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Fractionation and antioxidant properties of rice bran protein hydrolysates stimulated by *in vitro* gastrointestinal digestion

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ABSTRACT

Rice bran was used as a starting material to prepare protein concentrate through enzyme-assisted extraction. The hydrolysis of protein concentrate under *in vitro* gastrointestinal digestion (pepsin-trypsin system) greatly improved the antioxidant properties. Rice bran protein hydrolysate was further fractionated by membrane ultrafiltration (UF, F1: molecular weight (MW) <3 kDa, F2: MW 3–5 kDa, and F3: MW 5–10 kDa). Peptides with smaller MW possessed higher antioxidant activities ($P < 0.05$). UF showed a great efficacy to selectively separate the metal-chelating peptides. Tyrosine and phenylalanine had positive correlations with their DPPH & ABTS radicals scavenging activities and ferric reducing antioxidant power ($r > 0.831$). A major peptide fragment was detected at m/z 1088 by a MALDI-TOF mass spectrometry. There is high potential that antioxidative peptides from rice bran might also be produced in the gastrointestinal tract of the human body.

1. Introduction

Recently, findings of natural antioxidants have gained growing interest because the use of synthetic antioxidants is being strictly regulated due to their potential health hazards. Plant-based antioxidant peptides are increasingly being studied and considered as alternative antioxidants for food preservation and health protection. Rice bran is an underutilized component obtained from rice milling processes. According to world rice production, >800 thousand metric tons of rice bran is generated as by-product annually (USDA, 2016). Rice bran contains high quality protein with high amounts of essential amino acids, especially aromatic amino acids (9.46–11.41%) that act as strong antioxidants (Wang, Chen, Fu, Li, & Wei, 2017). There have been numerous reports about rice proteins and their bioactivities, such as their angiotensin converting enzyme (ACE)-inhibitory activity (Chen et al., 2013), their cholesterol-lowering effect (Yang, Chen, Xu, Nie, & Yang, 2013), and their antioxidant activities (Zhou, Sun, & Canning, 2012 and Phongthai, Lim, & Rawdkuen, 2016). However, macroproteins are generally inactive within the sequence of the original structure. Enzymatic hydrolysis is an effective method to expose and release bioactive peptides without affecting nutritional value. Various enzymes such as alkaline and neutral proteases (Zhou, Canning, & Sun, 2013), papain (Zhang et al., 2010), alcalase (Phongthai et al., 2016), flavourzyme, neutrase (Thamnarathip, Jangchud, Nitisinprasert, & Vardhanabhuti, 2016), and trypsin (Wattanasiritham, Theerakulkait, Wickramasekara, Maier, & Stevens, 2016) have been used to produce rice protein hydrolysates that possess antioxidant activities, including the ability to scavenge free radicals, inhibit lipid peroxidation, and chelate metal ions. These activities depend on many variables such as amino acid composition, degree of hydrolysis, molecular weight (MW), and peptides sequences due to the types of enzymes used (Saidi, Deratani, Belleville, & Amar, 2014). Therefore, there is interest in utilizing the high quality protein in rice bran, and there is great potential in the enzymatic hydrolysis method to add value to the protein with highly desired biological, functional, and nutritional properties.

Stimulating bioactivities in proteins through *in vitro* gastrointestinal (GI) digestion is being used extensively since it is rapid, inexpensive, safe, and is not bound by the same ethical restrictions (Xiao et al., 2014). The peptides are encrypted in the proteins and can be released by the GI tract in the human stomach and small intestine. The proteases in these areas have been reported to have abilities to induce peptide bioactivities from several protein sources such as from loach (*Misgurnus anguillicaudatus*) (You,

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Zhao, Regenstein, & Ren, 2010), salmon by-product (Ahn, Kim, & Je, 2014), radix isatidis (Xiao et al., 2014), coconut meat (Jin, Zhou, Li, Lai, & Li, 2015), amaranth (Orsini Delgado et al., 2016), pig meat (Simonetti, Gambacorta, & Perna, 2016), and fish skin gelatin (Ketnawa, Benjakul, Martínez-Alvarez, & Rawdkuen, 2017). However, different protein sources must be individually investigated since the differences in amino acid sequences/compositions influences their activities. In addition, many studies reported that the bioactivities of peptides are also related to their MW (Foh, Qixing, Amadou, & Xia, 2010; Girgih et al., 2015; Onuh, Girgih, Aluko, & Aliani, 2014; Saidi et al., 2014 and Wiriyaphan, Xiao, Decker, & Yongsawatdigul, 2015). Generally, peptides with low MW exhibit high bioactivities since they have a higher chance to cross the intestinal barrier and perform biological functions (Chi et al., 2015). In order to obtain those low MW active peptides, membrane fractionation is widely used over other methods for its productivity, lower capital investment, it's maintenance of product purity under ambient conditions, and for the ease of keeping the equipment clean (Saidi et al., 2014). Even if the enhancement of peptide bioactivities by digestive enzymes as well as the effect of MW has been vastly reported, there is no data available with regards to the use of membrane technology to fractionate rice bran protein hydrolysate into smaller fractions.

Based on the above rationale, the authors aimed to stimulate antioxidant activities of rice bran protein prepared by α-amylase-assisted extraction through *in vitro* GI digestion. This study also investigates protein patterns of the hydrolysates by SDS-PAGE, and the effects of fractionation by membrane ultrafiltration (UF) on the antioxidant activities of their fractions. Moreover, the UF fractions were analyzed for aromatic amino acid contents in order to investigate the correlation of their activities. Further, the fraction that possessed the highest antioxidant activity was further investigated for its MW distribution using MALDI-TOF mass spectrometry.

2. Materials and methods

2.1. Materials

Organic rice bran was supplied by Urmatt Ltd. (Chiang Rai, Thailand). α-Amylase (E.C.3.2.1.1, ≥500 units/mg protein) from *Bacillus licheniformis*; pepsin (E.C.3.4.23.1, ≥250 units/mg solid) from porcine gastric mucosa; trypsin (E.C.3.4.21.4, ~10,000 BAEE units/mg protein) from bovine pancreas; 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid; ABTS); 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate (ferrozine); 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox); 99% acrylamide; and 1,1-diphenyl-2-picryhydrazyl; DPPH) were all purchased from the Sigma-Aldrich Company (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) was purchased from VWR International Ltd (Lutterworth, England). Ferrous sulphate ($FeSO_4 \cdot 7H_2O$) was purchased from Ajax Finechem Pty Ltd (Auckland, New Zealand). Roti®-Mark standard protein-molecular weight marker was purchased from Carl Roth GmbH+ Co. KG (Karlsruhe, Germany). Tyrosine, tryptophan, and phenylalanine (99% purity) were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Enzyme-assisted extraction (EAE) of rice bran protein

Organic rice bran was defatted with 95% ethanol (1:5, w/v) according to the method of Phongthai et al. (2016). The slurry was stirred at room temperature for 1 h, and then centrifuged at 1500 rpm for 5 min. The precipitate was collected and re-extracted twice. The final rice bran fraction was dried overnight in the oven at 30 °C, and then passed through a 50-mesh sieve. Defatted rice bran (DFRB) was kept in a plastic zip lock bag at −18 °C before use in further experiments.

The single study of variables, including solid-liquid ratio, extraction time, and enzyme concentration, were studied in sequence. DFRB was mixed with distilled water with varying solid-liquid ratios of 0.5, 1.0, 1.5 and 2.0 g DFRB/10 mL, while the extraction time and enzyme concentration were fixed at 2 h and 5000 units, respectively. The slurry pH was adjusted to 6.2 (optimal pH for α-amylase), and the extraction was performed in a water bath at 55 °C (Tang, Hettiarachchy, Eswaranandam, & Crandall, 2003). Afterwards, the slurry pH was adjusted to 10.0 before further shaking for a period of 30 min. The slurry was then centrifuged at 10,000×*g* (4 °C) for 10 min. The supernatant was collected and the pH was adjusted to 4.5. After centrifugation, the precipitate was washed twice, adjusted to a pH of 7.0, and then freeze dried. The resulting dry powder was referred to as rice bran protein concentrates (RBPC).

In order to investigate the optimal extraction time (2, 3, 4, and 5 h), the solid-liquid ratio (1.0:10) and enzyme concentration (5000 units) were fixed. Additionally, to optimize the concentration of enzymes (2500, 5000, 10,000, and 15,000 units), other variables were fixed at the level that provided the highest protein recovery (1) and protein yield (2). Protein recovery and yield were calculated by the following equations. The final condition was used to produce RBPC for the next experiment.

$$\text{Protein recovery } (\%) = [(\text{Protein content in RBPC} \times \text{Gram of RBPC})/(\text{Protein content in DFRB} \times \text{Gram of DFRB})] \times 100 \quad (1)$$

$$\text{Protein yield } (\%) = (\text{Gram of protein powder obtained}/\text{Gram of DFRB used}) \times 100 \quad (2)$$

2.3. Alkaline extraction of rice bran protein

DFRB was mixed with distilled water (1:10, w/v), and then the pH was adjusted to 10 using 3 M sodium carbonate. The slurry was stirred using a stirrer at room temperature (25 °C) for 1 h. After centrifugation at 10,000*g*, 4 °C for 10 min, the supernatant was collected and adjusted to a pH of 4.5 using 3 M citric acid and centrifuged at the condition mentioned above. The precipitate was collected and adjusted to a pH of 7.0 and then freeze dried. The obtained powder was referred to as the "rice bran protein concentrate: RBPC"

2.4. In vitro digestion

The digestion of RBPC under *in vitro* gastrointestinal digestion (pepsin-trypsin system) was performed following the method of Wang et al. (2010) with some modification. Pepsin (enzyme: protein of 1:100, w/w) was added into the RBPC dispersions (pH 1.5, 1%, w/v), and the mixture was gently shaken at 37 °C for 120 min in an incubator shaker (IKA® Werke GmbH & Co. KG, Germany). The mixture was then neutralized with 1.0 M NaOH to terminate the enzyme activity. After, trypsin was added into the pepsin-digested mixture (enzyme: protein of 1:100, w/w). Following incubation at 37 °C for 120 min, the protein dispersion was heated at 95 °C for 10 min to stop the trypsin digestion. The slurries from pepsin and pepsin-trypsin digestions were centrifuged at 3,000 rpm for 10 min at room temperature. The supernatants were collected and dried by using a freeze dryer. The dried fractions, or