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Purification and Evaluation of Rice Bran Hydrolysates with Antimutagenicity

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Abstract A 3% suspension of heat-stabilized defatted rice bran was treated with papain, followed by inactivating the enzyme by heat, and centrifuged. The supernatant was subjected to ultrafiltration, and fractions with various molecular sizes, F1 (>30 kDa), F2 (10-30 kDa), F3 (5-10 kDa), F4 (3-5 kDa), and F5 (3 kDa<), were freeze-dried, and evaluated for antimutagenicity by Ames test using *Salmonella typhimurium* TA 100 against phenazine methosulfate. The F3 fraction containing highest antimutagenicity from ultrafiltration was separated into 6 fractions by DEAE-Sephadex A-25 ion-exchange column chromatography (F3-1–F3-6). Each fractions having protein contents were pooled, dialyzed, freeze dried, and evaluated for antimutagenicity. Among the six fractions, the F3-1, F3-2, and F3-6 fractions showed antimutagenicity, which were 80.2, 53.4, and 58.6% at concentration of 100 μg/plate, respectively. These F3-1, F3-2, and F3-6 fractions were subjected to Sephadex G-50 gel filtration column chromatography for further purification. Among the purified fractions, the F3-1-1, F3-2-2, and F3-6-1 fractions showed antimutagenicity of 84.5, 58.6, and 69.8% at concentration of 100 μg/plate, respectively. It is thought that these peptides can find application for nutraceutical and pharmaceutical products.

Keywords: rice bran hydrolysate, antimutagenic activity, ultrafiltration, papain

Introduction

Rice bran is an under-utilized milling co-product of rough rice. In 2005, approximately 223 million cwt of rough rice was produced in USA, resulting in about 22.3 million cwt of rice bran (1). The main components of rice bran are oil, protein, and carbohydrates. Rice bran protein has unique nutritional value, nutraceutical (2, 3) and functional properties (4). Its nutritional value is much higher than rice endosperm protein or protein from other cereals or legumes (5). Rice bran protein is also highly digestible (6). In addition to the high nutritional value, rice bran protein is a hypoallergenic food ingredient (7) and has anti-cancer activity (8). Rice bran undergoes heat treatment to preserve oil quality. The co-product of the oil extraction is the heat-stabilized defatted rice bran (HDRB). The HDRB costs approximately 2-4 cents/lb and has about 18-22% high quality protein (9). Value can be significantly enhanced to HDRB by producing biopeptides.

In the last decade in particular, interest in peptides derived from food proteins that have a bioactive role, such as lowering high blood pressure, anti tumor, and anti-microbials has grown considerably. Vis *et al.* (10) found that the ovalbumin (a major egg protein) hydrolysate had strong antimutagenicity. Naturally occurring antimutagens and anticarcinogens can be found in fresh fruits and vegetables, fermented food (11), and soybean pastes fermented by *Bacillus* strains (12). Shin and Lee (13) reported that commercial marinades also have antimutagenicity. It was reported that glycoproteins purified from a culture supernatant of lactic acid bacteria (LAB) showed strong antimutagenicity (14). Peptides in food can be formed during processing specially by fermentation and some of these peptides have intense biological activities. It was reported that high consumption of *miso* (Japanese fermented soypaste) decreased the rate of death from the incidence of stomach cancer (15). The peptides produced from *Lactobacillus bulgaricus* were reported to have anti-tumor activity (16). Abdelali *et al.* (17) reported that peptides/proteins from dairy products have very high antimutagenicity. Several peptides isolated from African frog, *Streptomyces*, and *Micromonospora* appeared to have not only anticancer but also antimicrobial activities (18). Peptides derived from hydrolysates of soybean proteins, soy sauce, and cheese slurries also exhibited anticancer activities (19).

The objectives of this study were to produce and purify peptides from HDRB, and evaluate peptides for antimutagenic activity.

Materials and Methods

Materials Heat-stabilized defatted rice bran (HDRB) was provided by Riceland Food Co. (Stuttgart, AR, USA). Food-grade papain W-40 was obtained from Amano Pharmaceutical Co., Ltd. (Nagoya, Japan). DEAE-Sephadex A-25 and Sephadex G-50 were purchased from Amersham Pharmacia Biotech Corporation (Uppsala, Sweden). The histidine-requiring strain of *Salmonella typhimurium* TA 100 (Xenomatrix Inc., San Diego, CA, USA) was used for the antimutagenic test. All growth ingredients used for of *S. typhimurium* TA 100 were

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purchased from Difco Lab. (Detroit, MI, USA). S9 mix from the liver of drug-treated Sprague-Dawley rats (male) used for metabolic activation in the Ames test was purchased from Kikkoman (Noda, Japan). All other chemicals used for antimutagenic test were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Enzymatic hydrolysis of HDRB A 3%(w/v) suspension of HDRB was prepared in distilled water (150 mL) and the pH adjusted to 8.0 with 1.0 M NaOH solution. The HDRB suspension was pre-warmed to 37°C and 18,000 units of papain added with shaking at 200 rpm. The sample was hydrolyzed at 37°C for 6 hr while pH was maintained constant by addition of 1.0 M NaOH solution when necessary. Following hydrolysis, the enzyme was inactivated by heat at 95°C for 3 min. Hydrolysates were centrifuged at 10,000×g for 30 min at 5°C, and the supernatant was subjected to fractionation.

Fractionation of HDRB hydrolysates by ultrafiltration HDRB hydrolysates were fractionated by ultrafiltration (A/G Tech. Co., Needham, MA, USA) through a series of spiral membranes of decreasing pore size. Hydrolysates were first passed through a 30 kDa molecular weight cut-off (MWCO) membrane. Permeates were then pumped subsequently through a 10, 5, and 3 kDa MWCO membrane. Fractions obtained were designated as F1 (>30 kDa), F2 (10-30 kDa), F3 (5-10 kDa), F4 (3-5 kDa), and F5 (3 kDa<). The fractions were dialyzed, freeze-dried, and evaluated for antimutagenic activity.

Purification of HDRB hydrolysates The ultrafiltrated fractions that demonstrated high antimutagenic activities were selected and purified on a DEAE-Sephadex A-25 (2.6×30 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 8.0). The column was then washed with the same buffer, followed by a 600 mL linear NaCl gradient (0–1.0 M) in the same buffer. Fractions of 5 mL/tube were collected using a fraction collector (Pharmacia Co., Uppsala, Sweden) at a flow rate of 0.85 mL/min, and monitored for absorption at 280 nm to determine protein content. Six fractions that showed protein contents were pooled, dialyzed, freeze dried, and then evaluated for antimutagenic activity. The three fractions out of six that demonstrated higher antimutagenic activity were further purified on a Sephadex G-50 (1.8×80 cm) equilibrated with the same buffer. Fractions were eluted at a flow rate of 0.66 mL/min to collect 5 mL/tube. Six fractions obtained through Sephadex G-50 were pooled, dialyzed, freeze dried, and evaluated for antimutagenic activity. The amounts of protein in each fraction collected were determined by the method of Bradford (20).

Mutagenicity and antimutagenicity testing in protein hydrolysates The antimutagenic effect of hydrolysates was assayed by Ames *et al.* (21) with slight modification. The histidine requiring stains of *S. typhimurium* TA100 were used for this test. For antimutagenic activity test, a final volume of 1 mL in a capped glass tube containing 50 μL of each fraction, 50 μL of phenazine methosulfate solution (PMS; mutagen, 200 μg/mL in dimethyl sulfoxide), 100 μL of an overnight culture of *S. typhimurium* TA 100 (10^8 CFU) cells, 300 μL of a 0.2 M sodium phosphate buffer (pH 7.4), and 500 μL of 4% S9 was used. The contents in the tube were mixed, capped, and pre-incubated at 37°C for 30 min with shacking at 200 rpm. Following incubation, an aliquot (250 μL) was added to the 3.0 mL of soft agar containing 0.05 mM histidine/biotin and plated on a minimal agar plate (Difco Lab.). After incubation for 48 hr at 37°C, the number of CFU was counted. The percentage inhibition of mutagenesis was calculated using the following equation: inhibition %=[(A-B)/(A-C)]×100, where A is the number of His^+ revertants induced by a mutagen in the absence of a sample, B is the number of His^+ revertants induced by a mutagen in the presence of a sample, and C is the number of spontaneous revertants in the absence of a mutagen. For the mutagenic test of the purified fractions, 50 μL of distilled water was used instead of the mutagen solution and all the other procedures were essentially the same as those described above. All the data represent the average of at least three experiments that were performed in triplicate.

Statistical analysis The data from the test for mutagenicity or antimutagenicity were analyzed by statistical comparisons of all pairs using Student's *t* test following one-way analysis of the variance in the Statistical Analysis System (SAS8.2; SAS Institute, Cary, NC, USA) (22). Statistical differences were determined at $p<0.05$.

Results and Discussion

The dose-response curve of PMS mutagenicity in test strain *S. typhimurium* TA100 is shown in Fig. 1. The range of dose tested was from 0 to 20 mg/plate. There was a linearly proportional dose-response relationship up to 10 mg/plate, so 10 mg/plate was chosen for antimutagenicity assay. The five fractions prepared from papain-treated HDRB by ultrafiltration were designated as F1 (>30 kDa), F2 (10-30 kDa), F3 (5-10 kDa), F4 (3-5 kDa), and F5 (<3 kDa). All five fractions from ultrafiltration were neither

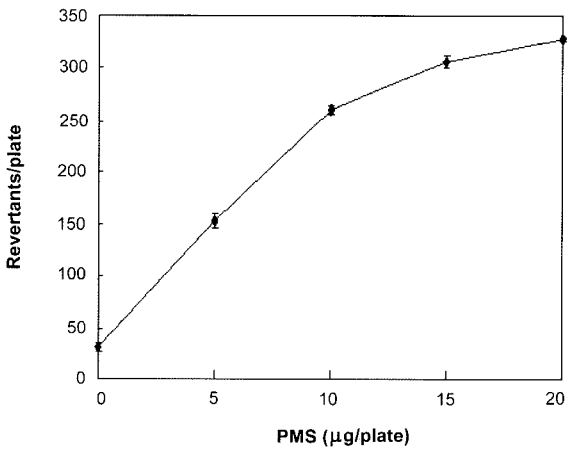

Fig. 1. Dose-response curve of PMS mutagenicity in *S. typhimurium* TA100. Each datum point is the average of 3 independent experiments ±SEM. Each dose was tested in triplicate for each experiment.

toxic nor mutagenic to *S. typhimurium* TA100, because the means from samples were not significantly different ($p>0.05$) from the mean of the negative control (Table 1). Although several proteins and peptides derived from food and lactic acid bacterial metabolites containing antimutagenicity have been reported (23, 24), there has been no report on rice bran hydrolysates. Until now, the mechanism of antimutagenic activity is not clear. Inhibition might occur either by physical binding or chemical reactions between peptides and mutagens. van Boekel *et al.* (25) concluded that limited proteolysis increased the number of reaction site between peptides and mutagens, and Vis *et al.* (10) reported that the exposure of inner protein groups by denaturation or hydrolysis can increase the antimutagenic capacity.

The antimutagenic activity of the ultrafiltrated fractions (500 μg/plate) on *S. typhimurium* TA100 against PMS (10 μg/plate) is shown in Table 2. Matar *et al.* (26) demonstrated that the smaller peptides from milk casein had stronger antimutagenic activities. Among five fractions from ultrafiltration, only the F3 (5-10 kDa) fraction showed the highest antimutagenic activity of 54.9%, while the antimutagenicity of F4 (3-5 kDa) and F5 (<3 kDa) fractions were 22.1, and 23.9%, respectively. The F3 fraction containing high antimutagenic activity was selected for further purification. The protein contents of each fraction through the purification steps are shown in Table 3. The protein contents of F3-1-1, F3-1-2, F3-2-1, F3-2-2, F3-6-1, and F3-6-2 were 93.2, 91.1, 90.5, 91.2, 93.3, and 90.6%, respectively (Table 3). The purified

Table 1. Effect of varying concentration of hydrolyzed rice bran fractions for mutagenic activities on *S. typhimurium* TA100[1)]

Fraction	Dose (μg/plate)	*His*$^+$ revertants/plate
F1	100	13±1.58[a2)]
	200	12±1.14[a]
	300	10±1.14[a]
	400	10±0.71[a]
	500	9±1.09[a]
F2	100	12±1.41[a]
	200	10±1.14[a]
	300	10±0.71[a]
	400	10±1.03[a]
	500	10±1.11[a]
F3	100	10±0.37[a]
	200	10±1.02[a]
	300	10±1.39[a]
	400	8±0.32[a]
	500	7±0.71[a]
F4	100	11±1.14[a]
	200	10±0.63[a]
	300	10±0.63[a]
	400	9±0.84[a]
	500	8±0.84[a]
F5	100	12±0.63[a]
	200	10±0.84[a]
	300	11±0.55[a]
	400	8±0.55[a]
	500	8±0.66[a]
Negative control		13±1.58[a]
Positive control		130±3.54[b]

[1)]The positive and negative controls indicate the number of CFU for *His*$^+$ revertants in the presence and absence of PMS, respectively.
[2)]Means within a column followed by the same letter are not significantly different ($p>0.05$).

Table 2. Antimutagenic activities of ultrafiltrated fractions of papain treated rice bran against PMS on *S. typhimurium* TA100[1)]

Fraction	Dose (μg/plate)	*His*$^+$ revertants / plate	Antimutagenic activity (%)
F1	500	117±1.41[a2)]	7.1
F2	500	110±1.84[b]	13.3
F3	500	63±1.41[c]	54.9
F4	500	100±3.18[d]	22.1
F5	500	98±2.70[d]	23.9
Negative control		12±1.41[e]	
Positive control		125±3.54[f]	

[1)]The positive and negative controls indicate the number of CFU for *His*$^+$ revertants in the presence and absence of PMS, respectively.
[2)]Means within a column followed by the same letter are not significantly different ($p>0.05$).

Table 3. Protein content and recovery of ultrafiltered, and column purified rice bran protein hydrolysates[1)]

Purification steps	Fraction	Protein (%)	Protein recovery (%)
HDRB		18.1	100
Ultrafiltration	F1	25.1	15.3
	F2	56.3	30.6
	F3	65.2	22.7
	F4	43.5	12.5
	F5	43.1	10.5
DEAE Sephadex A-50	F3-1	85.6	5.1
	F3-2	83.5	3.8
	F3-6	82.7	4.7
Sephadex G-50	F3-1-1	93.2	3.5
	F3-1-2	91.1	1.7
	F3-2-1	90.5	1.3
	F3-2-2	91.2	2.1
	F3-6-1	93.3	3.2
	F3-6-2	90.6	0.8

[1)]The data are significantly different from data of the control at a p value of <0.05.

fractions were tested for antimutagenic activity on *S. typhimurium* TA 100 against phenazine methosulfate using various amounts of up to 100 μg/plate (Table 4). The F3 fraction was subjected to DEAE-Sephadex A-25 ion-exchange column chromatography, and evaluated for anti-mutagenicity (Fig. 2). The F3 fraction was separated into six fractions containing protein (F3-1 through F3-6). Only three fractions (F3-1, F3-2, and F3-6) showed anti-mutagenic activities which were 80.2, 53.4, and 58.6% at concentration of 100 μg/plate, respectively (Table 4). The three fractions were further subjected to gel filtration on a Sephpadex G-50 column for further purification (Fig. 3). The fractions were designated F3-1-1, F3-1-2, F3-2-1, F3-2-2, F3-6-1, and F3-6-2 and analyzed for anti-mutagenicity (Fig. 3). Among these fractions, F3-1-1, F3-2-2, and F3-6-1 showed antimutagenic activity. The anti-mutagenic activities of F3-1-1, F3-2-2, and F3-6-1 were 84.5, 58.6, 69.8% at concentration of 100 μg/plate, respectively (Table 4). Although the antimutagenic activity of each fraction was the highest at concentration of 100 μg/plate, the antimutagenic activity of each fraction at concentration of more than 40 μg/plate was shown to have no significant difference ($p>0.05$). Purified peptides demonstrated higher antimutagenic activities (Table 4). The peptides from HDRB hydrolysis showed stronger antimutagenicity than the peptides from milk casein (25, 27).

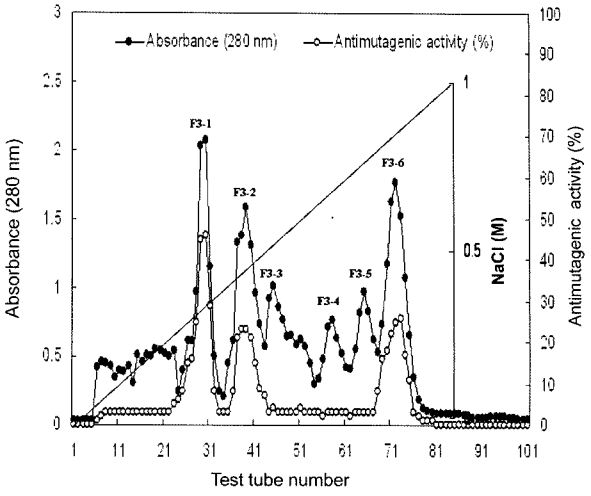

Fig. 2. DEAE-Sephadex A-25 ion-exchange column chromatogram of rice bran hydrolysate fractions (F3-1 - F3-6 obtained from F3) and their corresponding antimutagenic activity. (Test tube # 25 to 31 for F3-1; test tube # 35 to 43 for F3-2; test tube # 68 to 74 for F3-6).

Rice bran protein hydrolysates possess significant anti-mutagenic activity against phenazine methosulfate. Purification of rice bran protein hydrolysates using DEAE-

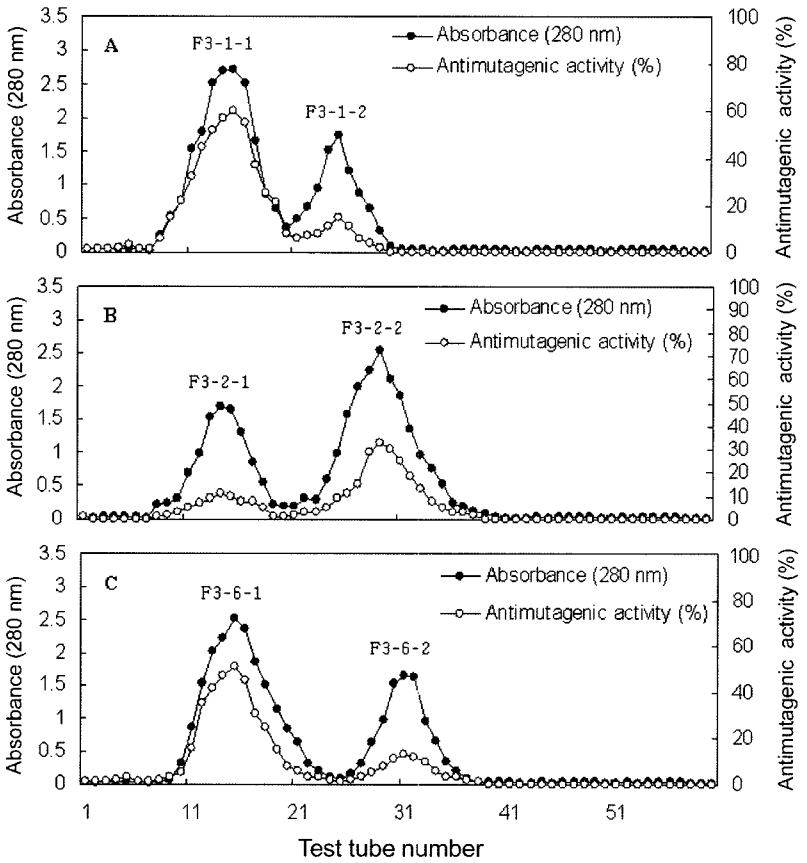

Fig. 3. Sephadex G-50 gel filtration column chromatograms of rice bran hydrolysate fractions and their corresponding antimutagenic activity. (Test tube # 9 to 20 for F3-1-1 from F3-1, A; test tube # 24 to 35 for F3-2-2 from F3-2, B; test tube # 11 to 21for F3-6-1 from F3-6, C.) A, B, and C represent samples applied from DEAE Sephadex A-25 column.

Table 4. Antimutagenic activities of the purified fractions against PMS on *S. typhimurium* TA100[1)]

Fraction	Dose (μg/plate)	*His*$^+$ revertants/plate	Antimutagenic activity (%)
F3-1	10	69±3.16[a2)]	53.4
	40	43±2.21[b]	75.9
	70	39±2.02[b]	79.3
	100	38±2.86[b]	80.2
F3-2	10	90±4.28[a]	35.3
	40	75±2.77[b]	48.3
	70	71±3.65[b]	51.7
	100	69±2.45[b]	53.4
F3-6	10	83±3.96[a]	41.4
	40	67±2.77[b]	55.2
	70	64±3.21[b]	57.8
	100	63±2.43[b]	58.6
F3-1-1	10	61±2.12[a]	60.3
	40	36±2.77[b]	81.9
	70	34±2.47[b]	83.6
	100	33±1.64[b]	84.5
F3-2-2	10	86±2.41[a]	38.8
	40	69±3.05[b]	53.4
	70	65±2.77[b]	56.9
	100	63±2.51[b]	58.6
F3-6-1	10	73±2.83[a]	50.0
	40	58±2.30[b]	62.9
	70	52±2.51[b]	68.1
	100	50±3.71[b]	69.8
Negative control		15±1.64	
Positive control		131±4.65	

[1)]The positive and negative controls indicate the number of CFU for *His*$^+$ revertants in the presence and absence of PMS, respectively.
[2)]Means within a same fraction followed by the same letter are not significantly different (p>0.05).

Sephadex A-25 column chromatography and Sephadex G-50 gel filtration column chromatography increased the protein content and antimutagenic activity. The F3-1-1 fraction gave the maximum antimutagenic activity. These peptides can find application in nutraceutical and pharmaceutical products.

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