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ORIGINAL PAPER

Development of a salmon protein hydrolysate that lowers blood pressure

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Abstract A salmon protein hydrolysate (SPH) was developed containing several angiotensin I-converting enzyme (ACE) inhibitory tripeptides the most abundant of which were Val-Leu-Trp, Val-Phe-Tyr, and Leu-Ala-Phe. Simulated digestion experiments showed that active constituents of SPH would survive in the digestive tract and be available for absorption into the bloodstream. In fact, ACE inhibitory activity was improved following simulated digestion suggesting that there were larger peptides in SPH that might contribute to bioactivity in vivo. A single oral dose (1,500 mg/kg body mass) of SPH significantly lowered blood pressure in spontaneously hypertensive rats (SHR). The treatment of SHR with either SPH fraction (<3,000 Da) or SPH fraction (>3,000 Da) reduced blood pressure. We conclude that the ability of SPH to lower blood pressure is due to a combination of ACE inhibitory tripeptides as identified, as well as additional unknown, peptide species that are generated during digestion of SPH in the gastrointestinal tract.

Keywords Angiotensin I-converting enzyme inhibitory peptide · Blood pressure · Nutraceutical · Functional food

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Introduction

Angiotensin-converting enzyme (ACE) functions in the renin-angiotensin system to increase blood pressure [1]. ACE catalyses the formation of angiotensin II (Ang II), a potent vasoconstrictor, from angiotensin I (Ang I) and inactivates bradykinin, a vasodilator. Inhibition of ACE is a first-line therapy for hypertension and congestive heart failure [2]. The pharmacological ACE inhibitors (e.g. captopril) are short peptide-based molecules that tightly bind to ACE at its active site and compete with Ang I for occupancy [3].

In addition to the synthetic compounds produced by the pharmaceutical industry, ACE inhibitors can be generated from animal and plant protein sources through the action of proteolytic enzymes [4]. Thus, protein digests containing ACE inhibitors have been produced from a variety of edible sources including milk, eggs, beef, fish, microalgae and seaweed [4–11] and represent potential nutraceutical and functional food ingredients. For example, proteolytic digestion of dried bonito muscle with thermolysin produces a hydrolysate with strong ACE inhibitory activity [12]. Bonito peptide hydrolysate (BPH) contains several short peptide species that contribute to overall activity ($IC_{50} \sim 40$ μg/mL) including a pentapeptide, Leu-Lys-Pro-Asn-Met (LKPNM; $IC_{50} = 2.4$ μmol/L), which yields the more active Leu-Lys-Pro (LKP; $IC_{50} = 0.32$ μmol/L) upon cleavage in the digestive tract [13]. An ultrafiltered version

of this hydrolysate is sold as an ingredient in the dietary supplement market in the United States as a product to help individuals maintain blood pressure within the normal range (PeptACE™, Natural Factors, Coquitlam, BC; Vasotensin™, Metagenics, San Clemente, CA). Ingestion of 1.5 g/day of the product was shown to lower blood pressure in mildly hypertensive patients [14]. Similarly, there are now milk protein-based products on the market [8, 10].

In the present study, we have developed a process to utilise waste protein from salmon (*Salmo salar*) to produce a hydrolysate containing ACE inhibitory peptides. The salmon protein hydrolysate (SPH) is produced through digestion of adherent protein from salmon rack, a by-product of the salmon aquaculture industry, with a neutral protease, followed by centrifugation to remove particulate matter and spray drying. We determined that SPH contains several ACE inhibitory tripeptides that contribute to overall in vitro activity (IC_{50} = 58.5 μg/mL). In addition, we show that SPH lowers blood pressure in spontaneously hypertensive rats (SHR) and that larger peptides present within the SPH contribute to its in vivo activity.

Methods

Reagents

Alcalase and Flavourzyme were purchased from Novozymes A/S (Bagsvaerd, Denmark). Fungal protease concentrate was purchased from Enzyme Development Corporation (New York, NY). Multifect Neutral and Protease GC106 were from Genencor International, Inc. (Rochester, NY). Protease S-Amano was obtained from Amano Enzyme USA (Elgin, IL). Rabbit lung acetone powder, porcine pepsin, bovine trypsin and chymotrypsin were purchased from Sigma Aldrich Chemical Company (Oakville, ON, Canada). ACE artificial substrate *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG) was purchased from Alpco Diagnostics (Windham, NH). BPH was obtained from Ocean Nutrition Canada, Ltd. (Dartmouth, NS, Canada). Parabens were purchased from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ). Wang resins, preloaded with Fmoc protecting groups, and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were from Novabiochem, Laufelfingen, Switzerland. All other reagents were of reagent grade.

Animals

SHR were obtained from Charles River Laboratories, Montreal, PQ, Canada (studies 1 and 2) and Beijing Vital River Laboratory Animal Company, Ltd. Beijing, China (study 3). The studies were carried out in full compliance with regulations of the Canadian Council on Animal Care and the Beijing Association for Laboratory Animal Science, respectively.

Preparation of salmon protein hydrolysate

Salmon rack (backbone and tail) were coarsely ground and homogenised in one volume of water. Parabens (0.013%) was added to inhibit microbial growth. The mixture was heated to 70 °C for 10 min to denature the protein and then cooled to 50 °C. The pH was adjusted to 8.0 with NaOH followed by addition of Protease S-Amano (2.6%, w/w). For the initial screening of enzymes for their utility in producing ACE inhibitory hydrolysates (Table 1), the various enzymes (Alcalase, Flavourzyme, Fungal protease concentrate, Protease GC106, Multifect Neutral, and Protease S-Amano) were tested near their optimal pH as specified by the manufacturers. The digestion proceeded for 7 h with constant stirring, followed by heat-inactivation of the enzyme (85 °C for 10 min). The digestion mixture was subjected to centrifugation of the combined solid/liquid phases followed by vacuum filtration through diatomaceous earth to remove particulate and residual lipid. The water-soluble phase was then evaporated to dryness or spray-dried to yield SPH. During the initial study to screen the various enzymes for utility, SPH was concentrated using Diaion HP-20 solid phase chromatography prior to the drying step. The HP-20 packing was conditioned with methanol followed by water. SPH was applied and passed through

Table 1 IC50 values of individual ACE inhibitory tripeptides identified in the SPH

Tripeptide	IC_{50} μg/ml (μM)	Content in the unprocessed SPH (μg/g)
Leu-Ala-Phe	13.7 (39.2)	3.6
Leu-Thr-Phe[a]	~30.7	
Ile-Ile-Phe	8.9 (22.7)	0.4
Leu-Ala-Tyr[a]	~32.9	
Ile-Ala-Tyr[a]	~57.5	
Val-Phe-Tyr	11.1 (26.0)	22
Tyr-Ala-Tyr	13.2 (31.8)	0.01
Val-Leu-Trp	10.0 (24)	280
Ile-Ala-Trp	3.7 (9.5)	0.5
Tyr-Ala-Leu[a]	~56.2	
Tyr-Asn-Arg	54.8 (121.0)	

Tripeptides were synthesised by manual Fmoc solid phase peptide synthesis procedure and their individual ACE inhibitory activities determined in vitro. IC_{50} values are expressed in μg/ml and μM units (μg/ml by tripeptide molecular weight)

[a] Purification was incomplete and therefore the results are reported only in μg/ml units. Tripeptide content in unprocessed SPH was determined by LC-MS using synthetic peptides as standards

the packing by gravity. The column was washed with water to remove unbound material. The column was then eluted with 100% ethanol to release the peptides. We subsequently investigated replacing this step with an ultrafiltration column (Pall Corporation, Mississauga, ON; MWCO 3,000 Da) as a means of increasing hydrolysate potency. The water-soluble phase was concentrated through a spiral wound, regenerated cellulose membrane to a pre-determined volume, collecting the filtrate (<3,000 Da) and re-circulating the retentate (>3,000 Da) back into the feed. The retentate was then diafiltered by adding water to the retentate at the same rate as permeate was being generated for a pre-determined number of retentate volumes.

LC/MS conditions

All LC/MS/MS experiments were performed using a Waters qTOF-1 with standard electrospray source in positive ion mode coupled to a Waters Alliance 2695LC. Typical conditions were a source cone voltage of 40 V, capillary voltage 3,200 V, desolvation temperature of 400 °C, and source block temperature of 120 °C. MS/MS experiments used a collision energy of 15 eV and Ar collision gas. Nitrogen gas was used for desolvation and nebulisation at 400 and 10 l/h, respectively. The separation was performed using an Agilent Zorbax C_8 column (2.1 × 150 mm) at 0.2 ml/min. The solvent gradient is described in Table 2. Data processing was performed using Waters Mass Lynx 4.0 software.

Determination of ACE inhibitory activity

ACE was extracted from rabbit lung acetone powder by vigorous resuspension into ACE buffer (100 mM Tris–HCl, 300 mM NaCl, 10 μM $ZnCl_2$, pH 8.3) followed by ultracentrifugation at 40,000×*g* [15]. Hydrolysates were tested for ACE inhibitory activity in vitro using a method previously described [16], which was modified to use a microplate reader. The enzyme (5–10 μl, ~5 milliunits) was added to 20 μl of a series dilution of hydrolysate in a 96-well plate. For calculation of IC_{50} the hydrolysate ranged in concentration from 1.7 to 1,160 μg/mL. The reaction was initiated by addition of 200 μL of substrate solution (FAPGG substrate diluted in ACE buffer) and monitored continuously at 37 °C at 340 nm in a spectrophotometric plate reader (SPECTRAmax 190, Molecular Devices, CA) for 10 min. Data were fitted to a four-parameter curve (SOFTmax PRO Software, Molecular Devices, CA) to calculate the concentration of hydrolysate that inhibits the enzyme by 50% (IC_{50}).

Table 2 LC gradient conditions for peptide separation using Agilent Zorbax C_8 column

Time (min)	Aqueous 0.05% TFA (%)	Methanol
0 min	95	5
90 min (linear)	10	90
100 min (hold)	10	90
100.1 min (step)	95	5
120 min (run stop)	95	5

Simulated digestion

Gastrointestinal digestion was performed as previously described [17] using simulated gastric and intestinal fluids (USP 25) in sequence. Pepsin and trypsin/chymotrypsinogen were added at substrate to enzyme ratios of 15:1 for 4-h incubations at 37 °C at pH 1.5 and 7.0, respectively, boiling the solutions to deactivate each reaction. Samples were lyophilised and tested for ACE inhibitory activity.

Determination of ACE inhibitory peptides

Hydrolysate fractionation involved standard techniques such as ethanol precipitation of proteins and large peptides, ultrafiltration using a 3,000 Da molecular weight cutoff and chromatographic isolation. Low molecular weight peptide fractions were subjected to gel filtration chromatography using a Tosohaas TSK-gel G3000PWXL column. Peptides were eluted with 10% aqueous methanol at a flow rate of 0.8 mL/min. The eluent was monitored by a UV/Vis diode array detector using the wavelength range from 192 to 450 nm. Major peaks were collected and assayed for ACE inhibitory activity. Those with the greatest potency were selected for peptide identification using LC–MS.

Peptide synthesis

Tripeptides were synthesised using manual Fmoc solid phase peptide synthesis using Wang resins pre-loaded with Fmoc protecting groups. Fmoc protecting groups were cleaved using piperidine and amino acid-coupled HBTU. Peptides were removed from the resin and side-chain deprotected using trifluoroacetic acid. Peptides were purified by semi-preparative HPLC and characterised by amino acid analysis and mass spectrometry. Purity was also confirmed by analytical HPLC.

Blood pressure measurement in SHRs

Blood pressure was measured in conscious rats by either a direct method (studies 1 and 2) or by the tail-cuff method (study 3). Study 1 investigated the blood-pressure-lowering ability of SPH prepared with two different proteases (Protease

S-Amano and Multifect Neutral). SHR, 10 weeks of age, were acclimatised for 4–7 days. Subsequently, they were randomised to control and test groups, four rats per group. Prior to the study, animals were anaesthetised with isoflurane and the left carotid artery was cannulated for monitoring blood pressure [18]. Briefly, a polyethylene catheter of PE-50 tubing (Intramedic, VWR Canlab) was filled with sterile heparinised saline solution and was inserted into the left common carotid artery and secured in place by 3–0 silk sutures placed around the catheter and carotid artery. The catheter was tunnelled subcutaneously and exteriorized through a skin incision in the inter-scapular region. To protect the catheter, and to allow the rats to move freely around the cage, the catheter was attached to a harness-swivel device (Stoelting Company, Wood Dale, IL). The surgical incisions were closed with a running stitch, and the harness-swivel device was sutured to the skin. Following surgery, the animals were allowed to recover in their cages for 2 days with food and water ad libitum. After recovery, the rats were given a single oral dose of SPH or vehicle (water) by gavage. Mean blood pressure measurements were taken directly from the carotid artery prior to dosing and again at 1, 2, 4, 6 and 8 h. The measurements were done using an IC monitoring device with sensors attached to the rats by the swivel and tether system [18]. This study was carried out in the facilities of KGK Synergize Inc. (London, ON).

In study 2 the blood-pressure-lowering ability of SPH prepared with Protease S-Amano and its low molecular weight ultrafiltrate (<3,000 Da; SPH-LM) were tested as described above, using six rats per group. A control group (water) and a positive control group (1,307 mg/kg BPH) were also run.

Study 3 compared SPH-LM with the corresponding high molecular weight fraction (>3,000 Da; SPH-HM) from ultrafiltration using the tail-cuff method to monitor blood pressure. SHR (10-week old) were acclimatised for 10–14 days after arrival with regular application of a tail-cuff to the animals so as to accustom them to the experimental manipulations. Those with systolic blood pressure higher than 150 mmHg were randomised to three groups, eight rats per group: SPH-LM, SPH-HM and control). Rats were given a single dose (1,500 mg/kg BW) of SPH-LM, SPH-HM or vehicle (water). Systolic blood pressure measurements were taken at time 0 as well as 1, 2, 4, 6, and 8 h after. Cuff pressure and the pulsations were captured by a data acquisition system.

Statistical analysis

The data are presented as mean ± standard error of the mean. For the studies in SHR, the data were analysed by repeated measures one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, wherein blood pressure at the various time points was compared to blood pressure at baseline for the group.

Results

Several commercial enzymes were screened for their ability to generate a SPH with ACE inhibitory properties. The digestions were carried out under optimal pH and temperature. Each SPH was subjected to HP-20 chromatography and tested for ACE inhibitory activity (Fig. 1). SPH prepared using Protease S-Amano was particularly potent at inhibiting ACE, exhibiting an IC_{50} of about 18 μg/ml. In the absence of HP-20 chromatography, its IC_{50} was 58.5 μg/ml (result not shown). Also of interest was the SPH from Multifect Neutral hydrolysis, which had an IC_{50} of about 50 μg/ml following HP-20 chromatography.

The SPHs prepared with Protease S-Amano and Multifect Neutral were tested in vivo for their ability to lower blood pressure using SHR. Animals were given a single dose of SPH (1,500 mg/kg) and mean carotid blood pressure was monitored for the next 6 h (Fig. 2). In animals treated with SPH prepared with Protease S-Amano there was a mild reduction in blood pressure, of about 11%, after 2 h that was maintained for the remainder of the measurement period. One-way ANOVA showed that the treatment produced a significant effect on blood pressure ($p < 0.05$); however, multiple-comparison testing (Dunnett's test) did not reveal differences between blood pressure at baseline and that of any of the time points. This lack of statistical significance was, in part, due to the small number of animals used in this study. Treatment of SHR with SPH prepared using Multifect Neutral did not have any effect on blood pressure and, therefore, we did not study this hydrolysate further.

The low molecular weight fraction of SPH prepared with Protease S-Amano was subjected to chromatographic separation by size exclusion chromatography with UV detection

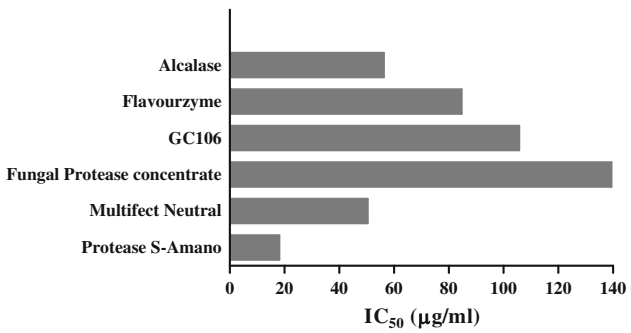

Fig. 1 ACE inhibitory activity of SPHs obtained using various proteases. Salmon protein hydrolysates were prepared using various proteases as described in text. IC_{50} values (μg/mL) are presented for each of the hydrolysates

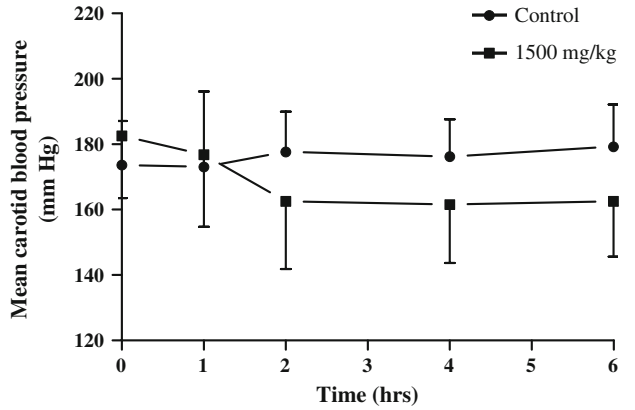

Fig. 2 The effect of SPH prepared with Protease-S Amano on mean carotid blood pressure in SHR. Results are the mean ± standard error of four animals for each group. SPH produced a significant effect on blood pressure (one-way ANOVA, $p < 0.05$); however, there were no statistical differences between the blood pressure at any of the time points and that at baseline (Dunnett's multiple comparison test)

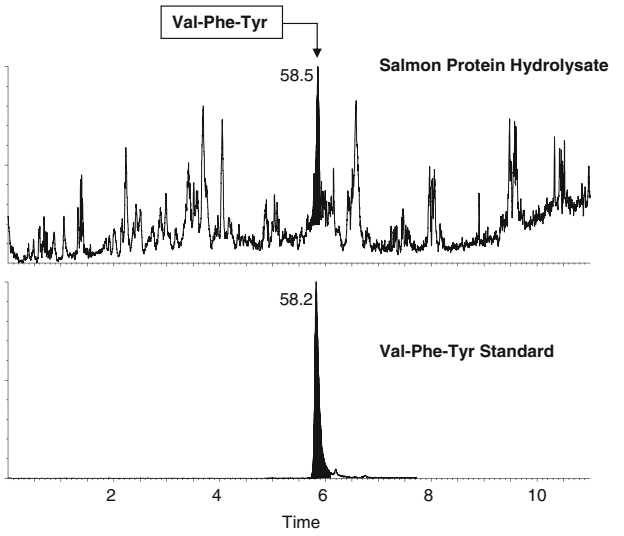

Fig. 3 LC/MS/MS chromatograms of *m/z* 428: total fragment ion current for **a** SPH prepared with Protease-S Amano; **b** synthesised standard of Val-Phe-Tyr. The protonated molecular ion of Val-Phe-Tyr is at *m/z* 417

over a broad wavelength range. The major fractions were collected and assayed for ACE inhibitory activity. Subsequently, the most active fractions were further analysed by LC/MS to reveal the identity of 11 putative ACE inhibitory tripeptides (Table 1). Of these tripeptides, most contain at least one branched-chain amino acid (valine, leucine, or isoleucine) and an aromatic amino acid (phenylalanine, tyrosine, and typtophan). In addition, for 6 of the 11 tripeptides, alanine is present in the second position. These peptides were synthesised and their purities determined by LC/UV. Each peptide was then assayed for ACE inhibitory activity (Table 1). The tripeptides Ile-Ala-Trp, Ile-Ile-Phe, and Val-Leu-Trp had the most potent activities against ACE with IC_{50} values of 9.5, 22.7 and 24 μM, respectively.

The concentrations in the unprocessed SPH of the six tripeptides which demonstrate the most potent ACE inhibitory activity were then estimated by LC/MS/MS. In these measurements, the concentrations of the synthesised tripeptide standards were corrected for their purity as determined by LC/UV. Figure 3a shows the complexity of the hydrolysate—this trace represents all fragment ions arising *m/z* 428 only (that of the protonated molecular ion for Val-Phe-Tyr). However, by comparison with the equivalent chromatogram of the standard (Fig. 3b) and by inspection of the MS/MS spectra themselves (Fig. 4), a clear identification of Val-Phe-Tyr is evident. Furthermore, by generating chromatograms of specific MS/MS fragment ions, much greater selectivity is possible. This can be seen in the example for the tripeptide Val-Leu-Trp (Fig. 5) where the chromatogram for fragmentation of the protonated molecular ion at *m/z* 417 to give the two major fragment ions *m/z* 205 and *m/z* 213 is shown. As was seen for Val-Phe-Tyr (Fig. 4) the mass spectrum of the synthesised Val-Leu-Trp closely matches that extracted from the LC/MS/MS trace of SPH (not shown). Similarly, the tripeptide Leu-Ala-Phe was unambiguously identified using both retention time and mass spectral matching. In determining the concentrations of the tripeptides in SPH (Table 1), LC/MS/MS traces like that shown in Fig. 5 with selected intense fragment ions, were used.

Although this data can only be considered an estimation of the peptide content, it gives a clear indication that three of these peptides (Val-Leu-Trp, Val-Phe-Tyr and Leu-Ala-Phe) were present in considerably higher abundance (3–300 μg/g) in the SPH than the other three peptides (all <1 μg/g). Considering this, and their measured low IC_{50} values, the three peptides Leu-Ala-Phe, Val-Phe-Tyr, and Val-Leu-Trp were thus likely responsible for a considerable fraction of the overall ACE inhibitory action of SPH.

For commercial production purposes it would be favourable to replace the HP-20 chromatographic step with an alternative concentrative step such as ultrafiltration. To investigate the feasibility of this, we subjected unprocessed SPH to an ultrafiltration step using a 3,000 Da MWCO. SPH prepared using this method had an IC_{50} against ACE of 37.3 μg/mL. We compared unprocessed SPH with this low molecular weight ultrafiltrate (SPH-LM) for efficacy in vivo (Fig. 6a). A single oral dose (1,500 mg/kg body mass) of unprocessed SPH resulted in a reduction of blood pressure by 14–15% from baseline that was evident within 1 h and maintained for the remainder of the 6 h monitoring period ($p < 0.05$, $n = 6$). Surprisingly, SPH-LM was not substantially more potent than the unprocessed SPH; producing a similar blood-pressure-lowering effect that was somewhat diminished at the 6 and 8 h time points.

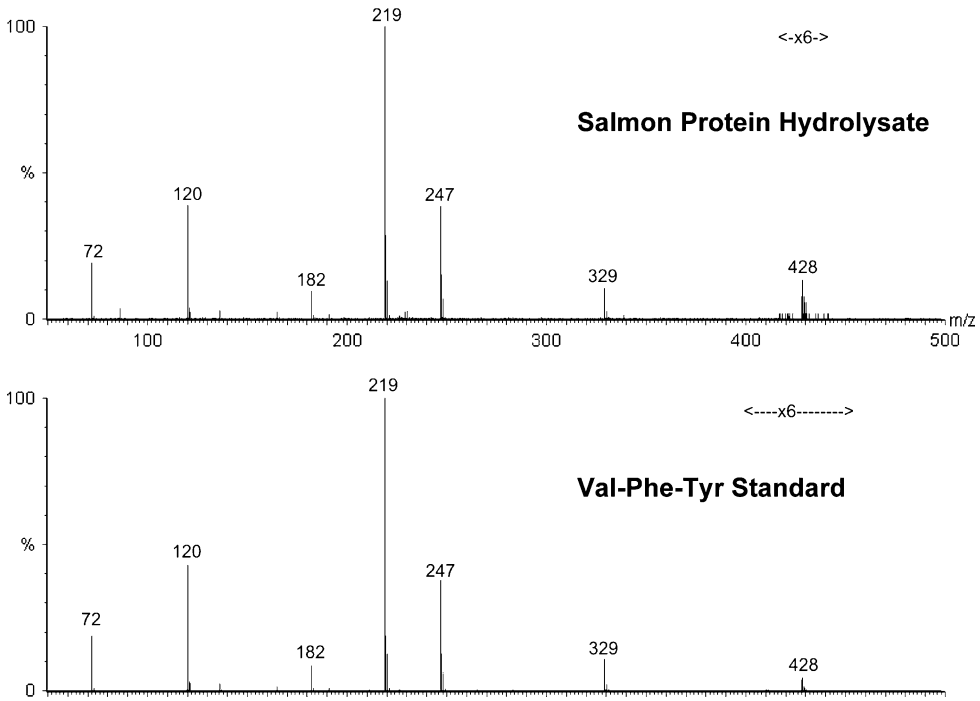

Fig. 4 MS/MS spectra of precursor ion *m/z* 428 for **a** SPH prepared with Protease-S Amano and **b** synthesised standard of Val-Phe-Tyr

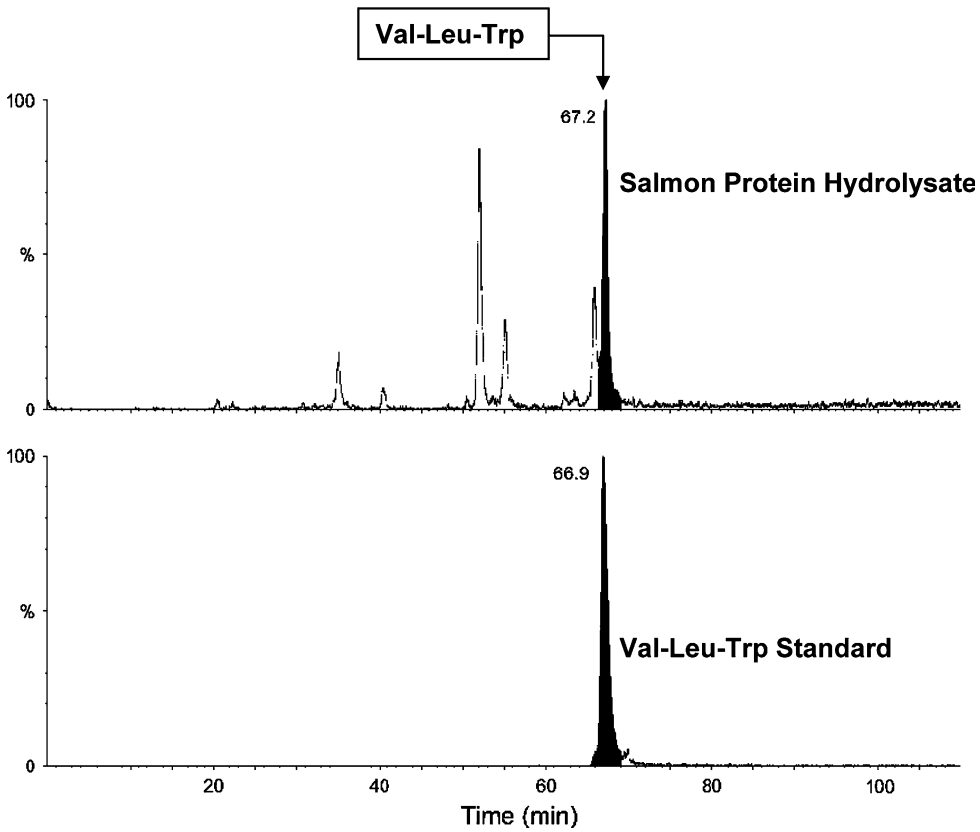

Fig. 5 LC/MS/MS chromatograms of *m/z* 417 → 205 + 213 for **a** SPH prepared with Protease-S Amano and **b** synthesised standard of Val-Leu-Trp. The protonated molecular ion of Val-Leu-Trp is at *m/z* 417

The effects of both these SPHs on blood pressure were comparable to that of BPH (Fig. 6b), a product whose preparation involves a 3,000 Da MWCO ultrafiltration step.

To study the possibility that there are larger molecular weight peptides in SPH that are contributing to blood-pressure-lowering activity in vivo, we subjected unprocessed SPH to sequential digestion with pepsin and trypsin/chymotrypsin

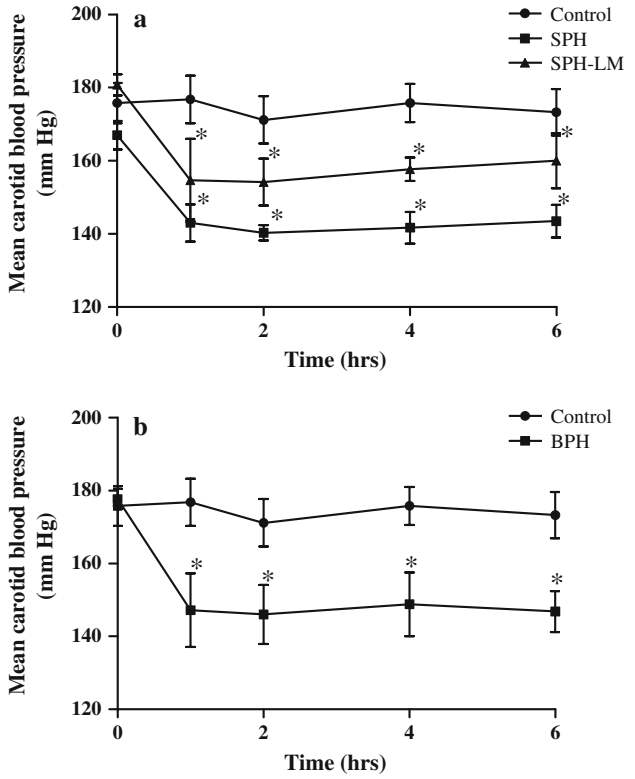

Fig. 6 The effect of SPH, SPH-LM and BPH on mean carotid blood pressure in SHR. **a** SPH-LM was prepared from SPH using ultrafiltration (MWCO 3 kDa). It was administered to SHR at a dose of 1,500 mg/kg body mass by oral gavage. **b** BPH was administered to SHR by oral gavage at a lower dose (1,307 mg/kg body mass) to account for its slightly higher total peptide composition compared to SPH. Control animals received an equivalent amount of water. Results are the mean ± standard error of six animals for each group. *Significantly different compared to the blood pressure at baseline (Dunnett's multiple comparison test, $p < 0.05$)

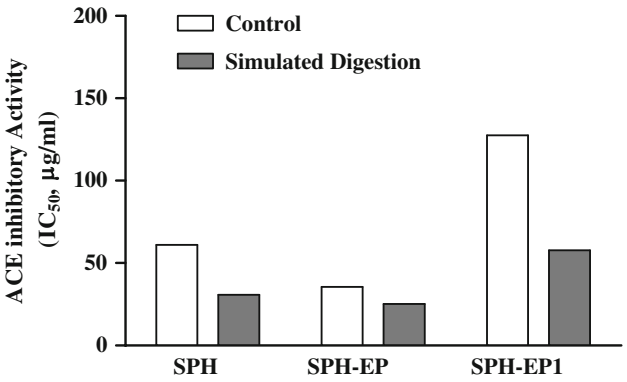

Fig. 7 Effect of simulated gastrointestinal digestion on the in vitro ACE activity of SPH. SPH and its ethanol-soluble and ethanol-insoluble fractions were incubated in simulated gastric fluid at pH 1.5 (with pepsin) for 4 h followed by incubation at pH 7 for 4 h (with trypsin/chymotrypsinogen). Controls were run in the absence of enzyme addition. Results are the average of two separate determinations

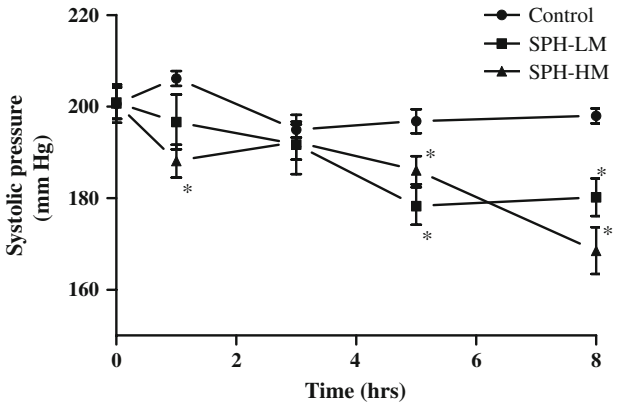

Fig. 8 Effect of SPH on blood pressure in spontaneously hypertensive rats. SPH-LM was prepared from SPH using ultrafiltration (MWCO 3 kDa). SPH-HM is the corresponding higher molecular weight fraction (>3 kDa). Each preparation was administered at a dose of 1,500 mg/kg body mass. Control animals received an equivalent amount of water. Results are the mean ± standard error of eight animals for each group.*Significantly different compared to the blood pressure at baseline (Dunnett's multiple comparison test, $p < 0.05$)

under conditions that simulate gastrointestinal digestion and measured the effect on ACE inhibitory activity (Fig. 7). We compared this to SPH that had been treated with ethanol to precipitate proteins and large peptides (SPH-EP) and its corresponding ethanol precipitate (SPH-EP1). Ethanol precipitation resulted in a considerable reduction of ACE activity (Fig. 7) indicating the likely pro-ACE activity of the larger peptides in SPH which are removed by this process. Furthermore, simulated digestion resulted in an increase in ACE inhibitory activity for each of the hydrolysates. Thus, for unprocessed SPH, SPH-EP and SPH-EP1 the IC_{50} for ACE inhibition was lowered from 61.1, 35.5, and 127.5 µg/mL to 30.7, 25.3, and 57.7 µg/mL, respectively, suggesting that the digestive process may give rise to peptide species that contribute significantly to blood-pressure-lowering properties of the SPH.

We investigated this possibility further, by comparing the ultrafiltered hydrolysate (SPH-LM) with its corresponding higher molecular weight fraction (>3,000 Da; SPH-HM; IC_{50} = 164.4 µg/ml) for blood-pressure-lowering ability in SHR (Fig. 8). Both preparations resulted in comparable blood-pressure-lowering in SHR.

Discussion

There is a growing body of literature on the physiological benefits of peptides that are released from food proteins through in vitro processing or during gastrointestinal digestion [19–21]. The generation of ACE inhibitory peptides from salmon protein as we report here is dependent on the correct choice of exogenous enzyme to use. Both Protease S-Amano and Multifect Neutral produced hydrolysates

with relatively potent ACE inhibitory activity. However, only the SPH prepared with Protease S-Amano had blood-pressure-lowering activity when tested in SHR, illustrating that in vitro ACE inhibitory activity is not always indicative of in vivo activity.

The strong ACE inhibitory activity of SPH prepared with Protease-S Amano was attributable to the presence of various peptide species of differing potency and abundance. We identified 11 tripeptides (Table 1) that appear to be the major contributors to this activity. Their amino acid sequences conform reasonable well with the structural requirements of ACE inhibitory peptides as recently reported by Wu et al. [22] in that a branched-chain amino acid (valine, leucine, isoleucine) is preferred at the amino terminus and an aromatic amino acid (phenylalanine, tyrosine and tryptophan) is preferred at the carboxyl terminus. Conversely, it is reported that the positively charged amino acids (e.g. arginine or lysine) are frequently found at the two position of ACE inhibitory peptides [22], whereas for the peptides disclosed here there is a neutral amino acid (alanine or branched-chain amino acid) at that position.

In an attempt to increase the potency of SPH, we concentrated the active peptides by removing higher molecular weight peptides through ultrafiltration; however, this process removed polypeptides that appear also to participate in blood-pressure-lowering in vivo since the effect of crude SPH was as effective, or more so, compared to ultrafiltered SPH (see Figs. 6 and 8). Based on simulated digestion of SPH (Fig. 7) we hypothesise that SPH contains a number of pro-peptides that become active during digestion, analogous to LKPNM of bonito muscle [13]. Consistent with our results is the observation that in vitro digestion of Wakame protein hydrolysate with gastrointestinal proteases increased its ACE inhibitory activity [9]. Certainly, bioactive peptides are produced in vivo, including ACE inhibitory peptides, following ingestion of milk proteins [19]. Thus, the blood-pressure-lowering effect of SPH appears to be a function of its peptide and polypeptide constituents, some of which become effective (or more effective) in the gastrointestinal tract.

We found that the use of Protease S-Amano, a commercial, food-grade enzyme is an effective proteolytic enzyme for generating SPH with blood-pressure-lowering activity and its use will be amenable to producing SPH at pilot and manufacture scale. The SHR study results presented here are promising, but preliminary. Further studies are required to determine whether SPH will produce a sustained reduction of blood pressure in SHR during chronic administration. Before proceeding to a study in humans, animal safety/toxicological trials are required. Of particular concern will be the dose required for effectiveness in humans. Even if we assume allometric scaling, the dose of 1,500 mg/kg body effective in these studies suggests that several grammes would be required for blood-pressure-lowering in humans. On the other hand, SPH has an IC_{50} value similar to that of BPH and produces comparable blood-pressure-lowering in SHR (Fig. 6). Since BPH has been shown to lower blood pressure in mildly hypertensive individuals at doses of 1.5–3.0 g/day [14, 23], a similar effective dose range might be expected for SPH in humans.

The goal of this research was to develop a SPH with blood-pressure-lowering properties suitable for use as a nutraceutical and as an ingredient in functional food products. In the latter case, flavour profile will be of paramount importance and additional process development may be required, for example, to remove peptides imparting bitter notes [24, 25]. However, it is envisaged that SPH could be produced as a dry ingredient for inclusion into flavourful foods such as seafood chowders, soups or crackers.

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