JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 1535–1542 1535
DOI:10.1021/jf9033199

Antioxidative and Angiotensin-I-Converting Enzyme Inhibitory Potential of a Pacific Hake (*Merluccius productus*) Fish Protein Hydrolysate Subjected to Simulated Gastrointestinal Digestion and Caco-2 Cell Permeation

Anusha G. P. Samaranayaka, David D. Kitts, 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

Pacific hake fish protein hydrolysate (FPH) with promising chemical assay based antioxidative capacity was studied for in vitro angiotensin-I-converting enzyme (ACE)-inhibitory potential, intestinal cell permeability characteristics, and intracellular antioxidative potential using the Caco-2 cell model system. FPH showed substrate-type inhibition of ACE with IC_{50} of 161 μg of peptides/mL. HPLC analysis revealed that different peptides were responsible for antioxidative and ACE-inhibitory activity. FPH inhibited 2,2′-azobis(2-amidinopropane) dihydrochloride-induced oxidation in Caco-2 cells at noncytotoxic concentrations. In vitro simulated gastrointestinal digestion increased ($P < 0.05$) antioxidative capacity; ACE-inhibitory activity of FPH remained unchanged, although individual peptide fractions showed decreased or no activity after digestion. Some FPH peptides passed through Caco-2 cells: the permeates showed 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity but no ACE-inhibitory activity. These results suggest the potential for application of Pacific hake FPH to reduce oxidative processes in vivo. Further studies are needed to assess prospective antihypertensive effects.

KEYWORDS: Pacific hake; fish protein hydrolysate; antioxidative; ACE-inhibitory; simulated gastrointestinal digestion; Caco-2 cell permeability; cytotoxicity

INTRODUCTION

Protein hydrolysates derived from different food sources, including fish (*1*−*4*), soy (*5*, *6*), egg white (*7*, *8*), and milk (*9*−*12*), have been reported to possess antioxidative properties. Recently, it has been demonstrated that protein hydrolysates exhibiting antioxidative potential may also contain peptides with other biological activities, such as angiotensin-I-converting enzyme (ACE)-inhibitory activity. Some examples of this include protein hydrolysates made from fermented milk (*12*), human milk and infant formula (*13*), chum salmon cartilage (*14*), and egg albumen (*8*), which contained both antioxidative and ACE-inhibitory peptides. A protein hydrolysate recovered from defatted *Brassica carinata* seed flour was shown to contain peptides with antioxidative, ACE-inhibitory, and hypocholesterolemic activities (*15*). These protein hydrolysates with multiple bioactivities could be useful in formulating functional food products that target reducing the symptoms of oxidative stress, hypertension, and possibly dyslipidemia, all common to coronary heart disease.

Numerous papers have also demonstrated different antioxidative capacities of peptides derived from a number of distinct marine species (*16*−*22*). Previous research conducted in our laboratory (*4*) showed that fish protein hydrolysate (FPH) with high antioxidative potential could be prepared from Pacific hake (*Merluccius productus*), which is an undervalued fish caught in the waters of the Northwest Pacific. The FPH was produced by 1 h of autolysis at 52 °C and pH 5.50 (*4*) through the action of cathepsin L-like endogenous proteases reported to be present in Pacific hake muscle (*23*). In vitro chemical antioxidant assays revealed that the FPH contained activity in both polar and nonpolar as well as an oil-in-water emulsion system (*4*). However, this and other studies have relied on the use of chemical-based antioxidant tests that do not have the capacity to establish the true efficacy of antioxidative activity of bioactive peptides. Moreover, there is little known about the potential for multiple bioactivity of protein hydrolysates derived from fish species, including Pacific hake.

Effects of simulated gastrointestinal (GI) digestion, intestinal cell permeability, and interaction with intracellular sources of oxidative stress are three important parameters to be taken into consideration in the determination of the bioavailability and bioactivity of food-derived peptides. Even though animal studies and human clinical trials are the best way to assess the in vivo efficacy, in vitro cultured cell model systems allow for rapid, inexpensive screening of the potential bioactive compounds for their bioavailability and metabolism. Human adenocarcinoma colon cancer (Caco-2) cell monolayers have been most commonly reported in the literature for studying intestinal permeability of bioactive compounds due to their similarity to the intestinal endothelium cells (*24*, *25*). Cell culture models can also be used to evaluate cytotoxicity of bioactive compounds at concentrations

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

Published on Web 01/19/2010

used to exert the desired bioactivity in the body, as well as to study the potential to inhibit intracellular oxidation and to reduce inflammatory responses by antioxidative compounds (*26*).

Study Objectives. In the present study, Pacific hake FPH that had previously been identified as a potential source of antioxidative peptides on the basis of in vitro chemical assays (*4*) was tested for its potential to also exhibit ACE-inhibitory capacity. In vitro simulated GI digestion of Pacific hake FPH was then employed to study the possible effects of digestion on antioxidative and ACE-inhibitory capacity. Furthermore, the Caco-2 cell monolayer permeability model (*27*) was used to investigate intestinal transport of Pacific hake digestion derived peptides and the potential bioactivity of those peptides that exhibited permeability.

MATERIALS AND METHODS

Materials. Pepsin, pancreatin, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), angiotensin-I-converting enzyme (ACE) from rabbit lung, hippuryl-histidyl-leucine tetrahydrate (HHL), 2,4,6-trinitrobenzenesulfonic acid (TNBS), trifluoroacetic acid (TFA), Hank's balanced salt solution (HBSS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Eagle's minimum essential mineral (EMEM) medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen Canada (Burlington, ON, Canada). Caco-2 (ATCC accession no. HTB-37) cells were obtained from the American Type Culture Collection (Manassas, VA).

Preparation of Fish Protein Hydrolysate. Fillets from 10 Pacific hake fish harvested off the coast of Vancouver Island, Canada (48.4° N 125.2° W), were used to prepare FPH. Whole fish (average weight = 671 ± 89 g) were transported on ice to the University of British Columbia Food Science Laboratory within 2 days after capture, individually packed in polyethylene bags, labeled, and frozen (−25 °C) until used. Fish were thawed in a cold room (4 °C) for 16 h, filleted, deskinned, and then ground twice using a grinder (BEEM Gigant, Butcher & Packer Supply Co., Detroit, MI) with a 4 mm screen to make fish mince.

FPH was prepared by autolysis as previously reported (*4*). Briefly, a slurry of fish mince in distilled water (at a weight ratio of 1:2) was autolyzed by incubation at pH 5.50 and 52 °C for 1 h; these temperature and pH conditions were previously reported to be optimal for activity of the endogenous proteases (*28*). After incubation, the slurry was heated in a boiling water bath (> 90 °C) for 15 min, centrifuged at 17000*g* for 20 min, and filtered through two layers of cheesecloth. The filtrates were adjusted to pH 7.0 and freeze-dried. Freeze-dried FPH samples were ground using a pestle and a mortar and then stored in sealed vials at −18 °C until used for further analysis.

Amino Acid Composition. FPH was analyzed for amino acid composition by the Advanced Protein Technology Center at The Hospital for Sick Children (Toronto, ON, Canada). Amino acid analysis was conducted on a Waters Pico-Tag HPLC system after precolumn derivatization with phenylisothiocarbamate of the samples hydrolyzed by acid (6 M HCl with 1% phenol at 110 °C for 24 h). Performic acid oxidation was performed before the Cys content was determined, whereas methanesulfonic acid hydrolysis was used in determining the Trp content. The results were used to determine both the total content of amino acids in the sample (expressed as percent of dry matter) as well as the relative amino acid composition (expressed as grams per 100 g of amino acids).

Ultrafiltration. A portion of FPH was fractionated further by ultrafiltration (UF) using an Amicon model 8400 UF unit (Millipore Corp., Billerica, MA) with Millipore membranes having molecular mass cutoffs of 10, 3, and 1 kDa. The fractions were collected as follows: > 10 kDa, peptides retained without passing through 10 kDa membrane; 3−10 kDa, peptides permeating through the 10 kDa membrane but not the 3 kDa membrane; 1−3 kDa, peptides permeating through the 3 kDa membrane but not the 1 kDa membrane; < 1 kDa, peptides permeating through the 1 kDa membrane. All fractions recovered were lyophilized and stored in sealed vials at −18 °C.

Simulated Gastrointestinal Digestion. In vitro pepsin−pancreatin digestion of FPH and selected UF fractions was performed by modification of the method of Garrett et al. (*29*). Sample (100 mg) was first dissolved in 10.0 mL of distilled water, and the pH was adjusted to 2.0 with 5 N HCl. Pepsin (enzyme to substrate ratio of 1:35 w/w) was added, and the mixture was incubated in a shaking incubator for 1 h 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 (enzyme to substrate ratio of 1:25 w/w) was added to the mixture, which was incubated again with shaking for 2 h at 37 °C, before being submerged in boiling water for 10 min to terminate the digestion. Aliquots of the digested sample (referred to as FPH-GI) were diluted to the appropriate concentrations of peptides for antioxidative capacity, ACE-inhibitory activity, and cell permeability assays, as well as for reversed-phase HPLC analysis, as described in the following sections.

Caco-2 Cell Permeability Assay. Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL), in a fully humidified atmosphere with 5% CO_2 at 37 °C. Cell culture medium was replaced every other day, and cells were subcultured weekly. Following confluence, Caco-2 cells (0.3 mL of 2×10^5 cells/mL) were seeded on the cell culture insert (0.32 cm^2, 0.4 μm pore size, BD Biosciences Canada, Mississauga, ON, Canada) with 0.8 mL of medium in each well of the 24-well cell culture companion plate. Cell culture medium was carefully changed every other day for at least 21 days until the Caco-2 cells were fully differentiated as monolayers. Monolayer integrity was monitored by measuring transepithelial electrical resistance (TEER) using a Millicell Voltohmmeter (Millipore Corp., Bedford, MA), both at the beginning and at the end of the assay. Cell viability measurements were also made before and after the experiment using the MTT assay (*30*).

Cell permeability measurements were conducted in triplicate. Only monolayers with TEER of at least 900 Ω were chosen for the transport study. After cells had been washed twice with HBSS buffer, 0.15 mL of FPH or GI-digested sample (5 mg/mL in HBSS) was added to the insert and 0.6 mL HBSS was added to the well. Normal culture conditions (i.e., pH 7.0 on both sides, in an incubator set at 37 °C) were maintained. After 2 h, permeates containing peptides passing through the cells into each receiving well were collected and used directly for HPLC analysis according to the procedure described below. Peptide concentration analysis was based on amino group content measured according to the TNBS method (*31*) as described by Liceaga-Gesualdo and Li-Chan (*32*). The efficiency of peptide transport expressed as percent permeability for each peak was calculated by considering the area under each fraction of the HPLC chromatogram as follows (*33*):

$$\%\ \text{permeability} = 100 \times [\text{peptide detected in receiver side}]/[\text{initial peptide added to the insert}]$$

Permeates were also used directly for the ABTS-radical cation decolorization assay, whereas an aliquot from each permeate was concentrated 7 times by freeze-drying and then reconstituting in HPLC-grade water to perform the ACE-inhibitory assay described below.

HPLC Analysis. HPLC analysis of FPH and FPH-GI as well as the permeates from the Caco-2 cell permeability assay was performed using an Agilent 1100 HPLC system equipped with a Jupiter C12 Proteo 90 Å column (250 × 4.6 mm, 4 μm, Phenomenex, Torrance, CA) set at 30 °C. The mobile phases consisted of 0.05% TFA in water (A) and 0.05% TFA in acetonitrile (B). A flow rate of 1 mL/min was used with the following gradient: 0% B at 0 min, increasing to 25% B at 25 min and 80% B at 30 min, and returning to 0% B at 38 min. Fifty microliters of 5 mg/mL sample was injected onto the column, and peptide peaks were monitored at 214 nm by a diode array detector.

Seven fractions were collected from HPLC of the FPH and FPH-GI samples, and the procedure was repeated 19 times to collect sufficient sample for subsequent antioxidative and ACE-inhibitory assays. Pooled fractions from the 19 HPLC runs were freeze-dried after the removal of acetonitrile under a stream of nitrogen, and each fraction was reconstituted in HPLC-grade water based on the relative peak area of the fraction. Calibration curves for the areas of each peak in the HPLC profile were obtained by injecting different aliquots (1, 2, 5, 10, and 10 μL) of the FPH and FPH-GI samples (at 5 mg/mL concentration) before permeation

through the Caco-2 cells. These calibration curves were used to calculate the amount of peptides that permeated through Caco-2 cells.

Antioxidative Assays. *Chemical Assays*. The antioxidative activity of FPH, UF fractions, FPH-GI, and fractions obtained after HPLC separation of FPH, FPH-GI, and the Caco-2 cell permeates of FPH and FPH-GI were measured by the ABTS-radical cation decolorization assay using the method described in Samaranayaka and Li-Chan (*4*). The Trolox equivalent antioxidative capacity (TEAC), which is the concentration of sample giving the same percent inhibition of absorbance at 734 nm of ABTS radical cation as 1 mM Trolox, was determined for FPH and FPH-GI samples by assessing percent inhibition of the absorbance of ABTS radicals by peptide solutions at four different final assay concentrations (6.67, 16.67, 33.33, and 66.67 μg/mL) in comparison to the values for a standard curve prepared with Trolox (0−20 μM final assay concentrations). Because the Caco-2 cell permeates were analyzed at one concentration, results were expressed as percent ABTS radicals scavenged instead of TEAC values. UF fractions were also analyzed further using the oxygen radical absorbing capacity (ORAC) assay described by Kitts and Hu (*34*) and Samaranayaka and Li-Chan (*4*).

Intracellular Antioxidative Activity by Caco-2 Cell Assay. An intracellular antioxidant assay was performed on FPH using Caco-2 cells maintained in DMEM, supplemented with 10% fetal bovine serum, penicillin (100 unit/mL), and streptomycin (100 μg/mL), following the procedure of Elisia and Kitts (*26*). Cells were incubated at 37 °C in a fully humidified environment under 5% CO_2, and Caco-2 cells at passage 40−60 were used for the experiments. Cells were subcultured at 2−3 days intervals before reaching 90% confluency.

AAPH-initiated intracellular oxidation in cultured Caco-2 cells was performed using black 96-well plates. Serial dilutions of FPH (0.625−5 mg/mL) in 100 μL of HBSS were added to cells (0.3 mL of 3.2×10^5 cells/mL) and incubated for 2 h at 37 °C. The FPH-containing solution was removed, and 100 μL of DCFH-DA probe (10 μM in HBSS) was added to the cells and incubated for 30 min. This was followed by the addition of 100 μL of AAPH (1 mM in HBSS) to the cultured cells after removal of the probe. Fluorescence readings ($\lambda_{excitation} = 485$ nm, $\lambda_{emission} = 527$ nm) were recorded using a microplate reader (Fluoroskan Ascent FL, Labsystem, Helsinki, Finland) at 0, 1, and 2 h after the addition of AAPH. The positive control consisted of cells with the DCFH-DA probe and the AAPH peroxyl radical initiator added but in the absence of FPH. The negative control consisted of cells exposed to only the DCFH-DA probe. All results were expressed according to the formula

$$\text{fluorescence} = \text{fluorescence}_{ti} - \text{fluorescence}_{t0}$$

where fluorescence_{ti} = fluorescence reading taken at time point "ti" during measurement and fluorescence_{t0} = initial fluorescence reading taken at the point when AAPH was added (time zero).

ACE-Inhibitory Activity Assay. ACE-inhibitory activity of FPH, FPH-GI, fractions obtained after HPLC separation of FPH and FPH-GI, and Caco-2 cell permeates of FPH and FHP-GI were determined according to the method described by Cinq-Mars and Li-Chan (*35*), with the following modification: samples were preincubated with ACE at 37 °C for 15 min before addition of the ACE substrate HHL. The 15 min preincubation time was based on results of a preliminary study in which FPH sample at 0.286 mg/mL concentration was preincubated with ACE for 0, 10, 20, 30, and 60 min (data not shown).

IC_{50}, which is the concentration of peptides required to inhibit ACE activity by 50%, was calculated for FPH and FPH-GI by performing the assay at four different final assay concentrations between 0 and 0.3 mg of solids/mL. The IC_{50} values were expressed in micrograms of peptides per milliliter by considering the total amino acid contents as a percentage of dry matter determined by amino acid analysis of FPH, >10 kDa, 3−10 kDa, 1−3 kDa, and <1 kDa UF fractions, which were 69.16, 97.49, 96.85, 87.31, and 53.00%, respectively.

The IC_{50} values could not be estimated for HPLC fractions because the actual assay concentration of peptides was not known, the fractions having been reconstituted on the basis of the average area under each fraction of HPLC chromatogram as described previously.

Cellular Cytotoxicity. Viability of Caco-2 cells after treatment with FPH and FPH-GI was assessed using the MTT assay, which measures the ability of functional mitochondria to catalyze the reduction of MTT to a formazan salt by mitochondrial dehydrogenases (*30*). Cytotoxicity tests were performed in triplicate with FPH and FPH-GI at different concentrations (dissolved in 100 μL of HBSS). Plates containing FPH and FPH-GI samples and Caco-2 cells were incubated for 24 h, followed by replacement of the medium with 100 μL of 0.5 mg/mL MTT. After further incubation for 4 h in the dark, 100 μL of 10% SDS dissolved in 0.01% HCl was added. The absorption at 540 nm was measured against a reference wavelength at 690 nm in a microplate reader (ThermoLabsystems Multiscan Spectrum, Chantilly, VA). The control sample consisted of Caco-2 cells treated with 100 μL of HBSS without FPH. Viability of treated cells was expressed as a percent of viable cells present in the control (*30*).

Statistical Analysis. All analyses were performed in triplicate. Statistical analyses were performed using the SPSS statistical program (SPSS 10.0, SPSS Inc., Chicago, IL). Tukey's test was used to evaluate differences among mean values for treatments at the $P < 0.05$ significance level.

Table 1. Antioxidative and ACE-Inhibitory Capacity of Pacific Hake Fish Protein Hydrolysate (FPH), Ultrafiltration (UF) Fractions, and in Vitro Gastrointestinal (GI) Digested Samples[a]

			ACE inhibitory activity	
sample	TEAC value[b]	ORAC value[b]	% inhibition[c]	IC_{50}[d]
FPH	262 ± 2 a	225 ± 17 a	55.06 ± 0.66 de	161
>10 kDa	251 ± 4 a	249 ± 10 a	18.05 ± 3.88 a	883
3−10 kDa	305 ± 4 b	255 ± 21 a	43.40 ± 6.70 c	319
1−3 kDa	312 ± 1 b	330 ± 18 b	66.91 ± 4.38 f	164
<1 kDa	240 ± 10 a	253 ± 17 a	55.92 ± 3.30 e	115
GI digested				
FPH-GI	376 ± 3 c	ND[e]	49.40 ± 2.10 cde	ND
1−3 kDa-GI	434 ± 17 d	ND	46.55 ± 2.19 cd	ND
<1 kDa-GI	370 ± 00 c	ND	33.33 ± 0.88 b	ND

[a] Average results from two replicate experiments analyzed in triplicate. Values within a column bearing different letters (a−f) are significantly different at $P < 0.05$. [b] Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbing capacity (ORAC) values expressed as micromoles of Trolox equivalents per gram of sample. [c] Percent inhibition of ACE was measured at 0.286 mg/mL sample concentration. [d] IC_{50} values are expressed in micrograms of peptide per milliliter. [e] Not determined.

RESULTS AND DISCUSSION

Antioxidative Activity of FPH and Fractions from Ultrafiltration or RP-HPLC. The results of the chemical-based ABTS and ORAC assays on ultrafiltered Pacific hake FPH fractions in the present study showed that peptides within the 1−3 kDa UF fraction recovered from FPH had the greatest antioxidative capacity (**Table 1**). These results are in agreement with former studies using other marine species that reported antioxidative peptides derived from FPH had molecular masses that ranged from 500 to 1800 Da (*2*, *3*, *22*, *36*−*38*).

Typical peptide profiles obtained using reversed-phase HPLC separation of FPH and FPH-GI (20 μL injection of 5 mg/mL of each starting solution onto HPLC) are shown in **Figure 1**. The most potent ABTS-radical scavenging capacity appears in peaks 4 and 6 (**Table 2**).

Effect of Simulated GI Digestion on Antioxidative Potential of FPH. Table 1 shows that FPH-GI, 1−3 kDa-GI, and <1 kDa-GI had higher TEAC values compared to FPH, 1−3 kDa, and <1 kDa, respectively. Sannaveerappa et al. (*39*) also reported that enzymatic breakdown of proteins present in an aqueous extract of herring under GI-like conditions could increase peroxyl radical scavenging activity and the potential to inhibit model LDL oxidation.

The concentrations of HPLC chromatogram peaks 4−6 were higher in FPH-GI than in FPH, whereas the content of more hydrophobic peptides (e.g., peak 7) decreased after GI digestion (**Figure 1**). Because no new major peaks appeared in the chromatogram of FPH-GI, the increased appearance of peaks

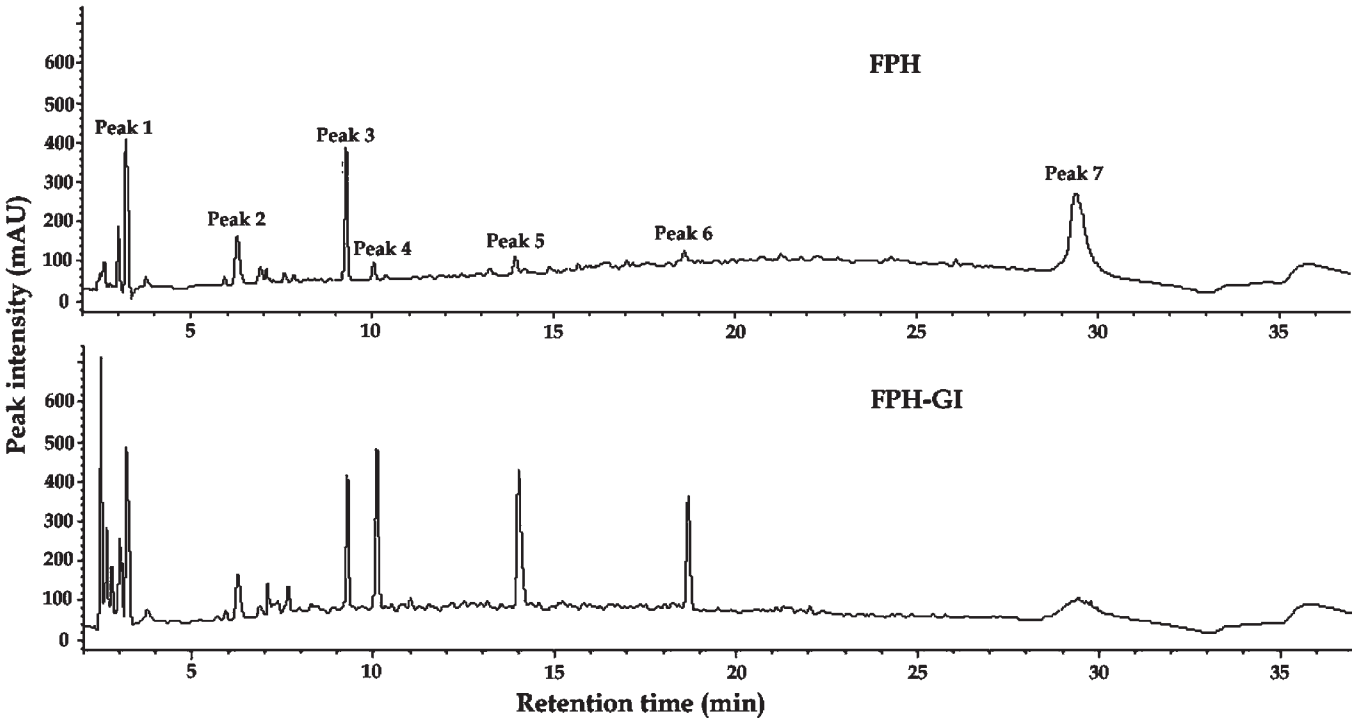

Figure 1. HPLC profiles of Pacific hake fish protein hydrolysate (FPH) and in vitro gastrointestinal (GI) digested FPH (FPH-GI) at similar sample concentration (20 μL injection of 5 mg/mL solution). Retention time ranges for the HPLC fractions collected (i.e., peaks 1−7) are 2.5−3.5, 6.0−7.4, 8.5−9.5, 9.51−10.3, 13−14.2, 18−19, and 29−37 min, respectively.

Table 2. Antioxidative and ACE-Inhibitory Capacities of HPLC Fractions Obtained from Pacific Hake Fish Protein Hydrolysate (FPH) and in Vitro Gastrointestinal (GI) Digested FPH[a]

		% ABTS$^{\bullet+}$ scavenged		% inhibition of ACE	
HPLC peak from **Figure** 1	retention time (min)	FPH	FPH-GI	FPH	FPH-GI
1	2.5−3.5	2.73 ± 0.12 a	2.73 ± 0.12 a	83.33 ± 1.56 a	48.19 ± 5.00 b
2	6−7.4	2.81 ± 0.00 a	2.90 ± 0.12 a	25.42 ± 1.56 a	7.96 ± 7.50 b
3	8.5−9.5	50.04 ± 0.24 a	70.67 ± 0.48 b	40.45 ± 3.44 a	38.90 ± 5.63 a
4	9.51−10.3	98.21 ± 0.12 a	97.95 ± 0.24 a	38.02 ± 1.25 a	28.29 ± 3.75 a
5	13−14.2	45.27 ± 0.24 a	43.39 ± 0.48 b	11.05 ± 3.13 a	13.04 ± 7.19 a
6	18−19	81.84 ± 0.36 a	83.55 ± 0.84 a	11.27 ± 0.31 a	19.89 ± 10.00 a
7	29−37	10.32 ± 0.48 a	6.05 ± 0.24 b	NA[b]	NA

[a] Values are mean ± standard deviation from triplicate analysis. Values within each property (antioxidant or ACE-inhibitory activity) in a row bearing different letters are significantly different at $P < 0.05$. [b] No inhibitory activity.

4−6 after pepsin−pancreatin digestion may have originated from products arising from the digestion of peptides present in peak 7.

ABTS-radical scavenging capacity of the collected fractions separated from samples with and without GI digestion both followed the same trend, with fraction 4 having the highest radical scavenging capacity followed by fraction 6 (**Table 2**). Furthermore, peak 3 showed a significantly higher ABTS radical scavenging capacity after GI digestion.

ACE-Inhibitory Activity of FPH and Fractions Obtained by Ultrafiltration or RP-HPLC. It is not uncommon for protein hydrolysates, or peptides recovered from them, to possess both antioxidative and ACE-inhibitory activities (*13*). The results of the present study showed that Pacific hake FPH had ACE-inhibitory activity, with an IC_{50} value of 235 μg of solids/mL, or 161 μg of peptides/mL (**Table 1**), when expressed on the basis of 69.16% amino acid content of FPH determined by amino acid analysis. A similar IC_{50} value of 165 μg of peptides/mL was reported previously for an FPH prepared using the same stock of Pacific hake captured near Vancouver Island, BC, but following a preincubation with an exogenous enzyme preparation (Protamex) at pH 6.5 and 40 °C for 125 min (*35*).

Comparison of the percent inhibition of ACE by UF fractions of FPH at 0.286 mg/mL sample concentration indicated highest ACE-inhibitory activity in the 1−3 kDa UF fraction (**Table 1**). When the ACE-inhibitory activity were compared as IC_{50} values expressed on a peptide rather than the total dry matter basis, the 1−3 kDa UF fraction, containing 87% amino acid content, had an IC_{50} value (164 μg of peptides/mL), which was similar to that of unfractionated FPH. The < 1 kDa UF fraction possessed the lowest IC_{50} value because only 53% of the dry matter was amino acids and/or peptides.

Of the seven fractions obtained by separating FPH using HPLC, the highest ACE-inhibitory potential was measured in peak 1 (**Table 2**). This finding indicates that peptides derived from Pacific hake FPH with potential blood pressure lowering capability are in fact different from peptides present in FPH that exhibited antioxidative activity. From the characteristic retention times separating the different fractions, it can be concluded that peptides contained in peak 1, eluting earlier and showing higher

ACE-inhibitory activity, are composed of relatively fewer hydrophobic amino acid residues and/or are of shorter chain length and therefore more polar in nature due to the presence of charged amino and carboxyl terminal groups. In comparison, peptides exhibiting the most potent ABTS-radical scavenging capacity appear in peaks 4 and 6 and would, in contrast, contain more hydrophobic residues and/or a longer chain length.

The fact that ACE is a dipeptidase enzyme makes it plausible for further hydrolysis reactions to occur, which could thereby affect the activity of ACE-inhibitory peptides in vivo. Depending on the outcome of GI digestion or other enzyme action in vivo, bioactive peptides can be classified as either true inhibitor (i.e., ACE-inhibitory activity remain unchanged), pro-drug (i.e., increased ACE-inhibitory activity), or substrate (i.e., decreased ACE-inhibitory activity) types of ACE inhibitors (*40*). Preincubation of FPH with ACE indicated that the ACE-inhibitory activity decreased with preincubation time when tested at 10 min and remained unchanged thereafter up to 60 min of preincubation at 37 °C (data not shown). Therefore, the FPH produced in this study was categorized as being a "substrate" type inhibitor with respect to the action of ACE. Because the ACE-inhibitory activity remains unchanged after 10 min, samples were preincubated with ACE for 15 min before the addition of HHL substrate when the ACE assay was performed during this study.

Effect of Simulated GI Digestion on ACE-Inhibitory Activity. ACE-inhibitory potential of FPH did not significantly change upon simulated GI digestion (**Table 1**). However, the effect of pepsin−pancreatin digestion was more pronounced for the partially purified ACE-inhibitory peptides present in the 1−3 and < 1 kDa UF fractions, where there was a significant decrease in ACE-inhibitory activity (**Table 1**). Moreover, a decrease in ACE-inhibitory activity was observed for peak 1 after in vitro GI digestion of the unfractionated FPH (**Table 2**). Further hydrolysis of ACE-inhibitory peptides into smaller peptides or free amino acids during GI digestion can result in a decreased relative ACE-inhibitory potential. During the present study, the ACE-inhibitory peptides originally present in FPH that were concentrated into 1−3 and < 1 kDa UF fractions (**Table 1**) and peak 1 (**Table 2**) during RP-HPLC separation might have been broken down into less active peptide fragments or amino acids upon pepsin−pancreatin digestion. Because the ACE-inhibitory activity is measured on the basis of total material in each fraction, another possibility for the decrease in activity of peak 1 after GI digestion can be the formation of new peptides without significant ACE-inhibitory activity but eluting in this retention time range (**Figure 1**), which could dilute the active peptides in this fraction. Furthermore, the ACE-inhibitory activity of peaks 4−6 did not significantly change upon GI digestion (**Table 2**), but the material in each peak greatly increased. It could therefore be suggested that new or more of the existing ACE-inhibitory peptides eluting in these retention time ranges might have formed during pepsin−pancreatin digestion. This could be the reason for not showing a significant change of the ACE-inhibitory activity of the unfractionated FPH upon GI digestion.

Diverse effects of simulated GI digestion on ACE-inhibitory activity have been reported. For example, two oligopeptides (IWHHT and IVGRPRHQG) isolated from a thermolysin digest of dried bonito exhibited enhanced activity following incubation with chymotrypsin and trypsin, respectively (*40*). Similarly, the IC_{50} value of Pacific hake FPH prepared by hydrolysis with Protamex was reduced to 90 μg of peptides/mL upon simulated GI digestion, compared to an IC_{50} value of 165 μg of peptides/mL for the undigested FPH (*33*). Studies by Wu and Ding (*41*) using soy protein hydrolysates reported a 10% increase in IC_{50} value following in vitro to GI digestion of the < 10 kDa UF fraction. The ACE-inhibitory activity of protein hydrolysates made from fermented milk (*42*), however, did not significantly change with in vitro pepsin pancreatin digestion. Furthermore, Miguel et al. (*43*) reported that the ACE-inhibitory activity of two ovalbumin-derived peptides of YAEERYPIL and RADHPFL decreased upon in vitro simulated GI digestion, even though the peptides were capable of exerting antihypertensive activity in spontaneously hypertensive rats (SHR). Therefore, given the diversity of outcomes reported for various hydrolysates and peptides, investigating the fate of bioactive peptides or precursor proteins and oligopeptides present in food during GI digestion and physiological uptake is an important step in screening for potential bioactive peptide sources.

Table 3. Caco-2 Cell Permeability of Peptides from Pacific Hake Fish Protein Hydrolysate (FPH) and in Vitro Gastrointestinal (GI) Digested FPH

	% permeation through Caco-2 cells[a]	
HPLC peak from **Figure** 1	FPH	FPH-GI
2	24.2 ± 0.8 a	20.3 ± 0.6 b
3	10.6 ± 0.3 a	8.4 ± 0.6 a
4	13.2 ± 1.6 a	5.3 ± 0.6 b
5	14.8 ± 0.9 a	3.1 ± 0.2 b
6	16.5 ± 0.3 a	8.9 ± 0.8 b

[a] Permeation (%) was calculated from the area of each peak in the HPLC chromatogram of FPH or FPH-GI analyzed before permeation and in the permeates after apical-to-basolateral permeation. Values shown are the mean ± standard deviation of three replicates. Values in a row bearing different letters are significantly different at $P < 0.05$.

Caco-2 Cell Permeate Analysis and Bioactivity Measurements. Analysis of cellular permeability of the FPH peptides was performed only on HPLC peaks 2−6 because the broad and complex nature of HPLC peaks 1 and 7 (**Figure 1**) made it difficult to reliably estimate the percentage permeability for those two peaks. The results demonstrated that certain components present in peaks 2−6 from FPH and FPH-GI could permeate the Caco-2 cell monolayer (**Table 3**). Quantification of peptide concentration in permeates using the TNBS assay indicated that 5.04% of FPH and 2.80% of FPH-GI (of the total amount of peptides loaded on the apical side of the Caco-2 cells) had passed through and were detected in the basolateral side of the cells. Although the permeability values expressed as percentage were lower for HPLC fractions of FPH-GI compared to FPH (**Table 3**), the total amounts transported for these components were actually higher in the permeates of FPH-GI than FPH, because the total content of these peaks was higher in FPH-GI than in FPH. This finding suggests that transport of peptides in the FPH-GI sample across the Caco-2 monolayer may have reached a saturation point.

In the present study, peptides from FPH and FPH-GI that were transported through Caco-2 cells possessed ABTS-radical scavenging activities of 5.78 ± 0.43 and 11.68 ± 1.01%, respectively, when the assays were conducted using 65 μL of the undiluted permeates. Therefore, permeated peptides from FPH-GI indicated higher antioxidative activity compared to the peptides permeated from the FPH. Both FPH and FPH-GI inhibited the intracellular oxidation of Caco-2 cells induced by AAPH substrate, with no significant difference between the two concentrations tested (0.625 and 1.25 mg/mL) (**Figure 2**). Because whole hydrolysate, instead of purified peptides, was used in these experiments to assess antioxidant activity of cell permeates and inhibition of intracellular oxidation, it is difficult to state whether the antioxidative peptides present in FPH could penetrate the intestinal cell membrane in intact form or after modification by brush border peptidases. Nevertheless, because HPLC of FPH and FPH-GI showed relatively high permeability of several

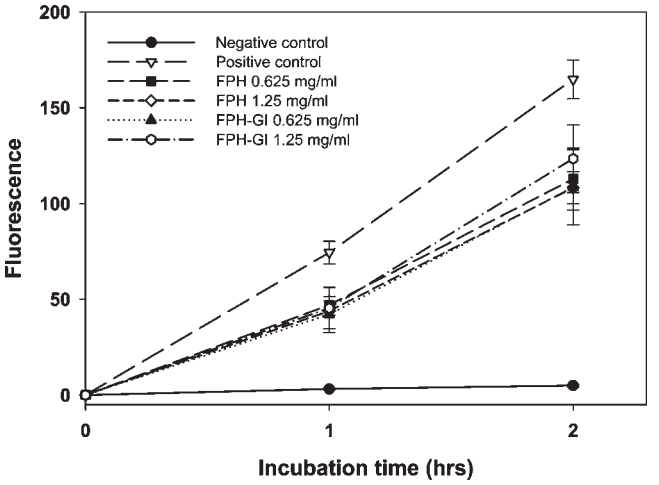

Figure 2. Inhibition of the 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced intracellular oxidation of Caco-2 cells by Pacific hake FPH and FPH-GI at 0.625 and 1.25 mg/mL sample concentrations. Values shown are the mean ± standard deviation of three replicates.

fractions (**Table 3**), including peaks 4 and 6, which exhibited antioxidative potential (**Table 2**), it can be speculated that these peptides permeated through the Caco-2 cell monolayer and effectively contributed to the measured antioxidative activity exhibited by the permeates.

Only a few studies at the present time have attempted to assess the antioxidative potential of peptide hydrolysates or isolated peptides using cell culture, animal models or human clinical trials. Rajapakse et al. (*18*) reported free radical scavenging activity of a peptide derived from fermented mussel sauce (e.g., HFGBPFH) in cultured human lung fibroblast cells. Antioxidative activity was concentration-dependent up to 75 μg/mL, with no further protection on cell survival obtained at higher peptide concentrations. A recent pilot human clinical trial using Seacure, a commercially available fermented fish product made by controlled yeast fermentation of Pacific hake, has also shown a reduction in the degree of small intestinal damage caused by the nonsteroidal anti-inflammatory drug indomethacin (*44*). This study suggested that glutamine present in FPH might have contributed to the observed antioxidant activity via stimulation of intercellular glutathione production. About 19.0% of the total amino acids present in FPH during the present study were also composed of glutamine and/or glutamic acid (**Table 4**), and therefore this compositional characteristic may be noteworthy in explaining further the in vivo potential for antioxidative functionality.

In the present study, although peptides that permeated through Caco-2 cells exhibited some degree of antioxidative activity, there was no similar measurable ACE-inhibitory activity of the permeates, even after concentration by ~7-fold. We suspect that the concentration of ACE-inhibitory peptides in permeates was too low to detect using our in vitro assay procedure. Alternatively, the ACE-inhibitory peptides may not have passed through Caco-2 cells, due either to physical limitation or, moreover, to the possibility that they were degraded to nonactive fragments by brush border peptidases. Our results (**Table 2**) show that most of the ACE-inhibitory peptides were concentrated into peak 1; nevertheless, results of HPLC analysis of Caco-2 cell permeate for both FPH and FPH-GI indicated that there was no detectable recovery of peptides which penetrated Caco-2 cells (data not shown). Our results are similar to findings reported by Vermeirssen et al. (*45*), who observed little or no ACE-inhibitory activity for GI-digested whey and pea proteins following transepithelial procedures using Caco-2 cell monolayer, even though the peptides

Table 4. Amino Acid Composition of Pacific Hake Fish Protein Hydrolysate

amino acid	composition (g/100 g)[a]
aspartic acid + asparagine	10.33
glutamic acid + glutamine	19.04
serine	4.00
glycine	4.52
histidine	1.91
arginine	7.97
threonine	4.03
alanine	6.26
proline	3.41
tyrosine	2.84
valine	4.78
methionine	3.12
isoleucine	3.67
leucine	9.82
phenylalanine	2.95
lysine	10.35
cysteine	0.88
tryptophan	0.15

[a] Expressed as grams per 100 g of amino acids. The total amino acid content expressed as a percentage of FPH dry matter was 69.16%.

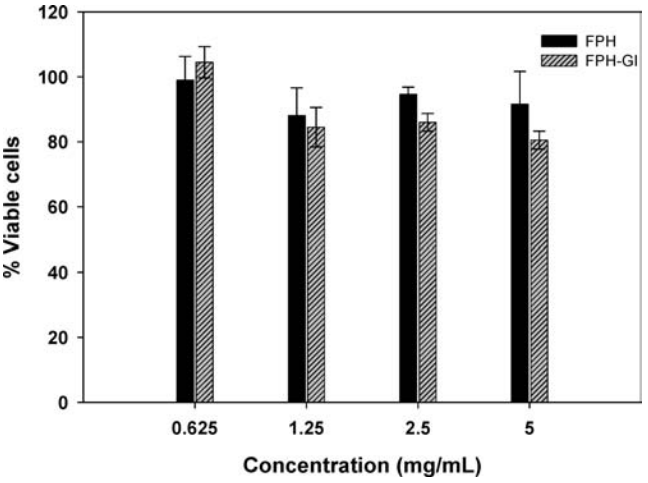

Figure 3. Viability of Caco-2 cells after incubation with Pacific hake FPH and FPH-GI at different concentrations. Values shown are the mean ± standard deviation of three replicates.

were found to be resistant to brush border peptidases. The pea digest did, however, exert a significant blood pressure lowering effect when injected at 50 mg of protein/kg of body weight into the femoral vein of spontaneously hypertensive rats (SHR) (*45*). Qian et al. (*46*) also reported that a peptide, characterized as WPEA-AELMMEVDP and isolated from tuna dark muscle, exhibited antihypertensive effect after oral administration to SHR. Three different transport routes, notably paracellular, fluid phase, and adsorptive transcytosis, are found to be involved in oligopeptide transport across the intestinal epithelium (*47*, *48*). Furthermore, the contribution of each route and the affinity of individual peptides to transport across the membrane will depend upon the relative molecular size and hydrophobicity as well as resistance to brush border peptidases (*47*, *49*).

Cellular Cytotoxicity. Cell viability ranged from 80 to 100% when cells were incubated for 24 h with 0.625−5 mg/mL FPH or FPH-GI (**Figure 3**). It is interesting to note that no effect on cell viability was observed at the lowest concentration of 0.625 mg/mL (**Figure 3**), whereas this concentration was sufficient to significantly inhibit oxidation of Caco-2 cells after 2 h of incubation at 37 °C (**Figure 2**). Furthermore, Pacific hake FPH showed

no toxicity to human hepatocellular liver carcinoma (HepG-2) cells when exposed at sample concentrations up to 1 mg/mL (results not shown). The antioxidative peptide VKAGFAWTANQQLS (1519 Da), isolated from tuna backbone protein hydrolysate, was also reported to have no cytotoxic effect when tested on human lung fibroblast (MRC-5) and human endothelial (ECV304) cells (*50*).

In summary, the results of this study using simulated GI digestion, Caco-2 cell permeation study, and intracellular antioxidant assay showed good potential of FPH from Pacific hake to act as an antioxidative agent, in vivo, at concentrations that were not toxic to the cells. Further work is underway using RP-HPLC−mass spectrometry to determine the sequences of peptides in the fractions with antioxidative capacity and to evaluate the mechanism of their antioxidative capacity. The ACE-inhibitory potential of FPH remained unchanged upon simulated GI digestion, but the Caco-2 cell permeate did not possess any ACE-inhibitory activity. Miguel et al. (*43*) suggested that inhibiting ACE activity is not the only mechanism by which peptides can act as antihypertensive agents. Even though the ACE-inhibitory activity of peptides may decrease upon the digestion by pepsin and pancreatic enzymes, or by brush border peptidases, the resultant peptide fragments might have the possibility to still contribute to hypotensive action by stimulating opioid receptors (*51*), acting on smooth muscles (*52*), or acting as antioxidants to reduce oxidative damages that are mediators in cardiovascular pathologies (*53*). Future studies using in vivo animal model systems and human clinical trials are therefore necessary to ascertain the required standards of evidence for both antioxidative and ACE-inhibitory potential of bioactive peptides derived from hake FPH before consumer use is recommended.

ACKNOWLEDGMENT

We thank R. de Silva and N. Knott of Pacific Fisheries Technologies Inc. and Steveston Seafood Direct Ltd., respectively, for providing Pacific hake and technical expertise. We give special thanks to Dr. Charles Hu and Xiumin Chen for assistance in performing the Caco-2 cell permeation study and intracellular oxidation assay, respectively. We greatly appreciate technical assistance by Imelda Cheung during HPLC fraction collection and by Satoko Nakayama during preliminary ACE-inhibitory assays.

LITERATURE CITED

(1) Amarowicz, R.; Shahidi, F. Antioxidant activity of peptide fractions of capelin protein hydrolysates. *Food Chem.* **1997**, *58*, 355–359.

(2) Wu, C. H.; Chen, H. M.; Shiau, C. Y. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Res. Int.* **2003**, *36* (9−10), 949–957.

(3) Jun, S. Y.; Park, P. J.; Jung, W. K.; Kim, S. K. Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein. *Eur. Food Res. Technol.* **2004**, *219*, 20–26.

(4) Samaranayaka, A. G. P.; Li-Chan, E. C. Y. Autolysis-assisted production of fish protein hydrolysates with antioxidant properties from Pacific hake (*Merluccius productus*). *Food Chem.* **2008**, *107*, 768–776.

(5) Chen, H. M.; Muramoto, K.; Yamauchi, F. Structural analysis of antioxidative peptides from soybean β-conglycinin. *J. Agric. Food Chem.* **1995**, *43*, 574–578.

(6) Pena-Ramos, E. A.; Xiong, Y. L. Antioxidant activity of soy protein hydrolysates in a liposomal system. *J. Food Sci.* **2002**, *67*, 2952–2956.

(7) Tsuge, N.; Eikawa, Y.; Namura, Y.; Yamamoto, M.; Sugisawa, K. Antioxidative activity of peptides prepared by enzymic hydrolysis of egg white albumin. *Nippon Nogeikagaku Kaishi* **1991**, *65*, 1635–1641.

(8) Davalos, A.; Miguel, M.; Bartolome, B.; Lopez-Fandino, R. Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. *J. Food Prot.* **2004**, *67*, 1939–1944.

(9) Suetsuna, K.; Ukeda, H.; Ochi, H. Isolation and characterization of free radical scavenging activities peptides derived from casein. *J. Nutr. Biochem.* **2000**, *11*, 128–131.

(10) Rival, S. G.; Boeriu, C. G.; Wichers, H. J. Caseins and casein hydrolysates. II. Antioxidative properties and relevance to lipoxygenase inhibition. *J. Agric. Food Chem.* **2001**, *49*, 295–302.

(11) Kitts, D. D.; Weiler, K. Bioactive peoteins and petides from food sources. Applications of bioprocesses used in isolation and recovery. *Curr. Pharm. Des.* **2003**, *9*, 1309–1323.

(12) Herńandez-Ledesma, B.; Miralles, B.; Amigo, L.; Ramos, M.; Recio, I. Identification of antioxidant and ACE-inhibitory peptides in fermented milk. *J. Sci. Food Agric.* **2005**, *85*, 1041–1048.

(13) Herńandez-Ledesma, B.; Quirós, A.; Amigo, L.; Recio, I. Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. *Int. Dairy J.* **2007**, *17*, 42–49.

(14) Nagai, T.; Nagashima, T. Antioxidative activities and angiotensin-I-converting enzyme inhibition of extracts prepared from chum salmon (*Oncorhynchus keta*) cartilage and skin. *Int. J. Food Properties* **2006**, *9*, 813–822.

(15) Pedroche, J.; Yust, M. M.; Lqari, H.; Megias, C.; Giron-Calle, J.; Alaiz, M.; Vioque, J.; Millan, F. Obtaining of *Brassica carinata* protein hydrolysates enriched in bioactive peptides using immobilized digestive proteases. *Food Res. Int.* **2007**, *40*, 931–938.

(16) Suetsuna, K. Separation and identification of antioxidant peptides from proteolytic digest of dried bonito. *Nippon Suisan Gakkaishi* **1999**, *65*, 92–96.

(17) Kim, S. K.; Kim, Y. T.; Byun, H. G.; Nam, K. S.; Joo, D. S.; Shahidi, F. Isolation and characterization of antioxidative peptides from gelatin hydrolysate of Alaska pollack skin. *J. Agric. Food Chem.* **2001**, *49*, 1984–1989.

(18) Rajapakse, N.; Mendis, E.; Jung, W. K.; Je, J. Y.; Kim, S. K. Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Res. Int.* **2005**, *38*, 175–182.

(19) Mendis, E.; Rajapakse, N.; Kim, S. K. Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *J. Agric. Food Chem.* **2005**, *53*, 581–587.

(20) Thiansilakul, Y.; Benjakul, S.; Shahidi, F. Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). *Food Chem.* **2007**, *103*, 1385–1394.

(21) Thiansilakul, Y.; Benjakul, S.; Shahidi, F. Antioxidative activity of protein hydrolysate from round scad muscle using Alcalase and Flavourzyme. *J. Food Biochem.* **2007**, *31*, 266–287.

(22) Kim, S. Y.; Je, J. Y.; Kim, S. K. Purification and characterization of antioxidant peptide from hoki (*Johnius belengerii*) frame protein by gastrointestinal digestion. *J. Nutr. Biochem.* **2007**, *18*, 31–38.

(23) An, H.; Seymour, T. A.; Wu, J.; Morrissey, M. T. Assay systems and characterization of Pacific whiting (*Merluccius productus*) protease. *J Food Sci.* **1994**, *59*, 277–281.

(24) Vermeirssen, V.; Augustijns, P.; Van Camp, J.; Opsomer, A.; Verstraete, W. *In vitro* intestinal transport and antihypertensive activity of ACE inhibitory pea and whey digests. *Int. J. Food Sci. Nutr.* **2005**, *56*, 415–430.

(25) Liu, R. H.; Finley, J. Potential cell culture models for antioxidant research. *J. Agric. Food Chem.* **2005**, *53*, 4311–4314.

(26) Elisia, I.; Kitts, D. D. Anthocyanins inhibit peroxy radical-induced apoptosis in Caco-2 cells. *Mol. Cell. Biochem.* **2008**, *312*, 139–145.

(27) Vermeirssen, V.; Van Camp, J.; Verstraete, W. Bioavailability of angiotensin-I-converting enzyme inhibitory peptides. *Br. J. Nutr.* **2004**, *92*, 357–366.

(28) 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.

(29) Garrett, D. A.; Failla, M. L.; Sarama, R. J. Development of an in vitro digestion method to assess carotenoid bioavailability from meals. *J. Agric. Food Chem.* **1999**, *47*, 4301–4309.

(30) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63.
(31) Adler-Nissen, J. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzene sulfonic acid. *J. Agric. Food Chem.* **1979**, *27*, 1256–1262.
(32) 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.
(33) Cinq-Mars, C.; Hu, C.; Kitts, D. D.; Li-Chan, E. C. Y. Investigations into inhibitor type and mode, simulated gastrointestinal digestion, and cell transport of the angiotensin I-converting enzyme-inhibitory peptides in Pacific hake (*Merluccius productus*) fillet hydrolysate. *J. Agric. Food Chem.* **2008**, *56*, 410–419.
(34) Kitts, D. D.; Hu, C. Biological and chemical assessment of antioxidant activity of sugar-lysine model Maillard reaction products. *Ann. N.Y. Acad. Sci.* **2005**, *1043*, 501–512.
(35) Cinq-Mars, C. D.; Li-Chan, E. C. Y. Optimizing angiotensin I-converting enzyme inhibitory activity of Pacific hake (*Merluccius productus*) fillet hydrolysate using response surface methodology and ultrafiltration. *J. Agric. Food Chem.* **2007**, *55*, 9380–9388.
(36) Je, J. Y.; Kim, S. Y.; Kim, S. K. Preparation and antioxidative activity of hoki frame protein hydrolysate using ultrafiltration membranes. *Eur. Food Res. Technol.* **2005**, *221*, 157–162.
(37) Je, J. Y.; Park, P. J.; Kim, S. K. Antioxidant activity of a peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. *Food Res. Int.* **2005**, *38*, 45–50.
(38) Ranathunga, S.; Rajapakse, N.; Kim, S. K. Purification and characterization of antioxidative peptide derived from muscle of conger eel (*Conger myriaster*). *Eur. Food Res. Technol.* **2006**, *222*, 310–315.
(39) Sannaveerappa, T.; Westlund, S.; Sandberg, A.-S.; Undeland, I. Changes in the antioxidative property of herring (*Clupea harengus*) press juice during a simulated gastrointestinal digestion. *J. Agric. Food Chem.* **2007**, *55*, 10977–10985.
(40) Fujita, H.; Yokoyama, K.; Yoshikawa, M. Classification and antihypertensive activity of angiotensin-I-converting enzyme inhibitory peptides derived from food proteins. *J. Food Sci.* **2000**, *65*, 564–569.
(41) Wu, J. P.; Ding, X. L. Characterization of inhibition and stability of soy-protein-derived angiotensin-I-converting enzyme inhibitory peptides. *Food Res. Int.* **2002**, *35*, 367–375.
(42) Hernandez-Ledesma, B.; Amigo, L.; Ramos, M.; Recio, I. Release of angiotensin converting enzyme-inhibitory peptides by simulated gastrointestinal digestion of infant formulas. *Int. Dairy J.* **2004**, *14*, 889–898.
(43) Miguel, M.; Aleixandre, M. S.; Ramos, M.; Lopez-Fandino, R. Effect of simulated gastrointestinal digestion on the antihypertensive properties of ACE-inhibitory peptides derived from ovalbumin. *J. Agric. Food Chem.* **2006**, *54*, 726–731.
(44) Marchbank, T.; Limdi, J. K.; Mahmood, A.; Elia, G.; Playford, R. J. Clinical trial: protective effect of a commercial fish protein hydrolysate against indomethacin (NSAID)-induced small intestinal injury. *Aliment. Pharmacol. Ther.* **2008**, *28*, 799–804.
(45) Vermeirssen, V.; Augustijns, P.; Van Camp, J.; Opsomer, A.; Verstraete, W. *In vitro* intestinal transport and antihypertensive activity of ACE inhibitory pea and whey digests. *Int. J. Food Sci. Nutr.* **2005**, *56*, 415–430.
(46) Qian, Z.-J.; Je, J.-Y.; Kim, S.-K. Antihypertensive effect of angiotensin I converting enzyme-inhibitory peptide from hydrolysates of Bigeye tuna dark muscle, *Thunnus obesus*. *J. Agric. Food Chem.* **2007**, *55*, 8398–8403.
(47) Satake, M.; Enjoh, M.; Nakamura, Y.; Takano, T.; Kawamura, Y.; Arai, S.; Shimizu, M. Transepithelial transport of the bioactive tripeptide, Val-Pro-Pro, in human intestinal Caco-2 cell monolayers. *Biosci., Biotechnol., Biochem.* **2002**, *6*, 378–384.
(48) Miguel, M.; Dávalos, A.; Manso, M. A.; Peña, G.; de, la.; Lasuncion, M. A.; Lopez-Fandino, R. Transepithelial transport across Caco-2 cell monolayers of antihypertensive egg-derived peptides. PepT1-mediated flux of Tyr-Pro-Ile. *Mol. Nutr. Food Res.* **2008**, *52*, 1507–1513.
(49) Shimizu, M.; Tsunogai, M.; Arai, S. Transepithelial transport of oligopeptides in the human intestinal cell, Caco-2. *Peptides* **1997**, *18*, 681–687.
(50) Je, J. Y.; Qian, Z. J.; Byun, H. G.; Kim, S. K. Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis. *Process Biochem.* **2007**, *42*, 840–846.
(51) Sipola, M.; Finckenberg, P.; Vapaatalo, H.; Pihlanto-Leppälä, A.; Korhonen, H.; Korpela, R.; Nurminen, M.-L. R-lactorphin and α-lactorphin improve arterial function in spontaneously hypertensive rats. *Life Sci.* **2002**, *71*, 1245–1253.
(52) Kuono, K.; Hirano, S.; Kuboki, H.; Kasai, M.; Hatae, K. Effects of dried bonito (*Katsuobushi*) and captopril, an angiotensin I-converting enzyme inhibitor, on rat isolated aorta: a possible mechanism of antihypertensive action. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 911–915.
(53) Touyz, R. M. Reactive oxygen species, vascular oxidative stress and redox signaling in hypertension: what is the clinical significance? *Hypertension* **2004**, *44*, 248–252.

Received for review September 20, 2009. Revised manuscript received December 4, 2009. Accepted December 29, 2009. This study was funded by a strategic grant from the Natural Sciences and Engineering Research Council of Canada.