JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Optimizing Angiotensin I-Converting Enzyme Inhibitory Activity of Pacific Hake (*Merluccius productus*) Fillet Hydrolysate Using Response Surface Methodology and Ultrafiltration

CRYSTAL D. CINQ-MARS AND EUNICE C. Y. LI-CHAN*

Food, Nutrition, and Health Program, Faculty of Land and Food Systems, 2205 East Mall, The University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4

The in vitro angiotensin I-converting enyzme (ACE) inhibitory activity of Pacific hake hydrolysates was investigated as a function of hydrolysis conditions, starting material variability, and ultrafiltration. Hake fillets were hydrolyzed using Protamex protease under various conditions of pH, hydrolysis time, and enzyme-to-substrate ratio (% E/S) according to a response surface methodology (RSM) central composite design. The hydrolysate produced at pH 6.5, 125 min, and 3.0% E/S had an $IC_{50}$ of 165 ± 9 μg of total solids/mL. ACE-inhibitory activity was not significantly different ($P < 0.05$) for hydrolysates produced using higher time–enzyme combinations within the model or from fish of different catches. Ultrafiltration (10 kDa molecular mass cutoff) resulted in an $IC_{50}$ value of 44 ± 7 μg of peptides/mL, 2.5 times more potent than the commercial product PeptACE Peptides ($IC_{50}$ = 114 ± 8 μg of peptides/mL). These results suggest that hydrolysates prepared with minimal fractionation from Pacific hake, an undervalued fish, may be a commercially competitive source of ACE-inhibitory peptides.

**KEYWORDS: ACE-inhibitory activity; hydrolysate; Pacific hake; *Merluccius productus*; response surface methodology; ultrafiltration; Protamex**

## 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 (*1*, *2*). Altered activity of bradykinin however, has been attributed to the onset of a persistent dry cough side effect (*1*).

Starting in the late 1980s, hydrolysates from food protein sources, including those from milk, soy, chicken, wheat, corn, and fish, have been shown to exhibit ACE-inhibitory activity (*2*, *3*). 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 of hydrolysate per day from dried bonito and experienced no adverse side effects (*4*). 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 (*5*), sardine (*6*), and pollack (*7*), have been shown to exhibit ACE-inhibitory activity in vitro, whereas those from fermented fish sauce (*8*), salmon (*9*), and sole (*10*) have exhibited antihypertensive efficacy in the spontaneously hypertensive rat (SHR) model, suggesting that this bioactivity is not necessarily species specific. Pacific hake (*Merluccius productus*), an abundant species off the west coast of Canada and the United States, has, however, never been investigated as a source of ACE-inhibitory peptides.

Undervalued for its poor fillet texture attributed to the occurrence of *Kudoa* spore infection (*11*), 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 (*12*), 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 (*13*). Response surface methodology (RSM) is a useful statistical tool to study such multivariable processes as it reduces the number of trials

* Corresponding author [telephone (604) 822-6182; fax (604) 822-5143; e-mail eunice.li-chan@ubc.ca].

10.1021/jf0713354 CCC: $37.00 
Published on Web 10/12/2007

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, the outcomes of hydrolysate functionality of which can be plotted as a predictive response surface (*14*). 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 (*15*) and van der Ven et al. (*16*) have successfully employed RSM to optimize emulsification and foaming capacities of shark protein hydrolysates and ACE-inhibitory activity of whey protein hydrolysates, 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 coefficient of determination ($r^2$) value (*14*).

Whereas 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) (*17*). 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 (*6*, *18*). However, at a 10 kDa cutoff, UF may be an industrially advantageous way to "enrich" the ACE-inhibitory potency of protein hydrolysates without compromising yield.

There has been great interest in recent years in using food proteins as sources of bioactive peptides, especially those with ACE-inhibitory activity (*1*–*3*). Most studies, however, have not considered the cost or abundance of starting material or demonstrated clear evidence or rationale for selection of hydrolysis conditions and the effects of starting material variability on final product bioactivity. Therefore, the objectives of this study were to evaluate the potential for utilizing Pacific hake as an abundant natural resource for generating ACE-inhibitory peptides, by assessing the effects of (*1*) hydrolysis conditions (using RSM), (*2*) starting material variability, and (*3*) UF separation on the ACE-inhibitory activity of Pacific hake fillet (PHF) hydrolysates in vitro. Furthermore, the ACE-inhibitory activity of these samples was compared to that of a commercially available product from bonito fish, PeptACE Peptides, to establish if further study was warranted to explore the potential of PHF as a commercially competitive source of ACE-inhibitory peptides.

## MATERIALS AND METHODS

**Materials.** Whole, raw, Pacific hake was provided by Steveston Seafood Direct Ltd. (Richmond, BC, Canada) during the summer catch seasons of 2004 and 2005 from the approximate area of 48.5° N latitude and 124–125° W longitude off the coast of Vancouver Island, BC. Fish were transported on ice by Pacific Fisheries Technologies Inc. (Delta, BC, Canada) to the University of British Columbia Food Science Laboratory within 2 days of capture. *Kudoa paniformis* infection levels were determined according to the method of Dawson Coates et al. (*19*) as modified by Samaranayaka et al. (*12*) prior to storage at −25 °C. Catch date, fish length, fish mass, and *K. 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 1**.

Alcalase 2.4L (protease from *Bacillus licheniformis*, 2.4 AU/g) and Flavourzyme 500L (protease from *Aspergillus oryzae*, 500 LAPU/g) were purchased from Sigma-Aldrich (St. Louis, MO). Protamex (1.5 AU/g) was a gift from Brenntag Canada Inc. (Langley, BC, Canada). All enzymes were produced by Novozymes (Bagsvaerd, Denmark). PeptACE Peptides, a product of Natural Factors Nutritional Products Ltd. (Coquitlam, BC, Canada), were purchased from Finlandia Pharmacy (Vancouver, BC, Canada). Hippuryl-L-histidyl-L-leucine (HHL as tetrahydrate) and ACE (from rabbit lung) were purchased from Sigma-Aldrich (St. Louis, MO). 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 $\mu$L aliquot and stored at −25 °C.

**Table 1.** Ranges of Catch Dates, Lengths, Masses, and *Kudoa paniformis* Spore Counts of Pacific Hake Used To Create Experimental Batches

| experiment batch | catch dates | no. of fish | length (cm) | body mass (g) | *K. paniformis* spore count[a] |
|---|---|---|---|---|---|
| preliminary | May 15, 2004 | 30 | 46–56 | 661–992 | $10^5$−$10^7$ |
| 1 | July 3–Sept 22, 2004 | 52 | 45–56 | 518–966 | $10^6$ |
| 2 | June 12–28, 2004 | 61 | 45–56 | 550–997 | $10^6$ |
| 3 | May 6–18, 2005 | 11 | 48–52 | 630–821 | $10^6$ |
| 4 | May 9–23, 2005 | 10 | 47–54 | 555–803 | $<10^4$ |

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

**Mince Preparation and Proximate Analysis.** Whole fish, grouped into batches as described in **Table 1**, were first thawed overnight at 4 °C, then filleted, ground, and homogenized as previously described (*12*). Mince was portioned into 150 g amounts, vacuum sealed, and stored at −25 °C until used for the hydrolysis trials. A 10 g portion of the homogenized mince was kept for proximate analysis. Moisture content and ash were determined according to AOAC Official Methods 930.15 and 942.05, respectively (*20*). The pH was determined according to the *Handbook of Meat Analysis* (*21*), and lipid was determined by using the Bligh and Dyer method (*22*). Nitrogen content was determined by combustion method AOAC Official Method 992.15 (*20*), 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.

**Preparation of Hydrolysates.** First, a 1:2 mince to water slurry was made by blending 150 g of prepared PHF mince with 300 mL of distilled water. The slurry was then preheated to boiling in a microwave oven at maximum power (800 W) for 2 min and then heated at 95 °C in boiling water with stirring for 10 min to inactivate endogenous enzymes. After cooling at room temperature to the desired temperature (55 °C for Alcalase 2.4L trials, 50 °C for Flavourzyme 500L trials, 40 °C for Protamex trials), the slurry was incubated in a water bath to maintain process temperature. The pH was adjusted to that desired using 6 N HCl or 6 N NaOH, after which commercial protease was added at the desired % E/S ratio. Sodium azide was also added in preliminary trials at a final concentration of 0.05% w/v to prevent microbial growth. The slurry was constantly stirred at 400 rpm with a 5 cm diameter propeller overhead stirrer (model 5VA, Eastern Industries, Hamden, CT). The pH and temperature were continually monitored, and the pH was kept constant by the addition of 6 N NaOH as hydrolysis progressed. After the desired process time, hydrolysis was terminated by submersion in boiling water for 15 min. The slurry was then centrifuged at 12000*g* at room temperature for 15 min. The supernatant was decanted through Whatman no. 4 filter paper, and the pH was 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 [$y = 97.796x + 0.572$, $r^2 = 0.999$, where $y$ is conductivity (mS/cm) and $x$ is molar concentration of NaCl]. The slurry supernatant solutions were freeze-dried to yield hydrolysate powder and stored at −25 °C. The mass of the salt, when calculated from the NaCl standard conductivity curve, was <0.5% of the hydrolysate mass. The % yield was calculated by using

$$[(\text{mass of dried powder} - \text{mass of salt})/\text{original mass of mince solids}] \times 100 \quad (1)$$

**Extent of Hydrolysis (EH).** The EH was determined by measuring amino groups using trinitrobenzenesulfonic acid (TNBS) according to

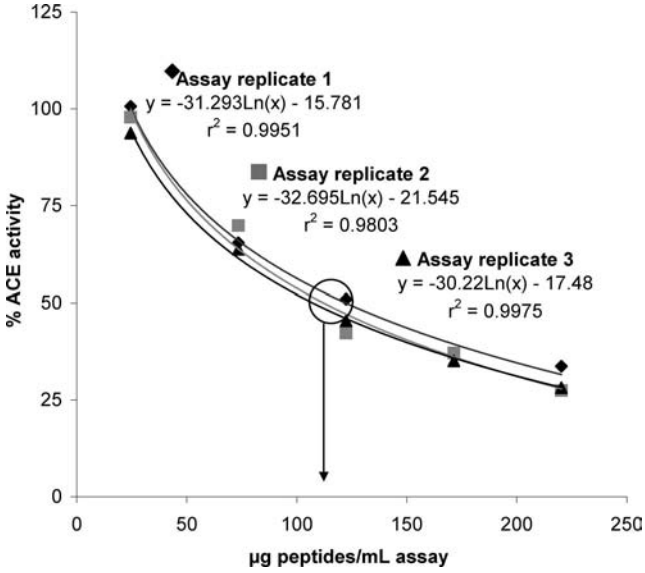


**Figure 1.** Example of $IC_{50}$ determination for PeptACE Peptides by regression analysis.

the method of Adler-Nissen (*23*) with modifications by Liceaga-Gesualdo and Li-Chan (*24*). Immediately prior to termination of hydrolysis, a 4 mL aliquot of the mince–water slurry was mixed with an equal volume of 24% trichloroacetic acid solution and centrifuged at 12100*g* for 5 min. An aliquot of the supernatant was then diluted 100 times with distilled–deionized water, and 0.2 mL of the diluted supernatant was added to 2.0 mL of 0.05 M sodium tetraborate buffer (pH 8.2) and 1.0 mL of freshly made 4.0 mM TNBS. After incubation in the dark at room temperature for 30 min, the reaction was terminated by the addition of 1.0 mL of 2.0 M $NaH_2PO_4$containing 18 mM $Na_2SO_3$, 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. Extent of hydrolysis was expressed as milliequivalents of free α-amino groups per liter of slurry, determined by comparison to a standard curve with 0.2–3.0 mM L-leucine.

**ACE-Inhibitory Activity.** ACE-inhibitory activity of hydrolysate samples was determined in triplicate according to a method adapted from that of Lo and Li-Chan (*25*) based on the ACE activity assay of Cushman and Cheung (*26*). In this assay, ACE activity is quantified by spectrophotometric absorbance at 228 nm (abs) produced by the liberation of hippuric acid (HA) from the synthetic substrate HHL. In the presence of an ACE inhibitor at a given concentration, ACE activity and HA production are depressed; therefore, percent ACE inhibition can be calculated as

$$1 - [(\text{abs of inhibitor-containing sample} - \text{abs of negative control})/(\text{abs of positive control} - \text{abs of negative control})] \times 100 \quad (2)$$

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 percent ACE inhibition values of five peptide concentrations, as shown in **Figure 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 h, after which 150 μL of 7.8 mM HHL was added and the solution was further incubated at 37 °C for 1 h. 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, 1 N) was used to terminate the reaction. To extract HA, 1.0 mL of ethyl acetate was added. The solutions were then vortexed for 30 s and microcentrifuged at 2000*g* at room temperature for 5 min. After centrifugation, 0.7 mL of the ethyl acetate layer was removed into a 10 mm diameter clear glass tube and the solvent was evaporated by heating for 30 min at 120 °C (Analog Dry Block Heater, VWR International Ltd., Delta, BC, Canada). The remaining HA residue was redissolved with 1.3 mL of distilled water, and the absorbance was read at 228 nm in a quartz cuvette.

**Ultrafiltration.** Ultrafiltration was carried out using an Amicon model 8400 stirred cell (Millipore Corp., Billerica, MA). The neutralized and filtered supernatant of the hydrolyzed mince–water slurry, as described above, was sequentially passed through Millipore membranes YM10, YM3, YM1, and YC05 (molecular mass cutoffs of 10 kDa, 3 kDa, 1 kDa, and 500 Da, respectively) under 40 psi of 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

$$[\text{mass retentate powder } \textit{or} \text{ mass filtrate powder}/\text{total mass of hydrolysate before ultrafiltration}] \times 100 \quad (3)$$

**Amino Acid (AA) Composition and Hydrolysate Ash Analysis.** Freeze-dried hydrolysate samples, from the slurry supernatant and its UF filtrates, were sent for AA analysis to the Advanced Protein Technology Centre at The Hospital for Sick Children (Toronto, ON, Canada). Using the Waters Pico-Tag system, samples were subjected to vapor phase hydrolysis by 6 N HCl with 1% phenol at 110 °C for 24 h, followed by precolumn derivatization of the hydrolysates using phenylisothiocyanate (PITC) and quantification by reverse phase HPLC using PITC-labeled amino acids as standards (*27*). Picomoles of individual amino acids detected were converted to mass quantities using their respective residue masses. 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 analyzed:

$$\mu\text{g of peptides/mL of assay} = (\%\text{mass AA}) \times (\mu\text{g of solids/mL of assay}) \quad (4)$$

Ash content of the hydrolysate solids was determined by using AOAC Official Method 942.05 (*20*).

**Statistical Analysis.** All assays were conducted in at least triplicate, and results are expressed as the mean ± standard deviation of measured values. 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 1**). 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 ($P < 0.05$).

## RESULTS AND DISCUSSION

**Proximate Analysis and Response Surface Model Factor Level Selection.** Results of the proximate analysis of Pacific hake used in this study, shown in **Table 2**, are in accordance with the reference values reported in the Nutrient Data Laboratory database (*28*). Lipid and crude protein contents were slightly lower than the reference, whereas moisture and ash contents were slightly higher than the reference. Given that Pacific hake is a migratory species, possible differences in diet composition may have led to some diversity in the proximate composition of the flesh.

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 of

**Table 2.** Proximate Analysis of Raw Pacific Hake (Whiting, *Merluccius productus*) Fillet Received from Steveston Seafood Direct Ltd. (Richmond, BC, Canada) As Compared to Values for "Whiting, Mixed Species, Raw" As Found in the USDA Nutrient Data Laboratory Database (*28*)

| | measured[a] (%) | reference (%) |
|---|---|---|
| pH | 6.86 ± 0.02 | not given |
| moisture | 82.50 ± 0.1 | 80.27 |
| ash | 1.47 ± 0.25 | 1.30 |
| total lipid | 0.91 ± 0.02 | 1.31 |
| crude protein | 15.6 ± 0.3 | 18.31 |
| carbohydrate | n/a | 0.00 |
| sum | 100.50 | 101.19 |

[a] Measured values are mean ± standard deviation of $n \geq 3$.

**Table 3.** Summary of Hydrolysate Yield and ACE-Inhibitory Activity of Hydrolysate Generated by Commercial Proteases in a 1:2 Pacific Hake Mince–Water Slurry

| sample | hydrolysis time (min) | % yield[a] | $IC_{50}$[b] (mg of solids/mL) |
|---|---|---|---|
| Alcalase2.4L | 0 | 35.3 | no inhibition effect |
| 2% E/S | 10 | 66.8 | 1.96 |
| (55 °C, pH 7.5) | 60 | 77.0 | 1.47 |
| | 240 | 86.0 | 1 |
| | 1440 | 99.7 | 0.7 |
| Alcalase2.4L | 0 | 29.4 | no inhibition effect |
| 4% E/S | 10 | 73.9 | 1.76 |
| (55 °C, pH 7.5) | 60 | 83.1 | 1.45 |
| | 240 | 91.0 | 1.21 |
| | 1440 | 100. | 1.17 |
| Flavourzyme500L | 0 | 27.0 | no inhibition effect |
| 2% E/S | 10 | 43.2 | >4.00 |
| (50 °C, pH 7.0) | 60 | 51.2 | >4.00 |
| | 240 | 62.5 | 3.62 |
| | 1440 | 75.8 | >4.00 |
| Flavourzyme 500L | 0 | 26.1 | no inhibition effect |
| 4% E/S | 10 | 47.6 | >4.00 |
| (50 °C, pH 7.0) | 60 | 59.1 | 3.36 |
| | 240 | 68.6 | 3.19 |
| | 1440 | 79.8 | >4.00 |
| Protamex | 0 | 22.0 | no inhibition effect |
| 2% E/S | 10 | 53.7 | 1.23 |
| (40 °C, pH 6.2) | 60 | 62.3 | 1.84 |
| | 240 | 72.9 | 2.23 |
| | 1440 | 87.0 | 2.33 |
| Protamex | 0 | 21.4 | no inhibition effect |
| 4% E/S | 10 | 59.8 | 1.6 |
| (40 °C, pH 6.2) | 60 | 81.3 | <1.00 |
| | 240 | 82.2 | 2.22 |
| | 1440 | 100. | 2.06 |

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

hydrolysate/mL) (*6*, *29–34*), could be generated from PHF. Second, commercial protease and hydrolysis variable ranges to use for further study by RSM were to be selected on the basis of yield and ACE-inhibitory activity of hydrolysates generated. As shown in **Table 3**, $IC_{50}$ values of <1.00 mg of 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 (*35*). The % E/S ratio range 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 min (±axial points) to include the decrease in ACE-inhibitory activity 1 h post hydrolysis as shown in **Table 3**. In the following RSM design, a total of 20 hydrolysis trials were conducted, including 6 replicates at the center point conditions of pH 6.5, 2.10% E/S, and hydrolysis time of 80 min (**Table 4**).

**Table 4.** Response Surface Methodology Central Composite Design Factor Levels for 20 Hydrolysis Trials and Their Corresponding Responses for Extent of Hydrolysis and Percent ACE-Inhibitory Activity[a]

| standard order | run order | pH | time | % E/S | extent of hydrolysis[b] (mequiv of free amino groups/L of slurry) | % ACE-inhibitory activity[b] |
|---|---|---|---|---|---|---|
| | | | | Corner Points | | |
| 1 | 12 | 5.8 | 35 | 1.00 | 79 ± 2 | 20.9 ± 5.4 |
| 2 | 9 | 7.2 | 35 | 1.00 | 74 ± 1 | 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 | 47 ± 0.3 | 4.5 ± 3.0[c] |
| 12 | 14 | 6.5 | 156 | 2.10 | 146 ± 10 | 42.2 ± 3.2 |
| 13 | 13 | 6.5 | 80 | 0.25 | 57 ± 0.6 | 9.0 ± 1.7 |
| 14 | 17 | 6.5 | 80 | 3.95 | 148 ± 5 | 36.8 ± 1.1 |
| | | | | Center 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 |

[a] Hydrolysates produced from batch 1 fillet as described in **Table 1**. [b] Results are the means ± standard deviation of triplicate assays. [c] Outlier data point in model (standardized residual >±3).

**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 the resulting hydrolysate, as shown in **Table 4**, two response surface models were generated (**Figures 2** and **3**). By stepwise elimination, factors that had a significant effect on EH were "time", "% E/S", "time$^2$", and "% E/S$^2$" (**Table 5**). Given a "square" model ($P < 0.01$), the equation of the response surface is

$$\text{mequiv free amino/L} = 1.32\text{time} + 47.2\%\text{E/S} - 0.00482\text{time}^2 - 6.36\%\text{E/S}^2 - 17.2 \quad (5)$$

By the same stepwise elimination process, factors that 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 5**). For the

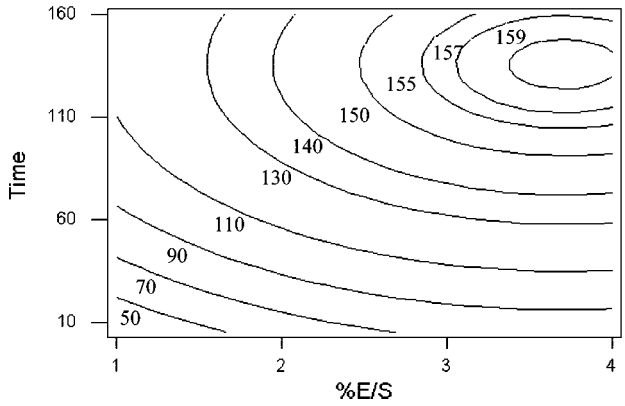


**Figure 2.** Response surface contour plot of extent of hydrolysis of Pacific hake hydrolysates (expressed as milliequivalents of free amino groups per liter of slurry) as a function of hydrolysis time (minutes) and enzyme-to-substrate ratio (percent). pH was held at 6.5. Hydrolysates were produced from batch 1 (**Table 1**).

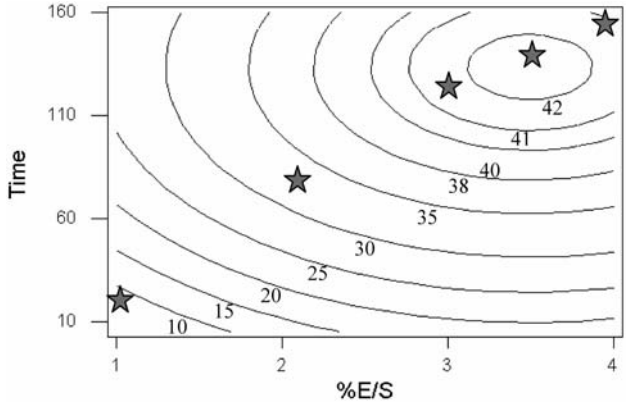


**Figure 3.** Response surface contour plot of ACE-inhibitory activity of Pacific hake hydrolysates (expressed as percent ACE inhibition at 200 $\mu$g of hydrolysate solids per milliliter) as a function of hydrolysis time (minutes) and enzyme-to-substrate ratio (percent). pH was held at 6.5. Hydrolysates were produced from batch 1 (**Table 1**). Stars indicate condition combinations chosen for model verification.

**Table 5.** Regression Coefficients, *p* Values, and Other Significance Statistics of RSM Prediction Models for the Extent of Hydrolysis and Percent ACE-Inhibitory Activity of Hydrolysates from Pacific Hake Fillet

| | extent of hydrolysis model | | % ACE-inhibitory activity model | |
|---|---|---|---|---|
| | coefficient | *p* value | coefficient | *p* value |
| factor | | | | |
| >intercept | −17.2 | 0.207 | 24.7 | 0.000 |
| pH | | | −6.08 | 0.003 |
| time | 1.32 | 0.000 | 0.388 | 0.000 |
| % E/S | 47.2 | 0.000 | 17.9 | 0.000 |
| time$^2$ | −0.00482 | 0.002 | −0.00145 | 0.022 |
| % E/S$^2$ | −6.36 | 0.011 | −2.56 | 0.016 |
| statistics of model significance | | | | |
| $r^2$ | 0.918 | | 0.884 | |
| linear regression | | 0.000 | | 0.000 |
| square regression | | 0.002 | | 0.010 |
| lack-of-fit | | 0.140 | | 0.014 |

purposes of plotting **Figure 3**, pH was held at the center value of 6.5 because, 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

$$\%\text{ACE-inhibitory activity (in the presence of 200}\mu\text{g of hydrolysate solids/mL assay)} = 0.388_{\text{time}} + 17.9\%\text{E/S} - 6.08\text{pH} - 0.00145\text{time}^2 - 2.56\%\text{E/S}^2 + 24.7 \quad (6)$$

The significant lack-of-fit term in this model, as shown in **Table 5**, indicates that the equation of the response surface may not accurately predict the true ACE-inhibitory activity in some regions of the model (*14*). This can be caused by a low standard deviation of the center point samples compared to the 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 min, 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, percent 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 min 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 and 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 et al. (*32*, *33*) in which prolonged hydrolysis of bovine blood plasma beyond 6 h with Alcalase, Neutrase, or Pronase E and of corn gluten beyond 8 h with Flavourzyme, respectively, resulted in a decrease in ACE-inhibitory activity. It is therefore likely to be 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 et al. (*16*), however, the RSM model included "pH$^2$" as significantly affecting ACE-inhibitory activity of whey protein hydrolysates produced using Corolase PP (a proteolytic enzyme preparation 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 nonphysiological 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.

**Model Confirmation and Assessment of Starting Material Variability.** Five RSM conditions, as shown in **Figure 3**, were chosen to verify the ACE-inhibitory activity predictions of center point (pH 6.5, 2.10% E/S, 80 min), the optimal region (pH 6.5, 3.00% E/S, 125 min, and pH 6.5, 3.50% E/S, 140 min), and the outer regions (pH 6.5, 1.00% E/S, 25 min; and pH 6.5, 3.95% E/S, 155 min). For model confirmation, predicted values were required to fall within the range of mean ± standard deviation of measured values. Two points in the optimal region

**Table 6.** Comparison of Extent of Hydrolysis and Percent ACE-Inhibitory Activity of Pacific Hake Hydrolysates from Different Mince Batches to Model Predictions at Selected Hydrolysis Conditions

| hydrolysis conditions | | | | mequiv/L of slurry | | % ACE-inhibitory activity[a] | |
|---|---|---|---|---|---|---|---|
| pH | % E/S | time (min) | batch[b] | exptl[c] | predicted | exptl[c] | predicted |
| 6.5 | 1.00 | 25 | 1 | 48.0 ± 0.7* | 53.5 | 11.7 ± 5.0 | 9.3 |
| | | | 2 | 50.1 ± 1.3* | | 11.1 ± 1.5 | |
| | | | 3 | 47.5 ± 3.2* | | 15.3 ± 2.5* | |
| | | | 4 | 49.9 ± 1.0* | | 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* | |
| 6.5 | 3.95 | 155 | 1 | 164 ± 4* | 158 | 39.8 ± 5.1 | 41.2 |
| | | | 2 | 163 ± 3* | | 40.8 ± 1.6 | |
| | | | 3 | 155 ± 5 | | 42.2 ± 2.5 | |
| | | | 4 | 159 ± 5 | | 39.9 ± 6.5 | |

[a] % ACE-inhibitory activity at 200 μg of hydrolysate solids/mL of assay. [b] Batches as per **Table 1**. [c] Results expressed as mean ± standard deviation of triplicate assays; values denoted by an asterisk do not confirm the model prediction.

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 *K. paniformis* spore count or catch date (**Table 1**).

As shown in **Table 6**, 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 for 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 min; and 3.50% E/S, 140 min) 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 min, 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 on lower ranges of process variables would be useful in more accurately predicting extent of hydrolysis in this region of steeply changing responses.

Nonetheless, because 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 min, 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.

**Effect of Ultrafiltration on $IC_{50}$ Value and Amino Acid Profile.** As shown in **Figure 4**, UF to <10 kDa significantly improved the ACE-inhibitory activity of hydrolysates compared to both unfractionated hake hydrolysate and PeptACE Peptides ($P < 0.05$) by approximately 70 and 58%, respectively. Comparatively, the decreases in $IC_{50}$ after filtration to <10 kDa found for cod frame hydrolysate and bovine albumin hydrolysate were only 10 and 20%, respectively (*29*, *30*). As such, UF to 10 kDa is particularly advantageous to PHF hydrolysates produced at the conditions of this study. Furthermore, a significant trend of decreasing $IC_{50}$ values (increasing ACE-inhibitory potency) is seen as longer peptides are removed (**Figure 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 of 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 five or fewer amino acid residues (*2*, *3*). Further UF treatment beyond 10 kDa would not be warranted in a commercial process for PHF hydrolysates because only an approximate 10% reduction in the $IC_{50}$ value is achieved with each successive filtration. Not only would this be very costly, but filtration to <1 kDa has been found to reduce yield to <9% (w/w dry basis) of unfractionated hydrolysate mass in the case of bovine albumin hydrolysates produced using Alcalase (*30*). At the optimized hydrolysis conditions employed in this study, ultrafiltration at a 10 kDa cutoff resulted in a maximum hydrolysate yield loss of only 5.4%, indicating that commercial UF treatment at 10 kDa is a reasonable option because ACE-inhibitory activity is also significantly enriched.

Also shown in **Figure 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 being destroyed. Therefore, within the ranges tested, there is

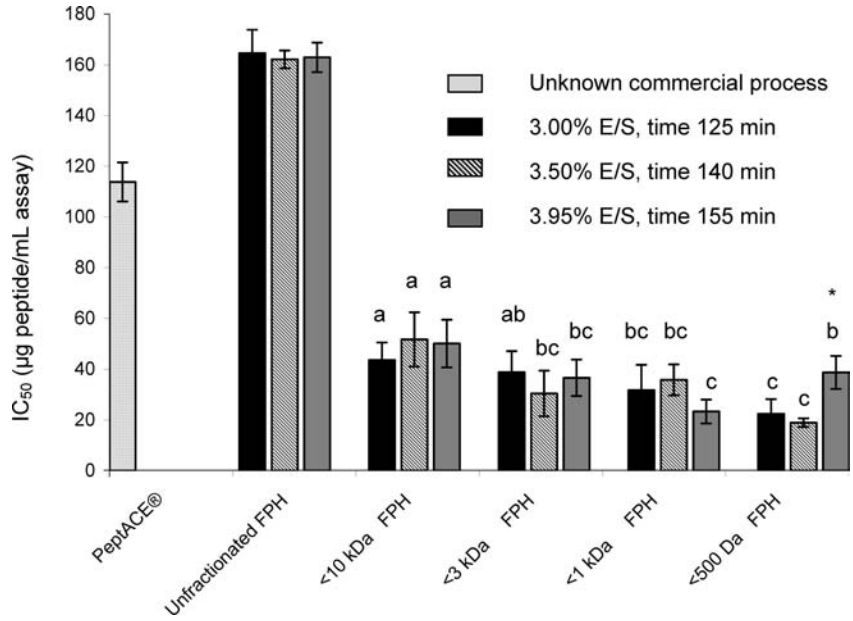


**Figure 4.** Comparison of ACE-inhibitory activity of PeptACE Peptides, Pacific hake hydrolysate, and Pacific hake hydrolysate ultrafiltrates, produced at three optimal hydrolysis conditions. Hydrolysates were produced from pooled fillet batches 1, 2, 3, and 4 (**Table 1**). Each bar represents the mean ± standard deviation of triplicate $IC_{50}$ values; bars with different letters within a hydrolysis condition group are significantly different ($P < 0.05$). Bar denoted by an asterisk is significantly different from others within the UF filtrate group.

**Table 7.** Amino Acid Profiles, Percent Mass of Amino Acids to Hydrolysate Solids, and Percent Ash of Commercial Product PeptACE Peptides, Unfractionated Pacific Hake Hydrolysate and Its Ultrafiltrates (Produced at pH 6.5, 3.00% E/S, and 125 min), and Unhydrolyzed Pacific Hake Fillet[a]

| amino acid recovered | PeptACE (%)[b] | unfractionated hydrolysate (%) | <10 kDa filtrate (%) | <1 kDa filtrate (%) | unhydrolyzed Pacific hake fillet (%) |
|---|---|---|---|---|---|
| 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 |
| 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/100 g)[c] | 61.2 | 54.7 | 53.7 | 38.6 | N/A |
| % ash to hydrolysate solids (g/100 g) | 4.2 | 11.6 | 12.8 | 24.3 | N/A |

[a] Hydrolysates produced from pooled fillet batches 1, 2, 3, and 4 (**Table 1**). [b] Calculated as percent moles of total moles of amino acids detected. [c] Without Trp or Cys.

no advantage, even if the hydrolysate is to be further processed by UF, to employ hydrolysis conditions greater than 3.00% E/S and 125 min to maximize ACE-inhibitory activity.

**Table 7** summarizes the amino acid profiles of unfractionated hake hydrolysate, its UF fractions, the commercial product PeptACE Peptides, and unhydrolyzed fillet. PHF hydrolysate has an AA profile comparable to that of PeptACE Peptides, 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 recent literature 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 tripeptides, and hydrophobic residues are common at each of the three C-terminal positions of oligopeptides (*2*, *3*). 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 by Pripp et al. (*36*). Furthermore, separation by UF did not grossly alter the AA profile of the hydrolysates, meaning that either peptides in all molecular mass ranges contain similar AA profiles or that the majority of the peptides fall below the 1 kDa molecular mass range. The latter

is more likely because, as discussed above, subsequent UF treatment did not result in a strong improvement in ACE-inhibitory activity either. In addition, amino acids 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 unhydrolyzed hake fillet (**Table 7**) and than is reported for Pacific hake (whiting) muscle (*37*), 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 because the individual ACE-inhibitory peptide sequences found in PHF hydrolysate are not yet known.

Ultrafiltration also resulted in an increase in nonpeptide materials compared to peptide mass. Specifically, as shown in **Table 7**, proportion of ash increased and peptide decreased per unit solids as hydrolysate was filtered to lower molecular mass cutoffs. However, because the fractions were collected as aliquots of filtrate and not as retentates, all filtrates contained the same concentration of salt. Therefore, percent 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 percent peptide content when $IC_{50}$ is expressed on a peptide versus solids basis, because 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. Hydrolysis using condition combinations beyond pH 6.5, 3.00% E/S, and 125 min 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. Ultrafiltration of the hydrolysate to enrich the <10 kDa fraction significantly decreased the $IC_{50}$ value to $44 \pm 7$ $\mu$g of peptides/mL.

Given that the coastal stock of Pacific hake is currently the most abundant groundfish population in the California Current system, with average annual commercial landings of 159,000 and 54000 metric tonnes in the United States and Canada, respectively, during 1996–2005 (*38*), the results of this study are significant in indicating good potential of using Pacific hake as a commercially competitive and reliable source of ACE-inhibitory peptides.

## ACKNOWLEDGMENT

We thank our summer and work study students (I. Cheung, T. Ho, and E. Leung) for their assistance in spore counting and Rey Interior at the Hospital for Sick Children Advanced Protein Technology Centre for performing the amino acid analyses.

## LITERATURE CITED

(1) Opie, L. H. *Angiotensin-Converting Enzyme Inhibitors: Scientific Basis for Clinical Use*; Wiley-Liss: New York, 1992; pp 20–60, 194–202.

(2) Meisel, H.; Walsh, D. J.; Murray, B.; FitzGerald, R. J. ACE inhibitory peptides. In *Nutraceutical Proteins and Peptides in Health and Disease*; Yoshinori, M., Shahidi, F., Eds.; CRC Taylor and Francis: Boca Raton, FL, 2006; pp 269–315.

(3) Li, G. H.; Le, G. W.; Shi, Y. H.; Shrestha, S. Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutr. Res.* **2004**, *24*, 469–486.

(4) Fujita, H.; Yamagami, T.; Ohshima, K. Effects of an ACE-inhibitory agent, katsuobushi oligopeptide, in the spontaneously hypertensive rat and in borderline and mildly hypertensive subjects. *Nutr. Res.* **2001**, *21*, 1149–1158.

(5) Kohama, Y.; Matsumoto, S.; Oka, H.; Teramoto, T.; Okabe, M.; Mimura, T. Isolation of angiotensin-converting enzyme-inhibitor from tuna muscle. *Biochem. Biophys. Res. Commun.* **1988**, *155*, 332–337.

(6) Matsui, T.; Matsufuji, H.; Seki, E.; Osajima, K.; Nakashima, M.; Osajima, Y. Inhibition of angiotensin I-converting enzyme by *Bacillus licheniformis* alkaline protease hydrolysates derived from sardine muscle. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 922–925.

(7) Je, J.; Park, P.; Kwon, J. Y.; Kim, S. A novel angiotensin I converting enzyme inhibitory peptide from Alaska Pollack (*Theragra chalcogramma*) frame protein hydrolysate. *J. Agric. Food Chem.* **2004**, *52*, 7842–7845.

(8) Ichimura, T.; Hu, J. N.; Aita, D. Q.; Maruyama, S. Angiotensin I-converting enzyme inhibitory activity and insulin secretion stimulative activity of fermented fish sauce. *J. Biosci. Bioeng.* **2003**, *96*, 496–499.

(9) Ono, S.; Hosokawa, M.; Miyashita, K.; Takahashi, K. Isolation of peptides with angiotensin I-converting enzyme inhibitory effect derived from hydrolysate of upstream chum salmon muscle. *J. Food Sci.* **2003**, *68*, 1611–1614.

(10) Jung, W.; Mendis, E.; Je, J.; Park, P.; Son, B. W.; Kim, H. C.; Choi, Y. K.; Kim, S. Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (*Limanda aspera*) frame protein and its antihypertensive effect in spontaneously hypertensive rats. *Food Chem.* **2006**, *94*, 26–32.

(11) Morrissey, M. T.; Hartley, P. S.; An, H. Proteolytic activity of Pacific whiting and effects of surimi processing. *J. Aquat. Food Prod. Technol.* **1995**, *4* (4), 5–18.

(12) Samaranayaka, A. G. P.; Ho, T. C. W.; Li-Chan, E. C. Y. Correlation of Kudoa spore counts with proteolytic activity and texture of fish mince from Pacific hake (*Merluccius productus*). *J. Aquat. Food Prod. Technol.* **2006**, *15* (4), 75–93.

(13) Kristinsson, H. G.; Rasco, B. A. Fish protein hydrolysates: production, biochemical, and functional properties. *Crit. Rev. Food Sci. Nutr.* **2000**, *40*, 43–81.

(14) Myers, R. H.; Montgomery, D. C. *Response Surface Methodology: Process and Product Optimization Using Designed Experiments*; Wiley: New York, 2002; pp 1–84, 235–302.

(15) Diniz, F. M.; Martin, A. M. Effects of the extent of enzymatic hydrolysis on functional properties of shark protein hydrolysate. *Lebensm. Wiss. -Technol.* **1997**, *30*, 266–272.

(16) van der Ven, C.; Gruppen, H.; de Bont, D. B. A.; Voragen, A. G. J. Optimization of the angiotensin converting enzyme inhibition by whey protein hydrolysates using response surface methodology. *Int. Dairy J.* **2002**, *12*, 813–820.

(17) Korhonen, H.; Pihlanto, A. Food-derived bioactive peptides–opportunities for designing future foods. *Curr. Pharm. Des.* **2003**, *9*, 1297–1308.

(18) Fujita, H.; Yoshikawa, M. LKPNM: a prodrug-type ACE-inhibitory peptide derived from fish protein. *Immunopharmacology* **1999**, *44*, 123–127.

(19) Dawson Coates, J. A.; Chase, J. C.; Funk, V.; Booy, M. H.; Haines, L. R.; Falkenberg, C. L.; Whitaker, D. J.; Olafson, R. W.; Pearson, T. W. The relationship between flesh quality and numbers of *Kudoa thyrsites* plasmodia and spores in farmed Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **2003**, *26*, 1–10.

(20) AOAC. *AOAC Official Methods of Analysis*, 16th ed.; Association of Official Analytical Chemists: Arlington, VA, 1995.

(21) Koniecko, E. S. *Handbook of Meat Analysis*, 2nd ed.; Avery Publishing Group: Wayne, NJ, 1985.

(22) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917.
(23) Adler-Nissen, J. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *J. Agric. Food Chem.* **1979**, *27*, 1256–1262.
(24) Liceaga-Gesualdo, A. M.; Li-Chan, E. C. Y. Functional properties of fish protein hydrolysate from herring (*Clupea harengus*). *J. Food Sci.* **1999**, *64*, 1000–1004.
(25) Lo, W. M. Y.; Li-Chan, E. C. Y. Angiotensin I converting enzyme inhibitory peptides from in vitro pepsin-pancreatin digestion of soy protein. *J. Agric. Food Chem.* **2005**, *53*, 3369–3376.
(26) Cushman, D. W.; Cheung, H. S. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* **1971**, *20*, 1637–1648.
(27) The Hospital for Sick Children, Advanced Protein Technology Centre–Amino Acid Analysis–Methods; http://www.sickkids.ca/APTC (accessed Aug 16, 2006).
(28) USDA. USDA National Nutrient Database for Standard Reference, release 17; http://www.nal.usda.gove/fnic/foodcomp/search (accessed Aug 15, 2006).
(29) Jeon, Y.; Byun, H.; Kim, S. Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. *Process Biochem.* **1999**, *35*, 471–478.
(30) Hyun, C. K.; Shin, H. K Utilization of bovine blood plasma proteins for the production of angiotensin I converting enzyme inhibitory peptides. *Process Biochem.* **2000**, *36*, 65–71.
(31) Pihlanto-Leppala, A.; Koskinen, P.; Piilola, K.; Tupasela, T.; Korhonen, H. Angiotensin I-converting enzyme inhibitory peptides of whey protein digests: concentration and characterization of active peptides. *J. Dairy Res.* **2000**, *67*, 53–64.
(32) Kim, S. K.; Byun, H. G.; Park, P. J.; Shahidi, F. Angiotensin I converting enzyme inhibitory peptides purified from bovine skin gelatin hydrolysate. *J. Agric. Food Chem.* **2001**, *49*, 2992–2997.
(33) Kim, J. M.; Whang, J. H.; Suh, H. J. Enhancement of angiotensin I converting enzyme activity and improvement of emulsifying and foaming properties of corn gluten hydrolysate using ultrafiltration membranes. *Eur. Food Res. Technol.* **2004**, *218*, 133–138.
(34) Vermeirssen, V.; Van Camp, J.; Verstraete, W. Fractionation of angiotensin I converting enzyme inhibitory activity from pea and whey protein in vitro gastrointestinal digests. *J. Sci. Food Agric.* **2005**, *85*, 399–405, 2005.
(35) *Protamex® Product Sheet*, Novozymes, Bagsvaerd, Denmark, 2004.
(36) Pripp, A. H.; Isaksson, T.; Stepaniak, L.; Sorhaug, T. Quantitative structure–activity relationship modeling of ACE-inhibitory peptides derived from milk proteins. *Eur. Food Res. Technol.* **2004**, *219*, 579–583.
(37) Benjakul, S.; Morrissey, M. T. Protein hydrolysates from Pacific whiting solid wastes. *J. Agric. Food Chem.* **1997**, *45*, 3423–3430.
(38) Helser, T. E.; Stewart, I. J.; Fleischer, G. W.; Martell, S. *Stock Assessment of Pacific Hake (Whiting) in U.S. and Canadian Waters in 2006*; The Pacific Fishery Management Council: Portland, OR, Feb 2006 (available online at http://www.pcouncil.org/groundfish/gfsafe0406/2006_hake_assessment_FINAL_ENTIRE.pdf (accessed Aug 16, 2007).

**Received for review May 7, 2007. Revised manuscript received August 17, 2007. Accepted August 31, 2007. Funding for this research was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC). C.D.C.-M was the recipient of the Julie Payette-NSERC Research Scholarship.**

JF0713354