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Improvement of functional properties and antioxidant activities of cuttlefish (*Sepia officinalis*) muscle proteins hydrolyzed by *Bacillus mojavensis* A21 proteases

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ABSTRACT

Functional properties and antioxidant activities of cuttlefish (*Sepia officinalis*) muscle protein hydrolysates, with different degrees of hydrolysis (DH from 7.3% to 18.8%), obtained by treatment with *Bacillus mojavensis* A21 alkaline proteases were investigated. Protein contents for all freeze-dried cuttlefish muscle protein hydrolysates (CMPHs) ranged from 80% to 86%. For the functional properties, hydrolysis by A21 proteases increased ($p<0.05$) protein solubility to above 78% over a wide pH range (2.0–11.0). However, the interfacial activities (emulsion activity index, emulsion stability index, foaming capacity and foaming stability) decreased with the increase of the DH. All CMPHs exhibited significant metal chelating activity and DPPH free radical-scavenging activity, and inhibited linoleic acid peroxidation. Antioxidant properties of protein hydrolysates increased with protein hydrolysis and the highest activities were obtained at DH of 16%. The IC_{50} values for DPPH radical-scavenging and metal chelating activities were found to be 0.52 ± 0.01 mg/ml and 0.67 ± 0.13 mg/ml. The obtained results suggested that functional properties and antioxidant activities of cuttlefish muscle protein hydrolysates were influenced by the degree of hydrolysis.

The composition of amino acids of undigested and hydrolyzed proteins was determined. CMPHs have a high percentage of essential amino acids such as arginine, lysine, histidine and leucine. They have a high nutritional value and could be used as supplement to poorly balanced dietary proteins.

1. Introduction

Functional properties can be defined as the overall physic-chemical behavior of performance of proteins in food systems during processing, storage and consumption (Hall & Ahmad, 1992). Many studies have demonstrated that functional properties of protein can be improved by enzymatic hydrolysis under controlled conditions (Quaglia & Orban, 1990). Hydrolysis potentially influences the molecular size, hydrophobicity and polar groups of the hydrolysate (Kristinsson & Rasco, 2000a). Protein hydrolysate has an excellent solubility at high degree of hydrolysis (Gbogouri, Linder, Fanni, & Parmentier, 2004; Shahidi, Han, & Synowiecki, 1995). High solubility of fish protein hydrolysate over a wide range of pH is a substantially useful characteristic for many food applications. Furthermore, it influences the other functional properties, such as emulsifying and foaming properties (Gbogouri et al., 2004; Kristinsson & Rasco, 2000a). However, a very high degree of hydrolysis can have enormously negative effect on the functional properties (Kristinsson & Rasco, 2000a). Greater emulsifying capacity and emulsion stability were obtained when DH was low for salmon by-product hydrolysate (Gbogouri et al., 2004) and sardine hydrolysate (Quaglia & Orban, 1990). Shahidi et al. (1995) also reported good foaming properties for capelin protein hydrolysates prepared by Alcalase at low DH.

Lipid oxidation is of great concern to the food industry and consumers because it leads to the development of undesirable off-flavors, odors, dark colors and potentially toxic reaction products (Lin & Liang, 2002). Furthermore, cancer, coronary heart disease and Alzheimer's diseases are also reported to be caused in part by oxidation or free radical reactions in the body (Diaz, Frei, Vita, & Keaney, 1997). To prevent foods from undergoing such deterioration and to provide protection against serious diseases, it is very important to inhibit lipid peroxidation occurring in foodstuffs and the living body. Antioxidants are used to preserve food products by retarding discoloration and deterioration as a result of oxidation. Antioxidant is defined as any substance that significantly delays or inhibits oxidation of a substance when present at low concentrations compared to that of an oxidizable substrate.

A part from their functionalities, protein hydrolysates from different sources, such as milk protein (Tong, Sasaki, McClements, & Decker, 2000), maize zein (Kong & Xiong, 2006), egg-yolk (Sakanaka & Tachibana, 2006), porcine proteins (Saiga, Tanabe, & Nishimura, 2003), yellow stripe trevally (Klompong, Benjakul, Kantachote, & Shahidi, 2007), yellowfin sole frame (Jun, Park, Jung, & Kim, 2004), herring (Sathivel et al., 2003), mackerel (Wu, Chen, & Shiau, 2003), sardinelle (Bougatef et al., 2010) and smooth-hound (Bougatef et al., 2009) have been found to possess antioxidant activity. The operational conditions employed in the processing of protein isolates, the type of

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protease and the degree of hydrolysis affect the antioxidant activity (Pena-Ramos & Xiong, 2002). Moreover, the utilization of proteins or their hydrolysates for food and/or cosmetic applications not only presents additional advantages over other antioxidants, but also they confer nutritional and functional properties (Moure, Domínguez, & Parajó, 2006).

Common cuttlefish (*Sepia officinalis*) is a cephalopod of the *Sepia* genus and found in abundance in some parts of the world. However, functional protein and antioxidant effect of protein hydrolysates from cuttlefish by protease treatment has not been reported yet for the purpose of food preservation and product development although cuttlefish contributes an important global source of protein, and its chemical constituents and nutritive value are worthy of mention.

The objectives of the present study were to investigate some functional properties and the antioxidant activity of cuttlefish muscle protein hydrolysates (CMPHs), prepared by treatment with alkaline proteases from *Bacillus mojavensis* A21.

2. Materials and methods

2.1. Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine (Ferrozine), butylated hydroxyanisole (BHA), α-tocopherol and linoleic acid were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). All other chemicals, namely ammonium thiocyanate, ferric chloride, EDTA and sodium hydroxide were of analytical grade.

2.2. Fish sample

Cuttlefish (*S. officinalis*), in the size range of 8–10 cuttlefish/kg was purchased from the fish market of Sfax city, Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w) and transported to the research laboratory within 30 min. The mantle was cleaned, deskinned and eviscerated and then stored in sealed plastic bags at −80 °C until used.

2.3. Enzyme

The crude proteases from *B. majavensis* A21 (Haddar, Bougatef, Agrebi, Sellami-Kamoun, & Nasri, 2009), was used for the production of hydrolysates. Protease activity was determined according to the method of Kembhavi, Kulkarni, and Pant (1993) using casein as a substrate. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions used.

2.4. Preparation of cuttlefish muscle protein hydrolysates

Cuttlefish (*S. officinalis*) muscle (500 g), in 1000 ml distilled water, was first minced using a grinder (Moulinex Charlotte HV3, France), and then cooked at 90 °C for 20 min to inactivate endogenous enzymes. The cooked muscle sample was then homogenized in a Moulinex® blender for about 2 min. The pH of the mixture was adjusted to the pH 10.0 (optimum pH of B. mojavensis A21 proteases). The hydrolysis reaction was started by the addition of the enzyme at a 3:1 (U/mg) enzyme/protein ratio. The reaction was conducted at pH 10.0, 50 °C for 4 h. During the reaction, the pH of the mixture was maintained constant by continuous addition of 4 N NaOH solution. After the required digestion time, the reaction was stopped by heating the solution for 20 min at 80 °C to inactivate enzymes. The cuttlefish muscle protein hydrolysates were then centrifuged at 5000 ×*g* for 20 min to separate insoluble and soluble fractions. Finally, the soluble phase was freeze-dried using freeze-dryer (Bioblock Scientific Christ ALPHA 1–2, IllKrich-Cedex, France) and stored at −20 °C for further use.

2.5. Degree of hydrolysis determination (DH)

The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds broken (h) to the total number of peptide bonds in the studied substrate (h_{tot}), was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis (Adler-Nissen, 1986) as given below:

$$DH(\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times Nb}{MP} \times \frac{1}{\alpha} \times \frac{1}{h_{tot}} \times 100$$

where B is the amount of base consumed (ml) to keep the pH constant during the reaction, Nb is the normality of the base, MP is the mass (g) of protein (N × 6.25), and α is the average degree of dissociation of the α-NH_2 groups released during hydrolysis expressed as:

$$\alpha = \frac{10^{pH-pK}}{1 + 10^{pH-pK}}$$

where pH and pK are the values at which the proteolysis was conducted. The total number of peptide bonds (h_{tot}) in a fish protein concentrate was assumed to be 8.6 meq/g (Adler-Nissen, 1986).

2.6. Chemical analysis

Moisture and ash content were determined according to the AOAC (1995) standard methods 930.15 and 942.05, respectively. Total nitrogen content of the substrate and selected hydrolysate products was determined by using the Kjeldahl method. Crude protein was estimated by multiplying total nitrogen content by the factor of 6.25. Lipids were determined gravimetrically after Soxhlet extraction of dried samples with hexane. All measurements were performed in triplicate. The protein and fat contents were expressed on a dry weight basis.

2.7. Amino acid analysis

For analysis of amino acids, the dry samples were dissolved in distilled water at 1 mg/ml and 50 μl of each sample were dried and hydrolysed in vacuum-sealed glass tube at 110 °C for 24 h in the presence of constant boiling 6 N HCl containing 1% (w/v) phenol and using norleucine as internal standard. After hydrolysis, samples were vacuum-dried, dissolved in application buffer and injected into a Beckman 6300 amino acid analyzer (Beckman Instruments Inc., Fullerton, Calif., U.S.A.).

2.8. Functional properties of CMPHs

2.8.1. Solubility

Solubility of CMPHs was carried out according to Tsumura et al. (2005) with slight modifications. Briefly, 200 mg of freeze-dried CMPHs was dissolved in 20 ml deionized distilled water and the pH of the mixture was adjusted to different values from 2.0 to 11.0 using 2 N HCl or 2 N NaOH solutions. The mixtures were stirred for 10 min at room temperature (25 ± 1 °C) and then centrifuged at 8000 ×*g* for 10 min. After appropriate dilution, the nitrogen content in the supernatant was determined by Biuret method (Gornall, Bardawill, & David, 1949). The nitrogen solubility of CMPHs, defined as the amount of soluble nitrogen from the total nitrogen, was calculated as follows:

$$\text{Nitrogen solubility}\,(\%) = \frac{\text{Supernatant nitrogen concentration}}{\text{Sample nitrogen concentration}} \times 100.$$

Solubility analysis was carried out in triplicate.