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Isolation and identification of antioxidant peptides from enzymatically hydrolyzed rice bran protein

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ABSTRACT

Khao Dawk Mali 105 rice bran protein (RBP) was fractionated into albumin (12.5%), globulin (13.9%), glutelin (70.8%) and prolamine (2.9%). The native and denatured RBP fractions were hydrolyzed with papain and trypsin for 3 h at optimum conditions. The RBP fractions and their hydrolysates were evaluated for their antioxidant activity by the Oxygen Radical Absorbance Capacity (ORAC) assay. The trypsin-hydrolyzed denatured albumin exhibited the highest antioxidant activity with an ORAC value of 4.07 μmol of Trolox equivalent (TE)/mg protein. This hydrolysate was separated by using RP-HPLC and three fractions with high antioxidant activity were examined by LTQ-FTICR ESI mass spectrometry. The MW of the peptides from these fractions were 800–2100 Da. and consisted of 6–21 amino acid residues. Most of the peptides from the fractions demonstrated typical characteristics of well-known antioxidant peptides. The results suggest that trypsin-hydrolyzed denatured rice bran albumin might be useful as a natural food antioxidant.

1. Introduction

Oxidation reactions play a significant role in the food industry because they cause loss of color, nutritional value and functionality as well as undesirable off-flavors and toxic compounds. Accumulation of toxic products may be dangerous to the health of consumers (Nawar, 1996; Paek, Jung, Nam, Shahioli, & Kim, 2001). An oxidation can generate free radicals that react with lipids and proteins to bring about food deterioration. In addition, free radicals generated in a biological system damage biomacromolecules such as DNA, protein and membrane lipid if the human body cannot control their formation or eliminate them. As a result, inflammation-induced oxidative stress and lipid peroxidation can induce various diseases, such as cancer, multiple sclerosis, and cardiovascular disease (Halliwell & Gutteridge, 1990). Therefore, it is important to inhibit oxidation reactions and formation of free radicals in food products and the living body. In food science, an antioxidant has been defined as any substance which significantly delays or inhibits the oxidation of a substrate when it is present in low concentrations compared to that of the substrate. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) may be added to food products to retard oxidation reactions. Though synthetic antioxidants are effective and inexpensive compared to natural ones, however, their applications are limited because many are suspected to be carcinogenic (Ito, Fukushima, & Tsuda, 1985; Madhavi, Deshpande, & Salunkhe, 1996). Therefore, there has been a great deal of interest in finding new antioxidants from natural sources to replace synthetic antioxidants for use in food.

Recently, many studies have reported that hydrolyzed proteins from various animal and plant sources possess antioxidant activity such as fish (Nazeer & Kulandai, 2012), soybean (Zhang, Li, & Zhou, 2010), cereal and legume (Zilic, Akıllıoglu, Serpen, Barac, & Gokmen, 2012), rice (Adebiyi, Adebiyi, Ogawa, & Muramoto, 2008; Adebiyi, Adebiyi, Yamashita, Ogawa, & Muramoto, 2009; Chanput, Theerakulkait, & Nakai, 2009; Kannan, Hettiarachchy, & Mahedevan, 2012; Kokkeaw & Thawornchinsombut, 2007), oat (Tsopmo, Cooper, & Jodayree, 2010), egg (Tanzadehpanah, Asoodeh, & Chamani, 2012; You, Udenigwe, Aluko, & Wu, 2010) and barley (Xia, Bamdad, Gänzle, & Chen, 2012). The antioxidant activity of these hydrolysates has been ascribed to the cooperative effect of a number of

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properties including their ability to scavenge free radicals, to act as metal-chelators and oxygen quenchers.

Rice bran is one of the most abundant co-products in the rice milling industry. It contains a substantial amount of protein ranging from 12% to 20%. Thailand produces rice bran annually around 2 million tons, about 40% of which is used to produce edible rice oil. The rest of this is used as feed for animals and contains 0.143–0.189 million ton of protein. Rice bran protein (RBP) is of high nutrition value and has nutraceutical properties (Saunders, 1990). Several studies have been carried out on RBP hydrolysates. For example, Kokkeaw and Thawornchinsombut (2007) prepared RBP hydrolysates using commercial proteolytic enzymes, Protex 6L. The product's maximal radical scavenging activity was only 27%. Chanput et al. (2009) reported that KDML 105 rice bran protein fractions (RBPFs) digested with pepsin and subsequently with trypsin revealed high antioxidant activity. Protein from the Japanese rice bran was fractionated and hydrolyzed with protease M, N, S, and P, resulting in peptides of 6–30 amino acid residues, show high antioxidant activity (Adebiyi, Adebiyi, Hasegawa, Ogawa, & Muramoto, 2009). However, little is known about the antioxidant activity of RBP, their hydrolysates and the structure of antioxidant peptides from RBP. Therefore, in the present study, we investigated antioxidant activity of RBP and their hydrolysates that were prepared by treatment with papain and trypsin. Further, the antioxidant peptides from hydrolysates with highest antioxidant activity were identified by tandem mass spectrometry (MS/MS).

2. Experimental

2.1. Materials and chemicals

KDML 105 rice bran was obtained from Patum Rice Mill and Granary Company, Ltd. (Bangkok, Thailand). The samples were packed in aluminum foil bags and kept at −18 °C until use. Fluorescein, DL-Dithiothreitol (DTT), 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Co. (St. Louis, MO, USA). Bovine serum albumin (BSA) and papain were purchased from Fluka BioChemika (Buchs, Switzerland). Trypsin and ProteaseMax were from Promega Corporation (Madison, WI, USA). All other chemicals used in the experiments were of analytical grade. Ultra filtration concentrator units (Vivaspin 20 MWCO: 5000) were from GE Healthcare Bio-Sciences AB (Uppsala, Sweden).

2.2. Preparation of rice bran protein (RBP)

KDML 105 full-fat rice bran was defatted by extracting twice with three volumes of hexane. The defatted rice bran was air-dried overnight in a fume hood, ground and sieved through a 0.5 mm screen.

Free phenolic compounds in defatted rice bran were extracted by shaking with 80% chilled ethanol (defatted rice bran: 80% chilled ethanol, 1:5) for 30 min. After centrifugation at 3600×*g* for 15 min, the supernatant was discarded, and the extraction was repeated two times (Adom & Liu, 2002). The residue was air-dried overnight in a fume hood, ground and kept at −20 °C until use.

RBP was fractionated according to the method of Chanput et al. (2009) with slight modification. The defatted rice bran (10 g) was first fractionated by extracting with distilled water for 60 min (rice bran: water, 1:6 w/v), filtered through nylon cloth (100 mesh) and centrifuged at 10,000×*g* for 30 min at 20 °C to obtain the albumin fraction (AF). The supernatants were pooled and ammonium sulfate was added at a final concentration of 70%. The resulting precipitate was collected, washed with distilled water to remove salt, and concentrated using ultra filtration concentrator units. The rice bran residue was extracted with 60 mL of 2% NaCl to recover the globulin fraction (GbF). The residue after extraction of globulin was further extracted with 0.1 N NaOH to yield the glutelin fraction (GlF). Then the residue was extracted with 70% ethanol to obtain the prolamin fraction (PF). To further improve the yield of the protein fractions, each extraction step was repeated with 40 mL solution.

AF was dialyzed in water, while GbF and GlF were dialyzed in 50 mM ammonium bicarbonate buffer pH 7.8 for desalting. Then, they were concentrated using ultra filtration cartridges with a MWCO of 5000 Da. The concentrated protein fractions were freeze-dried and stored at −20 °C until use. Crude protein content was determined by Bradford's method (Bradford, 1976).

2.3. Preparation of rice bran protein hydrolysates (RBPHs)

Aliquots of RBPFs were dispersed in 50 mM ammonium bicarbonate buffer pH 7.8 to obtain a protein concentration of 0.2% and hydrolyzed with trypsin either in native or denatured form. Denatured protein was obtained by adding ProteasMax and DTT to the protein solution, followed by incubation at 56 °C for 20 min, and then chloroacetamide was added (0.55 M), and the mixture incubated in the dark at room temperature for 15 min before hydrolysis. For papain hydrolysis, 20 mM phosphate buffer pH 7.0 was used and the hydrolysis was carried out as described for trypsin digestion. Hydrolysis was carried out at 37 °C for 3 h. The enzyme was rapidly inactivated by adding TFA to a final concentration of 0.5%. The mixture was centrifuged at 12,000×*g* for 20 min at 20 °C. The supernatant was freeze-dried and kept at −20 °C for further studies.

2.4. Determination of antioxidant activity

The antioxidant activity of the samples was examined using the ORAC assay. ORAC was measured according to the method of Ou, Hampsch-Woodill & Prior (2001) with slight modifications. The method measures the antioxidant scavenging activity against peroxyl radical generated by decomposition of AAPH at 37 °C. All reagents and samples were prepared in 75 mM of potassium phosphate buffer, pH 7.4. Briefly, 25 μL of buffer solution (blank), diluted sample solutions, or standards were manually transferred in triplicate to a 96-well flat bottom polystyrene microplate, followed by adding 150 μL of 8.16 nM fluorescein solution. The fluorescein solution and samples were incubated at 37 °C for 15 min directly in a microplate reader (TECAN model Infinite M200), and 25 μL of 153 mM AAPH was added. The mixture was incubated for 30 s before the initial fluorescence was measured with excitation, 485 nm; emission, 535 nm, and the fluorescence was recorded at 37 °C every min until the reading remained constant. Trolox solutions (6.25, 12.5, 25 and 50 μM) were used for defining the standard curve. All reaction mixtures of standards or samples were prepared in triplicate. The net area under the curve (AUC) was calculated as;

$$\mathrm{AUC} = 0.5 + f_1/f_0 + \cdots f_i/f_0 + \cdots + f_{34}/f_0 + 0.5(f_{35}/f_0)$$

where f_0 represents the initial fluorescence reading at 0 min and f_i represents the fluorescence reading at time *i*. Final ORAC values were calculated using the regression equation between Trolox concentration and the net AUC, and were expressed as μmol of TE/mg protein. The net AUC of samples were calculated as follows:

$$\text{net AUC} = \mathrm{AUC}_{\mathrm{sample}} - \mathrm{AUC}_{\mathrm{blank}}$$