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In vitro antioxidative activities of squid (*Ommastrephes bartrami*) viscera autolysates and identification of active peptides

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ABSTRACT

Squid viscera, one of the major by-products in squid processing, were hydrolyzed to generate bioactive autolysates using their endogenous proteases. *In vitro* antioxidative activities of squid viscera autolysates (SVAs) were evaluated. The SVAs demonstrated strong activity on scavenging 1,1-diphenyl-2-picrylhydrazil (DPPH) and hydroxyl radicals, as well as reducing power ability. The SVAs were purified using size exclusion chromatography and RP-HPLC. Nineteen peptides were identified in the active fraction SVAs3 by liquid chromatography-electrospray ionization/multi-stage mass spectrometry (LC-ESI-Q-TOF-MS/MS). Among these identified peptides, six peptides with relatively small molecular weights (700–800 Da), LLAPPER, FPGLADR, WVAPLK, FFNPVH, FNVVLK and LELPLK, were synthesized for assaying antioxidative activity *in vitro*. Peptide WVAPLK demonstrated strong scavenging effects on free radicals, with the IC_{50} values of 0.82 ± 0.08 mg/mL (or 1.14 ± 0.11 mM) for DPPH radical and 1.85 ± 0.04 mg/mL (or 2.60 ± 0.06 mM) for hydroxyl radical, respectively. The low molecular weight and hydrophobic residue W at the N-terminus and basic residue K at the C-terminus as well as specific residues P and L within sequence should play a key role for the high antioxidative activity of WVAPLK. The results suggested that the SVAs could be used as a source of antioxidants and peptides.

1. Introduction

Squid viscera, which are one of the predominant by-products and account for more than 20% of the whole body weight, are generated accompanying squid processing [1]. Squid viscera contain abundant natural proteins, lipids, and minerals [2]. The disposal of squid viscera will cause environmental pollution because of their high organic content [2,3]. Furthermore, the improper treatment of squid viscera will retard the instant processing for squid industry in market places [4]. Therefore, it is essential to develop efficient methods to convert squid viscera into more profitable and marketable products.

Enzymatic hydrolysis is considered as a safety approach to transform protein into functional hydrolysates [5]. Recently, protein hydrolysates produced from aquatic products and by-products have become popular in food industry due to utilization of these under-utilized marine sources into acceptable food supplements with enhanced functional properties [6–13]. In addition, aquatic products and by-products hydrolysates, such as abalone viscera [14], threadfin bream surimi processing byproduct [15], patin myofibrillar [16], grass carp skin [17], tuna dark muscle protein by-product [18] and bluefin leatherjacket heads [19], have also been proven to be good sources of antioxidant peptides.

Aquatic invertebrates and vertebrates viscera are rich sources of various enzymes, including proteases, lipase, and amylase [4,20]. Proteases represent one of the three largest groups of industrial enzymes, and play vital roles in food, pharmaceutical and leather industries, as well as in bioremediation [4]. The proteases of viscera have been found to possess pepsin, trypsin, chymotrypsin, collagenase and elastase [20]. In fact, aquatic invertebrates or vertebrates viscera have been reported to digest proteins to produce bioactive protein hydrolysates, such as autolysis-assisted Pacific hake protein hydrolysate [21], sardine viscera hydrolysate [22], and bovine muscle protein and gelatin hydrolysates digested by catfish viscera [23]. Hence, it is reasonable to believe that autolysis would be a much more economical and efficient way to resolve the problem of reutilization and valorization of aquatic byproducts in large scale

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compared with the conventional method of adding commercial proteases.

In a latest study by Arias-Moscoso et al. (2015), squid by-products were produced by autolysis using a mixture of endogenous proteases [24]. However, to the best of our knowledge, there are very few studies on the antioxidative activities of squid viscera autolysates. In our previous study, the autolysis conditions were optimized to prepare antioxidative squid viscera autolysates (SVAs) (data not published). In this study, the SVAs were evaluated for antioxidative activity using various assays *in vitro*, with the aim to assess the potential application used as natural antioxidants. Furthermore, the features of the most active peptide fraction were analyzed to investigate the possible influence of peptide composition on antioxidant properties.

2. Materials and methods

2.1. Materials and chemicals

Fresh squid viscera of North Pacific squid (*Ommastrephes bartrami*) were provided by Zhejiang Fudan Tourism Food Co., Ltd. Squid viscera were transported to our laboratory at 4 °C within 30 min. After removal of ink sac, the visceral masses were portioned, sealed separately in plastic bags (200 g), and stored at −20 °C until use. 1,1-diphenyl-2-picryl-hydrazil (DPPH) and reduced L-glutathione (GSH) were purchased from Shanghai Sigma Chemical Co., Ltd (Shanghai, China). L-carnosine was purchased from Aladdin Industrial Corporation (Shanghai, China). Acetonitrile and formic acid were of chromatographic grade, purchased from Thermo Fisher Scientific Co., Ltd (Shanghai, China). All other chemicals and reagents used in this study were of analytical grade.

2.2. Preparation of squid viscera autolysates (SVAs)

Frozen squid viscera were thawed with distilled water. The proximate composition of squid viscera used in this study was determined (water 73.06 ± 0.10%, crude protein 17.45 ± 0.69%, crude fat 8.08 ± 1.98% and ash 1.28 ± 0.01%) following the official methods of the AOAC [25]. Then, 100 g of squid viscera was mixed with distilled water at a ratio of 1:2 (w/v), and homogenized at a speed of 10,000 g for 1 min using a homogenizer (TM-767, Zhongshan, China). The pH of the mixture was adjusted to 7.0 using 6 M NaOH, followed by incubation at 50 °C for 100 min to autolysis. After autolysis, the mixtures were heated at 100 °C for 10 min to inactivate the endogenous enzymes, and then centrifuged at 5000*g* for 10 min (TD5A-WS, Changsha, China) to remove the insoluble substrate and upper layer of fat. The soluble squid viscera autolysates, designated as SVAs, were lyophilized, and stored at −20 °C for further study.

2.3. Determination of antioxidative activity of SVAs

2.3.1. DPPH radical scavenging activity assay

The abilities of SVAs and separated fractions or purified peptides to scavenge DPPH radical were evaluated according to our previous protocol [26] with slight modifications. Briefly, 600 μL of sample was mixed with 300 μL of 99.5% ethanol, followed by addition of 30 μL 0.02% DPPH (dissolved in 99.5% ethanol). After blending vigorously, the reaction mixtures were kept at room temperature for 60 min in dark. The absorbance of the resulting solution (A_S) was measured at 517 nm (721G-100 Vis-spectrophotometer, Shanghai, China). The DPPH radical scavenging activity was calculated with the following equation.

$$\text{DPPH radical- scavenging activity}(\%) = \frac{A_C - (A_S - A_B)}{A_C} \times 100$$

Where A_C was the absorbance of control with sample replaced by an equivalent volume of distilled water, A_B was the absorbance of blank with the same volume of 99.5% ethanol replacing DPPH solution.

2.3.2. Hydroxyl radical-scavenging activity assay

Hydroxyl radical scavenging activity was measured by the method as reported by De Avellar et al. [27] with slight modifications. First, the reaction mixture, including 70 μL of 0.75 mM 1, 10-phenanthroline, 140 μL of sodium phosphate (pH 7.4), 70 μL of $FeSO_4$, and 70 μL of sample, were mixed vigorously. Before incubating at 37 °C for 1 h, the mixture was added with 70 μL of 0.12% (v/v) H_2O_2, and then cooled quickly to room temperature with running tap water. The absorbance was determined at 536 nm (721G-100 Vis-spectrophotometer, Shanghai, China). The hydroxyl radical-scavenging activity was expressed as:

$$\text{Hydroxyl radical- scavenging activity}(\%) = \frac{A_S - A_P}{A_B} \times 100$$

Where A_S, the absorbance of sample; A_P, replacing sample with equivalent volume of distilled water; A_B, replacing sample and 0.12% (v/v) H_2O_2 with equivalent volume of distilled water.

2.3.3. Reducing power assay

The ability of sample to reduce Fe^{3+}/ferric cyanide complex to the ferrous form was determined according to our previous method [26] with minor modifications. In short, the reaction mixtures, including 100 μL of sample, 50 μL of 0.2 M sodium phosphate buffer (pH 6.6), and 50 μL of 1% (w/v) potassium ferricyanide, were incubated at 50 °C for 20 min. Subsequently, 50 μL of 10% trichloroacetic acid (v/v) was added to the mixture, and centrifuged at 3000*g* for 10 min (TGL-16C, Shanghai, China). The supernatant was added with 50 μL of 0.1% (w/v) ferric chloride, followed by vigorous mixing. After keeping at room temperature for 10 min, the absorbance of the reaction mixture was measured at 700 nm using a spectrophotometer (721G-100, Shanghai, China). An aliquot of 100 μL of distilled water instead of the sample was used as the blank. The higher the absorbance, the stronger the reducing power.

2.4. Amino acid composition analysis

Amino acid composition was determined for the SVAs. Ten milligrams of freeze-dried SVAs was previously digested with 15 mL of 6 mol/L HCl under a nitrogen atmosphere at 110 °C for 22 h. The digested samples were subsequently diluted to 25 mL by adding distilled water. Then, one milliliter of diluted digestions was completely dried under a nitrogen atmosphere. The dried sample was re-dissolved by adding 1.0 mL distilled water and subsequently dried under a nitrogen atmosphere. Finally, the dried sample was then re-dissolved in 1.0 mL of 0.02 mol/L HCl. After filtrating with a 0.45 μm filter membrane, 20 μL of sample filtration was used for assaying amino acid composition in an automatic amino acid analyzer (Hitachi L-8900, Tokyo, Japan). Results were determined as mg per g of sample.

2.5. Isolation and purification of antioxidative peptides from SVAs

2.5.1. Gel filtration chromatography

The lyophilized SVAs (0.5 g) was dissolved in 2 mL of distilled water, then fractioned on a Sephadex G 25 gel chromatography (1.6 × 50 cm, 5 μm) column pre-equilibrated and eluted with distilled water. The elution rate was 1.3 mL/min. Eluents were collected with an interval of 3 min. The absorbance was measured at 280 nm. All fractions were pooled, concentrated and lyophilized. The active fraction SVAs3 was collected for further purification. Molecular weight (MW) standards, bovine serum albumin (BSA)