Food Chemistry 122 (2010) 42–48

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Purification of antioxidative peptides prepared from enzymatic hydrolysates of tuna dark muscle by-product

Kuo-Chiang Hsu *

Department of Nutrition, China Medical University, No. 91, Hsueh-Shih Road, Taichung 40402, Taiwan, ROC

ARTICLE INFO

Article history:
Received 1 December 2009
Received in revised form 28 January 2010
Accepted 2 February 2010

Keywords:
Antioxidative activity
Protein hydrolysate
Tuna dark muscle
Orientase
Protease

ABSTRACT

To produce and identify bioactive peptides, two commercial enzymes, orientase (OR) and protease XXIII (PR) were used to hydrolyze tuna dark muscle by-product for up to 6 h, and the hydrolysates were evaluated for antioxidative properties. The results showed that, 60-min OR and 120-min PR hydrolysates possessed the highest antioxidative activity. Then, the protein hydrolysates were subjected to a Sephadex G-25 gel filtration chromatography, and the molecular weight of the peptide fractions which showed the highest antioxidative activity ranged from 390 to 1400 Da. The peptide fractions were further isolated using the two-step high-performance liquid chromatography (HPLC-1 and HPLC-2), and the amino acid sequences of the two antioxidative peptides from OR and PR hydrolysates were Leu–Pro–Thr–Ser–Glu–Ala–Ala–Lys–Tyr (978 Da) and Pro–Met–Asp–Tyr–Met–Val–Thr (756 Da), respectively. We thus conclude that antioxidative hydrolysates from tuna dark muscle by-product may be useful ingredients in food and nutraceutical applications.

1. Introduction

Protein-rich by-products from the seafood industry, especially dark muscles, have limited uses due to their dark colour, accessibility to oxidation and off-flavour. They are mainly processed into low market-value products, such as fish waste meal and fertilizer. Therefore, to recover the seafood proteins for further utilization, such as an application of enzymatic technology, may produce highly valuable products. Fish protein hydrolysates (FPH) have been reported to possess antioxidative and antihypertensive properties (Je, Park, Kwon, & Kim, 2004; Thiansilakul, Benjakul, & Shahidi, 2007a, 2007b). The bioactive peptides in FPH responsible for these properties are released upon endogenous and/or exogenous enzyme hydrolysis of fish proteins. Antioxidant activity has been reported for protein hydrolysates prepared from various fish sources such as capelin, mackerel, yellowfin sole, Alaska Pollack, Atlantic salmon, hoki, conger eel, and scad (Amarowicz & Shahidi, 1997; Berge, 2005; Je, Kim, & Kim, 2005; Je, Park, & Kim, 2005; Jun, Park, Jung, & Kim, 2004; Ranathunga, Rajapakse, & Kim, 2006; Thiansilakul et al., 2007a, 2007b; Wu, Chen, & Shiau, 2003). Most studies on purification of the antioxidative peptides from hydrolysates were carried out by gel filtration or ultrafiltration to determine the molecular weight distribution (Dong et al., 2008; Wang, Zhao, Zhao, & Jiang, 2007; Wu et al., 2003), however, the obtained peptides were mostly not further purified and unidentified.

Orientase, an endopeptidase prepared from *Bacillus subtilis*, is classified as both serine protease and metalloprotease; on the other hand, protease XXIII, an endopeptidase prepared from *Aspergillus melleus*, is classified as the mixture of aspartic protease, metalloprotease, serine protease and carboxypeptidase (Alder-Nissen, 1986). Orientase has broad specificity and acts mainly on Leu-, Phe-NH_2, and Tyr-COOH; meanwhile, protease XXIII has very broad specificity (Alder-Nissen, 1986). In previous studies, many commercial proteases, such as alcalase (Dong et al., 2008), flavourzyme (Thiansilakul et al., 2007a, 2007b) and papain (Wang et al., 2007), were used to obtain antioxidative peptides by hydrolyzing various protein sources. Alcalase, flavourzyme and papain were classified as serine, serine and cysteine proteases, respectively. Our choice of the two enzymes in this work was focused on their different specificity from other commercial proteases.

In Taiwan, canned tuna is the largest commercial canned fishery product, and the annual production quantity and value of canned tuna exceeds 1619 tons and 169 million NT dollars in 2008. Dark muscles were the by-products of canned tuna processing, mainly further used to produce fish waste meals and feeds. A research group has reported that antihypertensive and antioxidative peptides were obtained by hydrolyzing tuna dark muscle with pepsin which, an aspartic protease, mainly acted on the N-terminal aromatic amino acids (Qian, Je, & Kim, 2007; Je, Qian, Lee, Byun, & Kim, 2008). In our previous studies, we found that orientase and protease XXIII were used to produce highly antioxidative peptides by hydrolyzing tuna cooking juice (Hsu, Lu, & Jao, 2009; Jao & Ko, 2002). In this study, therefore, we tried to use the two commercial proteases, orientase and protease XXIII, to hydrolyze tuna dark

* Tel.: +886 4 22053366x7522; fax: +886 4 22062891.
E-mail address: kchsu@mail.cmu.edu.tw

0308-8146/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2010.02.013

muscle by-product and then identify the antioxidative activity and DPPH radical-scavenging capacity of the hydrolysates. Further, the antioxidative hydrolysates were isolated by gel filtration and two-step high performance liquid chromatography (HPLC) in sequence, and amino acid sequences of the antioxidative peptides were also determined.

2. Materials and methods

2.1. Sample preparation

Hsing-Yi Frozen Foods Industry Ltd. (Chiayi County, Taiwan) supplied the tuna dark muscle by-product. The whole tuna fish (*Thunnus tonggol*) was cooked by steam (100–105 °C) for 3–4 h, after which, the dark muscle by-product was vacuum-sealed in 400-ml polyethylene bags and then transferred on ice to our laboratory immediately, and then stored at −80 °C until used. The protein, lipid, ash and moisture contents of the dark muscle by-product were 25.3, 2.30, 1.29 and 71.3%, respectively, as determined according to the methods of AOAC (2000).

2.2. Chemicals and reagents

Orientase 90 N (specific activity of 70,000 units/mg solid), an endopeptidase prepared from *B. subtilis* was obtained in dry powder form from Hankyu Bioindustry Co. (Osaka, Japan). Protease XXIII (specific activity of 4.6 units/mg solid), an endopeptidase prepared from *A. melleus* was obtained in dry powder form from Sigma–Aldrich, Inc. (St. Louis, MO, USA). 1,1-diphenyl-2-picryhydrazyl (DPPH), 2,4,6-trinitrobenzenesulfonic acid (TNBS), L-ascorbic acid, butyl hydroxyanisol (BHA), α-tocopherol, thiocyanate, *N*-Formyl–Ala–Gly–Ser–Glu (Mr. 390), Bradykinin (Mr. 1400) and Biocytin–neuropeptide Y (Mr. 4500), trifluoroacetic acid (TFA), and acetonitrile were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Iron (II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$) was procured from Kanto Chemical Co. Inc. (Tokyo, Japan). Sephadex G-25 was produced by Pharmacia Biotech Co. (Uppsala, Sweden). Other chemicals and reagents used were analytical grade and commercially available.

2.3. Enzymatic hydrolysis

Fifty grammes of dark muscles were homogenized with 100 ml deionized water and preincubated at 37 or 50 °C for 20 min prior to enzymatic hydrolysis. The hydrolysis reaction was started by the addition of 2 ml enzyme solution (dissolved in deionized water) at an enzyme/substrate ratio of 1:100 (w/w). The reaction with orientase (OR) and protease XXIII (PR) were conducted at pH 7.0, 50 °C and pH 7.5, 37 °C, respectively, for up to 6 h. The pH of the mixture was maintained constant by using 1 N NaOH during hydrolysis. After hydrolysis, the hydrolysates were heated in a boiling water bath for 20 min to inactivate the enzymes. Hydrolysates were centrifuged (Centrifuge Himac CR21, Hitachi Ltd., Katsuda, Japan) at 12,000*g* for 15 min. A part of the supernatant was collected to determine the degree of hydrolysis, and the other part was freeze–dried (FDU-2000, EYELA freeze–dryer, Tokyo, Japan) and stored in a desiccator.

2.4. Measurement of degree of hydrolysis

According to the method of Benjakul and Morrissey (1997) with some modifications, the degree of hydrolysis (DH) of the hydralysate was determined as the ratio of the amount of α-amino acid released during hydrolysis to the maximum amount of α-amino acid in dark muscle. Properly diluted samples (125 μl) were mixed with 2 ml of 0.2125 M sodium phosphate buffer (pH 8.2), followed by the addition of 1 ml of 0.01% TNBS. The mixtures were then incubated in a water bath at 50 °C for 20 min in the dark. The reaction was terminated by the addition of 2 ml of 0.1 M sodium sulfite. The mixtures were cooled down at ambient temperature for 20 min. On the other hand, the maximum amount of α-amino acid in dark muscle was obtained by acid hydrolysis with 6 N HCl at 105 °C for 24 h. Then, the acid-hydrolyzed sample was filtered through Whatman filter paper No. 1 to remove the unhydrolyzed debris. The supernatant was neutralized with 6 N NaOH before α-amino acid determination. The absorbance was measured at 420 nm and α-amino acid was expressed in terms of L-leucine. The DH was calculated as follows:

$$\mathrm{DH}(\%) = [(L_t - L_0)/(L_{\max} - L_0)] \times 100$$

where L_t is the amount of α-amino acid released at time t; L_0 is the amount of α-amino acid in original dark muscle; $L_{\max}$ is the maximum amount of α-amino acid in dark muscle (Beak & Cadwallader, 1995).

2.5. Determination of antioxidative activities

2.5.1. DPPH radical-scavenging capacity

DPPH radical-scavenging capacity was estimated using the method of Yen and Wu (1999) with some modifications. The lyophilized samples were dissolved in 0.05 M phosphate buffer (pH 6.5) (3 mg/ml for hydrolysates; 1 mg/ml for gel filtration fractions; 150 μg/ml for HPLC-1 fractions and 100 μg/ml for HPLC-2 fractions). Four milliliter of the sample solution was added to a 1 ml solution of DPPH radical in methanol (final concentration of DPPH was 0.2 mM). In a single experiment, sample was replaced with 100 μg/ml L-ascorbic acid for comparative purpose. The mixture was shaken vigorously and allowed to stand for 30 min, and its absorbance was measured at 517 nm with a spectrophotometer (Hitachi U-2000, Tokyo, Japan). The percentage of DPPH radical-scavenging capacity was calculated as follows:

$$\text{DPPH radical-scavenging capacity}(\%) = [A_0 - (A_1 - A_s)]/A_0 \times 100$$

where A_0 is the absorbance of the control solution (containing only DPPH); A_1 is the absorbance of the DPPH solution containing samples; and A_s is the absorbance of the samples without DPPH.

2.5.2. Antioxidative activity

The antioxidative activity of tuna dark muscle hydrolysates was determined according to the ferric thiocyanate method (Chen, Muramoto, & Yamaguchi, 1995; Mitsuda, Yasumoto, & Iwami, 1966) with some modifications. Samples (3 mg/ml for hydrolysates; 1 mg/ml for gel filtration fractions; 150 μg/ml for HPLC-1 fractions and 100 μg/ml for HPLC-2 fractions) dissolved in 1 ml of deionized water were added to 1.0 ml of 0.1 M sodium phosphate buffer (pH 7.0) and 1.0 ml of 50 mM linoleic acid in ethanol (95%), then mixed in a 5 ml tube. In a single experiment, sample was replaced with 100 μg/ml BHA and α-tocopherol for comparative purpose. The test tubes were placed in the dark at 60 °C to accelerate oxidation. To 50 μl of the reaction mixture were added 2.35 ml of 75% ethanol, 50 μl of 30% ammonium thiocyanate, and 50 μl of 20 mM ferrous chloride solution in 3.5% HCl. After the mixture was stirred for 3 min, the peroxide value was determined spectrophotometrically at 500 nm. The number of days taken to attain an absorbance of 0.3 was defined as the induction period (Chen et al., 1995). The induction period (days) referred to the relative antioxidative activity of the samples.