Chemical characterization and anti-anaemia activity of fish protein hydrolysate from Saurida elongata

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Abstract: A low-value sea fish Saurida elongata was hydrolyzed using papain. Proximate composition analysis of the fish protein hydrolysate showed that the hydrolysate contained 84.7% crude protein, 7.1% ash and 3.5% fat. The determination of amino acid content by o-phthaldialdehyde pre-column derivatization reversed-phase high-performance liquid chromatography (RP-HPLC) showed that the hydrolysate contained 20 different amino acids, and the total percentage of eight essential amino acids was as high as 41.5% of the total amino acids, which meant that the hydrolysate could provide a good nitrogen source for human consumption. Peptides were separated by RP-HPLC using a C-18 column into more than 14 fractions. Molecular weight distribution of peptides was determined by size-exclusion HPLC, which indicated that the size of 74.8% of the peptides ranged from 1.0 to 5.0 kDa. Most importantly, the enzymatic hydrolysate from S elongata was shown to contain significant in vivo anti-anaemia activity on experimental anaemia models induced by blood loss or cyclophosphamide damage to hematogenic mechanism. To the best of our knowledge, this was the first time that the description of hydrolysis of S elongata and the indication of anti-anaemia activity in the hydrolysate have been reported.

Keywords: anti-anaemia; enzymatic hydrolysis; fish protein hydrolysate; Saurida elongata

INTRODUCTION

The use of fish protein hydrolysate (FPH) as ingredients for animal feed or human consumption dates back to the 1960–1970s.1–3 The enzymatic modification of inexpensive fishery sources to produce high-quality protein is of great interest to food scientists worldwide.4 Moreover, FPH can have not only nutritional but also functional and biological applications. Because some proteins possess a range of dynamic functional properties, FPH exhibits a wide spectrum of physicochemical behavior such as emulsifying activity, foamability and moisture sorption.5 Thus the modified proteins can be used as additives in food, beverage and cosmetic systems.6,7 During the last decade, several studies have shown that food protein hydrolysates or peptides possess biological properties such as the regulation of the immune system, gastrointestinal functions, blood pressure or mineral absorption.8 FPH produced from fish muscle, by-products and surplus has also been reported to contain biological activities. For example, angiotensin-converting enzyme (ACE) inhibitory activity was frequently reported from a variety of fish species. Kawasaki et al found that sardine muscle hydrolysate, prepared by using an alkaline protease, showed potential α-glucosidase inhibitory activity and significant antihypertensive effect on mild hypertensive subjects via ACE inhibition.9 Astawan et al found that Indonesian dried-salted fish hydrolysate, prepared by pepsin action, showed high ACE-inhibitory activity.10 Sardine autolysate and cod head hydrolysate powder were found to be able to inhibit nearly 30% of ACE activity.11 Proteolytic digestion of dried bonito muscle with thermolysin produced a hydrolysate with strong ACE inhibitory activity.12 Byun et al also reported that peptides derived from Alaskan pollack skin possessed ACE-I inhibitory activity.13

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Contract/grant sponsor: NSFC; contract/grant number: 40590390; 40590392
(Received 6 November 2003; revised version received 29 September 2004; accepted 8 February 2005)
Published online 2 June 2005

cholceystokinin. Bernet et al reported that FPH (Gabolysat PC60) had diazepam-like effects on stress responsiveness of the rats pituitary–adrenal system and sympathoadrenal activity. Fouchereau-Peron et al isolated an acidic fraction from a fish protein hydrolysate with calcitonin-gene-related-peptide-like biological activity. Kim et al isolated antioxidative peptides from gelation hydrolysate of Alaska Pollack skin.

The purpose of this paper was to discuss the chemical characterization and biological activity evaluation of an enzymatic hydrolysate obtained from an under-utilized sea fish Saurida elongata. The hydrolysate by papain under a defined set of hydrolysis conditions was shown to contain significant in vivo anti-anaemia activity.

MATERIALS AND METHODS

Materials
Fresh Saurida elongata, purchased from a local fish supplier in June, were frozen and stored at −30°C before preparation of hydrolysate and analysis. The enzyme papain, 70 000 IU g⁻¹ (EC 3.4.4.10) was a food grade protease purchased from Guangzhou Enzyme Products Plant (Guangzhou, China).

Chemicals
Cyclophosphamide was obtained from Tianping pharmacy factory (Shanghai, China; batch number: 9096010). Methanol and acetonitrile were of HPLC grade and obtained from Merck. All other chemicals were of analytical reagent grade either from Merck or Sigma. Water used in HPLC was purified by passing it through a Milli-Q treatment system (Millipore, Bedford, MA, USA).

Preparation of FPH
The preparation of FPH was according to our previous report with some modification. Raw S elongata was washed and minced. Some fish mince was sampled to test for moisture, protein, fat and ash. A 5-kg portion of mince was mixed 1:2.5 (w/w wet wt) with deionized water and homogenized in a blender for about 2 min. The mixture was then pre-heated in a water bath to 60 ± 1°C for optimal enzyme activity. Enzyme was added at enzyme:substrate ratio of 350 000 IU kg⁻¹ in the mince and a magnetic stirrer was used at low speed throughout the whole process of hydrolysis. After the mixture was incubated at 60 ± 1°C for 2 h the hydrolysate was stopped by heating the sample to above 90°C and keeping at this temperature for 30 min. The sample was cooled and then centrifuged at 8850 × g for 20 min. The supernatant was further filtered through a 0.6-μm membrane filter. After the total filtrate volume was measured, a 500-ml portion of the filtrate was then lyophilized and stored in a plastic desiccator at 4°C for proximate composition analyses and inorganic analyses, while the other filtrate was sealed and sterilized at 120°C for 30 min before used for HPLC analyses and biological activity test.

Proximate composition
Kjeldahl nitrogen analysis was used to determine the crude protein content in the raw material, the liquid hydrolysate and the freeze-dried FPH. Moisture content was measured using oven drying at 105°C. Ash was determined using AOAC standard method by heating the samples in the furnace at 550°C for 8–12 h. Fat was determined by the method of Soxhlet extraction.

Inorganic analysis
Freeze-dried FPH (approximately 1.5 g) was treated with 20 ml of concentrated nitric acid in a Kjeldahl tube. The mixture was allowed to stand overnight and then digested carefully on a hotplate until the production of red fumes of oxides of nitrogen ceased. After cooling, 10 ml of perchloric acid was added and the tube was heated again and allowed to evaporate to near dryness. Once the mixture cooled, it was transferred with 1.3 mol l⁻¹ HCl to a 50-ml volumetric flask and made up to the final volume for further use. Contents of Mg, K, Ca, Zn, Fe, Ni, Cr, Cu and Mn were determined by atomic absorption spectrometry on a Perkin-Elmer 3100 AAS (Norwalk, CT). Ammonium molybdate was added for color to the digested solution and then the content of P was determined by a colorimetric method with a Shimadzu UV-300 spectrophotometer (Kyoto, Japan).

Amino acid analysis
Free amino acids in hydrolysate were analyzed directly after pre-column derivatization with o-phthalaldehyde (OPA). The amino acid composition was determined after digestion of sample in 6 mol l⁻¹ HCl at 110°C for 24 h into free amino acids and pre-column derivatization with OPA. Hewlett-Packard (HP) (Palo Alto, CA, USA) 1050 HPLC system with a Hypersil ODS column from Phenomenex (125 × 4.0 mm ID, 5 μm particle size and 80 Å pore size, Torrance, CA, USA) was used. Mobile phases were 10 mmol l⁻¹ phosphate buffer (pH 7.2, solvent A) and a mixture of phosphate buffer (A)–methanol–acetonitrile 50/35/15 (v/v/v) (solvent B). A 25-min linear gradient from 0 to 100% of solvent B was used at a flow rate of 1.0 ml min⁻¹ and column temperature was kept at 40°C. The fluorescence detection was operated with initial excitation and emission wavelength of 340 and 450 nm which were changed at 21 min to 260 and 305 nm respectively.

Molecular weight distribution (size-exclusion chromatography, SEC)
Molecular weight distribution analysis was performed on a HP1100 liquid chromatography system equipped with quaternary solvent delivery system, diode-array UV-vis detection (DAD), autosampler and heated column compartment. Data acquisition and
integration were carried out using HP ChemStation. Separations were done at 25°C on a Biosep SEC-S2000 column (300 × 7.8 mm ID, 5 μm particle size and 145 Å pore size, Phenomenex, Torrance, CA) with a Phenomenex Biosep SEC-S guard column (75 mm × 7.8 mm ID). A buffer composed of 25 mmol l⁻¹ sodium phosphate (pH 6.8) and 0.25 mol l⁻¹ NaCl, filtered through a 0.45-μm filter, was used as mobile phase. The flow rate was 1.0 ml min⁻¹ and the effluent was monitored at 215 nm. FPH was filtered through a 0.45-μm Millex-HV filter before application to the column and the injection volume was 5 μl.

To calibrate the column and obtain a standard curve, five peptides of known molecular weight (MW) were used as standards. They were Cytochrome C (FW 12 400), Aprotinin (FW 6 500), Insulin chain B (FW 3 496), Angiotensin I (FW 1 296) and Morphiceptin (FW 5 22), all purchased from Sigma. A relationship between the retention time and log10 MW was established (R² = 0.965, p < 0.01), and it was fitted to a linear model of the formula: log₁₀ MW = 6.3979 - 0.2662Tᵣ where Tᵣ is the retention time in minutes. The molecular weight ranges of different fractions in SEC were based on the retention times of the fractions and determined using this standard curve.

Reversed-phase high pressure chromatography (RP-HPLC)
A HP1100 HPLC system described as above was used for peptide separation at 25°C. For separation, a Zorbax SB-C18 analytical column from Agilent Technologies (250 × 4.6 mm ID, 5 μm particle and 80 Å pore size, Palo Alto, CA, USA) was used. A gradient prepared from 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) was used as follows. The column was equilibrated initially with 0% B after which 5 μl of sample was injected and a gradient was then generated by increasing the concentration of B to 50% over 23 min. Detection was done at 215 nm and 280 nm and the flow rate was 1.0 ml min⁻¹.

In vivo anti-anaemia activity of FPH
Sample preparation
The FPH sample was diluted by adding 80 ml of distilled water to 100 ml of liquid FPH described above.

Animal models
Two experimental animal models were used in this paper to induce anaemia in mice by blood loss or cyclophosphamide damage to hematogenic mechanism. The biological activity evaluation of anti-anaemia of the FPH was carried out by these two animal models.

Mice
Fifty NIH mice, body weight 18 ~ 22 g, with an equal proportion of male to female were provided by Medical Use Animal Center of Sanitation Office (Guangdong Province, China). They were randomly assigned into five groups and each group has five male and five female.

Group 1: normal control. 10 healthy mice were fed under the same conditions without other treatment except feeding distilled water 20 ml kg⁻¹ body weight by gavage once a day and 0.5 ml of blood were collected from the retro-orbital sinus at the end of the experiment.

Group 2: anaemic control induced by blood loss, model 1. Blood (0.5 ml) was collected from the retro-orbital sinus at the beginning of the experiment and at the 8th day, every mouse was fed distilled water 20 ml kg⁻¹ body weight by gavage once a day for 7 days.

Group 3: blood loss induced anaemic and FPH-treated mice, trial 1. Blood (0.5 ml) was collected from the retro-orbital sinus at the beginning of the experiment and at the 8th day, every mouse was fed diluted FPH 20 ml kg⁻¹ body weight by gavage once a day for 7 days.

Group 4: anaemic control induced by cyclophosphamide damage to hematogenic mechanism, model 2. Each mouse was fed distilled water 20 ml kg⁻¹ body weight by gavage once a day for 9 days and additionally fed cyclophosphamide 100 mg kg⁻¹ body weight by gavage to induce anaemia at the 2nd, 3rd and 5th day. Blood (0.5 ml) was collected from the retro-orbital sinus at the 10th day.

Group 5: cyclophosphamide-induced anaemic and FPH-treated mice, trial 2. Each mouse was fed diluted FPH 20 ml kg⁻¹ body weight by gavage once a day for 9 days and additionally fed cyclophosphamide 100 mg kg⁻¹ body weight by gavage once a day for 9 days and at the 2nd, 3rd and 5th day additionally fed cyclophosphamide 100 mg kg⁻¹ body weight by gavage. Blood (0.5 ml) was collected from the retro-orbital sinus at the 10th day.

Hematology analysis
Hematologic values were analyzed to determine red blood cell (RBC), white blood cell (WBC) and hemoglobin (HGB) using an Abbott Cell-Dyn 3500 automated hematology analyzer (Abbott Diagnostic Division, CA, USA).

Statistical analysis
All tests of the proximate composition were done in triplicate and data were averaged. All the HPLC analysis methods including amino acids, RP-HPLC and size-exclusion HPLC were carefully established and the coefficient of variations were tested to be less than 2% by five consecutive injections. Data obtained in the animal experiment were expressed as

mean ± standard error. Comparisons among groups were performed using analysis of variance (ANOVA) and statistical software program SPSS version 10.0 was used. Differences were considered statistically significant if \( p < 0.05 \).

**RESULTS AND DISCUSSION**

**Preparation of FPH and its chemical composition**

In our previous work we have optimized the enzymatic hydrolysis conditions including temperature, enzyme/substrate (E/S) ratio, substrate concentration (fish/water ratio) and hydrolysis time. In the FPH preparation experiment the proteolysis of 5 kg of fish mince produced 14.0 l of liquid hydrolysate which consisted of 58.6 g l\(^{-1}\) dry matter. The contents of crude protein and free amino acid of the liquid FPH were 54.9 g l\(^{-1}\) and 4.05 g l\(^{-1}\), respectively. From the crude protein contents of the raw material (Table 1) and the liquid FPH, protein recovery was calculated to be 84.0% which was much higher than that in the other report about fish protein hydrolysis.

The freeze-dried FPH produced from *S. elongata* was a yellowish, fluffy and glossy powder and its proximate composition was listed in Table 1. In the freeze-dried FPH, 92.6% of dry matter was crude protein which consisted of 7.4% free amino acids and 92.6% peptides. The ash of the FPH in the present study was lower than that of the other FPH reported elsewhere. That was because papain used in the present experiment was a neutral enzyme and there was no need to adjust the pH by adding alkali or acid. When other enzymes such as alcalase or pepsin were used, the addition of alkali or acid would result in salts in the hydrolysate and thus higher ash content.

Table 2 showed 10 kinds of mineral elements determined in the FPH. Contents of K, P, Mg and Ca were very high, suggesting that the FPH could be a beneficial source of these minerals, in particular for populations and individuals with a low intake of Ca. Zn, Fe, Cr and Cu were essential microelements and Fe is of great interest because it is a kind of hematogenic material. However, the content of Fe in the FPH was normal compared with other foods rich in protein.

**Amino acid composition**

Amino acid composition of FPH was listed in Table 3. After hydrolysis under acidic condition, asparagine and glutamine were changed into aspartic and glutamic acids, respectively, and tryptophan had disappeared. So asparagine and glutamine values were included in the value of aspartic and glutamic acid, respectively, and the tryptophan in the peptides was not measured. The FPH contained 20 amino acids among which the most abundant were glutamic acid, aspartic acid, leucine and lysine, accounting for 42.6% of the total amino acid content. A comparison between the contents of hydrolyzed amino acids and that of free amino acids indicated that most of the amino acids were bound as residues in the peptides except taurine existing only in the state of the free amino acid.

The large percentage of eight essential amino acids accounted for 41.5% of the total amino acid, and lysine accounted for 7.2%. Table 4 showed that the contents of eight essential amino acids in the FPH were comparable with that of the amino acid
Fish protein hydrolysate from Saurida elongata

Table 4. Essential amino acid composition of FPH compared with the FAO/WHO pattern

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>FPH (g kg(^{-1}) crude protein)</th>
<th>FAO/WHO pattern(^a)</th>
<th>Amino acid score(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threonine</td>
<td>59.7(^c)</td>
<td>40</td>
<td>149</td>
</tr>
<tr>
<td>Valine</td>
<td>42.8</td>
<td>49.6</td>
<td>86</td>
</tr>
<tr>
<td>Methionine</td>
<td>24.8</td>
<td>35.0</td>
<td>71</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>NM</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>59.5</td>
<td>60.1</td>
<td>99</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>39.0</td>
<td>40.0</td>
<td>98</td>
</tr>
<tr>
<td>Leucine</td>
<td>65.7</td>
<td>70.4</td>
<td>93</td>
</tr>
<tr>
<td>Lysine</td>
<td>61.2</td>
<td>54.4</td>
<td>112</td>
</tr>
</tbody>
</table>

\(^a\) FAO/WHO pattern (1971).24
\(^b\) Amino acid score = (EAA of test protein/EAA of reference pattern) \times 100.
\(^c\) Threonine + histidine.
NM: not measured.

The first limiting amino acid in the FPH was methionine, whose quantity covered 71% of the FAO/WHO ideal pattern. Chemical scores of other essential amino acids ranged from 86 to 149. In addition, it was important that the FPH contained a high content (accounted for 19.5% of the total amino acids) of hydrophobic amino acids (leucine, lysine and isoleucine) because of their good effects on physical and functional properties of food proteins.25

Size-exclusion HPLC

From the size-exclusion chromatogram of FPH, peptides in the FPH were sorted into five fractions covering the ranges of 0–1.0 kDa, 1.0–3.0 kDa, 3.0–5.0 kDa, 5.0–8.0 kDa, and above 8.0 kDa. The relative areas of each fraction were given as a percentage of the total area. Table 5 shows the results of molecular weight distribution and the relative areas of each fraction. From the chromatographic data (Table 5), we observed that a high percentage of peptides in hydrolysate, up to 74.8%, sized from 1.0 to 5.0 kDa, while only a small percentage is above 8.0 kDa. Hydrolytic process of animal proteins by enzyme could generate molecules ranging from individual amino acids to peptides of various sizes14 and peptide length is thought to be closely related to biological activity.26

Reverse-phase HPLC

The RP-HPLC chromatograms of FPH are shown in Fig 1. At 215 nm the absorption of both the carbonyl groups and the peptide bonds are measured, while, at 280 nm, detection is chiefly based on the absorbance of phenyl conjugate effect. Therefore peptides containing amino acid residues of tryptophan, tyrosine and/or phenylalanine have strong absorbance at 280 nm. Fourteen fractions were obtained in the RP-HPLC as shown in Fig 1(b) but only eight of them were detected at 280 nm, shown in Fig 1(a). From Fig 1(a) we can draw the conclusion that the components of peaks 6, 7, 8, 9, 10, 11, 13 and 14 contained at least one of the above three amino acids, and in particular the component of peak 7 contained most aromatic grouping. In contrast, peaks 1, 2, 3, 4, 5 and 12 contained no aromatic amino acids because they showed no absorbance at 280 nm. The peptide-mapping of the RP-HPLC chromatogram, acting as a finger-print, was a valuable tool for establishing a reproducible hydrolysis experiment and controlling the quality of FPH.

In vivo anti-anaemia activity of the FPH

Hematological values, RBC, HGB and WBC in the five groups of mice are shown in Table 6. In comparison with the normal control (group 1), significant reductions of RBC, HGB and WBC in blood of anaemic mice caused by blood loss (group 2, model 1) were found (\(p < 0.01\)) and the same results were found in anaemic mice caused by cyclophosphamide damage to hematogenic mechanism (group 4, model 2). These hematological criteria confirmed that the two anaemia mouse models have been successfully established. After treatment with FPH, the counts of RBC, HGB and WBC in blood of group 3 (trial 1) restored to 94.5, 94.6 and 88.4% of the normal levels (group 1, normal control), respectively, and were significantly higher than that of the anaemic mice caused by blood loss (group 2, anaemic control, model 1) (\(p < 0.01–0.05\)). Similar to the above results, after treatment with FPH,

![Figure 1. RP-HPLC chromatograms of the FPH (a) detected at 280 nm, (b) detected at 215 nm.](image-url)
the counts of RBC, HGB and WBC in blood of group 5 (trial 2) restored to 95.6, 96.2 and 84.0% of the normal levels (group 1, normal control), respectively, and were significantly higher than that of the anaemic mice caused by cyclophosphamide intake (group 4, anaemic control, model 2) ($p < 0.05$). These results indicated that, in the present study, the FPH had significant anti-anaemia activity on anaemic mice caused either by blood loss or by cyclophosphamide intake. Nutritional anaemia and iron deficiency anaemia were commonly simulated by blood loss. The significant in vivo anti-anaemia activity of the FPH in trial 1 suggested that the FPH supplied 'raw material' for hematogenic action. The nutrition supplement, eg amino acids and mineral Fe, of the FPH for anaemic mice was helpful to improve the counts of RBC, WBC and HGB of normal cell function. By disrupting cellular DNA, cyclophosphamide is able to kill the cell and cells that divided rapidly, for example bone marrow cells are especially targeted by cyclophosphamide. Thus cyclophosphamide administration causes reduction in the production of blood cells by the bone marrow suppression leading to anaemia.$^{27}$ The anaemia caused by cyclophosphamide intake is generally difficult to return to normal level because it involves the restoration of marrow hematogenic function. The significant effect of FPH on the second anaemia model implied that biological activity might exist in the hydrolysate although the exact component(s) had not been discovered yet. This result suggested that the FPH could be used as a supplementary diet for patients who received cyclophosphamide chemotherapy to enhance their nutritional supplement and decrease the bone marrow suppression by cyclophosphamide.

**CONCLUSIONS**

A FPH was produced from *Saurida elongata* by enzymic hydrolysis under a defined set of conditions, which was shown to contain significant anti-anaemia activity. The in vivo anti-anaemia biological activity evaluation of the FPH was carried out on two kinds of anaemic mouse model induced by blood loss or cyclophosphamide intake. Experimental in vivo results proved that the FPH could significantly increase the levels of RBC, WBC and HGB of anaemic mice compared with that of the control group ($p < 0.01–0.05$), confirming that the FPH have significant anti-anaemia biological activity. Chemical characterization indicated that at least 14 peptides were fractionated from the hydrolysate by RP-HPLC and 74.8% of the peptides in the FPH ranged from 1.0 to 5.0 kDa by size-exclusion HPLC. Although the FPH contained various amino acids, especially rich in essential amino acids, it is hard to believe that the nutrition composition could result in so much significant anti-anaemia activity alone. It is reasonably concluded that the anti-anaemia activity was partly attributed to biological active peptide in the FPH. Further study is in progress to locate the activity in the FPH.

**ACKNOWLEDGEMENTS**

This work was supported by NSFC grants Nos 40590390 and 40590392 for which the authors are grateful.

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