the greatest challenges in developing nutraceutical and functional food products is proving the *in vivo* efficacy of its bioactive components. Animal studies and clinical trials are costly and require strict ethical consideration, so compounds thought to have positive bioactive potential must first be pre-screened by *in vitro* assay. Such compounds include angiotensin I-converting enzyme (ACE) inhibitory peptides of food protein hydrolysates, which act to lower blood pressure by preventing the formation of the vasoconstrictory agent angiotensin II and potentiating activity of the vasodilator bradykinin (Meisel and others 2006). Many protein hydrolysates and their isolated peptides have shown promising *in vitro* ACE-inhibitory activity, including those from casein, whey, chicken, bovine, wheat, corn, and fish sources (Li and others 2004).

However, not all ACE-inhibitory peptides may be effective in lowering blood pressure *in vivo*. For example a digest of dried bonito by pepsin-trypsin-chymotrypsin did not significantly lower blood pressure in the spontaneously hypertensive rat (SHR) model despite a potent *in vitro* ACE-inhibitory activity $IC_{50}$ of 41 μg/mL (Fujita and others 1995). On the other hand, at an oral dose of 1 mg peptide/kg body weight, PPK, with a relatively low *in vitro* potency of $IC_{50}$ >1000 μM, exerted a significant antihypertensive effect at 6 hours post-administration ($P<0.01$), while TNP, with an $IC_{50}$ of 207.4 μM, exerted a small but non-significant effect (Nakashima and others 2002). In addition, Captopril® (D-3-mercapto-2-methylpropanoyl-L-proline, Rubin and Antonaccio 1980), a peptide analog ACE-inhibitory drug, has demonstrated higher activity potency *in vitro*, yet the effects of some ACE-inhibitory peptides and Captopril® are comparable *in vivo* (Fujita and Yoshikawa 1999; Jung and others

[2] A version of this chapter will be submitted for publication. Cinq-Mars, C.D. and Li-Chan, E.C.Y. Evaluation of the angiotensin I-converting enzyme inhibitory activity of Pacific hake fillet hydrolysate after *in vitro* pepsin-pancreatin digestion. J. Agric. Food Chem.

2006). Therefore, it can be concluded that ACE-inhibitory peptides can be modified before reaching the target ACE molecules in the blood stream, which in turn may affect their *in vivo* efficacy.

Potential sites of ACE-inhibitory peptide modification by hydrolysis include the gastrointestinal (GI) tract and, given that ACE itself is a dipeptidase, the blood stream. Results of simulated GI digestion on soy protein isolate (Lo and others 2006) and pea protein (Vermeirssen and other 2003) showed that time spent in stomach and duodenum digestion phases significantly affects ACE-inhibitory $IC_{50}$ values of hydrolysates. In terms of isolated peptides, ACE-inhibitory oligopeptides, namely IWHHT and IVGRPRHQG from dried bonito, have been shown to increase in activity upon digestion with chymotrypsin and trypsin, while others, including YAEERYPIL and RADHPFL from ovalbumin hydrolysate, decreased in activity upon incubation with pepsin and pancreatin (Fujita and others 2000; Miguel and others 2006). Also, the ACE-inhibitory activity of hydrolysates from fermented milk products has been shown to not significantly change after simulated GI digestion (Hernandez-Ledesma and others 2004a). As such, both hydrolysates and individual ACE-inhibitory peptides are now being classified in the literature as 'pro-drug type', 'true-drug type', or 'substrate type' based on their increased, unchanged, or decreased ACE-inhibitory activity after simulated GI digestion, respectively.

This classification also applies to the effects on peptide ACE-inhibitory activity by incubation with ACE, where peptides that are hydrolysed by ACE to peptides of lower ACE-inhibitory activity are not considered true inhibitors, but rather as ACE substrates. 'True-drug' peptides and the products of 'pro-drug' peptides, upon contact with ACE, can be further classified according to 'inhibitor mode' as non-competitive or competitive inhibitors based on an inhibitor kinetics study and the Lineweaver-Burk plot (Li and others 2004). For example, in studies by Hernandez-Ledesma and others (2003) and Wu and others (2006), respectively, the $K_m$ value of ACE substrate was unchanged in the presence of ACE-inhibitory peptides from fermented whey (characteristic of a non-competitive inhibitor) while the inhibition activity of ACE-inhibitory peptide LRW isolated from pea protein was eventually overcome by increasing levels of ACE substrate (characteristic of a competitive inhibitor). As such, non-competitive inhibitors may exude a more steady acute antihypertensive effect *in vivo* as their inhibition activity is not affected by ACE substrate concentration.

To summarize, a useful way to predict *in vivo* efficacy, thereby deciding the need for further *in vivo* study, is to subject ACE-inhibitory hydrolysates to processes that simulate the potential sites of ACE-inhibitory peptide activity modification by hydrolysis. Therefore, the objectives of this study were to assess the effects of (1) simulated GI (sequential pepsin/pancreatin) digestion and (2) pre-incubation with ACE on the $IC_{50}$ values, and respective molecular weight profiles, of Pacific hake fillet (PHF) hydrolysate. To simulate potential activity in the blood stream, inhibitory mode of samples after GI digestion and ACE pre-incubation was also determined.

## 3.2 Materials and Methods

### 3.2.1 Materials

Freeze-dried hydrolysate powder, from the hydrolysis of PHF by Protamex® commercial protease, was obtained from the previous study and had been prepared as described in section 2.2.3 at the specific conditions of pH 6.5, enzyme-to-substrate (E/S) ratio 3.00%, and hydrolysis time 125 minutes, and had been fractionated by ultrafiltration as described in section 2.2.6. All hydrolysate samples were stored at –25° C.

Sodium bicarbonate (product S233-500) was purchased from Fisher Scientific Co. (Ottawa, ON). Pepsin (3300 U/mg, product P6887) and pancreatin (converts 25x weight of casein in 1 hr, product P7545) were purchased from Sigma-Aldrich Canada (Oakville, ON). All other materials used were as described in section 2.2.1.

### 3.2.2 *In vitro* pepsin-pancreatin simulated gastrointestinal digestion

Simulated GI digestion by *in vitro* pepsin-pancreatin hydrolysis was carried out in triplicate according to a developed method based on Hernandez-Ledesma and others (2004b) and Lo and Li-Chan (2005). Hydrolysate solids containing 225 mg peptides were mixed with 15 mL distilled-deionized water and pH adjusted to 2.0 with 5 N HCl. Pepsin was then added at an enzyme-to-substrate ratio of 1:35 w/w and incubated with shaking for 1 hour at 37° C. The pH was then adjusted to 5.3 with a saturated $NaHCO_3$ solution and further to pH 7.5 with 5 N NaOH. Pancreatin was then added at an enzyme-to-substrate ratio of 1:25 w/w and incubated with shaking for 2 hours at 37 °C. To terminate, the solution was submerged in boiling water for 10 minutes. The hydrolysate was freeze-dried and stored at –25° C.

### 3.2.3 ACE-inhibitory activity

In this study the effect of pre-incubation with ACE on the ACE-inhibitory activity of hydrolysate samples was investigated; therefore, the assay of Cushman and Cheung (1971), as described in section 2.2.5, was modified as follows. First, 30 μL of hydrolysate solution was mixed with 30 μL (2.5 mU) of ACE, or buffer as control, and incubated at 37° C for 1 hour. To

terminate pre-incubation ACE activity, samples were heated at 95 °C in boiling water for 5 minutes. After this pre-incubation step, 150 μL of 8.91 mM HHL and a fresh 30 μL (2.5 mU) aliquot of ACE was added and the solution was further incubated at 37° C for 1 hour. All assay components were dissolved in 0.025 M sodium tetraborate buffer containing 0.3 M NaCl (pH 8.3) for a total assay volume of 240 μL. HCl (250 μL, 1N) was used to terminate the reaction. In order to extract HA, 1.0 mL of ethyl acetate was added. The solutions were then vortexed for 30 seconds and centrifuged at 2000 x g for 5 minutes. After centrifugation, 0.7 mL of the ethyl acetate layer was removed into a 10 mm diameter clear glass tube and was evaporated by heating at 120°C for 30 minutes. The remaining HA residue was redissolved with 1.3 mL of distilled water and the absorbance read at 228 nm in a quartz cuvette. ACE-inhibitory activity with and without ACE pre-incubation was determined in triplicate.

### 3.2.4 MALDI-ToF mass spectrometry and amino acid analysis

MALDI-ToF (Matrix-assisted Laser Desorption/Ionization-Time of Flight) mass spectrometry analysis was conducted at the Laboratory of Molecular Biophysics Proteomics Core Facility, Michael Smith Laboratories, University of British Columbia (Vancouver, BC) using a Voyager-DE STR Workstation. A calibration matrix of α-cyano-4-hydroxycinnamic acid was used with an accelerating voltage of 20 000-25 000 V and acquisition mass range of 500-10,000 Da. Amino acid analysis of hydrolysate samples was carried out by the Advanced Protein Technology Centre at the Hospital for Sick Children (Toronto, ON) as described in section 2.2.7.

### 3.2.5 Study of inhibition kinetics

Creation of a Lineweaver-Burk plot for the determination of inhibition mode was carried out for hydrolysate samples according to the methodology proposed by Stauffer (1989). Activity of ACE in the assay was kept constant at 2.5 mU, while a range of concentrations of HHL in the final assay volume was set above and below the reported $K_m$ for HHL of 2.6 mM (Cushman and Cheung 1971), specifically, 12.5 mM, 4.63 mM, 2.50 mM, 1.71 mM, and 1.30 mM. Higher concentrations of HHL were not possible due to low solubility. Hydrolysate

samples were added at their respective $IC_{50}$ values (μg peptide/mL assay). ACE activity in the presence or absence of hydrolysate samples was determined using the assay for quantification of ACE inhibition as described in section 3.2.3 (Cushman and Cheung 1971; Lo and Li-Chan 2005, with modifications) and initial velocity ($V_o$) was taken as absorbance per minute at 228 nm over the total assay time of 1 hour. Dissociation constant ($K_i$) for hydrolysate samples was calculated by:

$$K_i \text{ value} = [\text{inhibitor}] / ((K_{app}/K_m) - 1.0) \qquad \text{[Equation 7]}$$

### 3.2.6 Statistical analysis

Using MINITAB version 12.21 (Minitab Inc., State College, PA), statistical significance of differences between means was evaluated by analysis of variance (ANOVA) using a General Linear Model with pairwise comparisons by Tukey's method ($P<0.05$).

## 3.3 Results and Discussion

### 3.3.1 Effect of *in vitro* pepsin-pancreatin simulated gastrointestinal digestion on ACE-inhibitory activity and molecular weight profile

In order to determine the 'inhibitor type' of the PHF hydrolysates, samples were first subjected to *in vitro* pepsin-pancreatin digestion. Hydrolysate samples were selected on the following basis: unfractionated hydrolysate to determine if digestion improves ACE-inhibitory activity, 10 kDa filtrate since it was the least 'intensive' UF treatment to show significant improvement in ACE-inhibitory potency (Chapter 2), 1 kDa filtrate to check if it is resistant to GI enzymes because of relatively short chain length, and PeptACE® in order to compare PHF hydrolysate to a commercial product.

As shown in Figure 3.1, after simulated GI digestion, unfractionated hydrolysate samples (PeptACE® and PHF hydrolysate) were significantly more inhibitory towards ACE, in that they had significantly lower $IC_{50}$ values, and therefore may be considered a 'prodrug-type'. Yet, PHF hydrolysate was still less potent than PeptACE®. On the other hand, simulated GI digestion lowered the ACE-inhibitory activity (increases the $IC_{50}$ value) of both ultrafiltrates, thus they can be considered 'substrate type'. As such, the <1 kDa fraction was not resistant to hydrolysis by GI enzymes. It is possible that since there were less large molecular weight peptides for the GI enzymes to act on as there were in the unfractionated samples, the enzymes may have been destroying the active low molecular weight peptides instead. Similar results were found by Wu and Ding (2002) in which the $IC_{50}$ value of <10 kDa soy protein hydrolysate increased from 65 μg protein/mL to 73 μg protein/mL upon *in vitro* pepsin-pancreatin digestion, although this change is less pronounced than for the PHF hydrolysate. In contrast, Hernandez-Ledesma and others (2004b) found that ACE-inhibitory activity of unfractionated whey infant formula hydrolysate decreased significantly at the intestinal stage of simulated GI digestion, while the activity of the <3 kDa filtrate remained constant. This underlines the need for assessing the effect of GI digestion on an individual case-by-case basis as protein sequence, hydrolysis process conditions, and commercial protease specificity all affect the hydrolysate produced, and in turn, the substrates and products of GI digestion. In the case of PHF hydrolysate produced using the process conditions of this study, the $IC_{50}$ value of the

unfractionated hydrolysate after simulated GI digestion was not significantly different from that of the <10 kDa filtrate, therefore UF treatment may not be necessary to produce potent ACE-inhibitory product.

It was hypothesized in the previous study as well that the ultrafiltrates contained peptides mainly in the low molecular weight range since sequential UF treatment from <10kDa to <1 kDa did not bring about drastic changes in $IC_{50}$ value or AA profile. Therefore, to test this hypothesis, molecular weight profiles were obtained by MALDI-ToF mass spectrometric analysis, and at the same time, information on the effect of simulated GI digestion on the molecular weight profile in relation to changes in $IC_{50}$ could be gathered. MALDI-ToF spectra for unfractionated PHF hydrolysate before and after simulated GI digestion, and for <10 kDa filtrate after simulated GI digestion are shown in Figures 3.2, 3.3, and 3.4, respectively. For comparison, MALDI-ToF spectra for the commercial product PeptACE® before and after simulated GI digestion are shown in Figures 3.5 and 3.6. A lower molecular weight limit of 500 Da exists for MALDI-ToF spectra due to interfering peaks attributed to the matrix.

Since few significant peaks were detected between 3.2 and 5 kDa, and no significant peaks were detected above 5 kDa, a common molecular weight range of 500 Da to 3.2 kDa for all spectra was selected for ease of comparison. The MALDI-ToF spectra confirm a high abundance of peptides in the < 1 kDa range for all samples tested, however, given that MALDI-ToF is a semi-quantitative analysis and that peak height is related to the ability of the species to desorb upon application of the laser, individual peptide concentrations as a proportion of the whole cannot be assigned. Similarly, peptides and proteins >10 kDa that may be present in the unfractionated PHF hydrolysate were not detected as significant peaks. Indeed, a % yield analysis of UF retentate obtained for PHF hydrolysate produced by Protamex®, as shown in Table 2.8, indicated that less than 6% of the total hydrolysate yield is greater than 10 kDa.

In comparing Figures 3.2 and 3.3, the unfractionated PHF hydrolysate sample before simulated GI digestion contains dominant peaks at approximately 1 kDa (~6-9 residues) and one at 588 Da (~4-5 residues), while the post-GI digestion sample contains two dominant peaks at 698 Da (~5-6 residues) and 849 Da (~6-8 residues) and the same peak at approximately 587 Da. As such, the improved ACE-inhibitory activity in the post-GI samples may be due to di- and tripeptides (<500 Da), these 3 detected peptides, or both. It is certain however, that GI digestion does change the molecular weight profile considerably, and with it the ACE-inhibitory

potency. Specifically, between the pre- and post-GI samples there is a loss of approximately 150 Da or 300 Da from the dominant peaks at 1 kDa. This corresponds to the cleavage of likely 1 or 2 amino acid residues, respectively, leaving the more ACE-inhibitory active product. This is also a positive outcome because smaller peptides are more likely to be absorbed by the small intestine by paracellular transport (Shimizu 1999). A study by Ruiz and others (2004) of simulated GI digestion on isolated ACE-inhibitory peptides from Manchego cheese also yielded dipeptide and single amino acid cleavage products. Using HPLC-MS/MS to identify the peptide fragments released by the action of GI enzymes, peptide VPSERYL was fully hydrolysed to Y, L, RY, VPSER, and YL, similarly resulting in an $IC_{50}$ value decrease.

Comparing Figures 3.3 and 3.4, since the <10 kDa filtrate is a subset of the unfractionated hydrolysate, the same dominant peaks remain after GI digestion (698 Da and 850 Da). Other common peaks exist as well (1283-1285 Da, 1510-1512 Da, 2296-2297 Da, 2721 Da). The commonality between these spectra is in accordance with their having the same $IC_{50}$, and is further evidence that UF processing of PHF hydrolysate is unnecessary due to modifications in the molecular weight profile, and ACE-inhibitory activity, during GI digestion.

Finally, Figures 3.5 and 3.6 show the MALDI-ToF spectra of the commercial ACE-inhibitory product PeptACE® before and after simulated GI digestion, respectively. The composition of PeptACE® is starkly different from the PHF hydrolysate with more than 6 significant peaks in a molecular weight range of 550-700 Da and 3 significant peaks at 834, 881, and 920, corresponding to peptides of approximately 4-6 residues; 2-3 less residues than the dominant peaks in the PHF hydrolysate sample. After simulated GI digestion of PeptACE® only two dominant peaks remain detectable below 1 kDa at 549 and 617 Da, indicating that many of the peptides contained in PeptACE® are subject to GI modification. This supports the observation of an overall 'pro-drug' activity. However, the peak at 617 Da occurs for the PeptACE® samples both before and after simulated GI digestion, indicating that the peptide is either resistant to GI modification, or more of this peptide is being formed by hydrolysis of larger peptides. Nonetheless, the amino acid sequence of the 617 Da peptide in the GI-digested PeptACE® sample compared to the sequence of the 698 and 850 Da peptides in the GI-digested PHF hydrolysate samples is a possible reason for their significant difference in ACE-inhibitory activity, although the additional contribution of peptides <500 Da to overall ACE-inhibitory activity is yet to be determined.

### 3.3.2 Effect of hydrolysate pre-incubation with ACE on ACE-inhibitory activity

Classification of ACE-inhibitory hydrolysates and their peptides into the three 'inhibitor types' also applies to the response of ACE-inhibitory peptides to contact (incubation) with ACE. In an attempt to ensure the hydrolysates in the assays were of the 'true inhibitor' type with respect to ACE prior to addition of HHL substrate, all assays performed thus far in these studies have been conducted with an ACE/peptide pre-incubation period. However, it was not yet known if the effect of pre-incubation with ACE differs between the different ultrafiltrates and the GI-digested samples.

As shown in Figure 3.7, results confirm that simulated GI digestion causes an increase in the ACE-inhibitory potency of PeptACE® and unfractionated PHF hydrolysate, but a decrease in the ACE-inhibitory potency of the UF fractions. With respect to pre-incubation with ACE, undigested <10 kDa PHF hydrolysate can be considered a 'substrate' type as there was a slight but significant decrease in ACE-inhibitory activity of the product. However, for *in vivo* efficacy it is more important that, after GI digestion, the ACE-inhibitory activity of the peptides is not significantly lowered, or is increased by contact with ACE in the blood stream. GI-digested PHF hydrolysate displayed the desired behaviour of a 'true-drug' type, as demonstrated by no significant change in the %ACE-inhibitory activity upon pre-incubation with ACE. These results therefore lend strong support for further *in vivo* study of the antihypertensive efficacy of PHF hydrolysate. The GI-digested PeptACE® was found to be a 'prodrug' type, since ACE-incubated samples showed a significantly higher %ACE-inhibitory activity. Peptides whose activity are increased with ACE pre-incubation have been shown to demonstrate a milder but more prolonged antihypertensive effect *in vivo*. For example LKPNM, shown to be cleaved *in vitro* by ACE to LKP, showed maximal decrease in blood pressure of SHR at 6 hours after oral administration, while LKP had a maximal effect at 4 hours, the lag period likely being attributed to *in vivo* conversion by ACE (Fujita and others 2000). Although not significant for the unfractionated PHF hydrolysate or ultrafiltrates as a whole (Figure 3.7), a higher ACE-inhibitory activity upon cleavage by ACE (i.e. 'pro-drug type') may exist for some of the peptides.

### 3.3.3 Inhibition mode of Pacific hake hydrolysate

Finally in order to determine the inhibition mode of the 'true-drug' type ACE-inhibitory hydrolysate samples as they would appear in the blood stream (i.e. the products of both GI digestion and exposure to ACE), PeptACE®, unfractionated PHF hydrolysate, and its <10 kDa filtrate after GI digestion and pre-incubation with ACE were used to construct a Lineweaver-Burk plot of inhibitor kinetics. While unfractionated PHF hydrolysate is the sample most practical for commercial production, the <10 kDa filtrate was also tested given their differing responses to simulated GI digestion in terms of ACE-inhibitory activity.

As shown in Figure 3.8, regression lines for the PeptACE® and <10 kDa PHF hydrolysate samples converge at the y-intercept with that for the reaction containing no inhibitor. This suggests a competitive inhibition by the hydrolysate samples, as a mixture of peptides, because inhibition can be overcome by increasing levels of substrate (Stauffer 1989). Wu and Ding (2002) also determined a competitive inhibition mode for <10 kDa hydrolysate of soy protein. However, simulated GI digestion of bonito protein has been shown to first produce competitive peptide substrates of ACE, and only after prolonged digestion at hours longer than physiological, are true inhibitors formed (Hasan and others 2006). This therefore emphasizes the essentiality of pre-hydrolysing proteins as sources of ACE-inhibitory peptides for commercial production, then, ideally allowing the GI tract to 'activate' the true inhibitory peptides. However, since the inhibition pattern for the GI digested unfractionated PHF sample did not follow the pattern of any conventional inhibition model (Stauffer 1989), with the regression curves of the sample and the control crossing before the y-axis, further interpretation was necessary to provide possible explanations as to its inhibition mode.

Constants $K_m$, $K_{app}$, and $K_i$ have been calculated for the HHL substrate, the HHL substrate in the presence of ACE-inhibitory hydrolyate, and the hydrolysate samples respectively, as shown in Table 3.1. The $K_m$ of 1.36 mM for HHL is less than 2.6 mM found by Cushman and Cheung (1971). However, this is likely because an incubation time of 1 hour has been used to calculate reaction velocity, rather than 30 minutes as used by Cushman and Cheung (1971), so the $V_{max}$ may have been suppressed by the average velocity over the entire hour. An hour incubation was required to allow the absorbance signal to be strong enough for accurate detection and the maximum HHL turnover over the hour was 8%.

In the presence of the <10 kDa PHF hydrolysate and PeptACE® samples, $K_{app}$ is greater than $K_m$ and $V_{app}$ is closely approaching $V_{max}$, which are characteristic of competitive inhibitors (Stauffer 1989). However, for the unfractionated PHF hydrolysate sample, $K_{app}$ is greater than $K_m$ but $V_{app}$ is greater than, rather than equal to, $V_{max}$. This behavior does not fit any conventional enzyme inhibitor patterns either, for example, non-competitive ($V_{app} < V_{max}$, $K_{app} = K_m$), partially non-competitive ($V_{app}$ approaching $V_{max}$, $K_{app} = K_m$), partially competitive ($V_{app}$ approaching $V_{max}$, $K_{app} > K_m$), or mixed ($V_{app} << V_{max}$ and $K_{app} > K_m$) (Stauffer 1989). No such inhibitory kinetics had yet been reported for ACE-inhibitory hydrolysates in the literature. However, given that the unfractionated PHF hydrolysate solution is a complex mixture of many different peptides, it not surprising that no clear net inhibition pattern was seen. Individual peptides may be acting as competitive, non-competitive, or mixed mode inhibitors, each with different affinities for the binding site. As a result, the data presented simply provides insight into the effect of ACE substrate concentration on net ACE-inhibitory activity, rather than a definitive classification of inhibition mode.

Finally, the $K_i$ of PeptACE® is lower than that of the <10 kDa PHF hydrolysate, meaning that ACE has a greater average affinity for the ACE-inhibitory peptides contained within the PeptACE® sample. This may be reflected in the higher %ACE-inhibitory activity of the PeptACE® samples after simulated GI digestion and pre-incubation with ACE (Figure 3.7). Unfortunately, a $K_i$ for the unfractionated PHF hydrolysate cannot be calculated since it is not a true competitive inhibitor, however its ACE-inhibitory activity is still closer to PeptACE® than the <10 kDa sample at 48% inhibition (Figure 3.7).

Overall, this study has demonstrated that, *in vitro*, unfractionated PHF hydrolysate exhibits a 'pro-drug' type ACE-inhibitory activity that is not significantly altered upon exposure to ACE. Exposure to pepsin-pancreatin simulated GI digestion changes the molecular weight profile to yield a peptide mixture with a lower overall $IC_{50}$ value of 90 μg peptides/mL assay. Given that no UF processing is needed to enrich the activity of the hydrolysate, this makes Pacific hake fillet an ideal source of ACE-inhibitory peptides for a nutraceutical or functional food product ingredient. However, true *in vivo* efficacy may only be speculated until *in vivo* studies are performed and the results of this study indicate that such studies are warranted.

## 3.4 Tables

**Table 3.1 Kinetics constants for HHL substrate and ACE-inhibitory hydrolysate samples, with ACE at 2.5 mU [1]**

| Inhibitor sample | $K_m$, or $K_{app}$ in the presence of inhibitor (µg HHL/mL assay) | $V_{max}$, or $V_{app}$ in the presence of inhibitor (abs. per min. at 228 nm) | Dissociation constant ($K_i$) (µg peptide/mL assay) |
|---|---|---|---|
| No peptide/ control | 1.36 | $1.06 \times 10^{-3}$ | -- |
| PeptACE® | 3.79 | $1.13 \times 10^{-3}$ | 35.8 |
| Unfractionated PHF hydrolysate | 4.88 | $1.41 \times 10^{-3}$ | N/A |
| < 10 kDa PHF hydrolysate | 2.37 | $1.00 \times 10^{-3}$ | 120 |

[1] $K_m$, $K_{app}$, $V_{max}$, and $V_{app}$ values based on the Lineweaver-Burk plot of Figure 3.8; $K_i$ calculated as

$K_i = [\text{inhibitor}] / ((K_{app}/K_m)-1.0)$

## 3.5 Figures

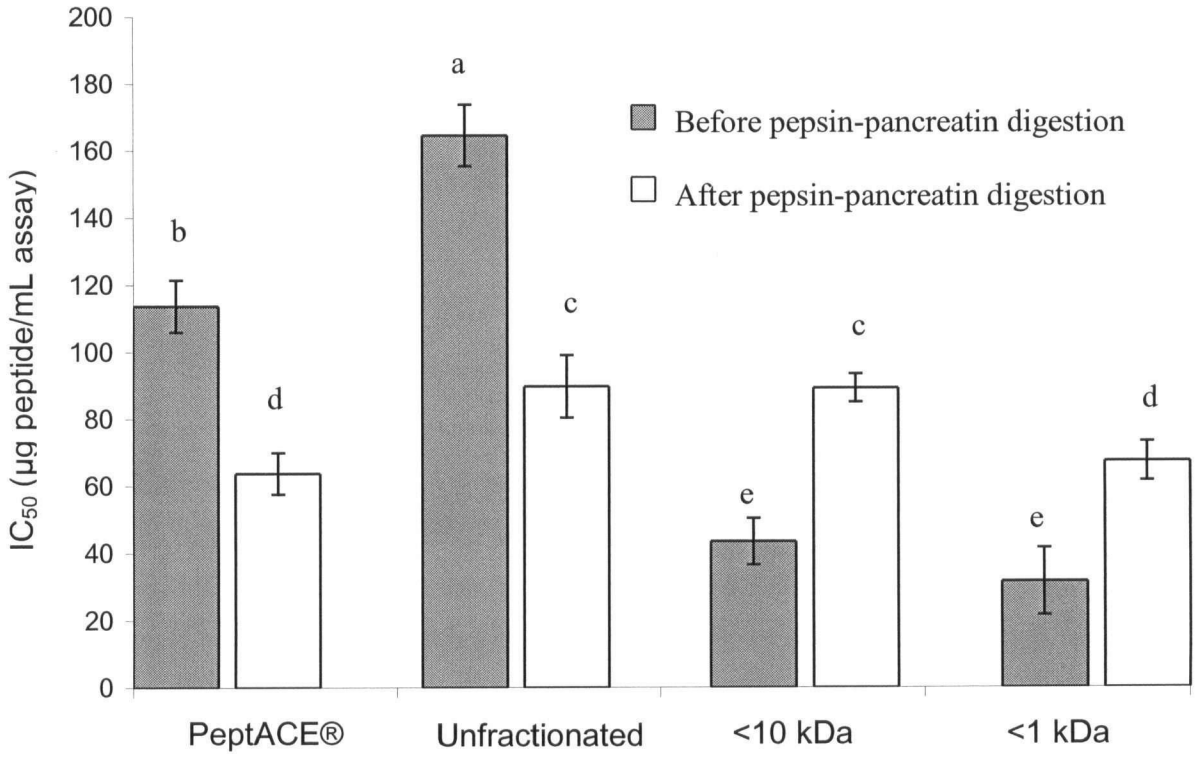


**Figure 3.1** **Comparison of ACE-inhibitory activity of PeptACE®, unfractionated Pacific hake fillet hydrolysate (hydrolysis conditions 6.5, 3.0%, 125 min), and its ultrafiltrates before and after simulated gastrointestinal (pepsin-pancreatin) digestion** [1]

[1] Each bar represents the mean ± standard deviation of triplicate $IC_{50}$ values; samples labeled with different letters are significantly different ($P<0.05$).

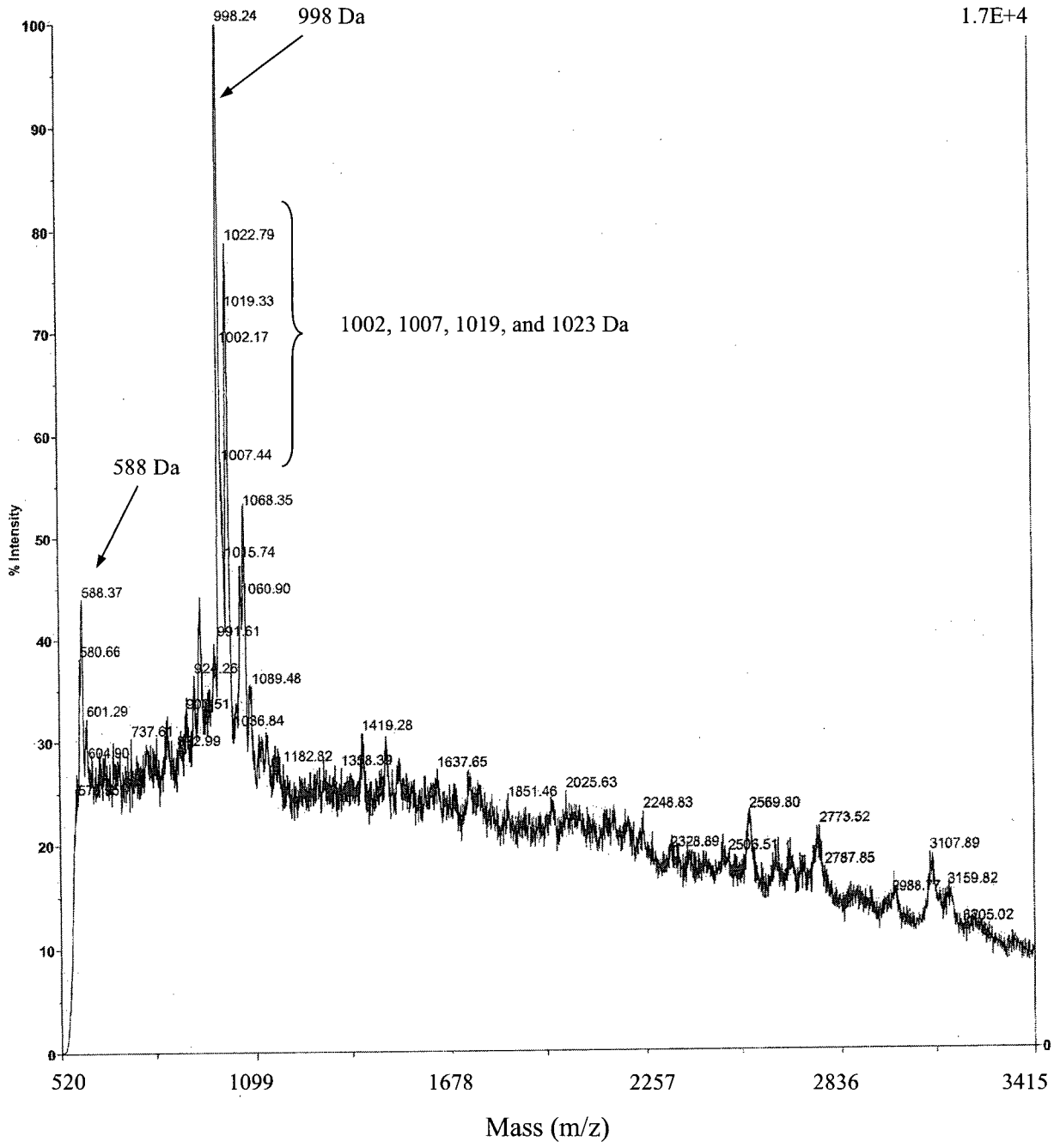


**Figure 3.2** **MALDI-ToF spectra of unfractionated Pacific hake fillet hydrolysate (from production conditions pH 6.5, 3.00% E/S, time 125 minutes) before simulated GI digestion**

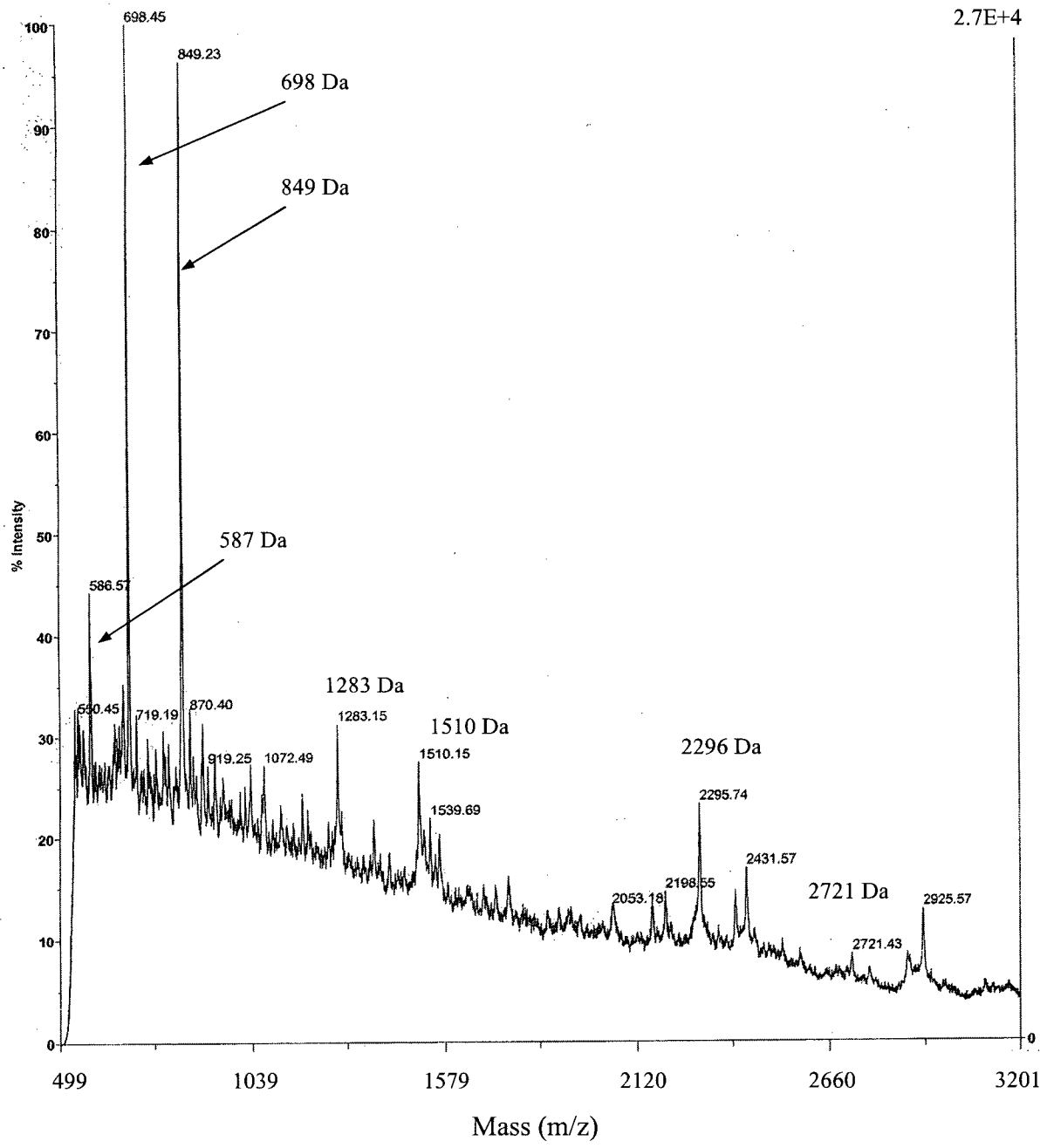


**Figure 3.3** **MALDI-ToF spectra of unfractionated Pacific hake fillet hydrolysate (from production conditions pH 6.5, 3.00% E/S, time 125 minutes) after simulated GI digestion**

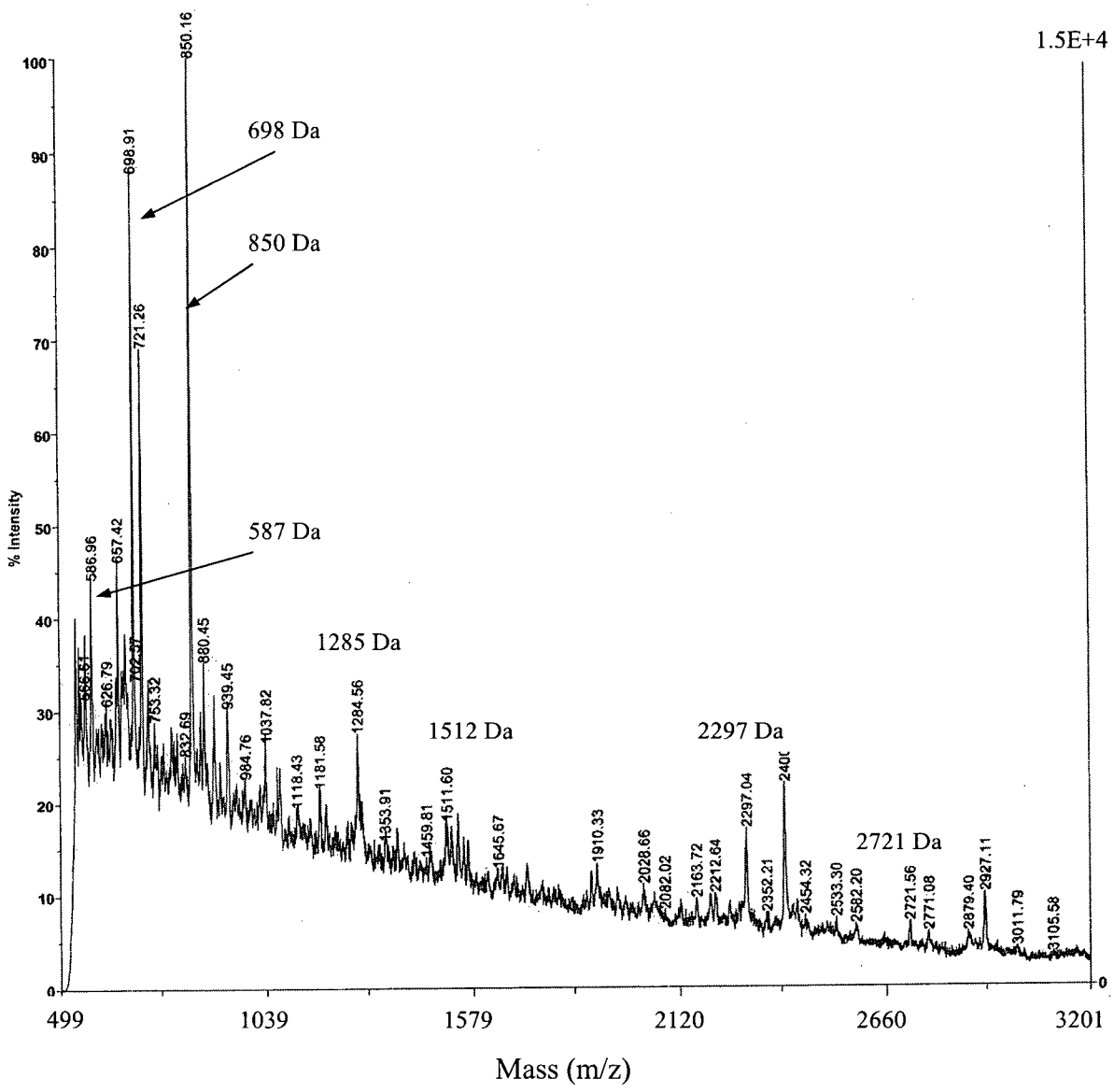


**Figure 3.4** **MALDI-ToF spectra of <10 kDa filtrate Pacific hake fillet hydrolysate (from production condtions pH 6.5, 3.00% E/S, time 125 minutes) after simulated GI digestion**

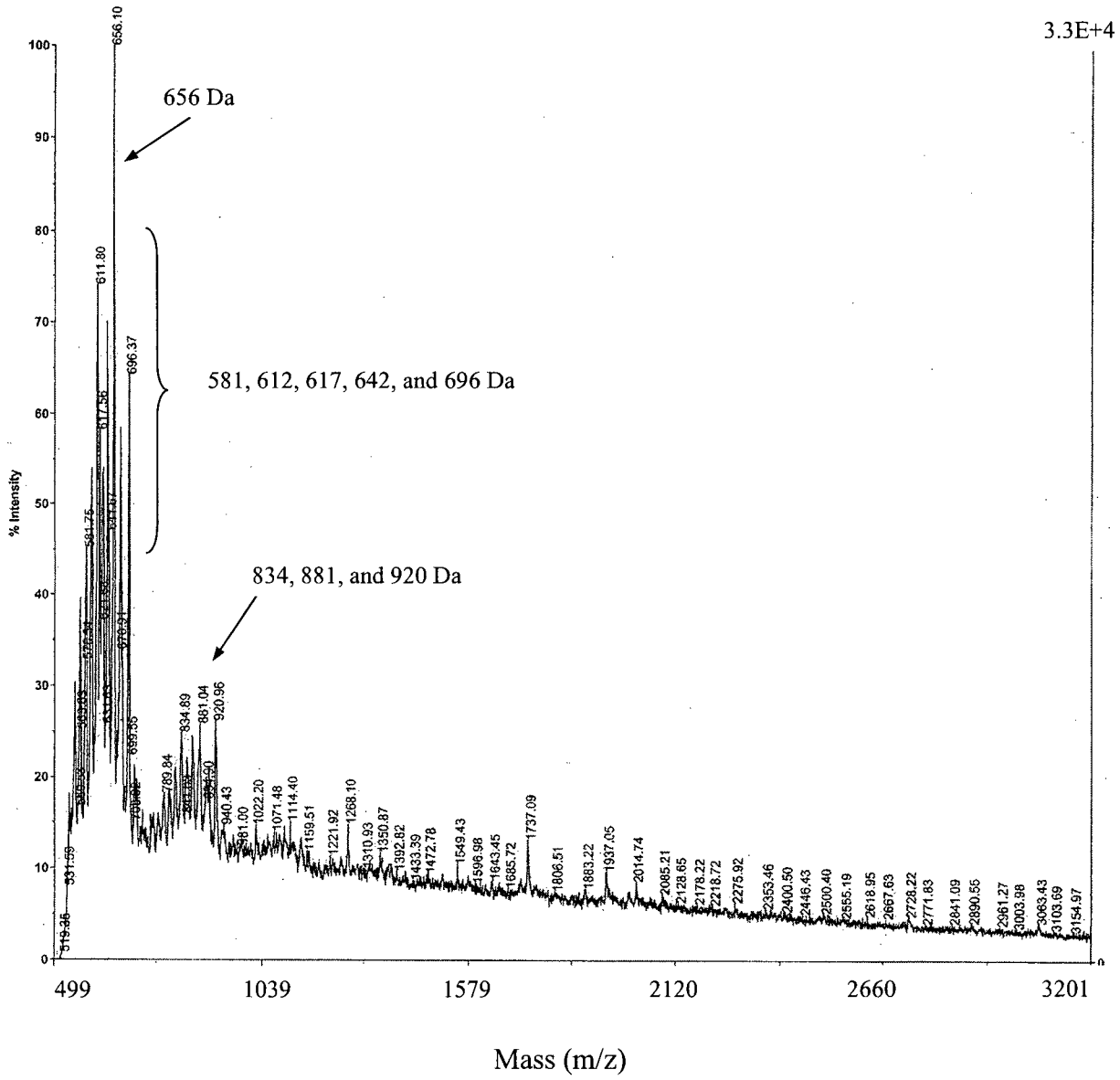


**Figure 3.5 MALDI-ToF spectra of unfractionated PeptACE® before simulated GI digestion**

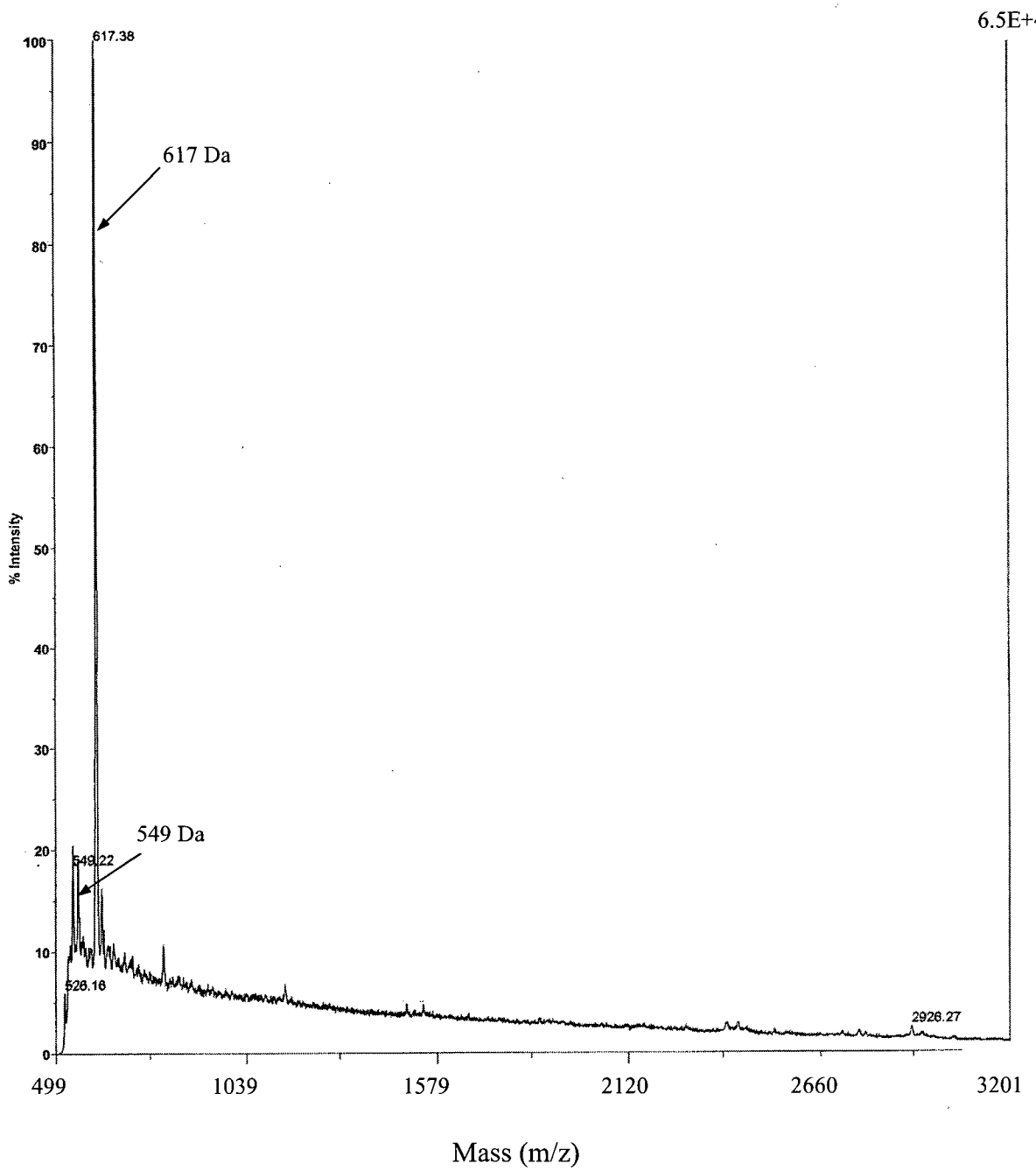


**Figure 3.6 MALDI-ToF spectra of unfractionated PeptACE® after simulated GI digestion**

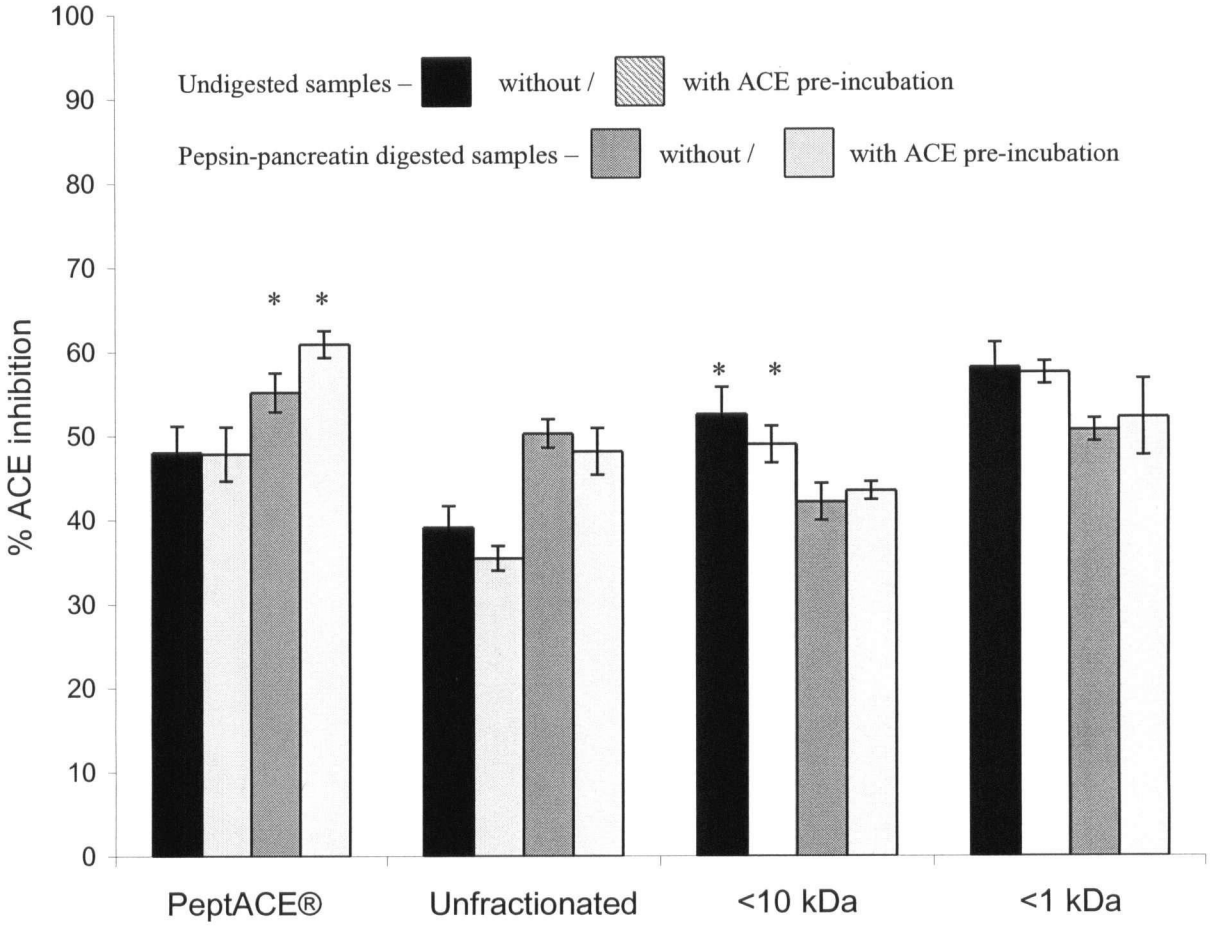


**Figure 3.7** **Effect of hydrolysate pre-incubation with ACE on the ACE-inhibitory activity of PeptACE®, unfractionated Pacific hake hydrolysate, and its ultrafiltrates (80 µg peptides / mL assay)** [1]

[1] Each bar represents the mean ± standard deviation of triplicate pre-incubation/without pre-incubation trials; bars of the same sample labeled with * are significantly different before and after pre-incubation with ACE ($P<0.05$).

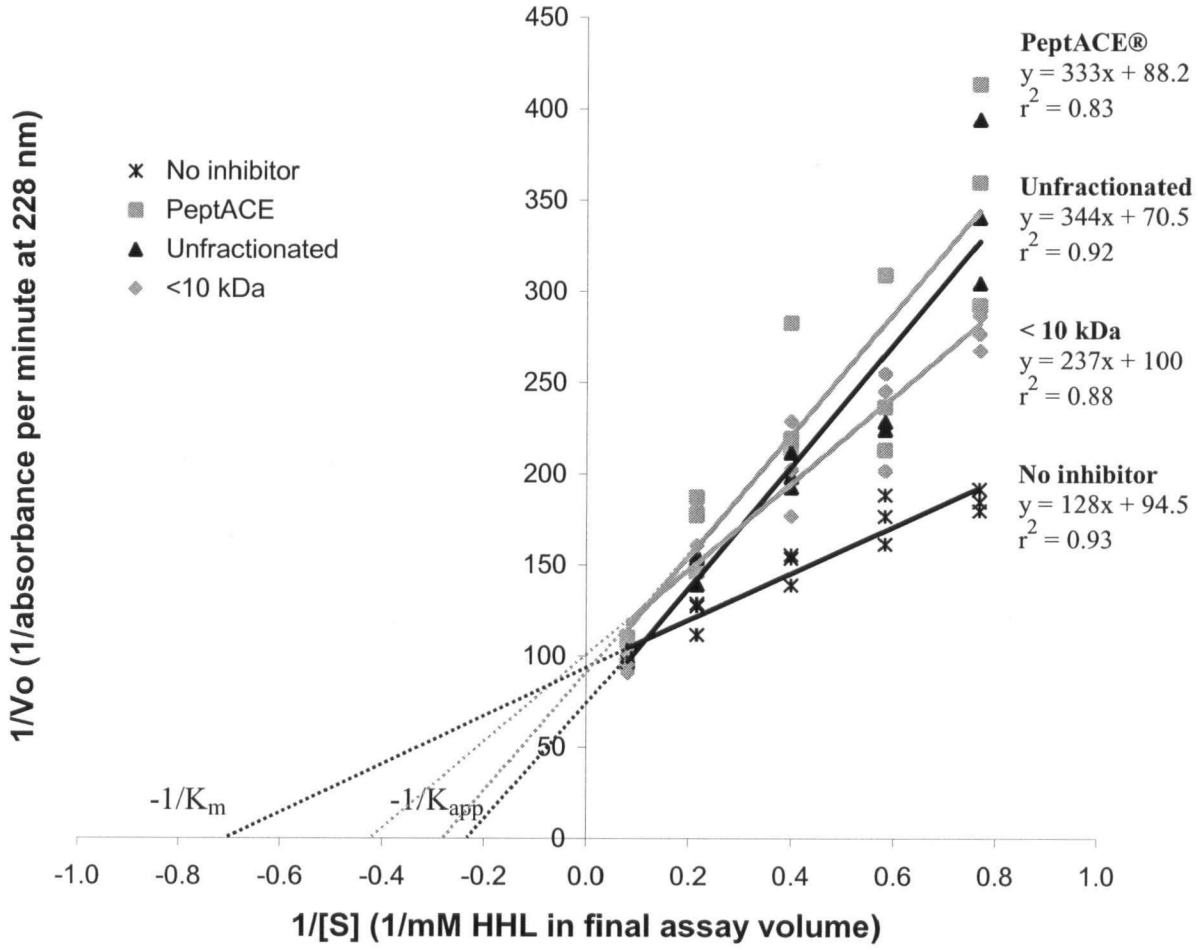


**Figure 3.8** **Lineweaver-Burk plot from triplicate assays of ACE activity in the presence of pepsin-pancreatin digested-ACE preincubated PeptACE® (64 µg peptide/ mL assay), unfractionated hake hydrolysate (90 µg peptide/mL assay), and <10 kDa hake hydrolysate (89 µg peptide/mL assay), at their respective $IC_{50}$ values**

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# CHAPTER IV Conclusion

## 4.1 Study findings and implications

Overall, the results of this thesis study have demonstrated that Pacific hake fillet (PHF) hydrolysate is a potential commercially competitive source of angiotensin I-converting enzyme (ACE) inhibitory peptides. Firstly, using Protamex® commercial protease, PHF hydrolysate with ACE-inhibitory activity comparable to other sources in the literature ($IC_{50}$ <200 μg peptides/mL) can be produced at conditions which minimize material and energy use compared to the conditions employed by other studies. For example, the optimal pH for Protamex® is 6.5 which is similar to the innate pH of the mince-water slurry (Novozymes 2004). This therefore reduces the need for pH adjustment compared to using Alcalase® or Pronase E® at pH 8 (Kim and others 2001), or CorolasePP® at pH 8-9 (van der Ven and others 2002). As well, Alcalase® and Pronase E® require a temperature of 50 °C, and CorolasePP® a temperature of 45 °C, while the optimal temperature for Protamex® is 40 °C, thereby reducing energy expenditure.

Further, enzyme addition and hydrolysis time beyond 3.0% (w/w substrate basis) and 125 minutes, respectively, did not result in improved ACE-inhibitory activity beyond $IC_{50}$ 165 ± 9 μg peptides/mL, therefore these conditions would be the maximum levels required in a commercial process. Comparatively, for an $IC_{50}$ of approximately 180 μg peptides/mL from corn gluten, Kim and others (2004) required 10% w/w substrate Flavourzyme® and a hydrolysis time of 8 hours. Conversely, Hyun and Shin (2000) using Alcalase® at a much lower enzyme addition level of 0.0005% w/w and a hydrolysis time of 4 hours, resulted in a significantly higher $IC_{50}$ value of 560 μg/mL. As such, the hydrolysis conditions and protease employed in this study achieve a balance between ACE-inhibitory activity and materials and energy used. However, it is not known if different enzyme-time combinations would have resulted in improved ACE-inhibitory potency in the latter studies, as process condition optimization was not performed. Finally, PHF variability in terms of catch date or *Kudoa paniformis* spore count did not affect ACE-inhibitory activity of hydrolysates produced, therefore implying that a commercial product of consistent quality would be attainable.

Another processing advantage of PHF hydrolysate as a source of ACE-inhibitory peptides is its behaviour upon simulated gastrointestinal (GI) digestion. While the ACE-inhibitory activity of ultrafiltered samples decreased after simulated GI digestion, that of the unfractionated hydrolysate increased, resulting in similar $IC_{50}$ values between the two samples and with the commercial product PeptACE®. Also reflecting the similar ACE-inhibitory activity after GI digestion were the molecular weight profiles of the PHF hydrolysate samples, as determined by MALDI-ToF mass spectrometry, in which the dominant peptide peaks in both samples were the same at 850 Da, 650 Da, and 587 Da. Therefore, since the net inhibitory activity of the unfractionated peptide mixture is 'pro-drug type', further fractionation processing is likely not necessary to produce a product with commercially competitive *in vivo* efficacy. Conducting an RSM study of commercial protease process factors in tandem with simulated GI-digestion may also help to identify further reductions in processing conditions and costs, while still attaining optimized ACE-inhibitory activity upon absorption. That is, a processing optimum may exist at lower %E/S and shorter time conditions than reported here, for which the hydrolysate produced would become 'activated' only after further hydrolysis by GI enzymes.

In addition to its activity as a 'pro-drug' inhibitor upon GI digestion, the net ACE-inhibitory activity of PHF hydrolysate was unaffected by pre-incubation with ACE, meaning that in the bloodstream it would likely act as a 'true-drug type'. On the same note, <10 kDa PHF hydrolysate acted predominantly as a competitive inhibitor, just as the peptide analogue drug Captopril® (Hernandez-Ledesma and others 2003). Yet it is still unknown if the active peptides in PHF hydrolysate would be absorbed in the intestine, how the presence of non-active peptides would affect absorption, and if ultrafiltration of the hydrolysate prior to digestion would affect either of these processes. In addition, all *in vitro* testing must be considered only as an estimation of what may happen *in vivo*, and antihypertensive efficacy can only be established by animal studies and subsequent clinical trials. For example, in the case of the commercial nutraceutical Katsuobushi Oligopeptide® from the thermolysin digest of dried bonito (Nippon Supplement 2006), the peptide LKP was found to have an *in vitro* ACE-inhibitory activity of only 8% compared to Captopril® (MW 217 g/mole), at $IC_{50}$ values of 0.3 and 0.022 μM, respectively (Fujita and Yoshikawa 1999). However, when administered orally in the spontaneously hypertensive rat model, LKP exhibited 91% of the antihypertensive activity compared to Captopril®, as compared by the moles/kg body weight required to

decrease systolic blood pressure by 10 mm Hg (an efficacy sufficient for the prevention of hypertension in borderline subjects) (Fujita and Yoshikawa 1999, Fujita and others 2001). As such, the results of this thesis study provide strong rationale for further investigation of ACE-inhibitory activity and antihypertensive efficacy of PHF hydrolysate *in vivo*.

## 4.2 Areas for further research and application

### 4.2.1 Study of absorption potential and other effects of the gastrointestinal tract

Before investing in animal studies or clinical trials, it would be helpful to first establish an *in vitro* estimation of PHF hydrolysate GI absorption and subsequent basolateral concentration. This has been performed using a Caco-2 cell culture monolayer in tandem with HPLC (Satake and others 2002) or by measuring ACE-inhibitory activity in the basolateral solution after a given time (Vermeirssen and others 2005). While this thesis study has focused on the effect of pepsin-pancreatin digestion on the ACE-inhibitory activity of PHF hydrolysate, activity of brush border peptidases may also further hydrolyze ACE-inhibitory peptides *in vivo*. Caco-2 cell culture study would allow these peptidases to interact with the hydrolysate sample prior to absorption. However, again, since Caco-2 cell cultures are known to have tighter intercellular junctions than intestinal cells *in vivo* (Vermeirssen and others 2004), these studies cannot replace the results of *in vivo* trials, and should therefore be viewed solely as rationale for further study.

### 4.2.2 Characterization of ACE-inhibitory peptides

As discussed, using unfractionated hydrolysate as a functional food ingredient is both economically advantageous from a production point of view, and allows for a greater margin in formulation than with the addition of isolated peptides. However, in order to be able to provide consumers with a standardized product, that is, with a known composition of active peptides and ACE inhibition modes, it is still important to characterize the peptides of highest ACE-inhibitory activity in the complex hydrolysate mixture. Since the most abundant peptides in the sample are not necessarily the most potent in ACE-inhibitory activity, affinity chromatography that selects for peptides preferably binding to ACE should be used. For example, using an anti-peptide antibody-coupled affinity column based on the ACE-inhibitory peptide antigen PTHIKWGD previously isolated from the hydrolysate of tuna meat, Hasan and others (2006) have recently isolated PNRIKYGD and PTHIKWGD, potent ACE-inhibitory peptides with $IC_{50}$ values of 4 μM and 8 μM, respectively, from the simulated GI-digest of bonito meat.

Once isolated and sequenced, peptides can be tested on an individual basis by *in vitro* assay, such as those employed in this thesis study, including simulated GI digestion, peptide-ACE pre-incubation, and determination of inhibition mode. Active peptides must then be synthesized in large enough quantities to test for efficacy in the spontaneously hypertensive rat (SHR) model and clinical trials. It will be, however, still difficult to ascertain other potential bioactive functions in a complex hydrolysate mixture, be it either potentially beneficial or detrimental. Cross-checking the sequences of peptides in the hydrolysate product with those found in databases such as Biopep (University of Warmia and Mazury, 2006) may help to identify peptides of bioactivities other than ACE-inhibition, such as those with opioid, immunomodulatory, antiamnestic, antithrombotic, antioxidative, antimicrobial, or even allergenic or toxic potential.

### 4.2.3 Additional mechanisms of antihypertensive efficacy

In addition to direct ACE-inhibitory activity, a recent study has found that peptides may also exert antihypertensive effects by means of another renin-angiotensin system (RAS) related mechanism. Specifically, Matsui and others (2005) found that peptide VY, previously demonstrated to lower blood pressure in mildly hypertensive subjects, additionally inhibited the proliferation of $Ca^{2+}$ channel stimulated-cultured vascular smooth muscle cells. In contrast, the proliferation of an angiotensin II-stimulated culture was not inhibited, thereby indicating that the antiproliferative activity of VY is specific as a $Ca^{2+}$ channel blocker. Therefore, not only would these peptides be acting to lower blood pressure by lowering production of the vasoconstrictor angiotensin II via ACE-inhibitory activity, but may also protect against vascular narrowing due to proliferation over-activity. In light of these findings, it would be worthwhile to investigate the antiproliferative activity of PHF hydrolysate in cell culture as a possible additional mechanism of antihypertensive efficacy.

### 4.2.4 Incorporation into functional foods

Products such as PeptACE® (Natural Factors Nutritional Products Ltd., Coquitlam, BC) and Kastsuobushi Oligopeptide® (Nippon Supplement 2006) have made ACE-inhibitory peptides available to consumers in a nutraceutical form, namely hydrolysate powder encased in

a gel capsule. Using ACE-inhibitory hydrolysates in functional foods however, poses many more challenges including palatability and formulation, yet may aid in reaching more consumers by being viewed less as a 'herbal remedy' or a 'drug' and more as part of their everyday diet.

First, use of protein hydrolysates in food formulation is often hindered by the bitter taste exhibited by some of the constituent peptides whose sequences would otherwise be buried in the native protein structure. While L-amino acids arginine, proline, leucine, phenylalanine, isoleucine, and tryptophan have a bitter taste as free residues, peptides are potentially more bitter when their amino acid composition and sequence best fits the dimensions and binding preferences of the bitter receptors on the tongue (Raksakulthai and Haard 2003). Intense bitterness has been associated with peptides that have at least two hydrophobic residues at the C-terminal, hydrophobic residues with side chains of at least 3 carbons, and sequences of arginine adjacent to proline (Raksakulthai and Haard 2003). Unfortunately, many of these amino acids and sequences are also strongly associated with ACE-inhibitory activity (Meisel and others 2006).

The use of exopeptidases in conjunction with endoproteases/endopeptidases has been shown to reduce the production of bitter hydrolysates (Raksakulthai and Haard 2003). As such, Protamex®, an endoprotease/exopeptidase mixture, was chosen for this thesis study. Comparatively, hydrolysate produced from threadfin bream using Alcalase®, an endoprotease mixture, exhibited bitterness as the dominant taste characteristic (Normah and others 2004), while Protamex® was found to generate salmon frame hydrolysate devoid of detectable bitterness (Liaset and others 2003). Nonetheless, a non-bitter taste profile cannot be assumed, therefore it is important that the taste profile of PHF hydrolysate be investigated and optimized concurrently with ACE-inhibitory activity prior to use in a functional food formulation.

Finally, as discussed above, absorption of ACE-inhibitory peptides in the small intestine is essential for *in vivo* antihypertensive efficacy. Studies have shown that the presence of other food components may modulate the absorption potential of bioactive peptides. These include capsianoside from sweet pepper, which increased tight junction permeability of Caco-2 cell monolayers (Shimizu 1999) and simple sugars and amino acids, whose sodium-coupled active transport though the mucosal cell layer created an increase in intracellular osmotic pressure that triggered further opening of tight junctions in isolated hamster and mice jejunum

(Pappenheimer and Volpp 1992). Incorporation of these substances into an ACE-inhibitory functional food could possibly be used to decrease the amount of active peptides needed in the formulation to exert the desired antihypertensive effect, as a greater portion of peptides would be absorbed in their active form compared to when ingested alone. Reducing the amount of hydrolysate in the formulation is also desirable to help avoid exceeding the threshold concentration of bitter peptides. In the same way, the molecular weight profile of the hydrolysate mixture, controlled by the hydrolysis process or ultrafiltration, may also affect the absorption potential of active peptides. By containing differing proportions of free amino acids, peptide sizes, or active versus non-active peptides, transporter proteins may become saturated, allowing the active peptides to be absorbed intact paracellularly (Matsui and others 2002; Vermeirssen and others 2004). Therefore, the effect of food formulation on absorption of ACE-inhibitory peptides from PHF hydrolysate is an area that should be investigated, and could be easily accomplished by incorporating additional components, or hydrolysates of differing molecular weight ranges, to the tandem HPLC/Caco-2 cell culture monolayer method of Section 4.2.1 (Satake and others 2002).

In summary, the results of this thesis study provide the first foundation on which to base further investigation of the ACE-inhibitory activity, absorption potential, and antihypertensive efficacy of PHF hydrolysate both *in vitro* and *in vivo*. Pacific hake, as an abundant, renewable, low-cost protein resource, is an ideal substrate for creating a value-added functional food ingredient, and with future research and product development, health benefits for the consumer may one day be realized.

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