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Stability of Mackerel (*Trachurus japonicas*) Hydrolysate with Iron-Binding Capacity in Simulated Gastrointestinal Fluids

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ABSTRACT

The aim of this study was to investigate the stability of mackerel (*Trachurus japonicas*) processing byproducts protein hydrolysate with iron-binding capacity *in vitro* simulated gastrointestinal systems. The changes in molecular weight distribution and iron-binding capacity were used for evaluating the stability of the hydrolysate in simulated gastrointestinal digestion. The molecular weight of mackerel hydrolysate with iron-binding capacity was mostly less than 1300 Da and composted mainly by tripeptides to undepeptides. The hydrolysate was stable in gastric or intestinal digestion separately for 5 h, or two-stage gastrointestinal digestion. The iron-binding capacity did not change significantly during gastrointestinal digestion. The mackerel processing byproducts hydrolysate had potential in iron fortification functional ingredients of food industry.

Key words: Mackerel, stability, gastrointestinal digestion, hydrolysate, iron-binding capacity

INTRODUCTION

Horse mackerel (*Trachurus japonicus*) is one of the most important fishery resources in the world. Its productivity is ranked as the third in all the single capture species of the world, with annual landings that exceeded 5-10 million tons in the past 10 years (Shi *et al*., 2008). The horse mackerel, which is extremely perishable and lower commercial value when compared to other marine fishes, mainly used for processing frozen fish sticks, segment, canned fish, fish meal and fish oil. These processing will produce a lot of byproducts, including head, skin, frame, viscera and others, which occupy about 50% of total weight and mainly are used for animal feeds (Cho *et al*., 2014). However, these mackerel processing byproducts are enriched in protein and maybe valuable source of bioactive peptides or functional foods ingredients, such as fish oil (Sahena *et al*., 2010), antibacterial peptides (Ennaas *et al*., 2015), gelatin (Khiari *et al*., 2011), rhamnose-binding glycoprotein (Terada *et al*., 2007), iron-binding peptides (Wang *et al*., 2013) and polyunsaturated fatty acids (Zuta *et al*., 2003).

Iron is one of the essential trace elements for people and animals and iron element in the metabolism of human body to participate in a variety of reactions (Hallberg *et al*., 1992). Iron is responsible for oxygen transport within haemoglobin. Meanwhile, iron is a vital substrate for haemoglobin production and sufficient iron stores are necessary to achieve and maintain adequate

levels of haemoglobin (Tay *et al.*, 2011). Mineral ions and peptide combination is absorbed by small intestine epithelial cells more easily than mineral ions alone (Schricker *et al.*, 1983). Therefore, the formation of food protein-derived mineral-chelating peptides has been extensively reported during the last decade (Guo *et al.*, 2014). The Fe bound to a casein peptide (β-CN (1-25)) improves Fe bioavailability in rats (Peres *et al.*, 1999) and Zn bound to yak milk casein hydrolysate improved Zn bioavailability (Wang *et al.*, 2011).

Recently more and more bioactive peptides are used for functional ingredients in food industry. However, these bioactive peptides will enter into the digestive tract with food consumption and the digest tract environment may affect these bioactive peptides or their biological activities. There are some reports about the stability of bioactive peptides in gastrointestinal digestion, such as ACE inhibitory peptides (Tavares *et al.*, 2011; Escudero *et al.*, 2014), antioxidative peptides (Megias *et al.*, 2009; Xie *et al.*, 2014; Tenore *et al.*, 2015), anticancer peptide (Bergeon and Toth, 2007) and mineral chelating peptides (Peres *et al.*, 1999; Wang *et al.*, 2011). However, the gastrointestinal stability of iron-binding peptides from mackerel enzymatic hydrolysate, has yet not been reported in the literature. The aim of our study was to evaluate the gastrointestinal stability of iron-binding peptides from mackerel protein hydrolysates.

MATERIALS AND METHODS

Materials: The horse mackerel (*Trachurus japonicus*) processing byproducts were provided by Zhoushan Fisheries Co., Ltd., China. The byproducts around enough ice were transported to laboratory and kept frozen at -20°C until used. The byproducts were thawed at 4°C for 24 h and then washed by running water. The washed byproducts were drained.

The pancreatin (from bovine pancrease, type II) and pepsin were purchased from Sinopharm Chemical Reagent Co., Ltd, China. Alcalase was purchased from Novozymes (China) Biotechnology Co., Ltd, China. Bovine serum albumin, cytochrome c from bovine heart, trypsin inhibitor from soybean (*Glycine max*) and vitamin B_{12} were purchased from Sigma Chemical Co., Ltd, USA. A hepta peptide, with amino acid of Ser-Val-Ala-Met-Leu-Phe-His and molecular weight of 804 Da, was purchased from Chinese Peptide Company (Hangzhou) to use for low molecular weight standard. All chemicals and reagents were of the highest grade available.

Enzymatic hydrolysis: Enzymatic hydrolysis conditions of horse mackerel processing product were according to Wang *et al.* (2013). Hydrolysis experiments were conducted in a 500 mL glass reactor. The mackerel processing byproducts were defatted by three-fold volume of isopropanol at 50°C for 12 h and then the sediment was dried at 80°C. Ten gram of defatted mackerel powder was dissolved in 20 mM pH 8.5 Tris-HCl buffer and placed in water bath at 55°C for 10 min. Alcalase was added at level of 16 U g^{-1} mackerel powder and stirred at 100 rpm. The hydrolysis reaction was stopped after 2 h by boiling the mixture for 10 min to inactivate the alcalase. Then the hydrolysate was centrifuged at 10000×g for 10 min at 4°C, the supernatant was collected and stored at -28°C until further use.

Stability in simulated gastrointestinal digestion: Stability of the mackerel hydrolysate peptides against *in vitro* gastrointestinal digestion was assessed using pepsin and pancreatin according to the method of Laparra *et al.* (2003) with some modifications. Pepsin solution in 6 M HCl (pH 2.0) was added to equality volume of 2.0 mg mL^{-1} mackerel hydrolysate peptides solution. The digestion was at 37°C for 1-5 h under continuous stirring. The pepsin solution in 6 M HCl was

boiling for 10 min and this solution acted as sample control. After digestion the mixture was boiling for 10 min to inactivate the pepsin. For intestinal digestion, 2% (w/v) pancreatin in pH 7.2 phosphours buffer was mixed with equality volume of 2.0 mg mL^{-1} mackerel hydrolysate peptides solution. The digestion was at 37°C for 1-5 h under continuous stirring. The pancreatin solution was boiling for 10 min and this solution acted as sample control. After digestion the mixture was boiling for 10 min to inactivate the pancreatin. For gastrointestinal digestion, the sample was first digest in pepsin solution for 2 h at 37°C and then the pH was adjusted to 7.2 with 1 M $NaHCO_3$ to inactivate the enzyme. Then, pancreatin solution was added and digests for 3 h at 37°C. The enzyme was inactivated in boiling water for 10 min. All samples were used for determination of molecular weight distribute and iron-binding capacity.

Measurement of peptide content and iron-binding capacity: The peptide content was determined by Folin-phenol method with bovine serum albumin as a standard to Lowry *et al.* (1951). The iron-binding capacity was measured by the spectrophotometric method according (Decker and Welch, 1990) with some modifications. The hydrolysate was diluted with 200 mM phosphate buffer (pH 8.0) to adjust the peptide content to 1.0 mg mL^{-1}. After the addition of 30 mM $FeSO_4 \bullet 7H_2O$ (including 0.1 M L-ascorbic acid), the mixture was stirred at room temperature for 30 min. Then the mixture was centrifuged at 10000×g for 10 min at 4°C to remove precipitates. The absorbance at 562 nm was determined after adding 2.75 mL distilled water, 1.0 mL acetate buffer solution (pH 4.5) and 0.25 mL 40 mM ferrozine reagent to the sample (1.0 mL). Distilled water was instead of sample as control. The iron-binding capacity was calculated as:

$$\text{Iron binding capacity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where, $A_{control}$ and A_{sample} are the absorbance at 562 nm of control and sample, respectively.

Determination of molecular weight distribution: The sample solution was filtered through 0.22 μm membrane and further used for determining the molecular weight by gel-filtration HPLC with a gel column (Waters Stragel HT5, 4.6×300 mm). The HPLC separation condition was as following: the mobile phase of 40% (v/v) acetonitrile including 0.1% (v/v) trifluoroacetic acid; flow rate of 1.0 mL min^{-1}; ultraviolet detection at 214 nm; column temperature of 40°C. The molecular weight standards were bovine serum albumin (66.7 kDa), trypsin inhibitor (20.1 kDa), cytochrome c (12.3 kDa), vitamin B_{12} (1355 Da) and a synthetic hepta peptide (804 Da), respectively.

Statistical analysis: Each data point was represented by the mean and standard deviation of three samples. The variance analysis (ANOVA) was conducted by Duncan's test at significance level of $p<0.05$.

RESULTS AND DISCUSSION

Molecular weight distribution of the hydrolysate: The defatted mackerel processing byproducts was hydrolyzed by alcalase in order to obtain iron-binding capacity peptides. The hydrolysate had iron-binding capacity of 44.2% and the degree of hydrolysis was 56.1%. Then the hydrolysate was filtrated by 0.22 μm and applied to determine molecular weight distribution on HPLC gel column. The result was shown in Fig. 1. The molecular weight of the iron-binding

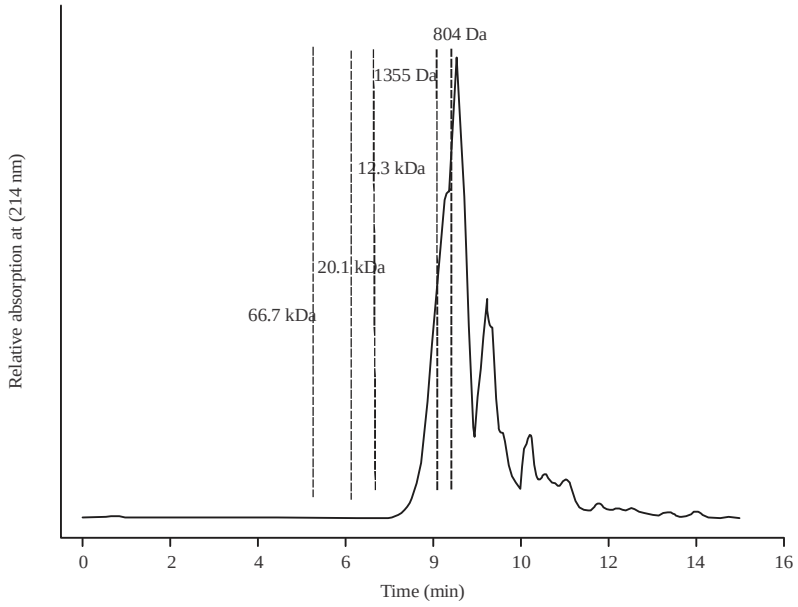

Fig. 1: Molecular weight distribution of mackerel hydrolysate with iron-binding capacity

peptides were mostly less than 1300 Da, especially from 300-1200 Da. It also suggested that the hydrolysate was composed mainly of mixed peptides of tripeptide to undepeptide, because the average molecular weight of amino acid residues in most proteins was 110. Many iron-binding peptides from enzymatic hydrolysis had small molecular weight. A small tripeptide with high iron chelating capacity, Ser-Cys-His (345 Da), was purified and identified from Alaska pollock skin hydrolysate (Guo *et al*., 2013). Kim *et al*. (2014) purified an iron-chelating peptide, amino acid sequence of Thr-Asp-Pro-Ile(Leu)-Ala-Ala-Cys-Ile(Leu), molecular weight of 802 Da, from spirulina protein hydrolysates. Lee and Song (2009) isolated an iron-binding nona-peptide, Asp-Leu-Gly-Glu-Gln-Tyr-Phe-Lys-Gly, molecular weight of 1055 Da, from porcine blood plasma protein hydrolysate with Flavourzyme. Huang *et al*. (2012) obtained a hepta-peptide (699 Da) with iron-binding ability from shrimp processing byproducts hydrolysate.

Stability in simulated gastrointestinal digestion: In order to study the resistance of the iron-binding peptides to gastrointestinal enzymes, the horse mackerel (*Trachurus japonicas*) protein hydrolysate peptides were subjected to a two-stage hydrolysis process, which simulated physiological digestion. The digests were analyzed by HPLC with the aim of identifying the fragments released by the action of gastrointestinal enzymes and also determined the iron-binding capacity by ferrozine method. First the hydrolysate was digested by simulated gastric fluid for 0-5 h, the molecular weight distributes and iron binding capacity were shown in Fig. 2 and 3, respectively. From Fig. 2, it was shown that artificial gastric fluid for as long as 5 h did not affected on the hydrolysate peptides distribution pattern, that was to say it was very stability to gastric digestion. The peptides peak time, peak height and peak type were almost perfectly similar to the blank group in the HPLC molecular distribute pattern. From Fig. 3, it was shown that the iron binding capacity of hydrolysate was slightly decreased at the first two hours during simulated gastric digestion. But the differences were not significant at $p<0.05$ level. That horse mackerel protein hydrolysate under the action of digestive enzymes in the stomach will still be able to

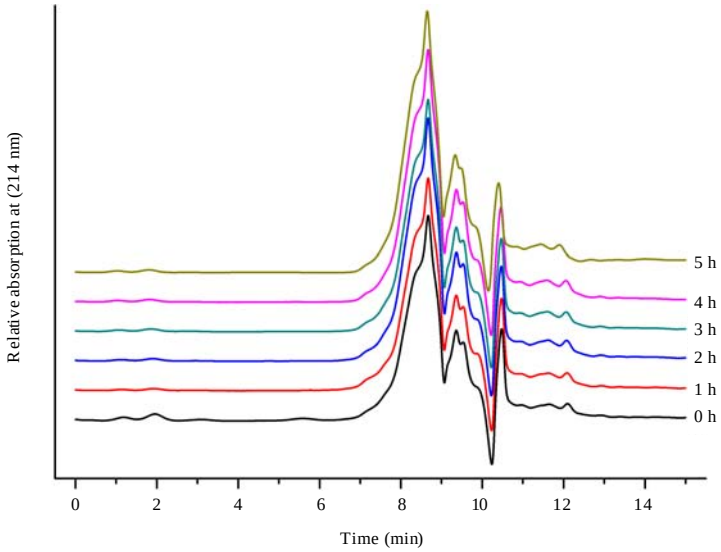

Fig. 2: Molecular weight distribution of mackerel hydrolysate in simulated gastric digestion

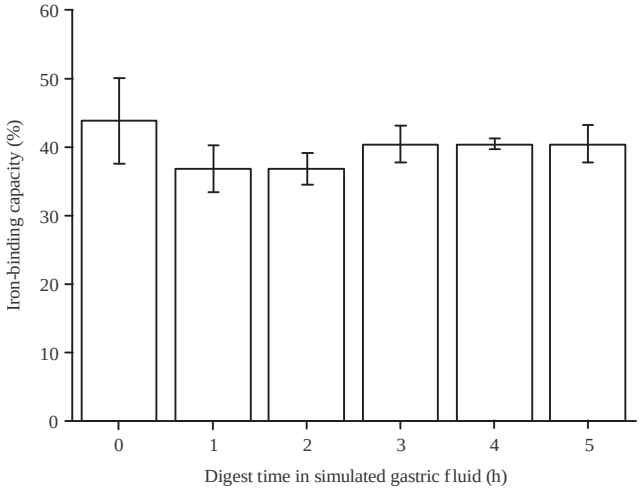

Fig. 3: Iron-binding capacity of mackerel hydrolysate in simulated gastric digestion

maintain its stability. It is known that the presence of proline makes amino acid sequences less susceptible to proteolytic enzymes (Ruiz *et al.*, 2004).

Then the hydrolysate was also digested by simulated intestinal fluid for 0-5 h, the molecular weight distributes and iron binding capacities were determined. The results were shown in Fig. 4 and 5, respectively. From Fig. 4, it was shown that the hydrolysate was relative stability in artificial intestinal fluid for 5 h and there were only very slightly changes in long kept-time, which might be caused by a little quantity amino acid produced from hydrolysate. The iron-binding capacity of hydrolysate during simulated intestinal digest process was not decreased significantly, shown in Fig. 5. All these information improved that the hydrolysate was stability to intestinal digest.

At last, the hydrolysate was also digested by simulated gastric fluid for 2 h and digested subsequently by simulated intestinal fluid for 3 h. The molecular weight distributes and iron

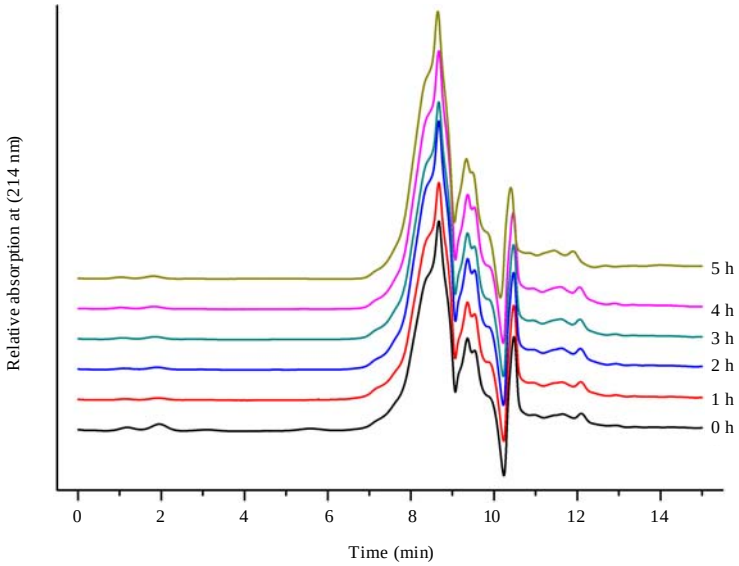

Fig. 4: Molecular weight distribution of mackerel hydrolysate in simulated intestinal digestion

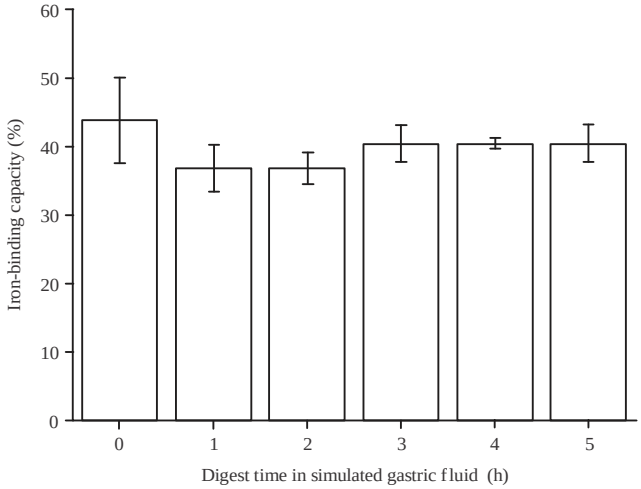

Fig. 5: Iron-binding capacity of mackerel hydrolysate in simulated intestinal digestion

binding capacities were determined, results shown in Fig. 6 and 7, respectively. From Fig. 6, it was shown that the hydrolysate was relative stability in artificial gastrointestinal digest and only very little peptides was digested into smaller peptides or amino acids. The iron-binding capacity was slightly decrease from 45.6-40.4% but the difference between these was not significant at $p<0.05$ level.

Obviously, the molecular weight distribute pattern and iron-binding capacity in gastrointestinal fluid improved that the mackerel protein alcalase hydrolysate was stability and it had potential for producing iron additives by enzymatic hydrolysis from horse mackerel protein. There was little information about stability of iron-binding peptides in gastrointestinal digestion. Wang *et al*. (2015) reported that three synthetic Zn-chelated-peptides were resistant to pepsin but not to pancreatin. Pancreatin can partly hydrolyse both peptides and Zn-peptide complexes but more than half of

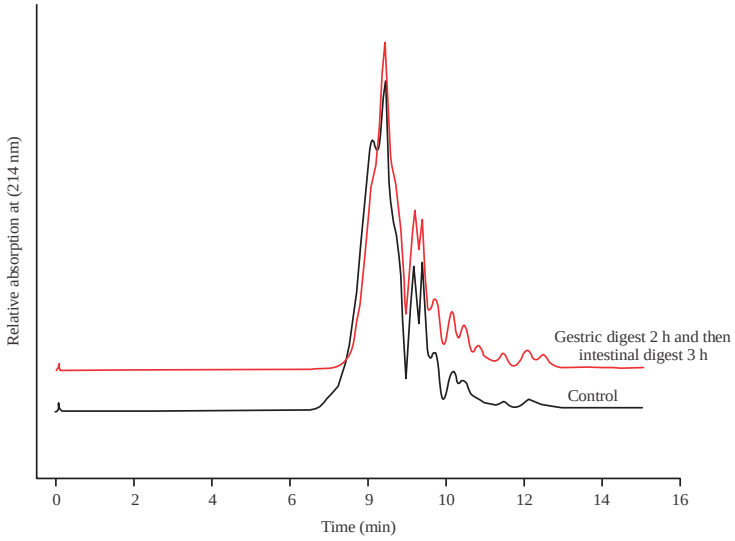

Fig. 6: Molecular weight distribution of mackerel hydrolysate in simulated gastrointestinal digestion

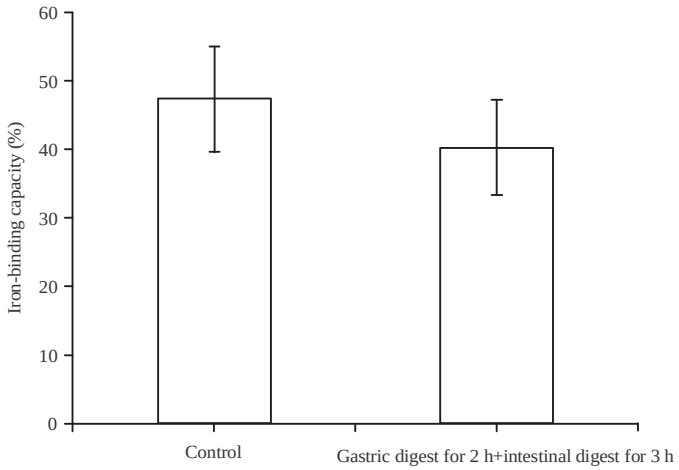

Fig. 7: Iron-binding capacity of mackerel hydrolysate in simulated gastrointestinal digestion

them remaining in their original form after gastrointestinal digestion. Wang *et al.* (2011) reported that zinc-chelating yak milk casein hydrolysate (YCH) made Zn more soluble under simulated intestinal conditions. Ait-Oukhatar *et al.* (2000) revealed that the dialyzable Fe could not release from Fe-β-CN (1-25) complexes *in vitro* digestion assay. Tavares *et al.* (2011) reported that two ACE-inhibitory small peptides from α-lactalbumin and β-lactoglobulin were not stable for gastrointestinal enzymes but the ACE-inhibitory effects were not severely affected. Escudero *et al.* (2014) revealed that five small peptides (500-629 Da) from Spanish ham were stable for gastrointestinal digestion and as well as the powerful ACE inhibitory activity.

CONCLUSION

This study has clearly demonstrated that the horse mackerel processing byproducts hydrolysate with high iron-binding capacity was stable to gastric and intestinal digestion. The iron-binding

capacity of hydrolysate and molecular weight distributions were was not significantly changed after gastrointestinal digestion. Results from this study indicated that it is feasible for iron-binding peptides from mackerel processing byproducts hydrolysate application in iron fortification food industry.

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