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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

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Fish protein hydrolysate (FPH) produced by incubation of Pacific hake fillet with 3.00% Protamex at pH 6.5 and 40 °C for 125 min demonstrated in vitro ACE-inhibitory activity (IC_{50} = 165 μg/mL), which was enhanced by ultrafiltration through a 10 kDa molecular weight cutoff membrane (IC_{50} = 44 μg/mL). However, after simulated gastrointestinal digestion, FPH and ultrafiltrate had similar ACE-inhibitory activity (IC_{50} = 90 μg/mL), indicating that FPH peptides act as "pro-drug type" inhibitors and that enrichment by ultrafiltration may be unnecessary. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry confirmed that the molecular weights of major peaks were <1 kDa regardless of ultrafiltration. ACE-inhibitory activities of digested hydrolysates were not significantly affected by preincubation with ACE ($P > 0.05$) and exhibited a competitive inhibitory mode. A permeability assay using fully differentiated colorectal adenocarcinoma (Caco-2) cells showed an apical to basolateral transport of peptides that ranged from ~2 to 20% after 2 h at 37 °C. Pacific hake fillet hydrolysates are a potentially bioavailable source of ACE-inhibitory peptides awaiting further in vivo study.

KEYWORDS: ACE-inhibitory activity; fish protein hydrolysate; Pacific hake; simulated gastrointestinal digestion; inhibitor type; inhibitor mode; Caco-2 cell transport assay; MALDI-ToF

INTRODUCTION

One of the greatest challenges in developing nutraceutical and functional food products is proving the in vivo efficacy of their bioactive components. Animal studies and clinical trials are costly and require strict ethical consideration, so compounds thought to have positive bioactive potential must first be prescreened by in vitro assay. Such compounds include angiotensin I-converting enzyme (ACE) inhibitory peptides of food protein hydrolysates, which act to lower blood pressure by preventing the formation of the vasoconstrictor angiotensin II and the breakdown of the vasodilator bradykinin (*1*). Many protein hydrolysates and the related isolated peptides have shown promising in vitro ACE-inhibitory activity, including those from casein, whey, chicken, bovine, wheat, corn, and fish sources (*2*). Recently, we reported that hydrolysates with ACE-inhibitory activity could be generated from Pacific hake (*Merluccius productus*) (*3*), one of the most abundant fish species harvested on the Pacific coast of North America, yet it is underutilized and under-valued due to endogenous proteolytic activity leading to unacceptable soft flesh. Furthermore, ultrafiltration using a 10 kDa cutoff membrane resulted in a peptide mixture with enhanced ACE inhibitory activity while reducing yield by only 5.4% or less, as compared to the unfractionated hydrolysates (*3*).

However, not all peptides exhibiting in vitro ACE-inhibitory activity may be effective for lowering blood pressure in vivo. For example, the digest of dried bonito obtained after pepsin−trypsin−chymotrypsin treatment (*4*) and the tripeptide TNP (*5*) did not significantly lower blood pressure in the spontaneously hypertensive rat (SHR) model despite potent in vitro ACE-inhibitory activity with IC_{50} values of 41 μg/mL and 207.4 μM, respectively. On the other hand, the tripeptide PPK, with a relatively low in vitro potency of IC_{50} >1000 μM, exerted a significant ($P < 0.01$) antihypertensive effect at 6 h postadministration, at an oral dose of 1 mg peptide/kg body weight (*5*). Moreover, comparable in vivo effects have been observed for some ACE-inhibitory peptides as compared to the peptide analog ACE inhibitor drug Captopril (D-3-mercapto-2-methylpropanoyl-L-proline), despite the higher in vitro activity potency of the latter (*6*–*8*). These studies indicate that in vitro tests should consider the possible modification of ACE-inhibitory peptides before reaching the target ACE molecules in the blood stream, which may affect in vivo efficacy.

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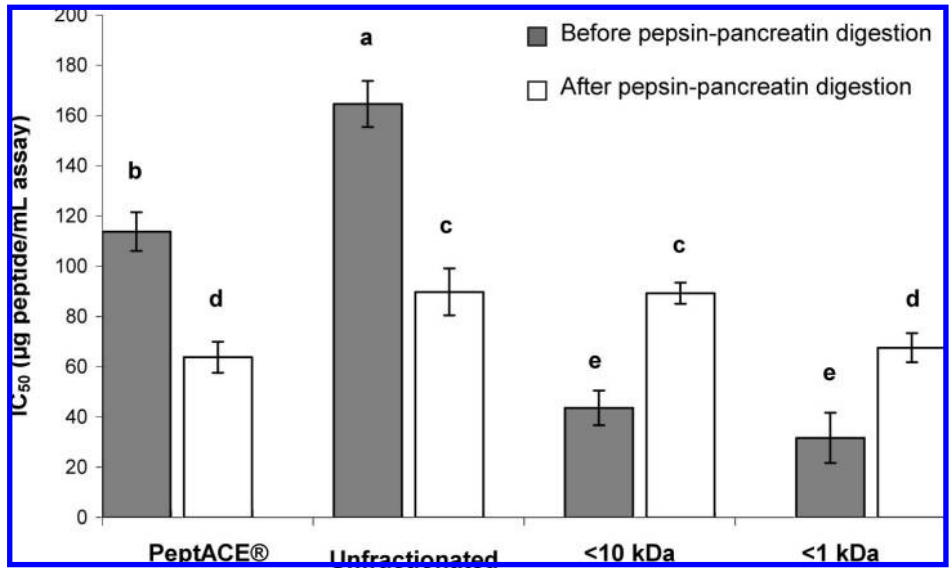

Figure 1. Comparison of ACE-inhibitory activity of PeptACE, unfractionated Pacific hake fillet hydrolysate (hydrolysis conditions: 3.00% E/S, pH 6.5, 40 °C, and 125 min), and its ultrafiltrates before and after simulated gastrointestinal (pepsin−pancreatin) digestion. Each bar represents the mean ± standard deviation of triplicate IC_{50} values; bars labeled with different letters are significantly different ($P < 0.05$).

Potential sites of ACE-inhibitory peptide modification by hydrolysis include the gastrointestinal (GI) tract and, given the fact that ACE itself is a dipeptidase, modification can also occur in the blood circulatory system. Results of simulated GI digestion on soy protein isolate (*9*) and pea protein (*10*) showed that time spent in stomach and duodenum digestion phases significantly affected ACE-inhibitory IC_{50} values of hydrolysates. In terms of isolated peptides, some ACE-inhibitory oligopeptides, namely IWHHT and IVGRPRHQG derived from dried bonito, were shown to increase in activity upon digestion with chymotrypsin and trypsin, whereas others, including YAEERYPIL and RADHPFL from ovalbumin hydrolysate, decreased in activity upon incubation with pepsin and pancreatin (*11, 12*). On the other hand, the ACE-inhibitory activity of hydrolysates from fermented milk products has been shown to not be changed significantly after simulated GI digestion (*13*). As such, both hydrolysates and individual ACE-inhibitory peptides are now being classified in the literature as "pro-drug type", "true-drug type", or "substrate type" based on an increased, unchanged, or decreased ACE-inhibitory activity after simulated GI digestion, respectively (*11*).

This classification also applies to the effects of preincubation with ACE on the peptide ACE-inhibitory activity, whereby peptides that are hydrolyzed by ACE to peptides of lower ACE-inhibitory activity are not considered true inhibitors but rather as substrates of ACE. True-drug peptides and the products of pro-drug peptides, upon contact with ACE, can be further classified according to "inhibitor mode" as noncompetitive or competitive inhibitors based on inhibitor kinetics and the Lineweaver–Burk plot (*2*). For example, in studies by Hernandez-Ledesma et al. (*14*) and Wu et al. (*15*), respectively, the K_m value of ACE substrate was unchanged in the presence of ACE-inhibitory peptides from fermented whey (characteristic of a noncompetitive inhibitor), whereas the inhibition activity of ACE-inhibitory peptide LRW isolated from pea protein was eventually overcome by increasing levels of ACE substrate (characteristic of a competitive inhibitor).

Another important factor that will determine the actual efficacy of orally consumed ACE-inhibitory peptides to lower blood pressure in vivo concerns the bioavailability or absorption efficiency across the intestinal epithelium. The human colorectal adenocarcinoma Caco-2 cell line has been widely accepted as an intestinal epithelial cell model for permeability measurements that may be applied to predict oral absorption of pharmaceuticals (*16*). Different routes of drug transport in Caco-2 monolayers can be compared to those in vivo and can be used to study the major absorptive mechanisms such as passive transcellular and paracellular transport, carrier-mediated influx, and efflux mechanisms (*17, 18*).

The objectives of this study were to assess the potential stability and absorption characteristics of ACE-inhibitory peptides in Pacific hake fillet (PHF) hydrolysates by utilizing in vitro assays. The effects of simulated GI (sequential pepsin/pancreatin) digestion and preincubation with ACE on the ACE inhibitory activity (IC_{50} values), inhibitor kinetics, molecular weight profiles, and transport in a Caco-2 cell monolayer model system were investigated. This research provides the basis for further investigation into the efficacy of bioactive peptides in PHF hydrolysates to lower blood pressure in vivo.

MATERIALS AND METHODS

Materials. PHF hydrolysate was prepared from Pacific hake fillet as previously described (*3*), using Protamex commercial protease at an enzyme-to-substrate (E/S) ratio of 3.00% at pH 6.5 and 40 °C for 125 min; the hydrolysate was fractionated by ultrafiltration through 10 or 1 kDa molecular weight cutoff (MWCO) membranes (*3*). All hydrolysate and ultrafiltrate samples were freeze-dried and stored at –25 °C. Pepsin (3300 U/mg, product P6887) and pancreatin (converts 25× its weight of casein in 1 h, product P7545) were purchased from Sigma-Aldrich Canada (Oakville, Ontario, Canada) and were produced by Novozymes (Bagsvaerd, Denmark). PeptACE Peptides, a product of Natural Factors Nutritional Products Ltd. (Coquitlam, British Columbia, Canada), was purchased from Finlandia Pharmacy (Vancouver, BC, Canada). Hippuryl-L-histidyl-L-leucine (HHL as tetrahydrate), angiotensin-converting enzyme (ACE, from rabbit lung), and MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl) tetrazolium bromide were purchased from Sigma-Aldrich (St. Louis, Missouri). ACE was dissolved and portioned in 0.025 M sodium tetraborate buffer containing 0.3 M NaCl (pH 8.3) to a concentration of 2.5 mU/30 µL aliquot, and the solution was stored at –25 °C. Human colorectal adenocarcinoma (Caco-2) cells were obtained from the American Tissue Culture

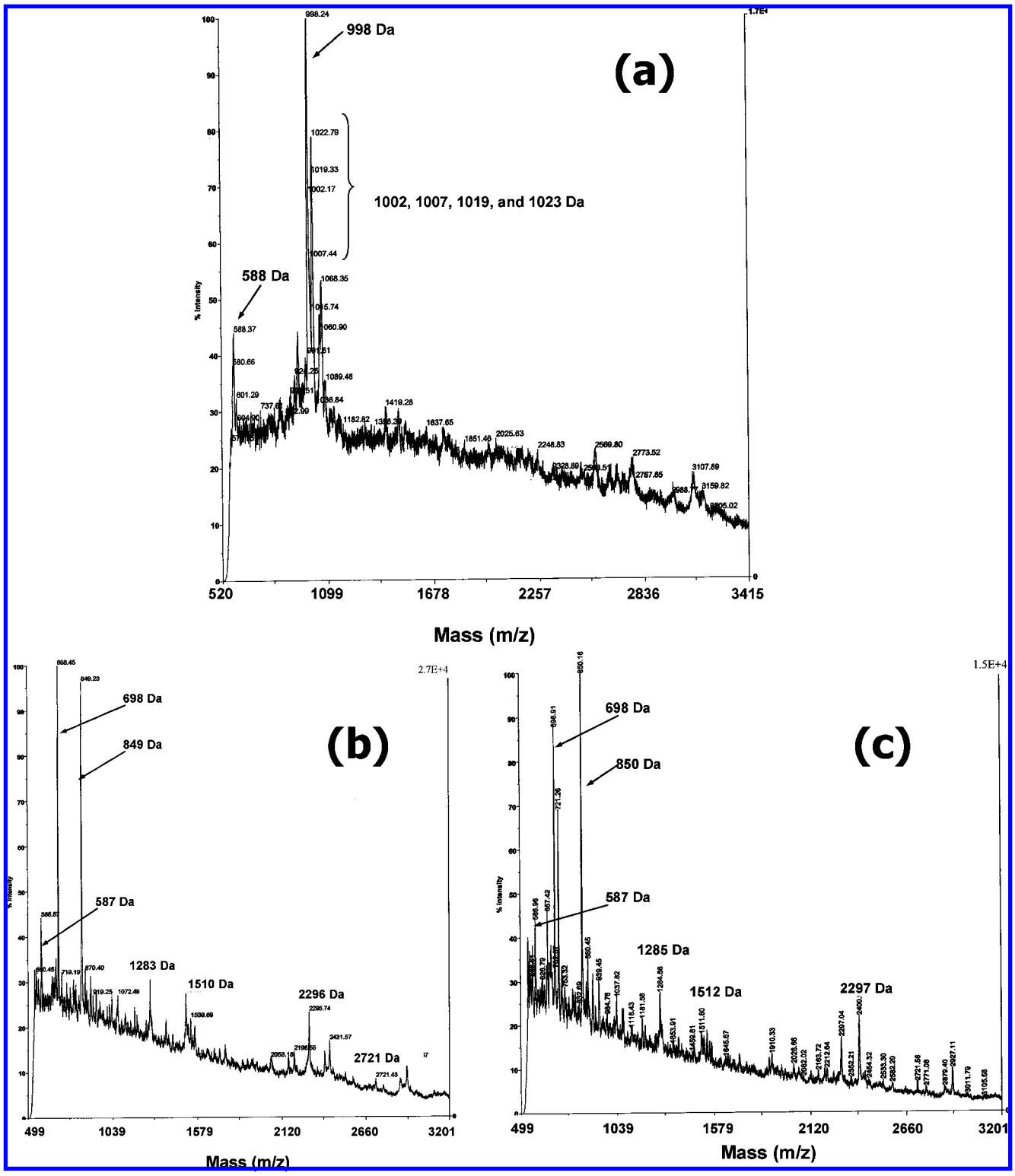

Figure 2. MALDI-ToF spectra of Pacific hake fillet hydrolysate and ultrafiltrate. (**a**) Unfractionated hydrolysate before simulated GI digestion, (**b**) unfractionated hydrolysate after simulated GI digestion, and (**c**) <10 kDa ultrafiltrate after simulated GI digestion.

Collection (Manassas, Virginia). Eagle's minimum essential mineral (EMEM) medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen Canada (Burlington, Ontario, Canada).

In vitro Pepsin−Pancreatin Simulated Gastrointestinal Digestion. Simulated GI digestion by in vitro pepsin−pancreatin hydrolysis was carried out in triplicate by modification of the methods of Hernandez-Ledesma et al. (*19*) and Lo and Li-Chan (*20*). Hydrolysate solids containing 225 mg of peptides were mixed with 15 mL of distilled–deionized water (ddH_2O), and the pH adjusted to 2.0 with 5 N HCl. Pepsin was then added (E/S 1:35 w/w), and the mixture was incubated with shaking 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 was added (E/S 1:25 w/w), and the mixture was further incubated with shaking for 2 h at 37 °C. To terminate the digestion, the solution was submerged in boiling water for 10 min. The hydrolysate was freeze-dried and stored at –25 °C.

ACE-Inhibitory Activity. The assay of Cushman and Cheung (*21*) was modified to enable investigation of the effect of preincubation with ACE on the ACE-inhibitory activity of hydrolysate samples. First, 30

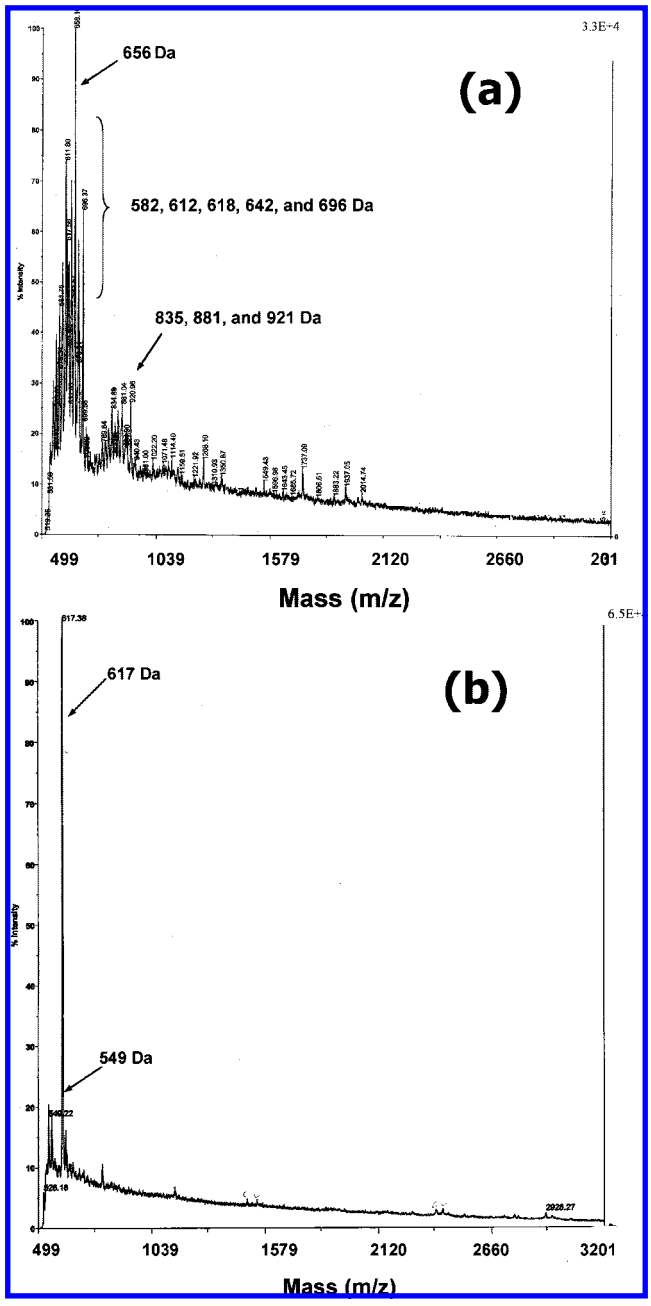

Figure 3. MALDI-ToF spectra of PeptACE Peptides (**a**) before and (**b**) after simulated GI digestion.

μL of hydrolysate solution was mixed with 30 μL (2.5 mU) of ACE, or buffer as control, and incubated at 37 °C for 1 h. To terminate the preincubation ACE activity, samples were heated at 95 °C in boiling water for 5 min. The ACE assay was then initiated by addition of 150 μL of 8.91 mM HHL and a fresh 30 μL (2.5 mU) aliquot of ACE and further incubation 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 240 μL. The reaction was terminated using HCl (250 μL, 1N), and 1.0 mL of ethyl acetate was added to extract hippuric acid (HA). The solutions were vortexed for 30 s and then centrifuged at 2000*g* 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 at 120 °C for 30 min. 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. ACE-inhibitory activity (%) with and without ACE preincubation was determined in triplicate. IC_{50} values were derived in triplicate from regression curves through % ACE inhibition values of five peptide concentrations.

MALDI-ToF Mass Spectrometry. Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-ToF) mass spectrometry analysis was conducted at the Laboratory of Molecular Biophysics Proteomics Core Facility, Michael Smith Laboratories, University of British Columbia (Vancouver, British Columbia). A saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA; from Sigma-Aldrich, Oakville, Ontario) in 50:50 acetonitrile: 0.1% trifluoracetic acid (TFA) in ddH_2O (v/v) was used as the MALDI matrix. Samples reconstituted in 0.1% TFA were serially diluted (1:1, 1:10, and 1:100) in the CHCA matrix, and 1.0 μl of each dilution was deposited on a stainless steel sample target and was allowed to dry at room temperature (dried droplet method). MALDI analyses were performed using a Voyager-DE STR delayed-extraction ToF mass spectrometer (Applied Biosystems, Framingham, Massachusetts) in the positive ion reflector and/or linear modes. The instrument is equipped with a nitrogen laser (337 nm) to desorb and ionize the samples. Spectra were obtained over the mass acquisition range of 50–500 Da in the reflector mode and 500–10 000 Da in the linear mode, with 50–100 laser shots per spectrum and 20 000V−25 000 V accelerating voltage. A low mass gate was set at 500 Da for analysis of the samples in linear mode to avoid interfering peaks attributed to the CHCA matrix. A close external calibration to bracket the mass range of interest was performed with CalMix1 and CalMix2 in the Sequazyme^{TM} peptide mass standards kit (PE Biosystems, Framingham, Massachusetts). The components (with masses in parentheses, expressed as average $(M + nH)n^+$) of the two calibration mixtures were des-Arg^1−bradykinin (905.05), Glu^1−fibrinopeptide B (1571.61), and neurotensin (1673.96) in CalMix1, and angiotensin I (1297.51), ACTH (clip 1–17) (2094.46), ACTH (clip 18–39) (2466.72), ACTH (clip 7–38) (3660.19), and bovine insulin (5734.59 and 2867.80 for singly and doubly charged signals) in CalMix2. Mass accuracy is specified at 50 ppm.

Study of Inhibition Kinetics. Construction of a Lineweaver–Burk plot for the determination of inhibition mode was carried out for hydrolysate samples according to the methodology described by Stauffer (*22*). Activity of ACE in the assay was kept constant at 2.5 mU, while a range of concentrations of HHL in the final assay volume was selected above and below the reported K_m for HHL of 2.6 mM (*21*), specifically at 12.5, 4.63, 2.50, 1.71, and 1.30 mM. Hydrolysate samples were added at their respective IC_{50} values (μg peptide/mL assay). ACE activity in the presence or absence of hydrolysate samples was determined using the assay for quantification of ACE inhibition as described above (*20, 21*), and initial velocity (V_o) was taken as absorbance per minute at 228 nm over the total assay time of 1 h. Dissociation constant (K_i) for hydrolysate samples was calculated by the following equation (*21*):

$$k_i \text{ value} = [\text{inhibitor}]/((k_{app}/k_m) - 1.0) \quad (1)$$

Caco-2 Cell Permeability Assay. Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia). Cells of passages 23–28 were used. Cells were cultured and maintained in a Petri dish with EMEM medium supplemented with 10% FBS and antibiotics (100 μg/mL penicillin and 100 IU/mL streptomycin) in fully humidified atmosphere with 5% CO_2 at 37 °C. Cell culture medium was replaced every other day.

For the cell permeability study, Caco-2 cells were seeded onto cell culture inserts (0.32 cm^2, 0.4 μ pore size, BD Biosciences Canada, Mississauga, Ontario) in a 24-well cell culture companion plate. Cell density on the insert (apical side) was 0.3 mL of 2×10^5/mL; 0.8 mL of EMEM was added to each well of the 24-well companion plate. Cell culture medium was carefully replaced every other day for at least 21 days, until the Caco-2 cells were fully differentiated as monolayers. Monolayer integrity was measured by transepithelial electrical resistance (TEER) with a Millicell ERS (electrical resistance system) (Millipore Corp., Bedford, Massachusetts, USA), both at the beginning and at the end of the absorption assay, after equilibrating the monolayers in transport buffers. Only monolayers with TEER of 900 Ω were chosen for the transport study. Cells were washed twice with Hank's buffered saline solution (HBSS) inside and outside the cell culture insert. Cell viability measurements were also made before and after the experiment using the MTT assay (*23*).

All experiments were conducted under iso-pH conditions (i.e., pH 7.0 on both sides) in an incubator set at 37 °C. For apical to basolateral

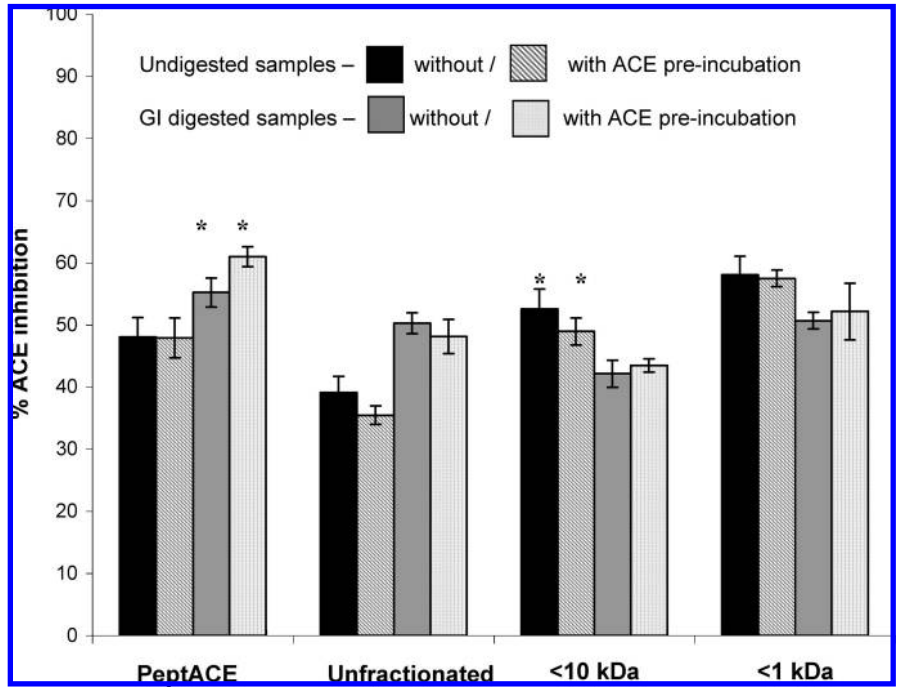

Figure 4. Effect of preincubation with ACE on the ACE-inhibitory activity of PeptACE Peptides, unfractionated Pacific hake hydrolysate, and its ultrafiltrates (80 μg peptides/mL assay). Each bar represents the mean $\pm$ standard deviation of triplicate preincubation or without preincubation trials; bars of the same sample labeled with an asterisk are significantly different before and after preincubation with ACE ($P < 0.05$).

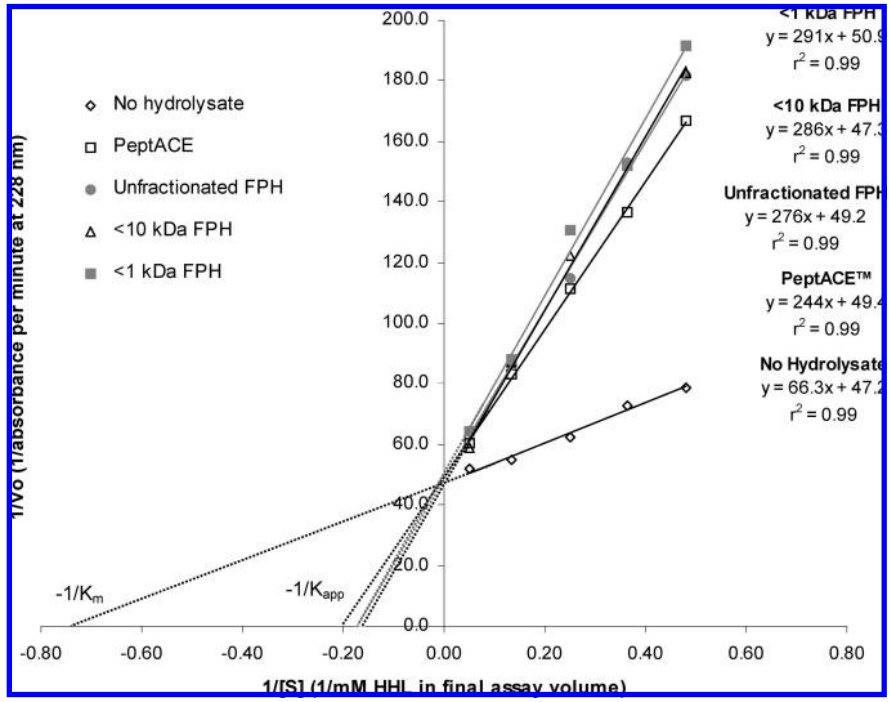

Figure 5. Lineweaver–Burk plot of ACE activity in the presence of PeptACE Peptides (64 μg peptide/mL assay), unfractionated Pacific hake hydrolysate (90 μg peptide/mL assay), <10 kDa ultrafiltrate (89 μg peptide/mL assay), and <1 kDa ultrafiltrate (68 μg peptide/mL assay) at their respective IC_{50} values. All samples were subjected to pepsin−pancreatin digestion and ACE preincubation, and the plot shows results from triplicate assays.

transport, 0.15 mL of PHF hydrolysate sample (10 mg/mL) was added to the apical side, and the insert was placed back into the 24-well plate with 0.6 mL of HBSS in each well. For basolateral to apical transport, 0.6 mL of PHF hydrolysate sample (10 mg/mL) was added to each well of the 24-well plate, and a cell culture insert with 0.15 mL of HBSS per insert was positioned in each well. The cell culture plate was incubated at 37 °C for 2 h, followed by sampling from either apical or basolateral side for HPLC analysis. All assays were performed in duplicate.

HPLC analysis of PHF hydrolysates sampled from the apical and basolateral sides was performed with an Agilent 1100 HPLC system equipped with a Jupiter C12 Proteo 90Å column (250 × 4.6 mm, 4 μ, from Phenomenex, Torrance, California) at 30 °C. The mobile phases, 0.05% trifluoroacetic acid (TFA) in water (A) and 0.05% TFA in acetonitrile (B), were used at a flow rate of 1 mL/min with the following gradient: 0% B at 0 min, increasing to 25% B at 25 min, 80% B at 30 min, and returning to 0% B at 38 min. The peptide peaks were monitored at 214 nm by a diode array detector. The content of peptide

Table 1. Kinetics Constants for HHL Substrate and ACE-Inhibitory Samples Assayed with ACE at 2.5 mU[a]

inhibitor sample[b]	K_m or K_{app} in the presence of inhibitor (μg HHL/mL assay)	dissociation constant (K_i) (μg peptide/mL assay)
control (no inhibitor)	1.41	
PeptACE Peptides	4.95	25.5
unfractionated PHF hydrolysate	5.62	30.1
<10 kDa ultrafiltrate from PHF hydrolysate	6.06	27.0
<1 kDa ultrafiltrate from PHF hydrolysate	5.71	22.3

[a] K_m, K_{app}, V_{max}, and V_{app} values are based on the Lineweaver–Burk plot of **Figure 5**. K_i was calculated as $K_i = [\text{inhibitor}]/((K_{app}/K_m) - 1.0)$ [b] Inhibitor samples were treated by simulated GI (pepsin−pancreatin) digestion and ACE-preincubation prior to the assay with ACE using HHL substrate.

in individual peaks was calculated from the calibration curve obtained for each respective peak of the PHF hydrolysate. The efficiency of transport, expressed as percent permeability, was calculated according to the following equation,

$$\text{Permeability } \% = 100 \times \frac{\text{peptide detected in receiver side}}{\text{initial peptide added to donor side}}$$

where "receiver" and "donor" refer to apical and basolateral sides, respectively, for basolateral-to-apical transport, and to basolateral and apical sides, respectively, for apical-to-basolateral transport.

Statistical Analysis. Using MINITAB version 12.21 (Minitab Inc., State College, Pennsylvania), statistical significance of differences between means was evaluated by analysis of variance (ANOVA) using a general linear model and pairwise comparisons by Tukey's method ($P < 0.05$). Lineweaver–Burk plots were prepared using Microsoft Excel 2000.

RESULTS AND DISCUSSION

Effect of In Vitro Pepsin−Pancreatin Simulated Gastrointestinal Digestion on ACE-Inhibitory Activity and Molecular Weight Profile. To classify the "inhibitor type" of the PHF hydrolysates, samples were first subjected to in vitro pepsin−pancreatin digestion. The samples included unfractionated PHF hydrolysate, the <10 kDa and <1 kDa ultrafiltrates (*3*), and the commercial product PeptACE Peptides from bonito fish.

As shown in **Figure 1**, PeptACE Peptides and the unfractionated PHF hydrolysate showed significantly greater ACE inhibitory activity (i.e., significantly lower IC_{50} values) after simulated GI digestion and may therefore be considered as prodrug type inhibitors. On the other hand, simulated GI digestion lowered the ACE-inhibitory activity (increased the IC_{50} value) of both 10 kDa and 1 kDa ultrafiltrates; thus, the bioactive peptides in the ultrafiltered samples can be considered as substrate type inhibitors. Because the ultrafiltrates contained less large molecular weight peptides than the unfractionated samples, it is possible that preferential hydrolysis of low molecular weight active peptides occurred, thus rendering them no longer or less active in ACE inhibition.

Similar results were reported by Wu and Ding (*24*) in which the IC_{50} value of the <10 kDa fraction of soy protein hydrolysate increased from 65 μg protein/mL to 73 μg protein/mL upon in vitro pepsin−pancreatin digestion. In contrast, Hernandez-Ledesma et al. (*19*) reported that ACE-inhibitory activity of unfractionated whey infant formula hydrolysate significantly decreased at the intestinal stage of simulated GI digestion, whereas the activity of the <3 kDa filtrate remained constant. This underlines the need for assessing the effects of simulated GI digestion on an individual case-by-case basis as protein sequence, hydrolysis process conditions, and commercial protease specificity all affect the composition and properties of hydrolysate produced and, in turn, the substrates and products of simulated GI digestion.

In the case of PHF hydrolysate produced using the process conditions of this study (*3*), after simulated GI digestion the IC_{50} values of unfractionated hydrolysate and the <10 kDa ultrafiltrate were not significantly different from each other, and both were slightly higher ($P < 0.05$) than the <1 kDa ultrafiltrate. However, ultrafiltration to <1 kDa on a commercial scale could be very costly. Furthermore, as indicated by the results shown in **Figure 1**, harnessing the ability of the GI enzymes to release the active peptides from the unfractionated hydrolysate rendered a product that was similar in potency to the <10 kDa ultrafiltered fraction, and that was only slightly less potent than PeptACE and the <1 kDa fraction. Therefore, ultrafiltration treatment of PHF hydrolysate may not be necessary to produce a potent ACE-inhibitory product.

In our previous study, based on the observation that sequential ultrafiltration treatment using membranes with MWCO of 10, 3, and 1 kDa only achieved approximately 10% reduction in IC_{50} values with each successive filtration, it was hypothesized that the ultrafiltrates contained peptides mainly in the low molecular weight range (*3*). Therefore, to test this hypothesis, molecular weight profiles were analyzed by MALDI-ToF mass spectrometry, and at the same time, information was gathered on the effects of simulated GI digestion on the molecular weight profiles in relation to changes in IC_{50}.

MALDI-ToF spectra for unfractionated PHF hydrolysate before and after simulated GI digestion, and for the <10 kDa ultrafiltrate after simulated GI digestion are shown in **Figure 2**, panels **a**, **b**, and **c**, respectively. For comparison, MALDI-ToF spectra for the commercial product PeptACE Peptides before and after simulated GI digestion are shown in **Figure 3**, panels **a** and **b**, respectively. These MALDI-ToF spectra confirm a high abundance of peptides in the ≤1 kDa range for all samples tested. However, given that MALDI-ToF is a semiquantitative analysis and that peak height is related to the ability of the species to desorb upon application of the laser, individual peptide concentrations as a proportion of the whole cannot be assigned. Very few peaks were detected between 3.2 and 5 kDa, and no significant peaks were detected above 5 kDa in any of the samples (data not shown).

In comparing **Figure 2**, panels **a** and **b**, the unfractionated PHF hydrolysate before simulated GI digestion contains dominant peaks at approximately 1 kDa (representing peptides with ~6–9 residues) and one at 588 Da (~4–5 residues), whereas the post-GI digestion sample contains two dominant peaks at 698 Da (~5–6 residues) and 849 Da (~6–8 residues) and the same peak at ~587 Da. As such, the improved ACE-inhibitory activity in the post-GI digested samples may be due to these three detected peptides and/or di- and tripeptides (<500 Da and undetected on the MALDI-ToF spectra). It is certain, however, that GI digestion does change the molecular weight profile considerably, and with it, the ACE-inhibitory potency. Specifically, between the pre- and post-GI digested samples there is a loss of approximately 150 or 300 Da from the dominant peaks at 1 kDa. This corresponds to the likely cleavage of 1, 2 or 3 amino acid residues, generating peptides with more potent ACE-inhibitory activity. This is also an important observation because smaller peptides are more likely to be absorbed by the small intestine by paracellular transport (*25*). A study by Ruiz et al.

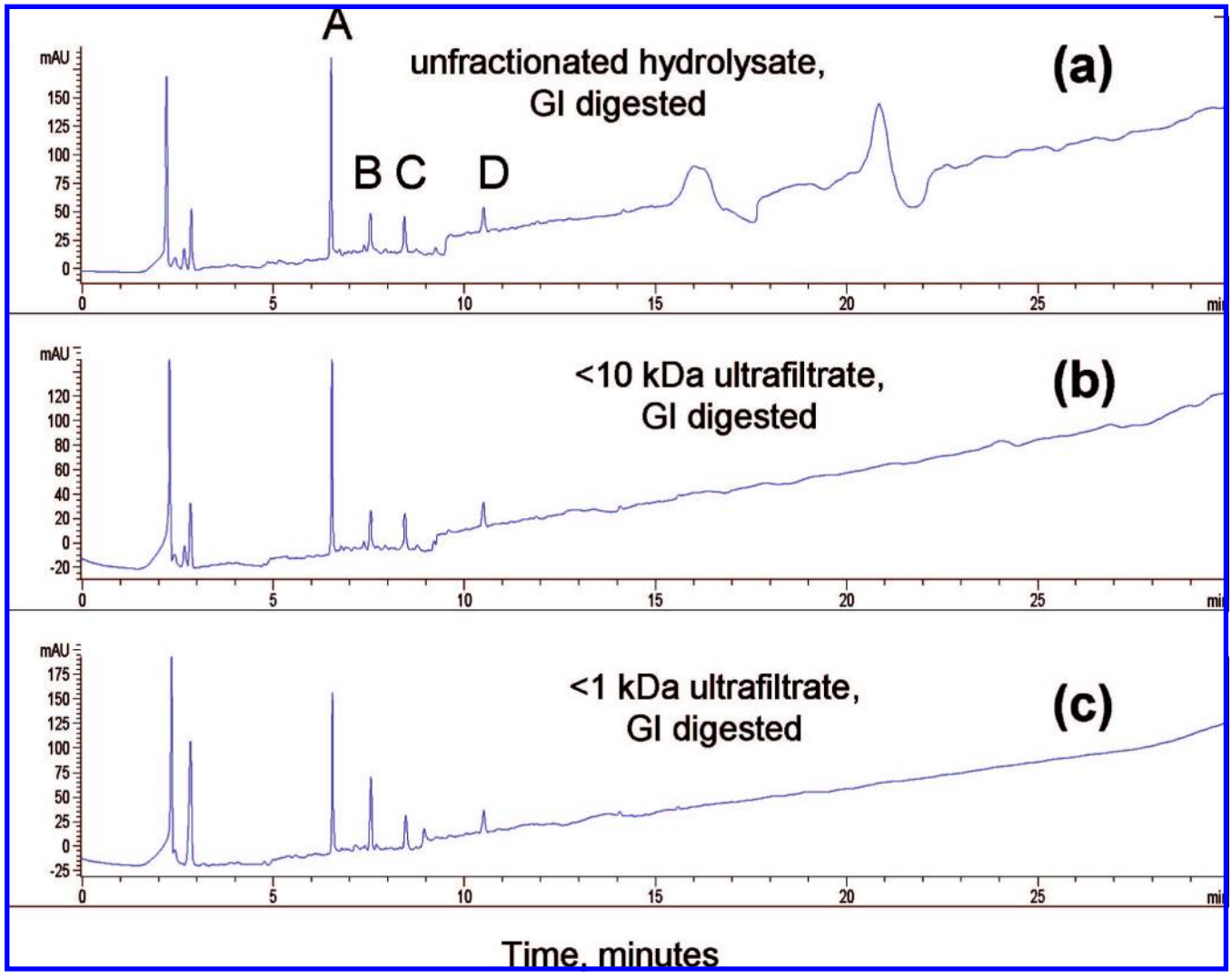

Figure 6. HPLC profiles of pepsin−pancreatin digested unfractionated Pacific hake hydrolysate, <10 and <1 kDa ultrafiltrate samples in the Caco-2 cell permeability assay.

Table 2. Permeability Values of Peptides in Unfractionated Pacific Hake Fillet (PHF) Hydrolysate and Ultrafiltrate Samples before and after Simulated GI Digestion Using Caco-2 Cell Monolayers[a]

		permeability (%)			
	sample	peak A	peak B	peak C	peak D
apical-to- basolateral	unfractionated PHF hydrolysate	5.1 ± 0.2	11.2 ± 1.8	12.7 ± 0.0	14.4 ± 2.3
	<10 kDa ultrafiltrate	6.4 ± 0.4	14.5 ± 0.3	14.2 ± 0.6	12.0 ± 4.8
	<1 kDa ultrafiltrate	2.2 ± 0.7	4.4 ± 1.4	19.9 ± 4.1	3.9 ± 0.3
apical-to- basolateral	unfractionated PHF hydrolysate, digested	10.3 ± 1.9	10.8 ± 2.7	16.5 ± 2.3	18.2 ± 3.3
	<10 kDa ultrafiltrate, digested	4.4 ± 0.9	4.1 ± 1.2	10.0 ± 1.7	8.8 ± 2.0
	<1 kDa ultrafiltrate, digested	3.1 ± 0.8	1.6 ± 0.4	7.1 ± 1.3	4.7 ± 1.1
basolateral-to-apical	unfractionated PHF hydrolysate, digested	0.9 ± 0.0	1.1 ± 0.1	0.9 ± 0.0	1.6 ± 0.1
	<10 kDa ultrafiltrate, digested	1.0 ± 0.2	1.1 ± 0.1	1.0 ± 0.3	1.5 ± 0.4
	<1 kDa ultrafiltrate, digested	1.4 ± 0.0	0.9 ± 0.0	1.1 ± 0.0	1.6 ± 0.0

[a] Values represent mean standard deviation ($n = 3$). The apical-to-basolateral, and basolateral-to-apical permeabilities for respective peaks A−D shown in the HPLC profile (**Figure 6**) are expressed as percentage transported after 2 h at 37 °C, calculated as 100 × [peptide detected in receiver side]/[initial amount of peptide loaded to donor side].

(*26*) on simulated GI digestion of isolated ACE-inhibitory peptides from Manchego cheese also yielded dipeptide and single amino acid cleavage products. Using HPLC-MS/MS to identify the peptide fragments released by the action of GI enzymes, the peptide VPSERYL was fully hydrolyzed to Y, L, RY, VPSER, and YL, resulting in a decreased IC_{50} value.

Comparing **Figure 2**, panels **b** and **c**, the MALDI-ToF spectra of the unfractionated hydrolysates and 10 kDa ultrafiltrate after simulated GI digestion show the same dominant peaks at 698 and 850 Da, and similar profiles of several smaller peaks, for example, 587, 1283–1285, 1510–1512, 2296–2297, and 2721 Da. The similarity between these spectra, in accordance with

the similar IC_{50} values of the digested hydrolysates and 10 kDa ultrafiltrate, provides further support for proposing that ultrafiltration processing of PHF hydrolysate may be unnecessary, because the modifications occurring during GI digestion could lead to peptide mixtures with similar molecular weight profile and ACE-inhibitory activity, whether or not ultrafiltration is performed.

Finally, **Figure 3**, panels **a** and **b**, show the MALDI-ToF spectra of the commercial product PeptACE Peptides before and after simulated GI digestion, respectively. The composition of PeptACE Peptides is starkly different from the PHF hydrolysate with more than 6 dominant peaks in a molecular weight range of 550–700 Da and another 3 peaks at 835, 881, and 921 Da. These peaks correspond to peptides of approximately 4–6 residues, that is, 2–3 residues less than the dominant peaks in the PHF hydrolysate. After simulated GI digestion of PeptACE Peptides, only two dominant peaks remain detectable below 1 kDa, namely, at 549 and 617 Da, indicating that many of the peptides contained in PeptACE Peptides are subject to GI modification, which may be related to the observation of an overall pro-drug activity for this product. However, the peak at 617 Da occurs both before and after simulated GI digestion of the PeptACE Peptides, indicating that this peptide is resistant to GI modification or that more of this peptide is being formed by hydrolysis of larger peptides.

Effect of Hydrolysate Preincubation with ACE on ACE-Inhibitory Activity. Classification of ACE-inhibitory hydrolysates and the related peptides into the three inhibitor types also applies to the response of ACE-inhibitory peptides to contact (incubation) with ACE. In an attempt to ensure that the hydrolysates in the assays were of the "true inhibitor" type with respect to ACE, all assays performed thus far in these studies were conducted with a preincubation period of ACE with peptides, prior to addition of HHL substrate. However, it was not yet known if the effect of preincubation with ACE would differ between the different ultrafiltrates and the GI-digested samples.

Figure 4 confirms that simulated GI digestion caused an increase in the ACE-inhibitory potency of PeptACE Peptides and unfractionated PHF hydrolysate, but a decrease in the ACE-inhibitory potency of the ultrafiltrate fractions. With respect to preincubation with ACE, undigested <10 kDa PHF hydrolysate can be considered a substrate type inhibitor because there was a slight but significant decrease in ACE-inhibitory activity of the product. However, for in vivo efficacy it is more important that, after GI digestion, the ACE-inhibitory activity of the peptides is not significantly lowered or is increased by contact with ACE in the blood stream. After GI-digestion, the unfractionated PHF hydrolysate as well as the ultrafiltrates all displayed the desired behavior of a true-drug type, as demonstrated by the absence of a significant change in the %ACE-inhibitory activity upon preincubation with ACE. Therefore, these results lend strong support for further in vivo study of the antihypertensive efficacy of PHF hydrolysate. The GI-digested PeptACE Peptides was found to exhibit pro-drug type inhibition, because ACE-incubated samples showed a significantly higher %ACE-inhibitory activity. Peptides whose activity are increased with ACE preincubation have been shown to demonstrate a milder but more prolonged antihypertensive effect in vivo. For example, the peptide LKPNM, shown to be cleaved in vitro by ACE to LKP, resulted in maximal decrease in blood pressure of SHR at 6 h after oral administration, although LKP had a maximal effect at 4 h, the lag period likely being attributed to in vivo conversion by ACE (*11*).

Inhibition Mode of Pacific Hake Hydrolysate. To determine the inhibition mode of the hydrolysate samples as they would appear in the blood stream (i.e., as the products of both GI digestion and exposure to ACE), analysis of inhibitor kinetics using a Lineweaver–Burk plot was conducted for PeptACE Peptides, unfractionated PHF hydrolysate, and its <10 and <1 kDa ultrafiltrate samples after GI digestion and preincubation with ACE. Although unfractionated PHF hydrolysate is the most practical sample for commercial production, the <10 and <1 kDa ultrafiltrates were also tested, given their differing responses to simulated GI digestion in terms of ACE-inhibitory activity.

As shown in **Figure 5**, the Lineweaver–Burk plot for all of the hydrolysate samples converge at the y-intercept with that for the reaction containing no inhibitor. This suggests a competitive inhibition by the hydrolysate samples, whereby inhibition can be overcome by increasing levels of substrate, and therefore there is no change in V_{max} (*22*). Wu and Ding (*24*) also determined a competitive inhibition mode for the <10 kDa fraction of soy protein hydrolysate. However, simulated GI digestion of bonito protein has been shown to first produce competitive peptide substrates of ACE, and only after prolonged digestion at hours longer than physiological are true inhibitors formed (*27*). This therefore emphasizes the essentiality of prehydrolysing proteins as sources of ACE-inhibitory peptides for commercial production, and then ideally allowing the GI tract to "activate" the true inhibitory peptides.

Table 1 shows the K_m, K_{app}, and K_i values calculated for the HHL substrate, the HHL substrate in the presence of ACE-inhibitory hydrolysate, and the hydrolysate samples, respectively. The K_m of 1.41 mM for HHL in this study is less than the 2.6 mM found by Cushman and Cheung (*21*). However, this is likely because an incubation time of 1 h has been used to calculate reaction velocity, rather than 30 min as used by Cushman and Cheung (*21*), so the V_{max} may have been suppressed by the average velocity over the entire hour. The one-hour incubation was required to yield sufficiently high absorbance readings for accurate detection; the maximum HHL turnover over the hour was 8%.

As expected for competitive inhibition, there was an apparent decrease in the affinity of the ACE enzyme for HHL substrate in the presence of the PHF hydrolysates and ultrafiltrates, as well as PeptACE Peptides, as indicated by K_{app} values that are greater than K_m, thereby reflecting the influence of the inhibitor dissociation constant K_i by the relationship $K_{app} = K_m(1 + 1/K_i)$. The K_i value of the unfractionated PHF hydrolysate is slightly higher than that of PeptACE Peptides, whereas the K_i value of the <1 kDa ultrafiltered fraction of PHF hydrolysate is lower than that of PeptACE Peptides (**Table 1**). These findings, in conjunction with the % ACE-inhibitory activity data shown in **Figure 4**, confirm that the most potent ACE-inhibitory activity of the PHF hydrolysate is contained within the low molecular weight fraction. However, as demonstrated by the MALDI-ToF spectra (**Figure 2**), the molecular weight profile of the pepsin-pancreatin digested unfractionated PHF hydrolysate contains mainly molecular weight peptides of less than <1 kDa. As such, simply increasing the dose of unfractionated peptides, and thus the low molecular fraction as well, to yield the same ACE-inhibitory effect would likely be much more cost-effective than extensive fractionation processing by ultrafiltration.

Caco 2-Cell Permeability Assay. Caco-2 monolayers exhibited a high TEER value (900 Ω), both before and after the exposure to PHF samples, indicative of monolayer integrity. Furthermore, no toxic effects with exposure of cells to PHF or

digestion products were observed in relation to mitochondrial enzyme activity in the MTT assay (data not shown).

Typical HPLC profiles of the unfractionated PHF hydrolysate and its <10 and <1 kDa ultrafiltrates investigated in the Caco-2 cell permeability assays are illustrated in **Figure 6**. The percentage transport of four of the peaks (A, B, C and D) is shown in **Table 2**. In general, the percent apical-to-basolateral transport of peptides in unfractionated PHF hydrolysate was less than 20% but was relatively higher for the more hydrophobic peaks (C and D) eluting later from the reverse-phase HPLC column than the earlier peaks (A and B). Simulated GI digestion also increased the permeability of peptides in Peaks A, C, and D in unfractionated PHF hydrolysate but decreased the transport of Peak B in both ultrafiltrates and of peak C in the <1 kDa ultrafiltrate. All four peaks from the predigested samples showed significantly higher apical-to-basolateral transport than basolateral-to-apical side transport, suggesting a greater absorptive potential after digestion.

In comparison, Satake et al. (*28*) reported that about 15% of the bioactive tripeptide VPP disappeared from the apical chamber by either hydrolysis or by transport, with only ~2% appearing in the basolateral solution after 60 min incubation. The apical-to-basolateral transport of VPP was slightly but not significantly higher than the basolateral-to-apical transport. Paracellular diffusion was identified as the main mechanism for the transport of the intact VPP peptide across the Caco-2 cell monolayer (*28*). Conversely, when assayed at a concentration of 10 mg/mL, Vermeirssen et al. (*29*) suggested that, at best, only small amounts of ACE inhibitory peptides from pea digest were transported through the Caco-2 cell monolayer but conceded that the sensitivity of their ACE inhibition assay may have been inadequate to detect active compounds in the basolateral samples. Although ACE inhibitory activity was detected in the basolateral compartments using higher peptide concentrations (50 mg/mL) of the pea digest, this activity was accompanied by a compromise in the monolayer integrity. Nevertheless, the pea digest exerted a significant blood pressure lowering effect when injected at 50 mg of protein per kg of body weight into the femoral vein of spontaneously hypertensive rats (*29*).

In the present study, the transport of PHF hydrolysate across the apical membrane was 10-fold higher than from the basolateral side, which indicates that the mechanism of intestinal absorption permeability could involve active components. Many active transport proteins are expressed in apical membranes of Caco-2 cells (*30*). The relatively high percentages of apical-to-basolateral transport values observed for the PHF hydrolysate samples in the current study (**Table 2**) suggest the merit of further research to investigate in vivo antihypertensive effects.

Overall, the in vitro findings obtained in this study have demonstrated that unfractionated PHF hydrolysate exhibits ACE-inhibitory activity in a manner that can be classified as a prodrug type with competitive inhibitor mode, the mechanism of which did not appear to be significantly altered upon exposure to ACE. Exposure to simulated GI digestion by pepsin and pancreatin changed the molecular weight profile to yield a peptide mixture with more potent inhibitory activity. An IC_{50} value of 90 μg peptides/mL was exhibited by both unfractionated hydrolysates and <10 kDa ultrafiltrate after simulated GI digestion, thus indicating that further processing of unfractionated PHF hydrolysate by ultrafiltration was not necessary to achieve that level of ACE inhibitory activity. The Caco-2 cell permeability assay showed that some peptide components of the hydrolysate were transported across the monolayer, thereby providing further evidence of absorption and resistance to proteolysis by cellular peptidases. These results suggest that the abundant yet under-utilized Pacific hake may be a good source for production of hydrolysates containing ACE-inhibitory peptides for application as nutraceutical or functional food product ingredients. However, true physiological efficacy may only be speculated pending in vivo studies, and the results of this study indicate that such studies are warranted.

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