

## Preparation and Characteristic of Iron-Binding Peptides from Shrimp Processing Discards Hydrolysates

Zhang-yan Ren, Guang-rong Huang, Jia-xin Jiang and Wen-wei Chen  
College of Life Sciences, China Jiliang University, Hangzhou, P.R.China Xueyuan  
Street 258, Xiasha High Education Area, Hangzhou, 310018, P.R. China

**Abstract:** The aim of this study was focused on preparation of iron-binding peptides from Shrimp Processing Discards (SPD) hydrolysate using response surface methodology (RSM) and characteristic of the iron-peptides complex. Six kinds of protease (tyrpsin, pepsin, protmex, flavourzyme, neutrase and alcalase) were used to hydrolysis the SPD protein and the trypsin hydrolysate showed the highest iron-binding activity. The factorial design experiments showed that pH, trypsin concentration and hydrolysis time affected significantly the iron-binding activity of hydrolysate. Then the trypsin hydrolysis conditions (time, enzyme concentration, pH) were optimized by RSM using central composition design for producing highest iron-binding activity. The pH 8.18, enzyme concentration of 500 U/ml and a hydrolysis time of 120 min were found to be the optimum conditions to obtain a higher iron-binding activity of 6.75 µg-EDTA/mg-protein, which is very closed to the three verified replicated experiments with 6.73 µg-EDTA/mg-protein. The amino acid composition of the iron-peptides complex was closed to FAO reference protein. The main compositions of the iron-peptides complex were iron of 11.1%, protein of 71.5% and ash of 19.3%. The peptides bound with iron were certified by infrared chromatography.

**Key words:** Amino acid composition, enzymatic hydrolysis, iron-binding activity, response surface methodology, shrimp processing discards

### INTRODUCTION

Iron is one of the essential mineral that can function as regulators, activators, transmitters, and controllers of various enzymatic reactions. Iron deficiency and specifically iron deficiency anemia, remains one of the most severe and important nutritional deficiencies in the world today. Iron deficiency with or without anemia has important consequences for human health and child development: anemic women and their infants are at greater risk of dying during the prenatal period; children's mental and physical development is delayed or impaired by iron deficiency; and the physical work capacity and productivity of manual workers may be reduced.

Many peptides released *in vitro* or *in vivo* from animal or plant proteins are bioactive and have regulatory function in human beyond normal and adequate nutrition (Jung and Kim, 2007a, b). The caseinphosphopeptides (CPP) (Meisel and Fitzgerald, 2003) and other peptides from the hydrolysates of milk (Vegarud *et al.*, 2000), whey (Kim *et al.*, 2007), sunflower protein (Megías *et al.*, 2007), Alaska Pollack (*Theragra chalcogramma*) fish protein (Jung *et al.*, 2006) and porcine blood plasma

protein (Chaud *et al.*, 2002) are found to possess mineral-binding activity.

China has been one of the world's largest shrimp producers since 1988, and have a rapid expansion recently (Xie and Yu, 2007). Zhejiang province, as a costal provincial capital in China, aquatic products processing industry was developed with an ineligible low comprehensive utilization of the processing discards. Shrimp Processing Discards (SPD), including heads, shells and tails, were micro-nutrient byproducts rich in amino acids for potential recommendations in the supplementation of animal and human diets (Bueno-Solano *et al.*, 2009). The crude protein contents were in the range of 9.3 to 11.6% and the iron content (7.29 mg/100 g) was present in the SPD (Heu *et al.*, 2003). Some mineral-binding peptides are isolated or purified from protease hydrolysates of hoki (Jung *et al.*, 2005) and crayfish (Inoue *et al.*, 2004) protein. By now, there has no report about the preparation of mineral-binding peptides from shrimp or its processing discards.

Hence, the present study was carried out to optimize the enzymatic hydrolysate with high iron-binding activity using response surface methodology (RSM) and study the characteristics of the iron-peptides complex.

Table 1: The hydrolysis conditions of six proteases and the iron-binding activity of hydrolysate

Enzyme	pH	Temperature (°C)	Hydrolysis time (h)	Iron-binding activity (µg-EDTA/mg-protein)
Trypsin	8.0	37	5	4.18±0.26 <sup>a</sup>
Pepsin	2.0	37	5	3.28±0.53 <sup>b</sup>
Protamax	7.5	50	5	1.44±0.22 <sup>c</sup>
Flavourzyme	7.1	50	5	3.00±0.34 <sup>b</sup>
Neutrase	7.1	50	5	1.40±0.23 <sup>c</sup>
Alcalase	8.0	50	5	1.59±0.19 <sup>c</sup>

Data presented in the table is the mean of three readings i.e. Mean±S.D. The values having the same superscript in a column do not differ significantly (p<0.05)

### MATERIALS AND METHODS

**Materials:** The SPD was purchased from Hangzhou Aquatic Markets, Zhejiang Province, China, during spring, 2010. The SPD were dried at 95°C for 24 h and smashed by the disintegrator. Then the dried SPD was milled and meshed through 0.1 mm sieve. The fined SPD powder was obtained and stored in 4°C. The commercial

protamax and flavourzyme were purchased from Novo (Novozymes, Denmark), the pepsin, neutrase and alcalase were purchased from Shanghai Bioscience Company (Shanghai, China) and the trypsin was purchased from Sinopharm Chemical Reagent Company (Shanghai, China).

**Enzymatic hydrolysis:** A 5.0 g SPD fine powder was dispended in 100 ml ultra-pure water and then the solution was adjusted to optimal pH of protease with 1N HCl or 1N NaOH. The SPD solution was hydrolyzed at optimal temperature for 3 h by six kinds of protease with a 20:1 substrate to enzyme ratio (w/w) at stirring condition. The optimal pH and temperature of six proteases used in this experiment was shown in Table 1. The pH of the reacted mixture was adjusted to optimal pH every 15 min with 1N HCl or 1N NaOH during hydrolysis. After hydrolysis, the protease was inactivated at 85°C for 15 min. The hydrolysate was centrifuged (5000 rpm, 10 min) to remove insoluble substrates and the supernatant was storied at -18°C till analysis.

Table 2: The five factors two-level factorial design for production of iron-binding peptides

Runs	Substrate concentration (S, %)	Enzyme concentration (ES, U/mL)	pH	Temp (T, °C)	Time (t, h)	Iron-binding activity (µg-EDTA/mg-protein)
1	2	200	7.1	45	6	12.15±1.18 <sup>a</sup>
2	2	200	8.5	45	1	11.55±0.76 <sup>a</sup>
3	2	2000	7.1	35	6	4.58±0.76 <sup>c</sup>
4	2	2000	8.5	35	1	9.01±0.50 <sup>b</sup>
5	8	200	7.1	35	1	2.61±0.41 <sup>d</sup>
6	8	200	8.5	35	6	2.62±0.67 <sup>d</sup>
7	8	2000	7.1	45	1	2.68±0.47 <sup>d</sup>
8	8	2000	8.5	45	6	1.91±0.54 <sup>d</sup>

Data presented in the table is the mean of three readings i.e., Mean±S.D; The values having the same superscript in a column do not differ significantly (p<0.05)

Table 3: Response surface methodology for production of iron-binding peptides

Runs	ES	pH	t	Iron-binding activity (µg-EDTA/mg-protein)
1	1000	7.8	3	6.11±0.089
2	1000	7.8	3	6.20±0.066
3	1000	9.0	3	7.16±0.061
4	500	8.5	4	7.03±0.069
5	1000	7.8	3	5.43±0.066
6	1840	7.8	3	5.16±0.150
7	1000	7.8	3	6.20±0.306
8	1000	6.6	3	0.68±0.080
9	1000	7.8	3	5.59±0.158
10	500	7.1	2	7.05±0.142
11	159	7.8	3	5.38±0.142
12	1000	7.8	1.3	6.22±0.187
13	1500	8.5	4	4.26±0.280
14	1500	7.1	4	4.19±0.120
15	1500	8.5	2	5.71±0.226
16	1500	7.1	2	4.04±0.130
17	500	7.1	4	4.06±0.086
18	1000	7.8	4.7	4.76±0.037
19	500	8.5	2	5.65±0.185
20	1000	7.8	3	3.96±0.209

Data presented in the table is the mean of three readings i.e., Mean±S.D

**Factorial design:** After enzyme choice experiments, trypsin was selected for next factorial design experiment. A five factors two-level factorial design was used to evaluate the effects of hydrolysis factors on iron-binding ability of hydrolysate. The five hydrolysis factors were substrate concentration, enzyme concentration, pH, temperature, and hydrolysis time, respectively. The factorial design experiment was shown in Table 2. After hydrolysis, the protease was inactivated at 85°C for 15 min. The hydrolysate was centrifuged (5000 rpm, 10 min) to remove insoluble substrates and the supernatant was stored at -18°C till analysis.

**Response surface methodology design:** The RSM (Box *et al.*, 1978) was applied to investigate optimum conditions of three variables pH (pH), hydrolysis time (t) and enzyme concentration (ES) on the iron-binding activity of the hydrolysates prepared using trypsin (Table 3). A three-level-three-factor Central Composition Design (CCD) was employed and a set of 20 experiments including 6 central points were carried out as shown in Table 3. According to the result of factorial design experiments and the labeled suitable enzymatic hydrolysis conditions of trypsin, the three levels of three variables were: pH (7.1, 7.8 and 8.5), t (2, 3 and 4 h) and ES (500, 1000 and 1500 U/mL), respectively. The measured variable response was iron-binding activity. The order of the experiments was fully randomized. The iron-binding activity was determined in triplicate. Data were analyzed by multiple regressions through the least-square method, to fit the following second-order equation:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=2}^3 \beta_{ij} X_i X_j \quad (1)$$

where,  $Y$  was the iron-binding activity,  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$  and  $\beta_{ij}$  were the constant, linear, quadratic and cross-product regression coefficients of the model, respectively; and where  $X_i$  and  $X_j$  represented the hydrolysis parameters. The mathematical analyses of data were conducted by the module of Expert Design V. 8.0 experimental design.

**Iron-binding activity determination:** The hydrolysate was demineralized by metal chelating resin (Amberlite IRC-748) and then was used for determining the iron-binding activity by the method of Decker and Welch (1990) with some modifications. Two milliliter of sample solution (1.0 mg protein/mL) was mixed with 2.0 mL distilled water and then was reacted with 0.1 mL of 1 mM  $\text{FeSO}_4$  and 0.2 mL of 5 mM 3-(2-pyridyl)-5, 6-bis(4-phenyl-sulfonic acid)-1, 2, 4-triazine (ferrozine) for 20 min at room temperature. The absorbance was read at 562 nm. The control was prepared in the same manner except that distilled water was used instead of the sample.

Ethylenediaminetetra-acetic acid (EDTA) was used as a positive control. All samples were prepared by triplicate and the iron-binding activity was measured in triplicate. The percentage of inhibition of ferrozine- $\text{Fe}^{2+}$  complex formation was given in Eq. (2):

$$\text{Ferrous chelating activity (\%)} = \left(1 - \frac{A_1}{A_0}\right) \times 100 \quad (2)$$

where,  $A_0$  was the absorbance of the control and  $A_1$  was the absorbance in the present of samples and EDTA. The iron-binding activity was expressed with equal EDTA per mg protein in the sample.

**Preparation of iron-peptides complex:** The hydrolysate was demineralized by metal chelating resin (Amberlite IRC-748) and then the iron-peptides complex was prepared by adding excessive  $\text{FeCl}_2$  solid particles under stirring for 2 h at room temperature. After time out, anhydrous alcohol was added to a final concentration at 85% (v/v) in the mixture solution and then was kept at 4°C for 1 h. The mixture was centrifuged (10,000 rpm) for 10 min at 4°C. The precipitation was washed with 95% (v/v) alcohol three times, and then dried under vacuum to obtain the iron-peptides complex powder.

**Composition analysis:** Moisture, protein and ash in the iron-peptides complex were determined according to the method of AOAC (2002). Iron content was determined according to Flame Atomic Absorption Spectrometry (FAAS) method (Bermejo *et al.*, 1997) after the sample ashed at 550°C. Amino acid composition was determined by HPLC using the method of Bidlingmeyer *et al.* (1984) after the samples hydrolyzed using 6 N HCl at 110°C for 24 h under vacuum.

**Infrared spectroscopy:** The 2 mg iron-peptides complex or peptides powders were grinded evenly with 200 mg KBr under infrared light to the size below 2.5  $\mu\text{m}$ . Then the transparent KBr piece was made at 60 MPa. The spectroscopy was analyzed using Thermo Nicole 380 at 400-4000 per cm.

**Statistical analysis:** Data and Statistical analysis were performed using scientific graphic and analysis computer software OriginPro (version 7) and data was expressed as mean  $\pm$  standard deviation of three experiments.

## RESULTS AND DISCUSSION

**Choice of the enzyme:** Several proteases had been used for the production of fish or shrimp protein hydrolysates. These included papain, pepsin, bromelin, trypsin, pancreatin, alcalase, neutrase, flavorzyme, protamax, pronase and microbial protease. However, the hydrolysis

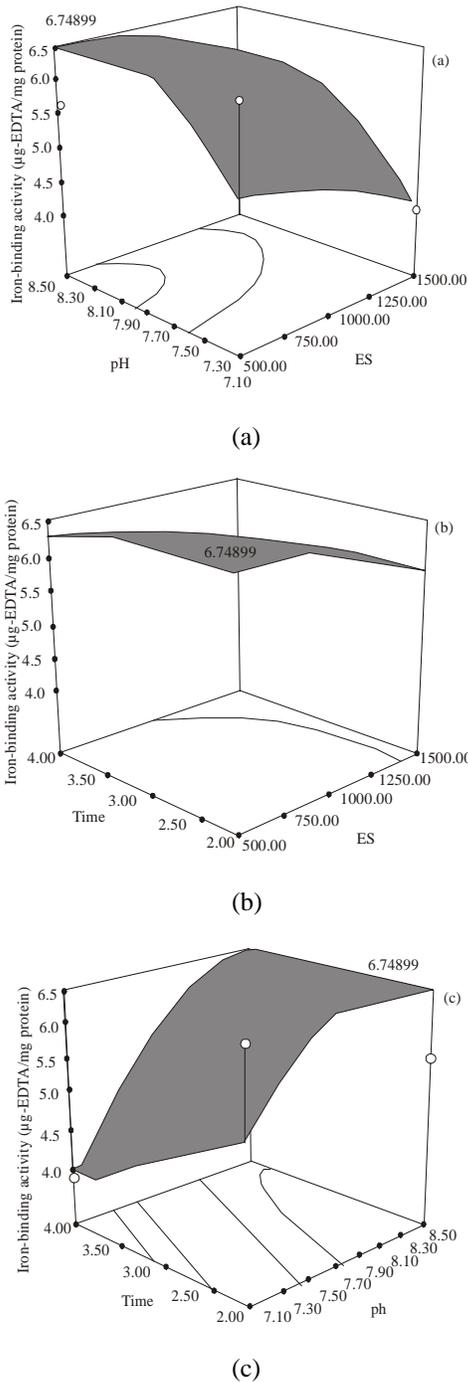


Fig. 1: Three dimensional response surface plots of the effects of enzyme concentration, pH and duration of hydrolysis on iron-binding activity of SPD hydrolysate

product quality or functional properties, such as antioxidative activity, angiotensin-converting enzyme (ACE) inhibitory activity, foaming ability, solubility and emulsifying ability, varied with the nature of the enzyme employed. In this study, six kinds of protease (trypsin,

pepsin, protamax, alcalase, neutrase and flavorzyme) were used to hydrolysis the SPD protein to obtain peptides with high iron-binding ability. The result was shown that trypsin might be the most suitable protease for producing iron-binding peptides from SPD protein, which was shown in Table 2. Trypsin had been used effectively for hydrolysis of the marine protein to obtain bioactive peptides, such as tuna backbone protein (Je *et al.*, 2007), marine rotifer *Brachionus rotundiformis* (Byun *et al.*, 2009), *Porphyra yezoensis* (Qu *et al.*, 2010), sardine protein (Kishimura *et al.*, 2006), cuttlefish (Kechaou *et al.*, 2009), hoki (*Johnius belengerii*) frame protein (Kim *et al.*, 2007) and Alaska Pollack (*Theragra chalcogramma*) backbone protein (Jung *et al.*, 2006).

**Optimization of hydrolysis parameters:** The most important parameters affecting the enzyme efficiency are pH (pH), temperature (T), enzyme concentration (ES), substrate concentration (S) and hydrolysis time (t). A five factors two-level factorial design was used to evaluate the effects of hydrolysis factors on iron-binding ability of hydrolysate. According to the optimal working conditions of trypsin, the studied pH and temperature ranged from 7.1 to 8.5 and 35 to 45°C, respectively. Enzyme concentration and substrate concentration ranged from 200 to 2000 U/mL and 2 to 8%, respectively. The hydrolysis time lasted between 1 and 6 hours. The design and result were shown in Table 2. The hydrolysis parameters of pH, ES and t were seemed to be the significant factors that influencing iron-binding ability of the hydrolysate. The relative coefficient of pH, ES, t were high ( $R^2 = 0.9664, 0.9598, 0.9598$ ). Therefore, these three hydrolysis parameters were selected to be optimized by RSM to produce hydrolysate with high iron-binding activity.

RSM was drawn to illustrate the influence of different hydrolysis factors such as pH, t and ES on iron-binding activity of the hydrolysate of SPD protein using trypsin. The experimental designs and results were shown in Table 3. The analysis of variance (Table 4) showed that the Prob>F value of the iron-binding activity was 0.1485, which demonstrates that the model itself was significant at a 85% confidence level.

According to the model regression analysis, the best explanatory model equation was given as Eq (3):

$$Y = 5.89 - 0.44 \times ES + 1.04 \times pH - 0.39 \times t - 0.14 \times ES^2 - 0.61 \times pH^2 - 0.059 \times t^2 + 0.021 \times ES \times pH + 0.039 \times ES \times t + 0.35 \times pH \times t \quad (3)$$

Table 4: Analysis of variance (ANOVA) for production of iron-binding peptides in the RSM quadratic model

Source	Value
DF	9
Sum of squares	25.99
F-value	1.99
Probe > F	0.1485

The 3D response surface was built using Eq (3) when one variable was fixed, and the other two were allowed to vary that was shown Fig. 1. Contour plots were generated from the polynomial model to illustrate the effect of each pair of independent variables on the ratio of iron-binding activity. The increment at the ratio of iron-binding activity was achieved by the decreasing in enzyme addition and the increment in reaction pH. When t factor was fixed, reaction pH showed an insignificant effect on the creation of iron-binding peptides. The optimum conditions of different independent variables were analyzed by using ‘Numerical Optimization’ of the Expert Design V.8.0 software. The results indicated that the optimal conditions were pH of 8.18, enzyme concentration of 500 U/mL and hydrolysis time of 2 h, and that the highest iron-binding activity was predicted to be 6.75 µg-EDTA/mg-protein. To confirm the accuracy of the model, triplicate hydrolysis experiments under the deduced optimum were conducted, and the iron-binding activity under the optimal hydrolysis conditions was average found to be 6.73 µg-EDTA/mg-protein, indicating that the response model was adequate to predict the reaction optimization.

**Compositions of iron-peptides complex:** The hydrolysate was demineralized by metal chelating resin

Table 5: Composition of the iron-peptides complex

Components	Percentage (%)
Protein	71.5±0.64
Iron	11.1±0.75
Moisture	4.8±0.27
Ash	19.3±0.91

Data presented in the table is the mean of three readings i.e. Mean±S.D

Table 6: Amino acid composition of the iron-peptides complex

Amino acid	Content (g/100 g)	Amino acid	Content (g/100 g)
Asp	15.47	Ile	2.13
Thr	4.22	Leu	3.28
Ser	5.10	Tyr	1.30
Glu	25.01	Phe	2.06
Gly	9.22	Lys	6.08
Ala	5.64	His	1.76
Cys	1.50	Arg	6.20
Val	3.60	Pro	4.09
Met	1.09		

Table 7: Essential amino acid composition of the iron-peptides complex and FAO requirement

Amino acid	Iron-peptides complex (g/100 g)	FAO Requirement *(g/100 g)
Thr	4.22	3.0
Tyr	1.30	2.8
Val	3.60	4.2
Met	1.09	2.2
Ile	2.13	4.2
Leu	3.28	4.8
Phe	2.06	2.8
Lys	6.08	4.2

\*: Recommended dietary allowances, 10th Edn., Edited by National Research Council, P-57, 1989

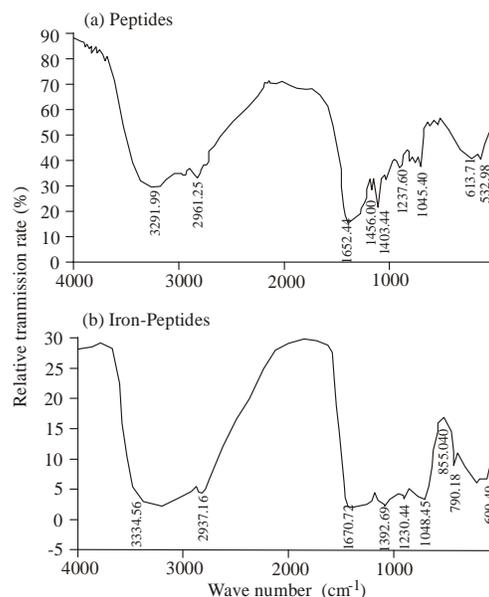


Fig. 2: Infrared spectra of peptides and iron-peptides complex

(Amberlite IRC-748) and then the iron-peptides complex was prepared by adding excessive FeCl<sub>2</sub> solid particles under stirring. Moisture, protein, iron and ash content in iron-peptides complex were determined and the result was shown in Table 5. It was shown that the iron-peptides complex was mainly composed by protein (71.5%) and iron (11.1%). The iron content in the prepared iron-peptides complex was similar to the EDTAFeNa, which was most used for iron intensification as food additive in food industry and the iron content in EDTAFeNa was 12.5 to 13.5% (EFSA, 2010). The amino acid composition of the iron-peptides complex was shown in Table 6. It was also comparable with the FAO requirement of essential amino acids composition in Table 7. The results in Table 6 and Table 7 indicated that the essential amino acid composition of the iron-peptides complex was very closed to FAO requirement. Therefore, the iron-peptides complex may be potential for one kind of iron fortified food additives.

**Infrared spectroscopy of iron-peptides complex:** The infrared spectra of the iron-binding peptide and the iron-peptide complex were shown in Fig. 2. In Fig. 2a, there displayed the C=O stretch near the wave number of 1650 per cm (1652, 1647) and the N-H stretch (3292 per cm) near the wave number of 3290 per cm, which were belonged to the aliphatic secondary amides. While near the wave number of 2959 per cm (2961), which belonged to the aliphatic hydrocarbons, displayed the asym -CH<sub>3</sub> stretching absorption. In Fig. 1b, the -CH<sub>3</sub> asymmetric stretching vibration occurred at 2960 per cm, and the other aliphatic hydrocarbons was occurred at 1230 and

1392 per cm. The -OH stretching in primary aliphatic alcohols was displayed at 3600-3200 per cm, -C-O-stretching and -OH deformation vibrations were at 1100-1000 per cm. Comparison of Fig 1a and b, it was shown that C=O probably binding iron transferred into C-O-Fe, therefore the wave number at 1652 and 1646 per cm were disappeared. The wave number at 1403 per cm in peptides and changed in iron-peptides complex shown that -COOH probably bound iron ion, turned into -COO-Fe. Therefore, iron might be bound to peptides with hydroxyl, carbonyl and carboxyl group. There had been reported that calcium might be bound to peptides with carbonyl or carboxyl group (Nemirovskiy and Gross, 2000) and iron might be bound to carboxyl and hydroxyl of casein hydrolysate peptides (Chaud *et al.*, 2002).

### CONCLUSION

The hydrolysis conditions for production of peptides with high iron-binding activity from SPD protein were optimized by factorial design and response surface methodology. The iron content in iron-peptides complex from shrimp processing discards hydrolysate was similar to EDTAFeNa. And iron binding to peptides was certified by infrared spectra. It may be potential for one kind of iron fortified food additives in the future.

### ACKNOWLEDGMENTS

The research work was supported by Zhejiang Provincial Natural Science Foundation of China (Y 3110278).

### REFERENCES

AOAC, 2002. Official Methods of Analysis, 16th Edn., Association of Official Analytical Chemists, Washington DC.

Bermejo, P., R. Domnguez and A. Bermejo, 1997. Direct determination of Fe and Zn in different components of cow milk by FAAS with a high performance nebulizer. *Talanta*, 45: 325-330.

Bidlingmeyer, B.A., S.A. Cohen and T.L. Tarvin, 1984. Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr.*, 336: 93-104.

Box, G.E., W.G. Hunter and J.S. Hunter, 1978. *Statistics for Experimenters*. John Wiley and Sons Inc., New York.

Bueno-Solano, C., J. López-Cervantes, O.N. Campas-Baypoli, R. Lauterio-García, N.P. Adan-Banteand D.I. Sánchez-Machado, 2009. Chemical and biological characteristics of protein hydrolysates from fermented shrimp by-products. *Food Chem.*, 112: 671-675.

Byun, H.G., J.K. Lee, H.G. Park, J.K. Jeon and S.K. Kim, 2009. Antioxidant peptides isolated from the marine rotifer, *Brachionus rotundiformis*. *Process Biochem.*, 44: 842-846.

Chaud, M.V., C. Izumi, Z. Nahaal, T. Shuhama, M.L. Bianchi and O. Freitas, 2002. Iron derivatives from casein hydrolysates as a potential source in the treatment of iron deficiency. *J. Agric. Food Chem.*, 50: 871-877.

Decker, E.A. and B. Welch, 1990. Role of ferritin as a lipid oxidation catalyst in muscle food. *J. Agric. Food Chem.*, 38: 674-677.

European Food Safety Authority (EFSA), 2010. Scientific Opinion on the use of ferric sodium EDTA as a source of iron added for nutritional purposes to foods for the general population (including food supplements) and to foods for particular nutritional uses. *EFSA J.* 8: 1414.

Heu, M.S., J.S. Kim and F. Shahidi, 2003. Components and nutritional quality of shrimp processing by-products. *Food Chem.*, 82: 235-242.

Inoue, H., T. Ohira, N. Ozaki and H. Nagasawa, 2004. A novel calcium-binding peptide from the cuticle of the crayfish, *Procambarus clarkia*. *Biochem. Biophys. Res. Comm.*, 318: 649-654.

Je, J.Y., Z.J. Qian, H.G. Byun and S.K. Kim, 2007. Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis. *Process Biochem.*, 42: 840-846.

Jung, W.K., P.J. Park, H.G. Byun, S.H. Moon and S.K. Kim, 2005. Preparation of hoki (*Johnius belengerii*) bone oligophosphopeptide with a high affinity to calcium by carnivorous intestine crude proteinase. *Food Chem.*, 91: 333-340.

Jung, W.K., R. Karawita, S.J. Heo, B.J. Lee, S.K. Kim and Y.J. Jeon, 2006. Recovery of a novel Ca-binding peptide from Alaska Pollack (*Theragra chalcogramma*) backbone by pepsinolytic hydrolysis. *Process Biochem.*, 41: 2097-2100.

Jung, W. and S.K. Kim, 2007. Calcium-binding peptide derived from pepsinolytic hydrolysates of hoki (*Johnius belengerii*) frame. *Eur. Food Res. Technol.*, 224: 763-767.

Kechaou, E.S., J. Dumay, C. Donnay-Moreno, P. Jaouen, J.P. Gouygou, J.P. Bergé and R.B. Amar, 2009. Enzymatic hydrolysis of cuttlefish (*Sepia officinalis*) and sardine (*Sardina pilchardus*) viscera using commercial proteases: Effects on lipid distribution and amino acid composition. *J. Biosci. Bioeng.*, 107: 158-164.

Kim, S.B., J.Y. Je and S.K. Kim, 2007a. Purification and characterization of antioxidant peptide from hoki (*Johnius belengerii*) frame protein by gastrointestinal digestion. *J. Nutr. Biochem.*, 18: 31-38.

- Kim, S.B., I.S. Seo, M.A. Khan, K.S. Ki, W.S. Lee, H.J. Lee, H.S. Shinn and H.S. Kim, 2007b. Enzymatic hydrolysis of heated whey: iron-binding ability of peptides and antigenic protein fractions. *J. Dairy Sci.*, 90: 4033-4042.
- Kishimura, H., K. Hayashi, Y. Miyashita and Y. Nonami, 2006. Characteristics of trypsins from the viscera of the true sardine (*Sardinops melanostictus*) and the pyloric caeca of arabesque greenling (*Pleuroprammus azonus*). *Food Chem.*, 97: 65-70.
- Megías, C., J. Pedroche, M.M. Yust, J. Girón-Calle, M. Alaiz, F. Millán and J. Vioque, 2007. Affinity purification of copper-chelating peptide from sunflower protein hydrolysates. *J. Agri. Food Chem.*, 55: 6514-8609.
- Meisel, H. and R.J. Fitzgerald, 2003. Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Curr. Pharm.* 9th December.
- Nemirovskiy, O.V. and M.L. Gross, 2000. Intrinsic Ca<sup>2+</sup> affinities of peptides: application of the kinetic method to analogs of calcium-binding site III of rabbit Skeletal Troponin. *C. J. Am. Soc. Mass Spectrom.*, 11: 770-779.
- Qu, W., H. Ma., Z. Pan., L. Luo., Z. Wang and R. He, 2010. Preparation and antihypertensive activity of peptides from *Porphyra yezoensis*. *Chem.*, 123:14-20.
- Vegarud, G.E., T. Langsrud and C. Svenning, 2000. Mineral-binding milk proteins and peptides; occurrence, biochemical and technological characteristics. *Brit. J. Nutr.*, 84: s91-s98.
- Xie, B. and K.J. Yu, 2007. Shrimp farming in China: Operating characteristics, environmental impact and perspectives. *Ocean Coast. Manage.*, 50: 538-550.