Food Chemistry 192 (2016) 34–42

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Gelatin hydrolysates from farmed Giant catfish skin using alkaline proteases and its antioxidative function of simulated gastro-intestinal digestion

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ARTICLE INFO

Article history:
Received 31 March 2015
Received in revised form 8 June 2015
Accepted 25 June 2015
Available online 26 June 2015

Keywords:
Alkaline proteases
Gastro-intestinal digestion
Gelatin
Giant catfish
Protein hydrolysates

ABSTRACT

This work aims to evaluate the ability of different alkaline proteases to prepare active gelatin hydrolysates. Fish skin gelatin was hydrolysed by visceral alkaline-proteases from Giant catfish, commercial trypsin, and Izyme AL®. All antioxidant activity indices of the hydrolysates increased with increasing degree of hydrolysis ($P < 0.05$). The hydrolysates obtained with Izyme AL® and visceral alkaline-proteases showed the highest and lowest radical scavenging capacity, while prepared with commercial trypsin was the most effective in reducing ferric ions and showed the best metal chelating properties. The hydrolysate obtained with Izyme AL® showed the lowest iron reducing ability, but provided the highest average molecular weight ($\geqslant$7 kDa), followed by commercial trypsin (2.2 kDa) and visceral alkaline-proteases (1.75 kDa). After *in vitro* gastrointestinal digestion, the hydrolysates showed significant higher radical scavenging, reducing ferric ions and chelating activities. Gelatin hydrolysates, from fish skin, could serve as a potential source of functional food ingredients for health promotion.

1. Introduction

Several fish processing by-products, that are usually eliminated as organic wastes, causing environmental problem, may contain high-quality functional compounds, such as collagen, PUFA, or calcium. The nutritional and medical benefits of these compounds suggest that they could be used, after processing, as ingredients in functional foods. Fish processing by-products, specifically viscera, are also an important source of proteases with interesting industrial applications. The most important proteolytic enzymes from fish viscera are the aspartic protease pepsin and the alkaline serine proteases trypsin, chymotrypsin, and elastase (EC. 3.4.21). The latter are optimally active at pH 7.5–10 and at a temperature of 35–45 °C, and unstable at lower temperatures and extreme pHs (Bougatef, 2013). Trypsin and trypsin-like enzymes demonstrate good potential for application in laundry detergents (Ktari et al., 2012), liquefaction of fish sauce (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2006), recovering of carotenoids from shrimp wastes (Sila, Nasri, Bougatef, & Nasri, 2012) and production of protein hydrolysates with antioxidant activities and/or angiotensin-I converting enzyme (ACE) inhibitory activity (Khantaphant, Benjakula, & Kishimura, 2011; Nasri et al., 2013; Phanturat, Benjakul, Visessanguan, & Roytrakul, 2010). Different authors have reported the extraction and characterisation of alkaline proteases, especially trypsin and trypsin-like enzymes, from viscera of different species, such as zebra blenny (Ktari et al., 2012, Nile tilapia (Unajak et al., 2012), grey triggerfish (Jellouli et al., 2009), goby (Nasri et al., 2012), Giant Amazonian fish (Freitas-Junior et al., 2012) and farmed Mekong Giant catfish (Ketnawa, Benjakul, Ling, Martinez-Alvarez, & Rawdkuen, 2013; Rawdkuen, Vannabun, & Benjakul, 2012).

Antioxidants are required to prevent lipid oxidation in food, avoiding the formation of toxic compounds and undesirable odours and flavours. Furthermore, oxidative stress, caused by excessive free radicals from human-body metabolism, has also been associated with the occurrence of several diseases, such as hypertension, cancer, diabetes, Alzheimer's and ageing (Rahman, 2007). Hence, it is crucial to inhibit the formation of free radicals and the oxidation of lipids occurring in living body and foodstuffs. Nowadays, there is a requirement to consume natural antioxidants instead of synthetic antioxidants, because of their potential hazard, so numerous

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[1] This Centre has implemented and maintains a Quality Management System which fulfils the requirements of the ISO standard 9001:2008.

http://dx.doi.org/10.1016/j.foodchem.2015.06.087
0308-8146/

researches on antioxidant fish protein hydrolysates have gained extensively interest.

Fish skin gelatin is extensively employed as an ingredient to improve the elasticity, consistency and stability of foods, but it can also be a source of antioxidant peptides. Thus, gelatin hydrolysates from brownstripe red snapper skin (Khantaphant et al., 2011), bigeye snapper skin (Phanturat et al., 2010), tuna and halibut skins (Alemán, Giménez, Montero, & Gómez-Guillén, 2011), sea bass skin (Senphan & Benjakul, 2014) and unicorn leather jacket skin (Karnjanapratum & Benjakul, 2014; Sai-Ut, Benjakul, Sumpavapol, & Kishimura, 2014) have been reported to possess antioxidative activity. However, the use of alkaline proteases from Giant catfish viscera, to produce gelatin hydrolysates, with potential nutraceutical/active functions has not been reported. These hydrolysates can serve as functional supplements in foods or drinks. Hence, this work aims to evaluate the ability of different alkaline proteases (trypsin, Izyme AL® and visceral proteases from Giant catfish viscera) to hydrolyse Giant catfish skin gelatin and yield active gelatin hydrolysates. As well, this work aims to investigate the effect of *in vitro* gastrointestinal digestion on the antioxidant activity of the obtained gelatin hydrolysates.

2. Materials and methods

2.1. Chemicals

Polyethylene glycol (PEG) 2000 g/mol, 2,2-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,4,6-Tripyridyl-s-riazine (TPTZ), 3-(2-pyridyl)-5,6-diphenyl-1,isulfonic acid (Ferrozine), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), BSA, aprotinin, vitamin B12, glycine, porcine pancreatin and porcine pepsin were obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Commercial proteases (porcine trypsin and Izyme AL®) were supplied by Novozymes (Bagsvaerd, Denmark). Hydrochloric acid, sodium hydroxide and tris-(hydroxymethyl)-aminomethane were from Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), Iron (II) sulphate heptahydrate ($FeSO_4{\cdot}7H_2O$), $FeCl_2{\cdot}7H_2O$, $FeCl_3{\cdot}4H_2O$, potassium persulfate ($K_2S_2O_8$) and other chemicals with an analytical grade were purchased from Panreac Applichem (Barcelona, Spain).

2.2. Raw materials

2.2.1. Giant catfish viscera

Viscera of 3-year-old farmed Mekong Giant catfish (*Pangasianodon gigas*) were obtained from Charun Farm, Chiang Rai, Thailand. They were packed in polyethylene bags, kept on ice and transported to the Food Technology Laboratory, Mae Fah Luang University, Chiang Rai, Thailand, within 60 min. Pooled viscera was immediately used for extraction of crude enzyme extract.

2.2.2. Skin gelatin

Gelatin powder from farmed Giant catfish skin was obtained according to Sai-Ut, Jongjareonrak, and Rawdkuen (2010). Briefly, washed skin was soaked in 0.2 M NaOH (skin:solution ratio of 1:10, w:v) at 4 ± 1 °C for 2 h with continuous gentle stirring. Alkaline-treated skin was then washed with tap water until pH < 7.5. The alkaline-treated skin was soaked in 0.05 M acetic acid with a skin:solution ratio of 1:10 (w/v), for 3 h at room temperature (25 ± 1 °C). Acid-treated skin was washed as previously described. The swollen fish skin was soaked in distilled water with a skin:water ratio of 1:10 (w/v) at 45 ± 1 °C for 12 h with continuous stirring to extract the gelatin. The mixture was then filtered using two layers of cheesecloth. The resultant filtrate was lyophilized and further grounded.

2.3. Amino acid analysis

Amino acid analysis was determined using the method described in Alemán, Pérez-Santin et al. (2011). The gelatin was dissolved (1 mg/ml) in ultrapure water, and further hydrolysed in vacuum-sealed glass at 110 °C for 24 h in presence of continuously boiling 6 N HCl, containing 0.1% phenol and norleucine (Sigma–Aldrich, Inc., St Louis, MO, USA) as internal standard. After hydrolysis, the sample was again vacuum-dried, dissolved in 0.2 M sodium citrate loading buffer (pH 2.2), and injected onto a Biochrom 20 amino acid analyser (Pharmacia, Barcelona, Spain).

2.4. Preparation of enzymatic extract from Giant catfish viscera

Visceral alkaline-proteases from Giant catfish viscera were extracted according to Ketnawa et al. (2013). The fish viscera was cut into small pieces and homogenised with extraction buffer (10 mM Tris–HCl pH 8.0, containing 10 mM $CaCl_2$) in the ratio of 1:5 (w/v) for 2 min. The mixture was centrifuged at 10,000×*g* (Avanti J-30I, Beckman Coulter, USA) for 10 min at 4 °C. The pellet was discarded and the supernatant was collected and referred to as "crude enzyme extract" (CE). CE was used as a raw material for extraction of alkaline proteases by aqueous two phase system (ATPS). The ATPS was prepared using the total volume of 40 ml by using a 50 ml graduated centrifuge tube. CE, PEG2000 and sodium citrate were sequentially added (70, 15 and 15%, w/w, respectively). The solutions were mixed thoroughly for 15 min using a Vortex mixer. Phase separation was achieved by centrifuging at 4000×*g* (LegendX1R, Thermo Fisher Scientific, Germany) for 10 min at 4 °C. After the top phase of 1st ATPS cycle was obtained, another 10% (w/w) of sodium citrate was added to the 2nd ATPS cycle. The upper phase from the 2nd ATPS was freeze-dried and stored at −20 °C until used. The obtained fraction was investigated for zymography and showed different bands that corresponded with different alkaline proteases, also including a trypsin-like enzyme (data not shown). The enzyme used in the experiments was referred to as viscera alkaline proteases and used abbreviation as GT.

2.5. Preparation of gelatin hydrolysates

The skin gelatin (3 g) was dissolved in 0.1 mM Tris–HCl pH 8.0 buffer solution (1:3; w/v) and subjected to enzymatic hydrolysis with visceral alkaline-proteases (hydrolysate GTH), commercial porcine trypsin (hydrolysate CTH) or Izyme AL® (hydrolysate IZH) for 2 h under optimal temperature (55 °C) and pH conditions (pH 8), according to the modification method presented by Giménez, Alemán, Montero, and Gómez-Guillén (2009). Eight units of enzymatic activity per gram of protein were used to hydrolyse the gelatin. The pH of the reaction was controlled using a pH-stat (TIM 856, Radiometer Analytical, Villeurbanne Cedex, France). The enzymes were inactivated by heating, at 95 °C for 10 min, and the samples were further centrifuged at 5000×*g* (Heraeus Multifuge 3L, DJB Labcare Ltd., Buckinghamshire, England) for 30 min. The supernatants were lyophilized and stored at −20 °C for further assays.

2.6. Degree of hydrolysis (DH) determination

The DH, defined as the percentage of peptide bonds cleaved with respect to the total number of peptide bonds, was calculated by the method described by Adler-Nissen and Olsen (1979) as follows:

$$DH = [(B \cdot N_b)/(\alpha \cdot M_p \cdot P_{tot})] \cdot 100$$

where B was the amount of alkali consumed to keep the pH constant during the reaction, N_b was the normality of the alkali, M_p was the mass of the protein substrate in the reaction (determined as $N \times 5.55$), α was the average degree of dissociation of the α-NH_2 groups released during hydrolysis ($\alpha = 10^{(pH-pka)}/1 + 10^{(pH-pka)}$), and P_{tot} was the total number of peptide bonds per unit weight of gelatin, calculated from the amino acid composition.

2.7. In vitro pepsin-pancreatin simulated gastrointestinal (GI) digestion

Simulated GI digestion was carried out according to You, Zhao, Regenstein, and Ren (2010) with a slight modification. The gelatin hydrolysates were re-dissolved (3% w/v) in distilled water and adjusted to pH 2.0 with 1 M HCl. Then, pepsin (4%, w/w of protein) was added. The mixture was incubated at 37 °C for 2 h. The pH was then adjusted to 5.3 with a 0.9 M $NaHCO_3$ solution and further to pH 7.5 with 1.0 M NaOH. Pancreatin was then added (10%, w/w of protein) and the mixture was incubated at 37 °C for 2 h. To terminate the digestion, the test tubes were kept in boiling water for 10 min. Aliquots were taken at 0 (gelatin hydrolysate), 2 (switch from pepsin to pancreatin) and 4 h (digested sample) during the *in vitro* digestion. They were cooled at room temperature and centrifuged at 11,000×*g* for 15 min. The supernatants were further lyophilized, kept in plastic tubes and stored at −20 °C before use.

The aliquots were analysed for nitrogen using a LECO FP-2000 nitrogen/protein analyser (LECO Corp., St. Joseph, MI, USA). Protein values were based on N (%) × 6.25.

2.8. Molecular weight distribution

The molecular weight distribution of gelatin hydrolysates was evaluated by size-exclusion chromatography (HPLC model SPE-MA10AVP, Shimadzu, Kyoto, Japan), by using a Superdex peptide PC 3.2/30 column (GE Healthcare Bio-Sciences, Barcelona, Spain) with a fractionation range between 7.0 and 0.1 kDa. An amount of 10 mg/ml powdered hydrolysates were dissolved in Milli-Q water, centrifuged at 9000×*g* for 5 min, and filtered at 0.45 μm for removing of contaminants before injection. The injection volume was 10 μl and the flow rate was 0.1 ml/min through the column temperature of 25 °C using 30% (v/v). Acetonitrile with 0.01% (v/v) trifluoroacetic acid in Milli-Q water was used as a mobile phase. Absorbance was monitored at both 214 and 280 nm. Molecular weight standards including bovine serum albumin (6.64 kDa), aprotinin (6.511 kDa), vitamin B12 (1.355 kDa), angiotensin II (1.046 kDa), hippuryl-L-histidyl-L-leucine (0.429 kDa) and glycine (0.075 kDa) were run through the column at the same condition and used for MW calculation. Plots of retention time for molecular weight standards were used to construct calibration curve, from which hydrolysate molecular distributions were computed.

2.9. Antioxidant activities of gelatin hydrolysates

Different assays (ABTS, FRAP and metal (ferrous) chelating ability) were performed, at least in triplicate, to study the antioxidant properties of the skin gelatin and the corresponding hydrolysates (GTH, CTH and IZH). The ABTS radical [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] scavenging capacity was determined according to Alemán, Giménez, et al. (2011). The stock solution of ABTS radical consisted of 7 mM ABTS in potassium persulfate 2.45 mM, kept in the dark at room temperature for 16 h. An aliquot of stock solution was diluted in Milli-Q water to obtain a working solution of ABTS radical with an absorbance of 0.70 ± 0.02 at 734 nm. A 20 μl aliquot sample (3.75 mg/ml) was mixed with 980 μl of ABTS reagent and then left to stand in the dark at 30 °C for 10 min. Absorbance values were read at 734 nm using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Japan). Results were expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC)/g of sample (gelatin or hydrolysate) based on a standard curve of vitamin C.

FRAP, a measure of the reducing power of samples, was performed according to Alemán, Giménez, et al. (2011) with some modifications. Sixty μl of sample (7.5 mg/ml) were incubated with 60 μl of Milli-Q water and 900 μl of FRAP reagent, containing 10 mM of TPTZ and 20 mM of $FeCl_3$ at 37 °C during 30 min. Absorbance values were read at 595 nm. Results were expressed as μmol Fe^{2+} equivalents/g of sample (gelatin or hydrolysate) based on a standard curve of $FeSO_4 \cdot 7H_2O$.

The Fe^{2+} chelating activity of the hydrolysates was measured according to Alemán, Giménez, et al. (2011). Briefly, 800 μl of sample (2.5 mg/ml) was mixed with 10 μl of 2 mM $FeCl_2$ and 20 μl of 5 mM Ferrozine. The mixture was kept at room temperature for 10 min prior to measuring the absorbance at 562 nm. Chelating ability was expressed as μmol EDTA equivalents/g of sample (gelatin or hydrolysate) based on a standard curve of EDTA.

2.10. Statistical analysis

All data was subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's multiple range tests. SPSS Statistic Program (Version 16.0) (SPSS Inc, Chicago, IL, USA) was used for data analysis.

3. Results and discussion

3.1. Amino acid composition

The amino acid composition of farmed Mekong Giant catfish gelatin was analysed (data not shown). The gelatin was rich in hydrophobic amino acids (642/1000 residues), being the most abundant, glycine (324/1000 residues), proline (121/1000 residues) and alanine (120/1000 residues); in contrast, the content of leucine (22/1000 residues), valine (21/1000 residues), phenylalanine (13/1000 residues), isoleucine (11/1000 residues), and methionine (10/1000 residues) was very low. Hydroxyproline accounted for 88/1000 residues. The number of imino acids (proline and hydroxyproline) in the Giant catfish skin gelatin was 209 residues per 1000 residues. Jongjareonrak et al. (2010) reported a similar amino acid composition of gelatin skin from the same fish species. It was rich in glycine (359 residues/1000 residues), alanine (106 residues/1000 residues), and proline (124 residues/1000 residues), while hydroxyproline accounted for 87 residues/1000 residues. The amount of imino acids (proline and hydroxyproline) was 211 residues/1000 residues. In another study, skin gelatin of grey trigger fish also contained a relevant amount of imino acids (176/1000 residues), whereas the content of serine was 40/1000 residues (Jellouli et al., 2009).

3.2. Effect of the different enzymes on the degree of hydrolysis

Enzymatic proteolysis is a valuable bioprocess to expose biological activities from original proteins, as some peptides generated from the hydrolysis possess stronger bioactivity compared to the native raw materials (Sila et al., 2015). In this work, the visceral alkaline-proteases, commercial trypsin and Izyme AL showed different abilities to hydrolyse the gelatin (Table 1). DH close to 10% was achieved when either visceral alkaline-proteases or

Table 1
Degree of hydrolysis achieved after enzymatic process of gelatin from Giant catfish skin.

Time (min)	Degree of hydrolysis (%)		
	Visceral alkaline-proteases (GTH)	Commercial trypsin (CTH)	Izyme AL (IZH)
10	6.21	8.83	0.81
20	8.08	8.83	0.81
40	9.12	8.88	0.82
60	9.67	9.22	0.82
90	9.91	9.95	0.82
120	10.14	9.97	0.90

commercial trypsin were used to hydrolyse the skin gelatin. In contrast, a negligible DH was obtained when gelatin hydrolysis was performed by Izyme AL®. All hydrolysis processes were characterised by a high rate of hydrolysis, during the initial stage (10–30 min), owing to a large number of peptide bonds available and a further slow down until reaching a stationary phase. The stability in the hydrolysis rate, observed in the later stage, was mainly due to a decrease in available substrate, enzyme auto-digestion and product inhibition (Khantaphant et al., 2011).

3.3. Antioxidative activities of gelatin hydrolysates

The gelatin hydrolysates showed antioxidant properties higher than those of the non-hydrolysed gelatin (Fig. 1). Moreover, the antioxidant activity of each sample increased throughout the hydrolysis process. This suggests that an increment in the hydrolysis of gelatin led to higher production of antioxidative hydrolysates/peptides. However, not only the DH achieved, but also the enzyme used, was extremely important in the antioxidative potency of the hydrolysates.

ABTS radical scavenging activity of the gelatin hydrolysates is depicted in Fig. 1A. All hydrolysates showed a significantly high Vitamin C Equivalent Antioxidant Capacity, much higher than that of the non-hydrolysed gelatin. IZH showed the significantly ($P \leqslant 0.05$) highest scavenging activity throughout all the hydrolysis processes. The results also showed that the radical scavenging potency and the DH (120 min) of each hydrolysate were inversely proportional, and also that the scavenging activity of each hydrolysate increased during the hydrolysis process (that is, as DH increased). This controversial result suggests that the radical scavenging activity mainly depends on the enzyme used to hydrolyse the gelatin.

Other authors have also found that DH and radical scavenging capacity are not directly correlated. Thus, Alemán, Giménez, et al. (2011) reported that trypsin hydrolysates of tuna skin and squid tunic showed lower DH (8.55, 11.7%) than those obtained with pepsin (28, 29%), but provided a higher ABTS activity. Wiriyaphan, Chitsomboon, and Yongsawadigul (2012) reported that a hydrolysate from by-products of threadfin bream prepared with pepsin (5% DH) showed the highest ABTS radical scavenging activity, FRAP, and inhibition of ß-carotene bleaching when compared with others obtained with *Virgibacillus* sp. SK33 proteinase (13% DH), Alcalase (27% DH) and trypsin (11% DH).

All protein hydrolysates showed a significantly higher ferric reducing ability (93–857 μmol Fe^{2+} equivalents/g sample) than that of the non-hydrolysed gelatin (4.09 μmol Fe^{2+} equivalents/g), as depicted in Fig. 1B. Alemán, Pérez-Santin, et al. (2011) also found a FRAP of different gelatin hydrolysates significantly higher than that of the non-hydrolysed gelatin, being this capacity independent of the enzyme used. The peptides generated from the hydrolysis of the Giant catfish skin gelatin, by different proteases, had different ability to provide electrons to the radicals. Significant increments in FRAP were observed in all hydrolysates along hydrolysis time; that is, when the DH increased ($P < 0.05$), suggesting that low molecular weight (LMW) peptides were mainly responsible of this activity. FRAP of CTH was found to be the highest ($P \leqslant 0.05$) at different times of hydrolysis (5.48–9.04 μmol Fe^{2+} equivalents/g sample), followed by that of GTH (5.25–6.37 μmol Fe^{2+} equivalents/g sample), while FRAP of IZH was the lowest (4.28–6.35 as μmol Fe^{2+} equivalents/g sample). Khantaphant et al. (2011) also reported an improvement in FRAP of different hydrolysates of skin of brownstripe red snapper when DH increased. Similar results were reported by Phanturat et al. (2010) with a hydrolysate from bigeye snapper muscle prepared using Alcalase, Neutrase and pyloric caeca extract.

The ferrous chelating activity of the hydrolysates, prepared using different proteases, at different time of hydrolysis, is shown in Fig. 2. The chelating activity against Fe^{2+} of all hydrolysates gradually increased when DH increased ($P \leqslant 0.05$). The hydrolysates showed a ferrous chelating activity significantly higher than that of the non-hydrolysed gelatin. The highest chelating activity was that of IZH, followed by those of CTH and GTH. From the results, generated peptides in all hydrolysates could effectively chelate the Fe^{2+}, leading to the retardation of initiation stage. Transition metals, such as Fe^{2+}, are well-known stimulants of lipid peroxidation and their chelation may help to retard the peroxidation and subsequently prevent food rancidity (Nasri et al., 2013). Therefore, chelation of metal ions by peptides in hydrolysates could retard the oxidative reaction. The highest chelating activity of the hydrolysates prepared with Izyme AL® was also the highest ABTS radical scavenging activity of the same hydrolysates.

It is known that the specific amino acid composition, structure and hydrophobicity of the peptides in hydrolysates are much more important factors than the DH to manifest antioxidant activity (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998; Wiriyaphan et al., 2012). Thus, the antioxidant activity of the gelatin hydrolysates could be ascribed to the specific composition in peptides and free amino acids (Wiriyaphan et al., 2012). Saiga, Tanabe, and Nishimura (2003) observed that the carboxyl and amino groups in the side chains of acidic (Glx, Asx) and basic (Lys, His, Arg) amino acids may play an important role in metal chelation by peptides. In this regard, the amount of acidic residues in the Giant catfish skin gelatin was high (115/1000 residues), while that of basic residues was lower (82/1000 residues, Table 1). Moreover, the presence of hydrophobic residues (i.e. Gly, Pro, Ala, Val and Leu) in hydrolysates has been linked to potent scavenging activities (Mendis, Rajapakse, & Kim, 2005). Thus, the abundance of Gly, Ala and Pro in the Giant catfish skin gelatin could help to enhance the solubility of peptides in lipids and hence facilitating their accessibility to hydrophobic radical species and to hydrophobic polyunsaturated fatty acids. Hydrophobic amino acids have been found in antioxidant peptides from skin gelatin (Alemán, Giménez, et al., 2011; Mendis et al., 2005), and Gly has been suggested to act as hydrogen donor (Mendis et al., 2005; Sarmadi & Ismail, 2010). However, most of researchers agree that the antioxidant activity of peptides may not be attributable to a single antioxidant mechanism, probably because the properties derived from the presence of different amino acids favour one mechanism over others. For instance, peptides rich in hydrophobic amino acids are expected to inhibit lipid peroxidation, both as proton donors to hydrophobic peroxyl radicals and as chelators of metal ions (Alemán, Giménez et al., 2011). Nonetheless, it is worth noting that not only the presence of some favourable amino acids, but also their correct positioning in the peptide sequence, is trivial to the antioxidative activity (Chen et al., 1998).

The DH could also significantly influence the antioxidative activity of the resulting hydrolysates, since DH greatly influences the peptide chain length as well as the exposure of the terminal

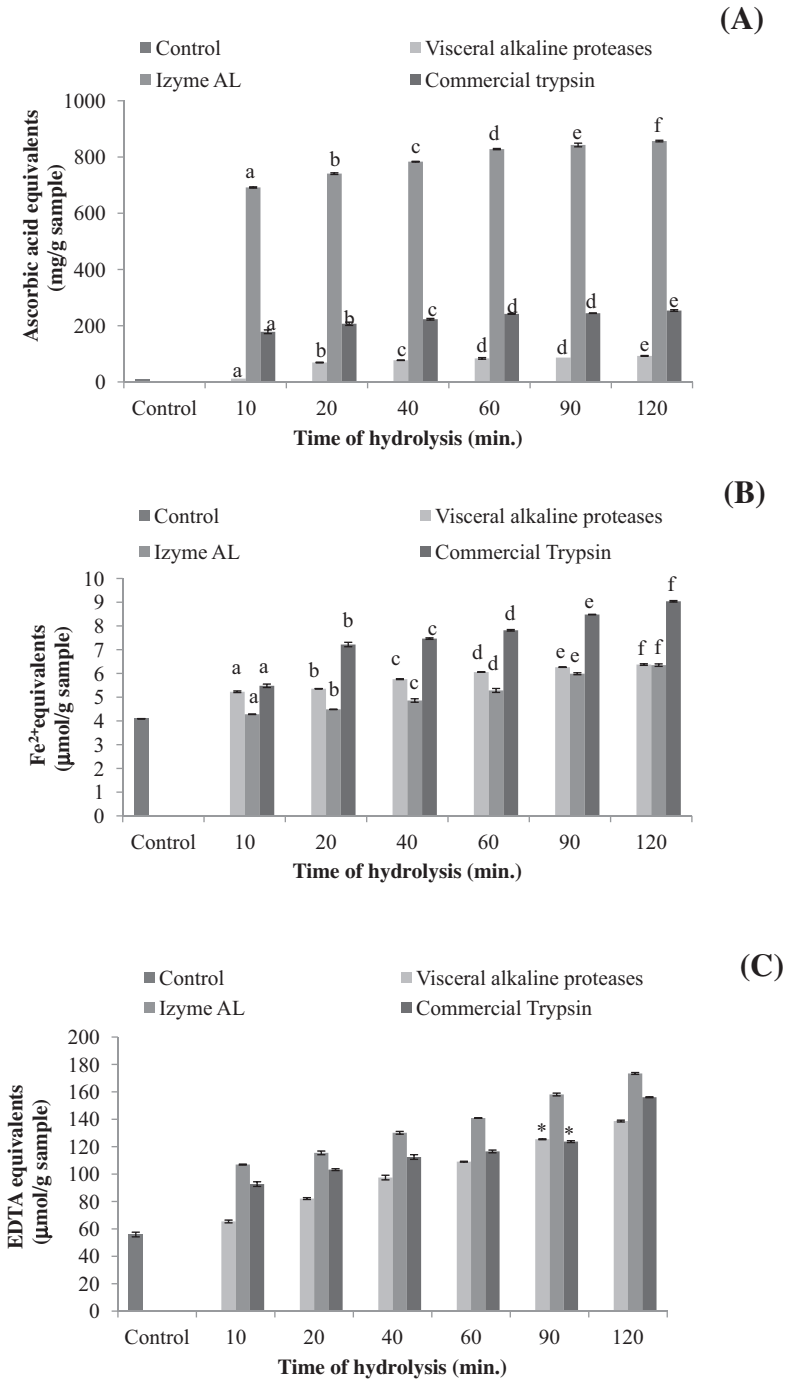

Fig. 1. ABTS radical scavenging capacity (A), ferric reducing ability (B) and metal ion chelating activity (C) of the control (non-hydrolysed skin gelatin) and the skin gelatine hydrolysates, (A) expressed as mg of ascorbic acid equivalents/g gelatin. (B) Expressed as mmol $FeSO_4{\cdot}7H_2O$ equivalents/g gelatin. (C) Expressed as µmol EDTA equivalents/g gelatin. Bars represent the standard deviation from triplicate determinations. Different letters (a, b, c. . .) indicate significant differences ($P \leqslant 0.05$) as a function of hydrolysis time and non-significant differences are highlighted with an asterisk.

amino groups of the products obtained. However, type of enzymes, the suitable ratio between substrate/enzymes or substrate/buffer solution and time of hydrolysis played a significant role as well.

The molecular weight distribution of the hydrolysates could also have an influence on their antioxidant activity, since lower molecular weight peptides were assumed to possess stronger antioxidant activity (Je, Park, & Kim, 2005; Phanturat et al., 2010). It was reported that antioxidant peptides mostly contain below the 20 amino acid residues per molecule and the lower the molecular weight, the higher their probability of crossing the intestinal barrier and exerting biological effects. The majority of the antioxidative peptides derived from food sources have molecular weights ranging from 0.5 to 1.8 kDa (Kim & Wijesekara, 2013; Wu, Chen, & Shiau, 2003). The varying MW distribution revealed considerable differences in the degree of protein breakdown depending on the enzyme used (Table 2 and Fig. 2). IZHs were mainly composed by peptides higher than 10 kDa (93–99%), followed by di/tri-peptides and free amino acids. These results are in accordance with the low degree of hydrolysis achieved and highlight the presence of proteins in the hydrolysates lightly

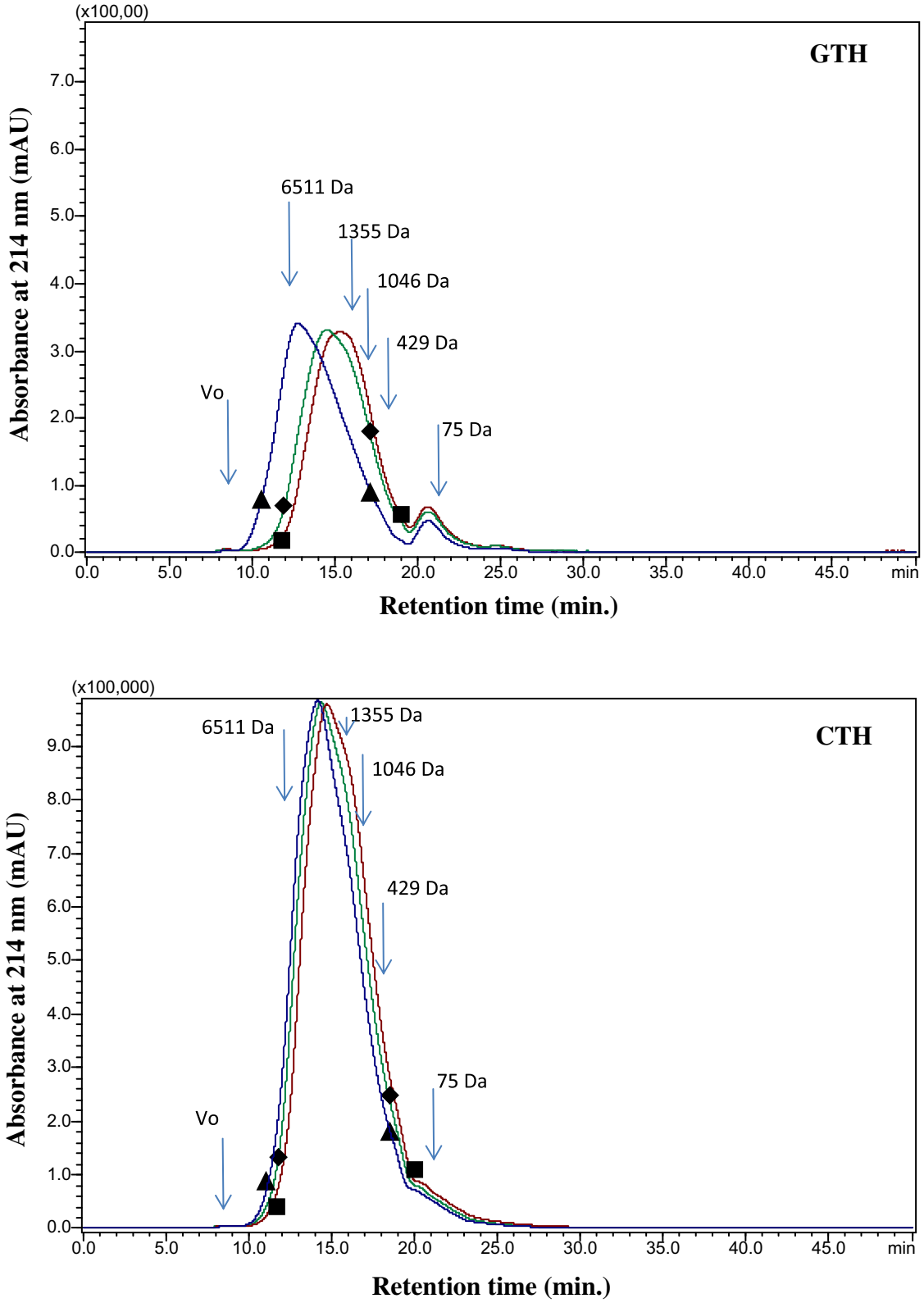

Fig. 2. Molecular weight distribution of gelatin hydrolysates obtained with different enzymes. Black line without symbols represent the molecular weight profile of Giant catfish gelatin (sample higher than 7 kDa and not retained by the column); (▲) hydrolysis at time 10 min; (♦) hydrolysis at time 60 min; (■) hydrolysis at time 120 min. The hydrolysates obtained with Izyme AL®(IZH) were not retained by the column (data not shown). Aprotinin (6.511 kDa), vitamin B12 (1.355 kDa), angiotensin II (1.046 kDa), hippuryl-L-histidyl-L-leucine (HHL, 0.429 kDa) and glycine (0.075 kDa) were used as molecular weight standards. BSA was not retained and eluted at 8.98 min, which was used to determine the void volume (V_o) of the column.

Table 2
Degree of hydrolysis, molecular weight distribution and fraction content of gelatin hydrolysates by different enzymes.

Enzymes	Time (min)	DH (%)	Molecular weight (kDa)	Amount (%)
Visceral alkaline-proteases (GTH)	10	6.21	5.528	100.00
	60	9.67	2.453	84.20
			0.139	15.80
	120	10.18	1.757	82.98
			0.139	17.02
Commercial trypsin (CTH)	10	8.83	2.897	100.00
	60	9.22	2.642	100.00
	120	9.97	2.212	100.00
Izyme AL (IZH)	10	0.81	>10.0	98.14
			0.091	1.86
	60	0.82	>10.0	99.34
			0.157	0.66
	120	0.90	>10.0	92.86
			0.159	7.14

DH: degree of hydrolysis.

hydrolysed. Peptides with MW below 3 kDa were predominant in the hydrolysates prepared with commercial trypsin for 10 min. The hydrolysate prepared with the viscera extract for 10 min was mainly composed of polypeptides (5.528 kDa). After 120 min of hydrolysis, the MW average decreased to around 1.757 kDa (82.98%). This mass range is similar to that reported by Wu et al. (2003), which found that the strongest antioxidant fraction of mackerel protein hydrolysate included small peptides (lower than 1.4 kDa) and free amino acids. Similarly, Je et al. (2005) found a correlation between antioxidant activity and the molecular weight of the peptides in Alaska Pollack frame protein hydrolysates, with greater antioxidant activity associated with peptides of low molecular weight (<1 kDa). ABTS radical scavenging capacity and ferrous ion chelating could not be well correlated with the molecular weight distribution of the hydrolysates, since the hydrolysate prepared with Izyme AL® showed the highest number of larger peptides, as compared to other hydrolysates (Table 2). This may be due to specific activity of Izyme AL®, which could yield potent antioxidant peptides and denoting, once again, the importance of the peptide sequence, rather than the whole hydrolysate molecular weight distribution (Giménez, Alemán, Montero, & Gómez-Guillén, 2009). Numerous studies have reported that peptides from gelatin hydrolysates showing high antioxidant activity have varying MW, e.g., 0.156–1.7 kDa. Phanturat et al. (2010) studied the ABTS radical scavenging activity of fractions from bigeye snapper skin gelatin hydrolysates, and found that the fraction containing peptides with a MW of 1.7 kDa showed the highest antioxidative activity. Senphan and Benjakul (2014) reported ABTS radical scavenging capacity of a gelatin hydrolysate from seabass, mainly in fractions containing molecules of 0.156, 0.364 and 1.505 kDa. Zhang, Duan, and Zhuang (2012) identified two low MW peptides (0.317 and 0.645 kDa) in a gelatin hydrolysate from tilapia skin that showed hydroxyl radical scavenging activities.

The results suggested that there was a difference in the amount and size of proteins or peptides generated by the different hydrolysis processes. Besides, skin hydrolysate most likely contained certain peptides with radical scavenging activity, which could terminate radical chain reaction (Senphan & Benjakul, 2014). Different researchers have reported that the molecular weight of peptides is also believed to play a key role in chelating metals. Klompong, Benjakul, Kantachote, and Shahidi (2007) pointed out that metal-chelating ability and molecular weight had a linear relationship, and that metal chelating ability increased with decreasing molecular weight. Nevertheless, Megías et al. (2007) reported that if peptide length is too short, chelation is unstable. It is worth noting the considerably high metal chelating activity of IZH, as compared to that of GTH or CTH, denoting once again the importance of the presence of specific peptide sequences, rather than the whole hydrolysate molecular weight distribution.

3.4. Stability in gastro-intestinal model system, molecular weight profiles and antioxidant activity

The resistance of bioactive peptides against gastro-intestinal proteases (such as pepsin, trypsin, chymotrypsin, etc.) is a pre-requisite for their action *in vivo* and exploitation for human functional foods. Indeed, several peptides resistant to gastrointestinal digestion can be absorbed in their intact form through the intestine (Daniel, 2004). Therefore, the stability of GTH, CTH and IZH during *in vitro* gastro-intestinal digestion was studied (Table 3). After 4 h of digestion, GTH, CTH and IZH were mainly composed of peptides of around 1.602, 1.392, and 0.366 kDa, respectively, that is, the simulated GI digestion of the hydrolysates did not digest them completely. Although they seemed to resist the simulated gastric digestion, important modifications in the antioxidant activity of the digests were found (Fig. 3). The hydrolysates (mainly CTH) showed an increase in the ABTS radical scavenging activity after GI digestion (7000–11,400 folds). The antioxidant activity of CTH increased mainly during the gastric digestion, and thereafter this activity slightly increased up to the end of digestion. In contrast, the ABTS radical scavenging activity of IZH increased slightly during gastric digestion and significantly during the intestinal digestion. The FRAP activity of the samples was affected in a different way by the GI digestion. Thus, the intestinal digestions increased FRAP activity of mainly IZH and GTH, and decreased such activity of CTH. After GI digestion, FRAP activity of IZH augmented about 80%, while the activity of CTH and GTH was similar to that determined before digestion. The metal ion chelating ability of GTH and IZH decreased noticeably during the gastric digestion. Thereafter, during the intestinal digestion, the chelating ability of GTH increased about 450%, however, the activities of IZH and CTH decreased significantly. After the simulated GI digestion, only the sample GTH remained the metal ion chelating ability observed before digestion.

The results depicted in Fig. 3 suggest that the molecules responsible for the antioxidant activities tested are very different. As well, the results suggest that there are not a direct correlation between the MW of the peptide fractions from the hydrolysates and their antioxidant activities. It was demonstrated that the antioxidant activity of peptides depends not only on their MW, but also on other factors, such as amino acid composition, sequence and configuration of peptides (Chen et al., 1998). In addition, the

Table 3
Molecular weight distribution of gelatin hydrolysates after *in vitro* gastro-intestinal digestion.

Enzymes	Digestion condition	MW (kDa)
Visceral alkaline-proteases (GTH)	Before gastric digestion	1.757
	After gastric digestion	1.757
	After intestinal digestion	1.602
Commercial trypsin (CTH)	Before gastric digestion	2.212
	After gastric digestion	1.740
	After intestinal digestion	1.392
Izyme AL (IZH)	Before gastric digestion	>10.0
	After gastric digestion	>10.0
	After intestinal digestion	0.366

MW: molecular weight.

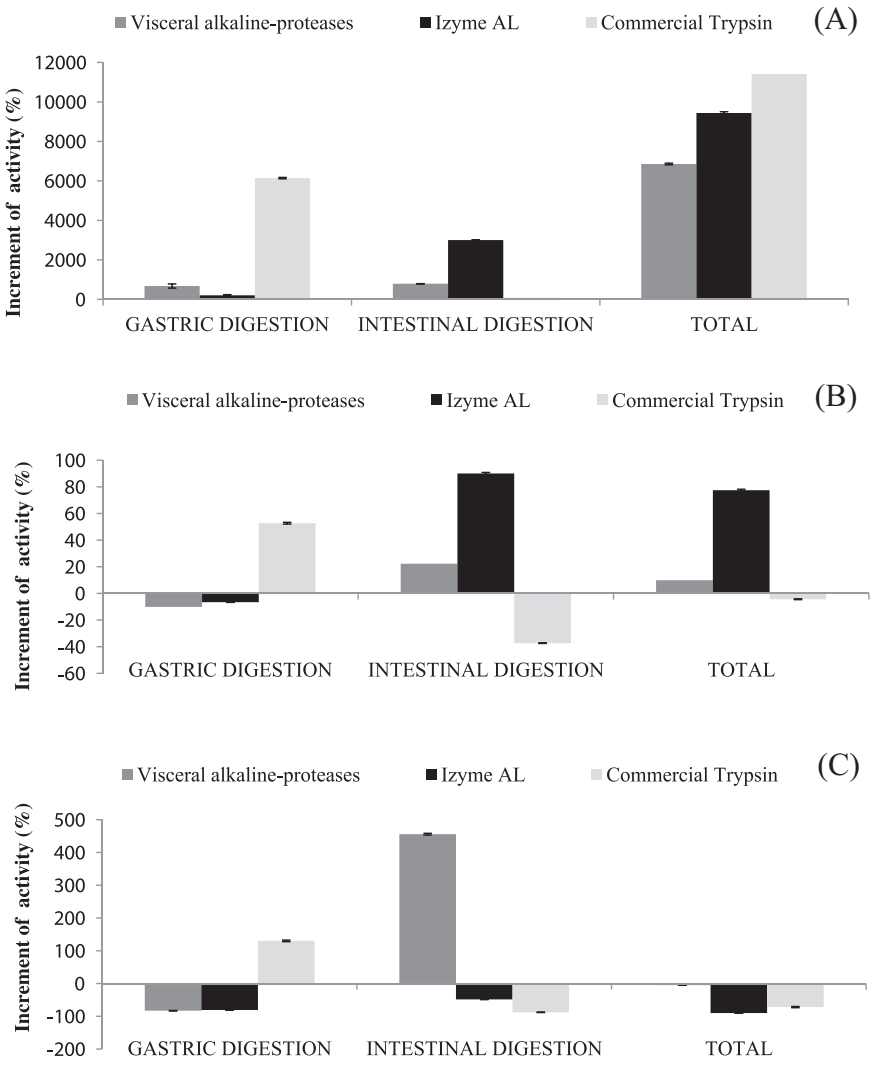

Fig. 3. Increments in the antioxidant activity of the hydrolysates (ABTS (A); FRAP (B); metal ion chelating (C)) after gastric-, intestinal-and total *in vitro* digestion. Bars represent the standard deviation from triplicate determinations.

mechanism of action of antioxidants in various test systems and the localisation of antioxidants in various phases of food or biological systems could both affect the results of antioxidant assays.

Several reports have also reported an increase of antioxidant activity of protein hydrolysates after being digested in a simulated model system (Khantaphant et al., 2011; Nasri et al., 2013; Senphan & Benjakul, 2014). Khantaphant et al. (2011) showed that DPPH radical scavenging activity of brownstripe red snapper hydrolysate increased within the first hour of simulated digestion, and FRAP increased throughout the digestion process. In another study, Senphan and Benjakul (2014) found a slight increase in ABTS radical scavenging activity of sea bass skin hydrolysate and chelating activity during pepsin digestion, but sharp increases within the first 20 min under duodenal conditions. However, when incubation time ran from 80 to 180 min, no changing in ABTS radical scavenging activity was observed, but an increase in metal ion chelating activity was observed. Nasri et al. (2013) reported that β-carotene-linoleate bleaching activity of goby fish hydrolysate slightly increased after pepsin and duodenal digestion. Additionally, Nalinanon, Benjakul, Kishimura, and Shahidi (2011) found that ABTS radical-scavenging activity of ornate threadfin bream hydrolysate increased by about 7–12% after being digested by pancreatin for 30–60 min.

4. Conclusion

In summary, this work demonstrates that visceral alkaline proteases and trypsin-like enzymes (Izyme AL® and porcine trypsin) were useful for the production of antioxidant hydrolysates from Giant catfish skin gelatin. The DH was not wholly related to the antioxidant activity of the resulting hydrolysates. The molecular weight profile of selected gelatin hydrolysates indicated that gelatin proteins were hydrolysed into peptides with smaller molecular weights and free amino acids, which is also reflected by the DH. The simulated gastrointestinal digestion of the gelatine hydrolysates significantly improved their antioxidant activity. These hydrolysates could be potential ingredients with interest in functional foods.

Acknowledgements

This study was financed by the Spanish Ministry of Economy and Competitiveness under project AGL2011-2760. Ms. Sunantha Ketnawa was funded by the Royal Golden Jubilee Ph.D. Program (Grant No. PHD 0113/2554) from the Thailand Research Fund. The TRF Distinguished Research Professor Grant was acknowledged. Authors are grateful to the Analysis Service Unit facilities of ICTAN for the analysis of total nitrogen.

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