International Food Research Journal 23(4): 1614-1622 (2016)
Journal homepage: *http://www.ifrj.upm.edu.my*

Biofunctional and physicochemical properties of fish scales collagen-derived protein powders

[1*]Manikkam, V., [2]Mathai, M.L., [3]Street, W.A., [1]Donkor, O.N. and [1]Vasiljevic, T

[1]Advanced Food Systems Research Unit and [2]Centre for Chronic Diseases
College of Health and Biomedicine, Victoria University, P.O. BOX 14428, Melbourne, VIC 8001, Australia
[3]Geelong Food Co-products Cluster, P.O. Box 842, Gisborne, VIC 3437, Australia

Article history

Received: 2 July 2015
Received in revised form: 25 December 2015
Accepted: 13 January 2015

Keywords

Angiotensin converting enzyme
Bioactive peptides
Enzymatic hydrolysis
Fish wastes
Particle size
Trypsin inhibitory activity

Abstract

Fish waste, such as scales, is a rich source of proteins, which can be applied in various commercial applications. Enzymatic hydrolysis for example simulated gastrointestinal digestion (SGID) can release physiologically active peptides with the potential to benefit consumers' health. Powdered (PH) and agglomerated (AH) hydrolysates prepared from fish scales-derived collagen were investigated for their physiological and biofunctional properties. Having a higher protein and moisture content, PH showed greater solubility and digestibility than AH. *In vitro* SGID significantly impacted on the studied inhibitory activities. The released peptides (RP) of PH after completed digestion, exhibited higher angiotensin converting enzyme inhibitory (ACE-I) activity (73.65%) compared to AH. Both preparations showed similar trypsin inhibitory (TI) activities, 44.33% and 47.11% respectively. In contrast, the antioxidant activities of the hydrolysates were very low upon SGID. Physicochemical properties of these preparations apparently affected their *in vitro* physiological properties, which were further modulated through SGID.

Introduction

On an annual basis worldwide, more than 50% of the total catch of the 100 million tons of fish harvest is discarded as underutilised fish and fish processing wastes, including scales (Blanco *et al*., 2007). The waste is created due to poor technological properties, low commercial value and reduced functional features. Such wastes are either thrown back into the sea, causing a serious marine environmental issue (Blanco *et al*., 2007); or they are rejected as by-products, traditionally being utilised for the production of fertilizer and animal feed (Korhonen and Pihlanto, 2006). Importantly, these discarded materials consist of nutritionally valuable compounds, in terms of their protein and mineral composition. Thus, these natural marine resources could effectively be developed into novel compounds, such as proteinaceous hydrolysates or bioactive peptides, by employing advanced food technologies, such as enzymatic hydrolysis (Möller *et al.*, 2008). Manikkam *et al*. (2015) highlighted that human gut enzymes are able to hydrolyse protein fragments from marine sources to produce a range of small peptides acquiring essential biofunctionalities. Therefore, this process of enzymatic hydrolysis is a potential improvement in the area of food science and sustainability as it firstly, aids in minimising marine wastes and maintaining a sustainable aquatic environment, and secondly, enables the production of food hydrolysates with physiological functions, which may aid in the prevention and/or management of human diseases.

The development of enzymatic hydrolysates, with potential ACE-I activity (Fahmi *et al.*, 2004) and antioxidant capacity (Lin and Li, 2006) from fish wastes of different fish species had led to maximising protein recovery. Moreover, fish wastes, such as scales are indeed valuable sources of collagen. Marine scales-derived collagen has been widely known for its cosmetic, anti-aging and food technological applications (Ogawa *et al*., 2004). Besides, the collagen-derived hydrolysates produced by commercial enzymes exerted high anti-free radical activity and showed greater potential to decrease blood pressure (Morimura *et al.*, 2002). Furthermore, Zhu *et al*. (2010) concluded that dietary supplementation with marine collagen derived peptides could offer protection against diabetes and hypertension; and could supply novel bioactive peptides in functional foods.

Metabolic disease, such as obesity and its related comorbidities, like hypertension, diabetes,

*Corresponding author.
Email: *vasambal.manikkam@live.vu.edu.au*

cardiovascular diseases, amongst others, are current medical concerns that are reaching pandemic levels worldwide. Obesity, a life-style-dependent condition, may be prevented by increasing satiety and monitoring the renin-angiotensin system (Mathai *et al*., 2008). Captopril and perindopril are specimens of synthetic ACE inhibitors that have successfully aided in reducing fat mass, body weight and blood pressure of rats (Mathai *et al*., 2008). However, their side effects provide important substitutions to naturally derived ACE inhibitors from food sources, since the latter are natural, safer as well as fairly inexpensive; and may form part of a daily balanced diet.

Alternatively, the inhibition of certain enzymes involved in nutrients' metabolism, may fundamentally assist in the management of obesity. One plausible approach could include trypsin inhibition since it may assist in the production of cholecystokinin (CCK), induced by a trypsin-sensitive CCK-releasing peptide to suppress appetite (Herzig *et al.*, 1996). A current lack of information relevant to TI potency of fish hydrolysates and their RP upon digestion requires further attention. Obesity is also a risk factor for releasing reactive oxygen species, oxidising lipids within the body and causing progressive aging, inflammation and increased risk of cancers, such as prostate and breast carcinomas (Giovannucci, 2007). Therefore, a need to explore the anti-oxidant activity of fish scales derived hydrolysates, as a very important property to maintain health, is necessary.

Currently on the market, more than 500 commercial health products with claimed bioactive peptides are available. Two of which include 1) Nutripeptin; a cod hydrolysate for lowering glycemic index and 2) Fortidium LIQUAMEN; a fish autolysate exhibiting multiple effects, such as, reducing oxidative stress, lowering glycemic index and anti-stress (Guérard *et al*., 2010). There is a need to use marine waste to develop more commercial health products to combat metabolic diseases. Owing to the ongoing wastage of fish scales within the fish industry, there has recently been a mounting interest in exploring their possible uses as functional food ingredients or nutraceuticals, aiming at beneficially influencing human health. For instance, sea bream scales were used to produce ACE-I peptides, with amino acid sequences of Gly-Tyr, Val-Tyr, Gly-Phe and Val-Ile-Tyr. They were analysed for their hypotensive effects in spontaneously hypertensive rats (Fahmi *et al*.,2004). However, there is limited information about the ACE-I peptides derived from fish scales collagen.

Therefore, this study aimed at investigating the physicochemical properties as well as the potential of two fish protein powers, derived from the collagen of fish scales, to simultaneously act as i) an ACE inhibitor, ii) a trypsin enzyme inhibitor, and iii) an anti-oxidant or free radical scavenger. The effects of the digestive proteases involved in an *in vitro* simulated digestion on the bio-functionalities of these powders were also evaluated.

Materials and Methods

Materials

Angiotensin-I-converting enzyme from rabbit lung (A6778), Hippuryl-Histidyl-Leucine (HHL), captopril, trifluoroacetic acid (TFA), trichloroacetic acid (TCA), acetonitrile, pepsin (from porcine stomach mucosa), bile extract (porcine), pancreatin (porcine pancreas), trypsin (Type II-S from Porcine pancreas), Nα-Benzoyl-L-arginine-4-nitroanilide hydrochloride (BAPNA), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), vitamin C, Bradford Reagent and bovine serum albumin (BSA) were all purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Ethyl acetate, glacial acetic acid and dimethyl sulfoxide were from Merck Pty Ltd (Darmstadt, Germany). All the other chemicals used for the preparation of buffering solutions were of analytical grade. Fish scale collagen-derived protein powders were kindly supplied by Eastern P/L (Geelong, Victoria, Australia).

Scanning electron microscope (SEM) analysis

Prior to the SEM analysis of the powders' morphology, a representative amount of each product was placed on a two-sided adhesive carbon tape to dry in a vacuum oven overnight. Desiccated samples were then gold-coated (Neo Coater MP – 19020NCTR, JEOL, Japan) for 8 and 10 min for PH and AH respectively, to generate a gold plasma around the samples. Imaging was carried out using a bench top SEM (Neo Scope JCM – 5000, JEOL, Japan).

Protein and moisture content determination

The protein content was analysed by the Kjeldahl method (AOAC 955.04) and the moisture content was assayed by oven drying at 105^{o}C (AOAC 990.19).

Particle size distribution (PSD) pattern analysis

A 0.1% (w/w) dispersion was prepared from each sample. The refractive index of the hydrolysate dispersions was measured by a refractometer (ATAGO Co. Ltd, Tokyo, Japan). The dispersions were left hydrated overnight with constant stirring on a magnetic stirrer (Industrial Equipment & Control Pty. Ltd, Australia) in a cold room (4^{o}C). The PSD patterns of the hydrolysates were then determined

using a Malvern Zetasizer Nano ZS Instrument (Malvern Instruments, Worcestershire, UK) at 25°C.

Solubility

The solubility of the undigested samples was determined according to Dissanayake *et al.* (2012). Briefly, a 0.1% solution (w/w), prepared from each product was vortex-mixed (Chiltern Scientific Instrumentation Ltd, United Kingdom) and immediately centrifuged at 1865 g (Sorvall® RT7) for 15 min. Protein contents of the supernatants were determined by the Kjeldahl method. The % solubility was calculated as follows,

$$\text{Solubility, \%} = \left[\frac{\text{Protein content in supernatant}}{\text{Protein content of original sample}}\right] \times 100 \quad 1)$$

Digestibility

The protein digestibility assay was conducted according to Foh *et al.* (2011), with slight modifications. Powders, approximately 10 mg, were digested in 5 ml trypsin solution (0.1 mg/ml in 100 mM Tris-HCl buffer, pH 7.6). The suspension was incubated at 37°C for 2 h and the process was stopped by adding 2.5 ml of 50% TCA. The mixture was allowed to stand for 30 min in a cold room at 4°C and centrifuged at 1865 g (Sorvall® RT7) for 20 min. The resultant precipitate was dissolved in 2.5 ml NaOH. Protein content was determined by the Kjeldahl method. Protein digestibility was determined using equation 2:

$$\text{Protein digestibility, \%} = \left[\frac{\text{Protein content in original sample}}{\text{Protein content in TCA precipitate}}\right] \times 100 \quad 2)$$

Simulated gastrointestinal digestion (SGID)

The *in vitro* SGID process was a modified version of Medenieks and Vasiljevic (2008). After the pepsin digestion, the pH was increased to 8.0 with 1 M $NaHCO_3$ before the addition of 5 ml trypsin enzyme solution (EC 232-650-8) (0.2 g trypsin in 10 ml 0.1 M potassium phosphate buffer), followed by shaking incubation (InnovaTM 4230, New Brunswick Scientific, Edison, NJ. USA) at 55°C for 1 h at 100 rpm. To simulate the small intestinal digestive conditions, the pH of the partially digested samples was adjusted to 6.3 with 1 M HCl, after which, 3 ml pancreatic/bile solution was added, following 2 h shaking incubation at 37°C at 100 rpm. To monitor the bioactivities after each digestive treatment, an aliquot of digest was removed, submerged into a boiling water bath (RATEK Instruments) for 15 min to halt all enzymatic reactions. After cooling on ice, all samples were centrifuged at 4 000 g (Beckman J2-HS centrifuge, JA-20 rotor, Palo, Alto, CA, USA) for 15 min, and the supernatant fractions were filtered using a 0.45 µm pore size filter (Schleicher & Schuell GmbH, Germany) into clean tubes and were stored at -18°C for further analyses.

Determination of ACE inhibitory activity

The ACE-I activity, involving the application of ACE (1 unit/10 ml) and its substrate HHL (5 mM), was spectrophotometrically assayed according to Donkor *et al.* (2007). The resulting residue of hippuric acid was dissolved in 1 ml deionised water and its concentration was determined by measuring the absorbance at 228 nm, using UV/Visible spectrophotometer against MilliQ water as the blank. The extent of inhibition was calculated using equation 3:

$$\text{ACE inhibitory activity, \%} = \left[1 - \left(\frac{C-D}{A-B}\right)\right] \times 100 \quad 3)$$

Where,

A = Absorbance in the presence of ACE and without the ACE-I component

B = Absorbance without the ACE-I component

C = Absorbance with ACE and the ACE-I component

D = Absorbance without ACE and with the ACE-I component

The ACE inhibition was also expressed in terms of the IC_{50} value, representing the protein concentration (mg/ml) in the sample required to inhibit 50% of the ACE activity.

Determination of trypsin inhibitory (TI) activity

The TI activity of the undigested powders and their digests was determined according to Medenieks and Vasiljevic (2008). Briefly, 250 µl of samples were pre-incubated at 37°C for 10 min with 625 µl of BAPNA solution. This was followed by the addition of 250 µl trypsin enzyme solution before incubating for 10 min at 37°C. The reaction was terminated by adding 250 µl of 30% glacial acetic acid and vortex-mixed. The absorbance of each sample was read at 410 nm. The extent of inhibition was calculated using Equation 3.

DPPH radical scavenging activity (RSA)

The antioxidant capacity of the products and their digests was evaluated by measuring the free RSA according to Donkor *et al.* (2012), with slight modifications. Briefly, 4.0 ml DPPH solution (0.075 mM in methanol) was added to 0.1 ml diluted (in 1 ml methanol) undigested sample and the digests followed by 30 min incubation in the dark, after which, the absorbance was read at 517 nm with a Pharmacia UV spectrophotometer. The antioxidant activity was calculated as percent inhibition, using

Equation 4:

$$\text{Inhibition}, \% = \left[1 - \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of blank}}\right)\right] \times 100 \quad 4)$$

Reverse-phase HPLC analysis of released peptides

The non-digested powders and the peptides released upon the SGID were profiled using a Varian HPLC (Varian Analytical Instruments, Walnut Creek CA, USA) equipped with a reverse-phase C-18 monomeric column - 5µm, 300Å, 250mm x 4.6mm and a guard column (Grace Vydac, Hesperia, CA, USA) (Donkor *et al.* 2007). Samples were applied using a 10 µl injection loop. The peptides were eluted by a linear gradient from 100% to 0% solvent A [0.1% TFA in deionised water] in solvent B (0.1% TFA in 90%, v/v, acetonitrile in deionised water) over 90 min. All samples and mobile phase solvents were filtered through a 0.45 µm membrane filter. Peptide separations were conducted at a flow rate of 0.75 ml/min. The eluted peptides were monitored at 214 nm using a Varian 9050 variable wavelength UV/Vis detector.

Statistical analysis

The experiments were arranged to explore the influence of three digestive enzymes, namely pepsin, trypsin and pancreatin on the inhibitory activities of the two fish protein powders. The *in vitro* gastrointestinal digestion of each sample was conducted in triplicate. The readings of the inhibitory activities of each replicate were taken three times, indicating that the mean of nine readings was considered. The results were analysed using a General Linear Model (GLM) procedure by the Statistical Analysis System (SAS). All data were analysed by a two-way ANOVA using the SAS software. The level of significance was set to $P < 0.05$.

Results and Discussion

Morphology of fine (PH) and granulated (AH) fish powders

Figure 1 illustrates the electromicrographs obtained for each sample. The PH particles demonstrated highly porous spherical shapes with uneven surfaces (Figure 1A). The appearance of cracks and holes on the surfaces was also obvious. In comparison to AH, which showed irregular network of structures (Figure 1B), PH displayed a smoother matrix. The differences observed could be due to processing treatments employed during their manufacture. There is currently very little information relevant to the structural conformation or morphology of hydrolysates produced from fish scales collagen. Thus, comparing our results with previous studies is difficult since our study is the first to look into the morphological properties of fish scales collagen-derived powders.

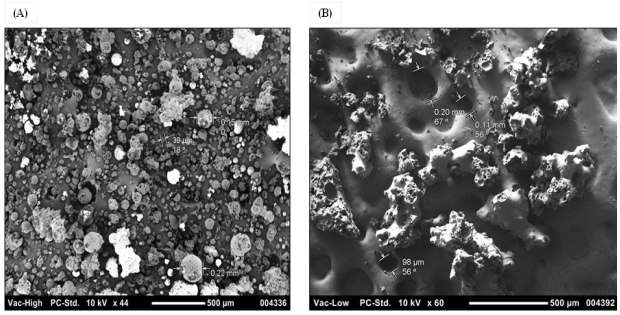

Figure 1. Scanning electromicrographs of commercially produced fish protein powders. (A) Powdered hydrolysate (PH): highly porous spherical structures with uneven surfaces, and the appearance of cracks and holes on the surfaces were obvious; (B) Agglomerated hydrolysate (AH): irregular network of structures, with rough surfaces were observed. The sizes of the spheres and holes as well as the angles at which they are positioned, are marked with arrows in (A) and (B)

However, in the study of Foh *et al*. (2011), fresh minced meat and hot water dip hydrolysates exhibited smoother matrix compared to their concentrates, showing aggregates of packed flake-like structures. Zhang *et al.* (2006) revealed a significant difference between the surface morphology of collagen and gelatin. Collagenous samples demonstrated fibril networks with rough membranous structure, whereas the gelatin membrane without fibril networks appeared only as smooth structure on the surface. Such demonstrated differences due to manufacturing of these products may also play a role in their solubility, subsequent digestion and release of the physiologically important peptides.

Physicochemical properties

In our study, the physicochemical properties of the fish protein powders have been characterised by their protein and moisture contents, solubility and digestibility, as presented in Table 1, in addition to their PSD pattern. The PH showed protein content of 71.24 ± 0.00% whereas AH contained substantially less proteins (44.52 ± 0.23%). The difference in protein content is likely caused by the processing of these protein powders. Moreover, the high protein content of PH could be a result of the solubilisation of protein during hydrolysis and the removal of insoluble undigested non-protein substances (Benjakul and Morrissey, 1997).

Furthermore, the types of enzymes used for the enzymatic hydrolysis in the production of the fish protein powders might have impacted on the protein

Table 1. Selected physicochemical properties of fish scale-collagen derived hydrolysates

Physicochemical properties of hydrolysates	Samples	
	Powdered hydrolysate	Agglomerated hydrolysate
Protein content, %	71.24 ± 0.00[a]	44.52 ± 0.23[b]
Moisture content, %	7.26 ± 0.08[a]	6.87 ± 0.14[b]
Solubility, %	48.15 ± 0.48[a]	18.98 ± 0.34[b]
Digestibility, %	40.76 ± 0.48[a]	11.94 ±0.48[b]

Values are represented as the mean ± standard deviation
Small letters within same row represent the significant difference (P<0.05)

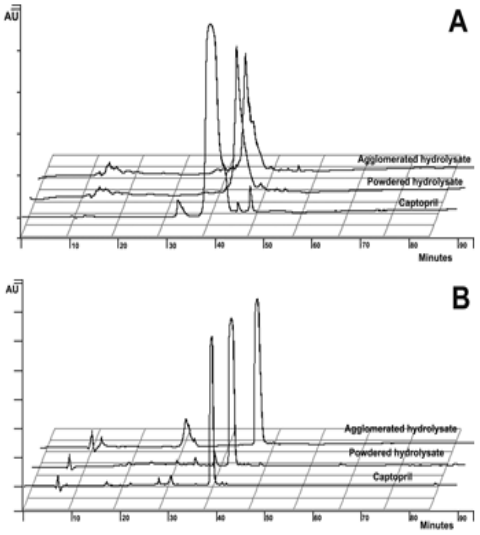

Figure 2. RP-HPLC profiles of peptides released by SGID from the fish-scales collagen-derived powders. (A) peptide profiling of undigested samples; (B) peptide profiling of released peptides after simulated digestion

content of the final product. This is due to the fact that proteolytic enzymes are site-specific enzymes, indicating that the types of polypeptide fragments released upon hydrolysis are dependent upon their specificity (Damodaran *et al*., 2008). For instance, Muzaifa *et al.* (2012) found that protein content of hydrolysates prepared by Alcalase enzyme (82.66 ± 1.36%) differed from that of Flavourzyme-derived hydrolysate (73.51 ± 3.53%). In our study, the moisture content played no apparent role in higher protein concentration of the samples with PH having a higher moisture content of 7.26 ± 0.08% compared to AH, 6.87 ± 0.14% (Table 1).

From the PSD pattern (Figure not shown) of the fish protein powders, no obvious differences in PSD between the two products were observed, with all particles being below 10 nm in size. It can also be deduced that PH and AH had approximately 15% and 25% of particle size greater than 1 nm, respectively. Moreover, for the certainty of the PSD measurements, the refractive index of the sample dispersions were determined to be 1.38 for both AH and PH. Particle size is an important parameter that governs the functionality of a protein hydrolysate. One such functionality is solubility (Table 1), which differed ($P < 0.05$) between PH and AH.

Solubility of hydrolysates greatly depends on the water-protein interactions within the matrix. Enzymatic treatment during the production of the hydrolysates may affect water holding capacity as demonstrated by Kristinsson and Rasco (2000). Moreover, Muzaifa *et al.* (2012) reported significant difference between the solubility of hydrolysates derived from Alcalase and Flavourzyme. Similarly, Damodaran *et al*. (2008) reported that the types of enzymes used in the production of hydrolysates may impact on their solubility. In our study, after centrifugation of AH and PH, more pellet was observed for AH, an indication of lower solubility of AH compared with PH. Another factor that might have affected the solubility of our samples is hydrophobicity. Damodaran *et al.* (2008) mentioned that proteins with higher hydrophobic amino acids will show a lower solubility. Based on this theoretical concept, it can be suggested that in our study, AH is more hydrophobic due to its lower solubility and aggregated (agglomerated) form. Further studies should look into the hydrophobicity of our samples to better understand the change in their solubility.

Whilst digestibility is another important functional property of a fish protein hydrolysate, it is also crucial in the determination of the nutritive quality of a protein, since it affects the bioavailability of its amino acids. In our study, the *in vitro* protein digestibility of the samples was evaluated by the release of TCA-soluble nitrogen. As indicated in Table 1, AH had a significantly lower digestibility ($P < 0.05$) than PH, a similar trend to its solubility. Temperature used during enzymatic hydrolysis, one factor that may affect protein digestibility; might have impacted on the digestibility of AH during its manufacturing process (Guo *et al*., 1999). In contrast, the higher protein solubility of PH might have improved its digestibility. Moreover, protein conformation, the processing techniques, enzymes involved during hydrolysis, and anti-nutritional factors, such as trypsin inhibitors are several factors that may influence the digestibility of hydrolysates (Damodaran *et al.*, 2008).

Table 2. ACE-inhibitory activity and the IC_{50} values of the undigested hydrolysates, their enzymatic digests and the released peptides after completion of SGID

Digestion stages	ACE inhibition (%)			IC_{50} (mg/mL)		
	Powdered hydrolysate	Agglomerated hydrolysate	Captopril	Powdered hydrolysate	Agglomerated hydrolysate	Captopril
Undigested	23.04^{Bc}	75.45^{Ab}	96.64^{Aa}	0.33^{Ba}	0.12^{Db}	0.07^{Dc}
Pepsin digests	33.33^{Dc}	53.35^{Ca}	40.66^{Db}	0.23^{Ca}	0.14^{Cc}	0.19^{Bb}
Trypsin digests	56.37^{Cb}	65.97^{Ba}	62.65^{Ca}	0.36^{Aa}	0.29^{Ab}	0.35^{Aa}
Pancreatin digests	66.84^{Bc}	75.51^{Ab}	84.53^{Ba}	0.31^{Ba}	0.28^{Ab}	0.21^{Bc}
Fragments after completion of SGID	73.65^{Ab}	69.57^{Bc}	95.99^{Aa}	0.14^{Da}	0.16^{Ba}	0.10^{Cb}
SEM		1.62			0.01	

The means present average of 6 independent observations (n≥6); SEM – pooled standard error of the mean
Small letters represent the significant differences (P<0.05) within a row for same parameter
Capital letters represent the significant differences (P<0.05) within a column

Peptide profiling

The qualitative profiles of the undigested samples and the peptides released upon SGID, as analysed by RP-HPLC, are illustrated in Figure 2A and 2B, respectively. The retention times of undigested PH, AH and captopril were between 40 and 50 minutes (Figure 2A). Similarities in peak shape and retention times were observed for digested captopril and PH (around 40 minutes; Figure 2B), indicating that PH may contain peptides with similar chemical structure to captopril. However, it would require further purification and sequencing to confirm. Moreover, the retention times of the observed peaks for all the undigested (Figure 2A) and digested (Figure 2B) samples were very similar. This could possibly indicate that the types of peptides released could be comparable but differed in concentrations. Further isolation, purification and sequencing techniques should be performed to identify and characterise the potent released peptides.

Bio-functionalities of undigested AH, PH, enzymatic digests and RP after completion of SGID

ACE-I activity

Table 2 presents the extent of *in vitro* ACE inhibition by undigested PH, AH, captopril, digests and RP. Captopril is a modified proline dipeptide and the first compound developed in the ACE-I class of anti-hypertensive drugs. Undigested AH exhibited a significantly higher ACE-I activity of 75.45% ($P < 0.05$) compared to PH, 23.04%. Apparently, the low solubility and larger particle size of AH did not impact on ACE-I activity.

Bioactive peptides containing 3-20 amino acid residues per molecule are generally inactive when encrypted in the amino acid sequence within a large protein molecule (Möller *et al*., 2008). The biologically active peptides will exert physiological functions only after cleavage from the parent protein. Upon enzymatic hydrolysis, such as during gastrointestinal digestion, proteins are broken down into oligopeptides before further conversion into shorter peptides. In this regards, digestion is an important process that possibly releases these bio-functional peptides, acting beneficially on the target organ in the human body, after complete absorption (Vermeirssen *et al*., 2004).

The ACE inhibition of the crude pepsin-digests of PH, AH and captopril were 33.33%, 53.35% and 40.66%, respectively. Moreover, an increase in activity against ACE in the trypsin and pancreatin digests of all samples was observed. Trypsin cleaves peptide bonds in non-polar amino acids. The peptides derived from tryptic hydrolysis can have C-terminus amino acids such as Valine, Alanine, Leucine, Proline, Tyrosine, Phenylalanine, Histidine and Tryptophan. Trypsin-derived peptides may inhibit ACE with a high affinity to substrates having C-terminus amino acids such as Alanine, Histidine, Leucine, Proline and Valine (Jung *et al*., 2006).

The ACE inhibition by RP from the PH and AH obtained after complete digestion were 73.65% and 69.57%, respectively. As depicted in peptide profiling (Fig. 2B), the RP of PH appeared to have a similar retention time as that of captopril. Collagen contains large amounts of hydroxyl-proline as a constituent amino acid (Morimura *et al*., 2002), thereby the strong ACE-I activity of RP from PH in comparison to AH. Moreover, the conditions of the *in*

Table 3. Trypsin inhibitory activity of the undigested hydrolysates, their enzymatic digests and the released peptides after completion of SGID

Digestion stages	Trypsin Inhibition (%)		IC_{50} (mg/mL)	
	Powdered hydrolysate	Agglomerated hydrolysate	Powdered hydrolysate	Agglomerated hydrolysate
Undigested	14.33^{Db}	27.70^{Ca}	$0.54C^{a}$	0.31^{Cb}
Pepsin digests	18.15^{Cb}	32.20^{Ba}	0.43^{Da}	0.23^{Db}
Trypsin digests	12.74^{Db}	31.47^{Ba}	1.72^{Aa}	0.60^{Ab}
Pancreatin digests	33.82^{Bb}	49.01^{Aa}	0.62^{Ba}	0.44^{Bb}
Fragments after completion of SGID	44.33^{Aa}	47.11^{Aa}	0.24^{Ea}	0.22^{Da}
SEM	1.32		0.05	

The means present average of 6 independent observations (n≥6); SEM – pooled standard error of the mean
Small letters represent the significant differences (P<0.05) within a row for same parameter
Capital letters represent the significant differences (P<0.05) within a column

vitro digestion process, such as enzyme preparations, temperatures, pH and incubation time might impact on the degree of hydrolysis and the resultant ACE-I activity of the samples.

The decline in the inhibitory activity of RP from AH after the pancreatin digests could likely be due to further degradation by these enzymes (Donkor *et al.*, 2007). Proline residues are often resistant to degradation and may often pass from the small intestine into the blood circulation as short peptides (Korhonen and Pihlanto, 2006). Further studies would be necessary to confirm the amino acid sequence of these peptides after purification.

ACE-inhibitory peptides have been identified and isolated from various food sources, such as probiotic yoghurt (Donkor *et al*., 2007). However, these studies used bacterial and various commercial proteolytic enzymes (such as Alcalase, Flavourzyme, etc) for the production of ACE-I peptides; thus, comparison between previous reports and the current study is very difficult. Moreover, there is very limited information regarding ACE-I peptides of hydrolysates derived from the collagen of fish scales. Therefore, more research is required to establish these effects in order to maximise the utilisation of these marine wastes.

Trypsin inhibitory activity

The TI potency of the undigested AH and PH were 27.70% and 14.33%, respectively (Table 3). Similar to ACE-I activity, TI was increased by SGID. In general, AH digests showed higher inhibitory activity compared to those of PH. The lower inhibitions of PH digests may be attributed to a different peptide profile and disappearance of bioactive peptides during proteolysis. Likewise, Donkor *et al.* (2007) reported the appearance and disappearance of some potent peptides during the storage of probiotic yoghurt. Gobbetti *et al*. (2000) stated similar findings. However, the current study indicated that there was no significant (P>0.05) difference between the TI activities of the RP of AH and PH (47.11% and 44.33%), respectively.

It has previously been hypothesised that high protein food sources influence the secretion of CCK at a higher rate compared to low protein sources (Blom *et al*., 2006). The current study showed an inverse correlation between the protein content and trypsin inhibition, which was observed in the case of PH (Table 3). Medenieks and Vasiljevic (2008) investigated the TI activity of peptides released by SGID of myofibrillar proteins extracted from fresh and frozen fish. Their study demonstrated that TI activity was greatly affected by state of fish (fresh, frozen) and season of catch (winter, summer). Their findings indicated that environmental factors and processing conditions may impact on peptides release as well as TI activity, which may have also played a major role in the observed differences in our study.

DPPH radical scavenging activity (DPPH RSA)

Our results reported low free DPPH-RSA for PH and AH (data not shown). Enzymatic hydrolysates of collagen from fish scales were found to be weak antioxidants in previous studies (Morimura *et al*., 2002), in line with our observations. Several factors such as protein sources, enzyme specificity, proteolytic activity, structure and molecular weight as well as amino acid composition are known to affect antioxidative activities of protein hydrolysates (Nagai *et al*., 2014). Peptides containing cysteine amino acids were reported to be responsible for high antioxidant activity. Thus, the low antioxidant activity

in our study might be due to poor sources of cysteine amino acid. Conversely, trypsin-derived peptides of Hoki *(Johnius belengerii)* skin hydrolysates exhibited high DPPH RSA (Mendis *et al*. 2005). The tryptic gelatin hydrolysates were found to be rich sources of amino acids constituents Gly (37.5%), Pro (9.3%), Glu (8.8%), Ala (8.6%), Arg (7.1%) and Hyp (5.98%). Similarly, croaker *(Otolithes ruber)* muscle protein hydrolysate scavenged 59.7% DPPH; and the peptide sequence of the purified fraction responsible for the antioxidant activity contained cysteine. The presence of cysteine could be an associated link to the antioxidant capacity of the peptide (Nazeer *et al*., 2012). Thus, it appears that antioxidant peptides could be released from fish collagen but additional research should be conducted to explore potential mechanisms for their release.

Conclusion

Our study demonstrated that these two fish protein powders were likely prepared by two different processing methods resulting in variations of their protein content and morphology, which may have impacted on their solubility and digestibility. Furthermore, *in vitro* SGID has improved the ACE and trypsin inhibitory activities of the released peptides of the products, but not the antioxidant capacity. Moreover, the physicochemical properties such as the high protein content, the fine particle sizes as well as the corresponding high solubility and digestibility of the PH could have contributed to the significantly high ACE and trypsin inhibitory activities of its released peptides. However, further purification and sequencing of the released peptides of the PH are required to confirm if the latter could possibly be utilised as a potential ACE inhibitor. As a whole, the production of hydrolysates derived from marine wastes such as scales may deliver health benefits to consumers, whilst minimising aquatic wastes and maintaining an ecological oceanic environment. At the same time the processing techniques should be linked to the intended use since the assessed samples showed quite different physiological functionalities.

Acknowledgements

Vasambal Manikkam is a recipient of an Australian Postgraduate Award (Industry) supported by an ARC Linkage Grant (LP0991005) in collaboration with Geelong Food Co-Clusters (GFCC), Geelong and Victoria University, Australia. The author Manikkam wishes to extend her appreciation to Dr. Nicholas Milne, Research Fellow from the Institute for Sustainability and Innovation (ISI), Victoria University, for training and assistance with the scanning electron microscope. Mr Robert Lancaster is also acknowledged by the authors for offering the samples as a kind gift.

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