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Analytical Methods

Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry

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Abstract

Grass carp muscles were hydrolyzed with various proteases (papain, bovine pancreatin 6.0, bromelain, neutrase 1.5MG and alcalase 2.4L) to extract antioxidant peptides. The hydrolysates were assessed using methods of hydroxyl radical scavenging ability and lipid peroxidation inhibition activity. Hydrolysate prepared with alcalase 2.4L was found to have the highest antioxidant activity. It was purified using ultrafiltration and consecutive chromatographic methods including ion-exchange chromatography, multilayer coil high-speed counter-current chromatography, and gel filtration chromatography. The purified peptide, as a potent antioxidant, was identified as Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val (966.3 Da) using RP-HPLC connected on-line to an electrospray ionization mass spectrometry. As well, it was found that basic peptides had greater capacity to scavenge hydroxyl radical than acidic or neutral peptides and that hydrophobic peptides contributed more to the antioxidant activities of hydrolysates than the hydrophilic peptides. In addition, the amino acid sequence of the peptide might play an important role on its antioxidant activity.

Keywords: Grass carp muscle; Antioxidant peptides; High-speed counter-current chromatography; Electrospray ionization-mass spectrometry

1. Introduction

Oxidation is an essential reaction in all living organisms. The formation of free radicals and other reactive oxygen species (ROS) is unavoidable during the oxidative metabolic process. These reactive radicals play an important role in signal transduction (Hancock, Desikan, & Neill, 2001). However, excess free radicals can cause destructive effects on living tissues and foodstuffs (Wang, Zhao, Zhao, & Jiang, 2007). Among all ROS, hydroxyl radical is considered to be the most reactive and capable of damaging almost any compound it comes in contact with in the living cells (Castro & Freeman, 2001). The free radical chain reaction can initiate peroxidation of membrane lipids, which has been shown to be associated with many diseases (Halliwell, 2001; Halliwell & Whiteman, 2004). Lipid peroxidation that occurs in food products is responsible for the development of unacceptable flavour and taste, decrease in shelf life and the formation of potentially toxic reaction products (Pihlanto, 2006). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), t-butylhydroquinone (TBHQ) and propyl gallate have been widely used in foodstuffs to delay the deterioration caused by oxidation (Wanita & Lorenz, 1996). However, they are restricted in some countries due

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to their potential health hazard (Becker, 1993; Branen, 1975). As a result, the demand for safe and naturally occurring antioxidants as alternatives to synthetic ones has grown steadily for many years.

In recent years, the antioxidant activity of bioactive peptides generated from the digestion of various proteins has attracted much attention. The peptides from milk (Blanca, Ana, Lourdes, & Isidra, 2007), soybean (Gibbs, Zougman, Masse, & Mulligan, 2004), rice bran (Parrado et al., 2006), oil seeds (Aluko & Monu, 2003), eggs (Sakanaka, Tachibana, Ishihara, & Juneja, 2004) and porcine (Saiga, Tanabe, & Nishimura, 2003) have all been shown to exhibit antioxidant activity. In addition, aquatic products and by-products have also proven to be good sources of antioxidant peptides. For example, studies on peptides from capelin protein (Amarowicz & Shahidi, 1997), mackerel protein (Wu, Chen, & Shiau, 2003), jumbo squid skin (Mendis, Rajapakse, Byun, & Kim, 2005), hoki frame protein (Kim, Je, & Kim, 2007) and yellowfin sole frame protein (Jun, Park, Jung, & Kim, 2004) have reported the presence of significant antioxidant activities. However, to the best of our knowledge, little research has been done on the antioxidant properties of freshwater fish derived peptides. Grass carp, one of the high yield freshwater fish, currently comprises up to 35–40% of the total freshwater fish species in China. However, the development of value-added products from grass carp has not been fully exploited yet. Therefore, preparation of bioavailable antioxidant peptides from grass carps may be one way of producing high-value food ingredients from this under utilized fish.

The antioxidant activity of peptides is closely related to their amino acid constituents and their sequences (Chen, Muramoto, Yamaguchi, Fujimoto, & Nokihara, 1998). It is believed that the antioxidant peptides possess some metal-chelation or hydrogen/electron donating activity, which could make them interact with free radicals and terminate the radical chain reaction or prevent their formation (Wang et al., 2007). Hydrophobic amino acids and one or more residues of His, Pro, Met, Cys, Tyr, Trp, Phe and Met are believed to enhance the activities of the antioxidant peptides (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Da'valos, Miguel, Bartolome', & Lo'pez-Fandi∼no, 2004; Herna′ndez-Ledesma, Da′valos, Bartolome′, & Amigo, 2005). However, some recent findings have shed new light on this subject. For example, the Glu-Leu residue in peptides was recently reported to play an important role in radical scavenging (Jun et al., 2004). Also, the known antioxidant peptide, Gln-Gly-Ala-Arg, does not contain any of the above mentioned proton-donating amino acid residues in its sequence (Li, Chen, Wang, Ji, & Wu, 2007). It appears that more research is needed to clarify the structure–function relationship of peptides.

As an effective separation technique, high-speed counter-current chromatography (HSCCC) has been successfully applied to separate natural products and compounds including flavonoid (Peng, Fan, & Wu, 2005), coumarin (Shibusawa, Haqiwara, & Chao, 1997) and saponin (Du, Jerz, & Waibel, 2003). For peptides, the use of HSCCC has been mainly concentrated with the separation of standard mixtures of free peptides (Ma & Ito, 1997), while little has been done on the separation of peptides in hydrolysates.

The objective of this study is to assess and identify or characterize antioxidant peptides from grass carp which is an under utilized freshwater fish. Besides, using HSCCC to separate peptides in hydrolysates can provide useful information for the isolation of peptides derived from other proteins.

2. Materials and methods

2.1. Material

The healthy grass carps (*Ctenopharyngodon idellus*, 822 ± 147 g in weight and 40.8 ± 2.8 cm in length) were obtained from a local market in Guangzhou, China. Grass carps (without head, tail, skin, bone, internal organs and blood) were filleted and minced in a MM12 mincer (Shaoguan Food Machine Co., China). The minced material was frozen and stored at −20 °C for further use. Five food-grade enzymes used for hydrolysis experiments (Papain, bovine pancreatin 6.0, bromelain, neutrase 1.5MG and alcalase 2.4 L) were provided by Novo Nordisk Co. (Beijing, China) and Mingyuan Co. (Guangzhou, China). Protein standard mixtures used for molecular weight calibration by gel filtration chromatography were supplied by AmershamBiotech (GE, Piscataway, NJ, USA). Amino acid standard mixtures, glutathione (GSH), oxidized glutathione (GSSH), methyl tert-butyl ether (MTBE), phenylisothiocyanate (PITC) and acetonitrile were all HPLC-grade and purchased from Sigma (Beijing, China). Alpha-deoxyribose (2-deoxy-D-ribose) was purchased from Fluka (Stockholm, Sweden). All other reagents were analytical-grade.

2.2. Preparation of grass carp muscle hydrolysates

Frozen minced grass carp muscles (1250 g) were thawed and mixed with deionized water (1250 ml). The mixture obtained was divided into five equal fractions. Each of the fractions was adjusted to the required pH with 0.01 M NaOH and heated in a water bath to the required temperatures (Table 1). Papain, bovine pancreatin 6.0, bromelain, neutrase 1.5MG and alcalase 2.4L were dissolved in deionized water, respectively, and added in the proper proportions based on their activities (Table 1). The hydrolysis reaction was performed in a shaking incubator (New Brunswick Scientifics C24, China). At the end of the hydrolysis period, the mixtures were heated in boiling water for 10 min to inactivate the proteases. The hydrolysates were centrifuged in a GL-21M refrigerated centrifuge (Xiangyi Instrument Co. Ltd., Changsha, China) at 4125 g for 30 min and the supernatants were lyophilized and stored in a desiccator for further use.