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Purification of a Hepta-peptide with Iron Binding Activity from Shrimp Processing By-products Hydrolysates

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Abstract: The aim of this study was focused on isolation and purification of an iron binding peptide derived from shrimp processing by-products. The shrimp processing by-products was hydrolyzed by Flavourzyme to produce iron binding peptides hydrolysate. Then the hydrolysate with iron binding ability was isolated and purified by SP-Sepharose Fast Flow cation exchanger, Sephadex G-25 gel filtration and reversed phase high-performance liquid chromatography (RP-HPLC), respectively. A peptide of molecular mass 699 Da with strong iron binding ability was obtained and identified by mass spectrometry (MALDI-TOF-TOF) to be LPTGPKS after analysis and alignment in database. Results indicated that the production of iron-binding peptides from shrimp processing by-products supported further applications for high-value bioproducts in health care product.

Keywords: Amino acid sequence, enzymatic hydrolysis, flavourzyme, iron binding peptide, isolation

INTRODUCTION

Peptides derived from proteins where they belonged to have been found to be physiologically active or bioactive. Via separation and purification by various chromatography technology, the peptide with highest bioactive can be obtained. Various peptides with significant respective bioactive have been identified after chromatography and mass spectrometry, for example, antioxidative peptides (Hsu, 2010; Lee *et al*., 2010; Kim *et al*., 2009; Zhang *et al*., 2009), antibacterial or antimicrobial peptide (Cytryńska *et al*., 2007), mineral-binding milk peptides (Vegarud *et al*., 2000), calcium-binding peptides (Jiang and Mine, 2000; Jung *et al*., 2006; Jung and Kim, 2006), iron-binding peptides (Lee and Song, 2009; Kim *et al*., 2007; Wu *et al*., 2008), copper-chelating peptide (Megías *et al*., 2007) and other novel bioactive peptides (Ghassem *et al*., 2011).

Shrimp processing by-products was a potential source of bioactive molecules, its crude protein content ranging from 9.3 to 11.6% (Heu *et al*., 2003). Shrimp-originated peptides were found out successively, for instance, anti-lipopolysaccharide factor peptide (Lin *et al*., 2010), antimicrobial peptide (Rolland *et al*., 2010), calcium-binding peptides (Huang *et al*., 2011; Inoue *et al*., 2004), Angiotensin Converting Enzyme (ACE) inhibitory peptides (Ghassem *et al*., 2011).

A quarterly update based on the GLOBEFISH databank (Globefish, 2010) in 2010 showed shrimp (HS03) imports into China totaled 29271 tons valued at USD 15 million, up 25.5% in quantity and 41.1% in value on a year to year basis. The shrimp processing by-products were used to product sauce, feed (Leal *et al*., 2009) or carotenoid (Sachindra and Mahendrakar, 2005; Sowmya *et al*., 2011) and chitin (Valdez-Peña *et al*., 2010) etc., while only a few study on iron-binding peptide from shrimp by-products were reported Huang *et al*. (2011). This study focused on the purification of a hepta-peptide with iron-binding activity from shrimp processing by-products hydrolysate.

MATERIALS AND METHODS

Materials: The shrimp processing by-products were delivered on October, 2010 from Hangzhou Beijiping Aquatic Co. Ltd, Zhejiang, China. The samples were packed in polyethylene bags and placed in ice. Then the samples were transported to the research laboratory within 60 min. The samples were rinsed three times with distilled water and dried at 65ºC under vacuum for 24 h. The dried samples were smashed into fine powder and passed through 150 mesh (0.1mm) sieve then stored in sealed container at 4ºC. Flavourzyme (1500 U/mg) was purchase from Novo (Novozymes, Denmark). Chemicals and solvents were of analytical and HPLC grade.

Preparation of enzymatic hydrolysates and degree of hydrolysis (DH): The sample was dispended in ultra-pure water into 5% (w/v) mixture and the pH was adjusted to 7.0 with 1N HCl or 1N NaOH. Then the sample was hydrolyzed at 50ºC for 7h by Flavourzyme with enzyme concentration of 400 to 6400 U/mL. The hydrolysis reaction was stopped by heating the

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solutions at 85ºC for 15 min to inactivate Flavourzyme. The hydrolysates were then centrifuged at 5000 g for 10 min to remove insoluble substrates and the supernatant was stored at 4ºC till analysis, less than 3 days. The DH was estimated by determination of free amino groups by reaction with Trinitrobenzenesulfonic acid (TNBS) (Adler-Nissen, 1979):

$$\mathrm{DH}(\%) = \frac{H_t - H_0}{H_{\max} - H_0} \times 100$$

where, H_t was the amount of a specific liberated amino acid at time t, H_0 was the amount of the specific amino acid in the original substrate (blank) and H_{max} was the maximum amount of the specific amino acid in the substrate obtained after hydrolysis.

Determination of iron binding ability: The hydrolysates were diluted one half with distilled water, then demineralized using macroporous resin (Amberlite IRC-748I sodium form) to exclude the interference of divalent iron and mixed with equal volume of 200 mM phosphate buffer (pH8.0). After the addition of 30 mM $FeSO_4 \cdot 7H_2O$ (including 0.1 M L-ascorbic acid), the solution was stirred at room temperature for 1 h and the reaction mixture was centrifuged at 8000 g for 10 min to remove precipitates. The iron content in the supernatant was determined using a chromomeric method with orthophenanthroline reagent (Krishna *et al*., 1970). The absorbance at 510 nm was determined after adding 2.75 ml distilled water, 1 mL acetate buffer solution (pH4.5) and 0.25 mL orthophenanthroline reagent to the sample (1.0 mL). The protein content was determined using a chromomeric method with Folin-phenol (Lowry *et al*., 1951), using bovine serum albumin as a standard. The iron-binding capacity was expressed as the binding iron quantity (mg) per unit of protein (mg).

Purification of iron binding peptide: The hydrolysate was demineralizated and concentrated at 50ºC, 1000 g under vacuum, filtrated by 0.45 μm filter film, then loaded onto a SP Sepharose Fast Flow (GE health, USA) cationic exchange column (16×500 mm, H&E, China) every 0.6 ml per loading. Elution was performed on solution A (20 mM sodium acetate buffer, pH 4.0) and solution B (20 mM sodium acetate buffer solution including 1 M NaCl, pH4.0) with a gradient of 0-100% B at flow rate of 2.0 mL/min. Meanwhile the eluation was monitored at 280 nm and the absorption peaks were fractionated.

The protein content and iron-binding capacity of the fractions were determined. The highest iron-binding fraction was pooled and concentrated, then loaded onto a Sephadex G-25 (GE health, USA) gel filtration chromatography column (10×600 mm), eluted with distilled water at flow rate of 1.0 ml/min and monitored at 214 nm. The highest iron-binding fraction was pooled and lyophilized. The sample powder was dissolved in distilled water and loaded onto reversed phase high-performance liquid chromatography (RP-HPLC) with a C_{18} reverse-silica gel column (20×250 mm, Shim-pack PRC-ODS). Elution was performed on A (0.5% trifluoroacetic acid (TFA) in water) and B (0.5% TFA in 30% acetonitrile) with a gradient of 0-15% B at 4.0 mL/min for 30 min.

The highest iron-binding fraction from RP-HPLC column was pooled and lyophilized for analytical HPLC. The sample powder was dissolved in distilled water and was loaded onto an analytical C_{18} reverse-silica gel column (4.6×250 mm, Shim-pack ODS-SP). Elution was performed on A (0.5% TFA in water) and B (0.5% TFA in 30% acetonitrile) with a gradient of 0-15% B at 4.0 mL/min for 30 min. The highest iron-binding fraction was pooled and lyophilized.

Peptide sequence identification: Molecular Weight (MW) of the purified iron binding peptide was determined using an MALDI-TOF-TOF tandem mass spectrometer and sequenced using De Novo Explorer. A 0.5 μL sample was loaded to the target plate then natural drying, added 0.5 μL 0.5 g/L CHCA (0.1% TFA dissolved in 50% acetonitrile) to be analyzed until its natural dried. Another target plate was only added 0.5 μL 0.5 g/L CHCA (0.1% TFA dissolved in 50% acetonitrile) set as blank. The mass spectrometer analysis was performed by 4700 proteomics analyzer (Applied Biosyetems, USA). The wavelength of NdYAG laser was 355 nm, accelerating voltage was 20 kV, data acquisition was executed by [M+H] and auto acquisition mode. The hydrolyzed myoglobin fraction was chosen to the instrumental calibration as external standards. Peptide Mass Fingerprint (PMF) of matrix and sample scanning m/z was ranged from 100 to 3000 Da. Compared to the matrix PMF spectrum, different peptide fraction ion was further decomposited using MS/MS. Mass bellowed 150 Da was prior checked, several residues was preliminary deducted then sequencing by De Novo Explorer in 4700 explore. Reflector positive parameter was selected in MS: CID (off), mass rang (700-3200 Da), Focus Mass (1200 Da), Fixed laser intensity (6000), Digitizer: Bin Size (1.0ns). 1KV positive parameter was selected in MS/MS : CID (on), precursor Mass Windows [Relative 80 resolution (FWHM)], fixed laser intensity (7000), Digitizer: Bin Size (0.5 ns).

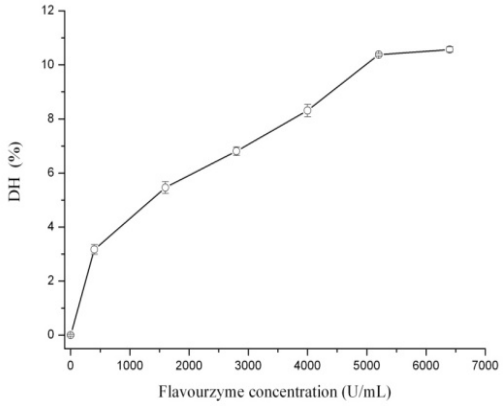

Fig.1: The effect of flavourzyme addition on the DH of shrimp processing by-products hydrolysate

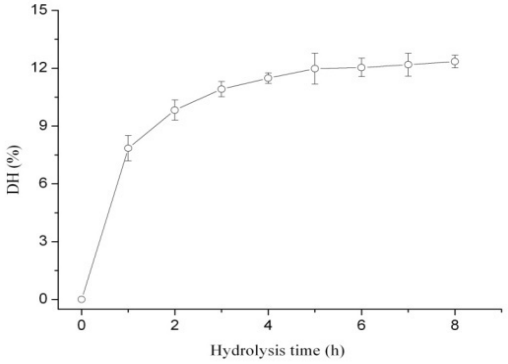

Fig. 2: The hydrolysis curve of shrimp byproducts by flavourzyme

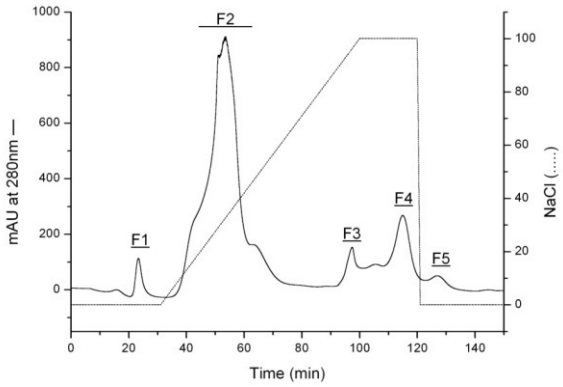

Fig. 3: The SP sepharose fast flow chromatography of shrimp processing by-products hydrolysates by flavourzyme. The column (16×500 mm) was washed with 20 mM of sodium acetate buffer (pH 4.0) and then eluted with a step gradient of NaCl as indicated. The flow rate was 2 mL/min, and elution was monitored at 280 nm. mAU (continuous line), NaCl concentration (broken line)

Table 1: The iron binding ability of shrimp by-products hydrolysates fractions during isolation and purification procedure

Fraction	Iron binding ability (mg-Fe/mg-protein）
F1	0.619
F2	0.052
F3	0.000
F4	0.238
F5	0.000
F11	0.518
F12	0.530
F121	2.930
F122	5.130
F123	18.08

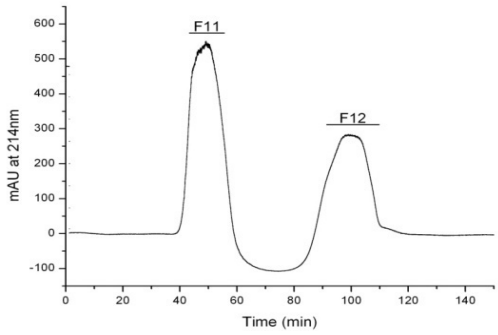

Fig. 4: The sephadex G-25 chromatography of F1 fraction. The column (10×600 mm) was washed with distilled water at the flow rate of 1.0 ml/min and elution was monitored at 214 nm

Statistical analysis: Data and Statistical analysis were performed using scientific graphic and analysis computer software OriginPro (version 7) and data was expressed as mean±SD of three experiments.

RESULTS AND DISCUSSION

Preparation of enzymatic hydrolysates: The enzymatic hydrolysate of sample was prepared by Flavourzyme at pH 7.0 and 50ºC. During hydrolysis, the DH was determined. The Flavourzyme addition quantity affected the DH, shown in Fig. 1. It showed that hydrolysis with 5200 U/ml enzyme additive was suitable in preparing hydrolysates and the DH reached 10.5% at this condition. Also, the hydrolysis time affected the DH, shown in Fig. 2. The DH was increased dramatically in the primary hydrolysis stage and after 5 h, it was tend to become stable, about 11.5%. So, hydrolysis time of 5 h was selected to preparation of shrimp by products hydrolysates for further study.

Purification of iron binding peptide: Throughout the purification process, iron binding activity was assessed using chromomeric method with orthophenanthroline reagent to determine the free iron after addition of ferric

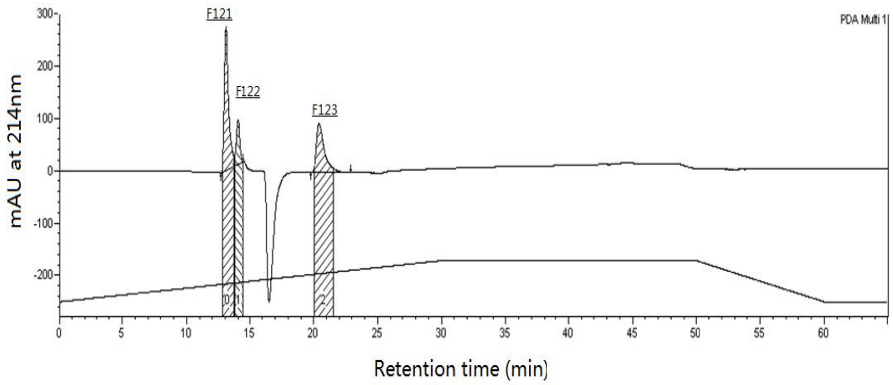

Fig. 5: Semi-prepared reversed-phase HPLC chromatography of fraction F12 derived from sephadex G-25 gel filtration. The column (20 mm×250 mm, shim-pack PRC-ODS) were equilibrated with solvent A (0.5% trifluoroacetic acid in deionized water) and eluted with a linear gradient of solvent B (0.5% trifluoroacetic acid in 30% acetonitrle). The flow rate was 4.0 ml/min and detection was 214 nm

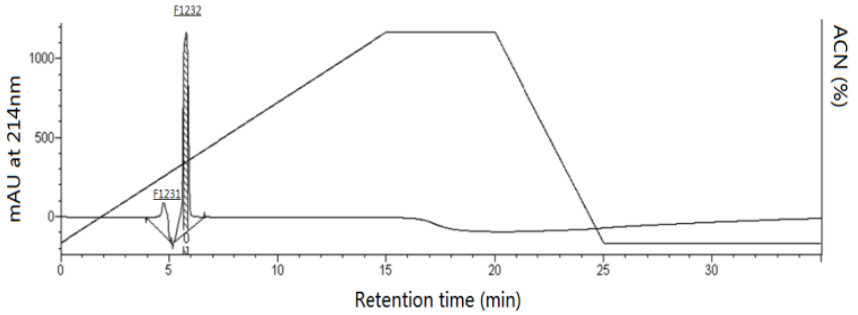

Fig. 6: Analytic RP-HPLC chromatography of fraction F123 obtained from semi-prepared RP-HPLC.

sulfate into sample solutions. Ion exchange chromatography was used as the first purification step and SP-Sepharose Fast Flow cation exchanger was loaded onto a SP-Sephadex C-25 column. Adsorbed fractions were eluted with a linear gradient of NaCl (0-1 mol/L) during 120 min (Fig. 3). The purification result showed that five major peaks were obtained. Each fraction was pooled and the protein content and iron-binding capacity were determined subsequently, shown in Table 1. The fraction F1 had highest iron-binding capacity of 0.619 (mg-Fe/mg-protein), whereas fraction F3 and F5 was lower than limit of determination. The fraction F1 was collected and lyophilized. Then it was dissolved in distilled water and loaded onto a gel filtration chromatography on Sephadex G-25 column. The fraction was separated into two distinct fractions, F11 and F12 (Fig. 4). The fraction F12 showed higher iron binding ability, 0.53 (mg-Fe/mg-pro). Then, F12 was pooled and lyophilized, dissolved with distilled water into 20 mg/mL, loaded onto a semi-prepared C_{18} RP-HPLC. Three major peaks (F121, F122 and F123) were obtained and collected by auto-collector, shown in Fig. 5. Each fraction was pooled and the protein content and iron-binding capacity was determined subsequently (Table 1). It shown that fraction F123 had the highest iron-binding capacity of 18.08 (mg-Fe/mg-protein). Thus fraction F123 was pooled and concentrated, then loaded onto an analytic C_{18} RP-HPLC for purity determination (Fig. 6). Fraction F1232 (marked as 0) was displayed purity index equaled to 1.00000 in the Shimadzu HPLC analytic software. Then the fraction F1232 was collected to amino acid sequence analysis.

Peptide sequence identification: The amino acid sequence of fraction F1232 was analysed using an MALDI-TOF-TOF tandem mass spectrometer and sequenced using De Novo Explorers. The result was shown in Fig. 7 and a novel hepta-peptide was discovered. The mass weight of the hepta-peptide was 699Da and the amino acid sequence was LPTGPKS (Leu-Pro-Thr-Gly-Pro-Lys-Ser).

The alignments was submitted using protein BLAST tool on The National Center for Biotechnology Information (NCBI), extracelluar solute-binding protein family 1 (Alicyclobacillus acidocaldarius LAA1),

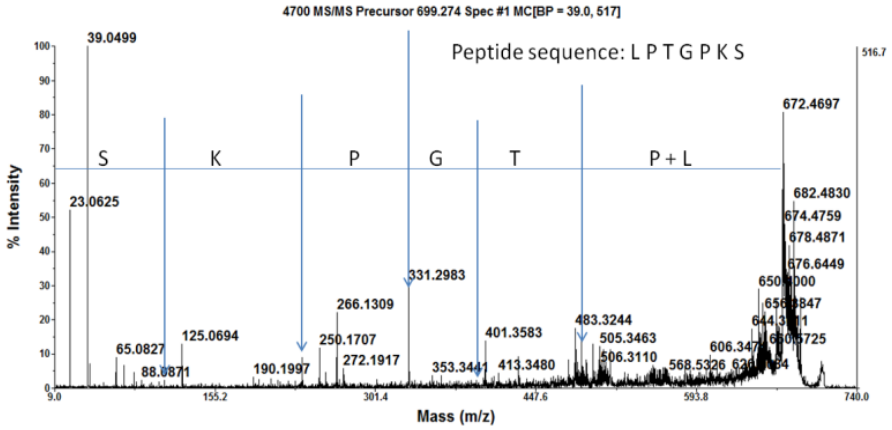

Fig. 7: Amino acid sequence deducted and sequencing by De Novo Expolorer in 4700 explorer

NCBI accession No.ZP_03495377.1 was found with 85% query coverage, certain extracelluar solute-binding capacity may the hepta-peptide had and recent study shown Met, Tyr and Ser were binding site of the phosphorylate minerals (Houben *et al*., 1999). Pro and Hyp were typical amino acid of collagen composition, thus Ser and Pro in hepta-peptide may have relationship with iron-binding site. Lys contributed to absorption of calcium for its acidity and iron and calcium belonged to the essential divalent minerals.

CONCLUSION

This study discovered iron binding bioactive peptide could be derived from shrimp by-products hydrolysates. The fractions of these hydrolysates were separated and purified via column chromatographic and two-step HPLC procedure. Then the amino acid sequence of the strongest iron binding peptide was identified by MALDI-TOF-TOF to be LPTGPKS. It was suggested that the amino acid of Ser, Lys and Pro in the sequence might contribute to the iron-binding capacity. Results from this study indicated that it was feasible to produce natural iron binding peptides from shrimp processing by-products by enzymatic hydrolysis.

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