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Purification and identification of a novel heteropolysaccharide RBPS2a with anti-complementary activity from defatted rice bran

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Abstract

A novel heteropolysaccharide RBPS2a with anti-complementary activity was obtained from defatted rice bran by hot water extraction, ethanol precipitation, and purified by gel chromatography after anion-exchange chromatography. This fraction exhibited more potent anti-complementary activity than other polysaccharide fractions. RBPS2a was eluted as a single symmetrical narrow peak on high-performance gel-permeation chromatography (HPGPC) and the average molecular weight was 90,000 Da. We found RBPS2a contained 86.7% polysaccharide and 8.7% protein. The amino acid pattern showed that RBPS2a contained large amount of glutamic acid, arginine, aspartic acid, lysine, and alanine. The molar content of the above five amino acids constituted 59.31% of the total amino acids. Gas chromatography of absolute acid hydrolysate of RBPS2a suggested that it was composed of arabinose, xylose, glucose and galactose with a molar ratio of 4:2:1:4. The Fourier-transform infrared spectra (FT-IR) and ^{1}H, ^{13}C NMR spectroscopy analysis revealed that RBPS2a had a backbone consisting of β-(1→3)-linked D-galacopyranosyl residues substituted at O-2 with glycosyl residues composed of α-D-xylose-(1→4)-α-D-arabinose-(1→ and α-D-glucose-(1→4)-α-D-arabinose-(1→ linked residues. Furthermore, some of the fractions extracted and purified from defatted rice bran exhibited strong anti-complementary activity. Among these fractions, the purified polysaccharide RBPS2a had the highest activity.

Keywords: Rice bran; Heteropolysaccharide; Anti-complementary activity

1. Introduction

Rice is consumed in its polished form as a staple food in many countries. Rice bran is the outer layer of brown rice, obtained as a by-product of the rice milling industry. It has been under-utilized as a human food and has traditionally been used primarily in feeds (Spears, Grieshop, & Fahey, 2004). Rice bran is a good source of protein, fat and carbohydrates, in addition to several valuable phytonutrients, antioxidants, vitamins and minerals (Asaf, Basil, Winston, & Larry, 1997; Gurpreet & Sogi, 2007; Orthoefer, 1996; Qureshi, Mo, Packer, & Peterson, 2000). Studies have repeatedly shown that rice bran gives interesting health benefits (Azizah & Yu, 2000; Juan et al., 2006).

Rice bran polysaccharides have drawn the attention of chemists and immunologists in recent years because of the immune-modulation and anti-tumour properties. Many biologically active polysaccharides extracted from rice bran appeared to elicit excellent physiological properties in maintaining health and preventing diseases. For example, an arabinogalactan isolated from rice bran has anti-tumour properties in gastrointestinal carcinoma and colon cancer (Cummings, Bingham, Heaton, & Eastwood, 1992; Takeshita et al., 1992). Rice bran hemicelluloses have a

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significant effect in increasing the peripheral blood lymphocytes (Takenaka & Itoyama, 1993), enhancing the immune function (Tzianabos, 2000), reducing thymus atrophy in rats (Takenaka, 1992), and improving the peripheral utilization of insulin (Hikino, Takahashi, Oshima, & Konno, 1988). MGN-3, a modified arabinoxylane from rice bran is a potent biological response modifier that is able to enhance natural killer (NK) cell activity in cancer patients, to increase T and B cell mitogen response upon ingestion, and has a strong anti-HIV activity (Ghoneum, 1998; Ghoneum & Gollapudi, 2003).

Anti-complementary activity is an immune-modulating activity, which is responsible to the immunological defense system. The complement system plays an important role in the host defense against foreign invasive organisms such as bacteria, fungi, and viruses (Yamagishi, Tsuboi, & Kikuchi, 2003). However, some smaller molecules involved complementary cascade could induce the release of mediators from mast cells and lymphocytes (Na, Byung, Hyeong, Jong, & Jae, 2005). Aberrant activation of these molecules may cause a variety of inflammatory diseases (Vogt, 1985). Therefore, the ability to modulate complement activity would clearly be beneficial in the therapy of inflammatory diseases. Data published revealed that many kinds of polysaccharides have shown strong capability on anti-complementary (Samuelsen, Paulsen, Wold, Knutsen, & Yamada, 1998; Zhang, Kiyohara, Matsumoto, & Yamada, 1997). It has been reported that polysaccharides from different resource have different anti-complementary activities in vitro, depending on their structural features. However, little attempt has been made to study the polysaccharides isolated from the defatted rice bran and their anti-complementary activity.

The aim of our study was to investigate the separation, bioactivity-directed fractionation, purification and identification of heteropolysaccharide RBPS2a. The anti-complementary activities of these major polysaccharide fractions isolated from defatted rice bran were also evaluated.

2. Materials and methods

2.1. Materials

Defatted rice bran was obtained from Hangzhou Zhonggu Grain & Oil Co., Ltd. (Zhejiang Province, China). It was squeezed and ground, pass through 1 mm sieve, and then stored at 4 °C. Bovine serum albumin, glucuronic acid and all the sugar standards were purchased from Sigma Chemical Company (USA). α-Amylase was obtained from Novozymes Company (Denmark). Sheep erythrocytes and Rabbit anti-sheep erythrocyte antibodies were obtained from Wuxi Huisheng Regent Company (Jiangsu Province, China). The other chemicals were analytical grade and purchased from Sinopharm Chemical Regent Company (China). Normal human serum (NHS) was obtained from healthy adults.

2.2. Extraction of polysaccharide

Defatted rice bran was extracted with water in a ratio of 1:15 (w/v) at 90 °C for 2 h and retreated two more times. The extracts were centrifuged at 4000*g* for 20 min. The supernatant was concentrated in a vacuum rotary evaporator at 45 °C. The proteins and starch in the extract were removed using the Sevage reagent and α-amylase, and then three volumes of 95% ethanol were added to the extract for 24 h at 4 °C with vigorous stirring. The precipitate was collected by centrifugation at 8000*g* for 10 min and dissolved with distilled water. Then the soluble part was freeze-dried. Water-soluble crude polysaccharide RBPS was obtained.

2.3. Separation and purification of the polysaccharides

Sample RBPS was dissolved in distilled water and left overnight, the soluble part was separated by a Q-Sepharose big beads anionic resin (Pharmacia AP, Sweden) column (45 × 3 cm, i.d.). Stepwise elution was done with a discontinuous gradient of water, 0.3 and 0.5 M NaCl at pH 7.0 and the flow rate was 1.5 ml/min. Nine millilitre fractions was collected in one tube and the absorbance at 280 nm for protein and 490 nm for carbohydrate using UV detector and phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Pebers, & Smith, 1956) were monitored respectively.

Four fractions, RBPS1, RBPS2, RBPS3 and RBPS4 were collected, dialyzed against deionised water (molecular weight cut-off 3500 Da) and lyophilized. The main anti-complementary activity was detected in the fraction RBPS2. Further purification of RBPS2 was implemented with gel filtration by a Sepharose CL-6B (Pharmacia AP, Sweden). Two fractions of RBPS2a and RBPS2b were separated and then lyophilized for anti-complementary activity test and the subsequent structural analysis.

2.4. Homogeneity and M_rs

The molecular weight of RBPS2a was determined by HPGPC (high-performance gel-permeation chromatography) with a Waters HPLC system, including two serially linked Ultrahydrogel™ Linear column (300 mm × 7.8 mm i.d. × 2), a waters 2410 interferometric refractometer detector and an on-line degaser. The sample was eluted with 0.1 M sodium acetate buffer at a rate of 0.9 ml/min. The column was calibrated with standard T-series Dextran (T-2000, T-580, T-190, T-70, T-10, Sigma). Data were analyzed with Millennium 32 Software (Waters, USA).

2.5. Protein contents and analysis of hydrolyzed amino acid

The protein content of protein-bound polysaccharide RBPS2a was measured by the method of Lowry (Lowry, Rosebrough, Farr, & Randall, 1951), using bovine serum albumin as the standard. The protein concentration of column fractions was determined by measuring the absorption

at 280 nm. Amino Acids in RBPS2a were determined with a Hitachi 835-50G automatic amino acid analyzer (Hitachi Ltd., Tokyo, Japan). The hydrolysis of RBPS2a was done in a sealed ampoule for 24 h at 110 °C using 1 ml of 6 M HCl solution under vacuum. The hydrolysate was evaporated and then the dried residue dissolved in 0.02 M HCl. The sample was filtered through a 0.45 μm nylon filter before being injected into the amino acid analyzer.

2.6. Fourier-transform infrared spectra (FT-IR)

Nicolet Nexus FT-IR spectrometer was used for detecting functional groups. The purified polysaccharide RBPS2a was ground with KBr (spectroscopic grade) powder and then pressed into pellets for FT-IR measurement in the frequency ranging from 4000 to 400 cm^{-1}, FT-IR spectra of the materials were obtained at a resolution of 8 cm^{-1} (Kumar, Joo, Chio, Koo, & Chang, 2004).

2.7. Analysis of monosaccharide compositions and total carbohydrate

Total carbohydrate of RBPS2a was measured according to the phenol-sulphuric acid method mentioned above. The uronic acid content was determined by measuring the absorbance at 525 nm using *m*-hydroxybiphenyl colorimetric procedure and with glucuronic acid as the standard (Blumenkrantz & Asboe, 1973).

The polysaccharide RBPS2a (10 mg) was dissolved in 10 ml of 2 M trifluoroacetic acid (TFA) and hydrolyzed at 120 °C for 6 h in a sealed glass tube. The solution was then evaporated and then addition of methanol to the residue. Derivation was then analyzed according to the method of Guentas et al. (2001). Alditol acetates of the reduced sugars and authentic standards (glucose, mannose, galactose, xylose, rhamnose and arabinose with inositol as the internal standard) were prepared with AC_2O at 100 °C. The derivatives were analyzed by gas chromatography (Shimadzu GC-2010, Japan) equipped with OV1701 capillary column (30 m × 0.32 mm i.d.) and a flame-ionization detector (FID). The operation was done using the following conditions: H_2: 47 ml/min; air: 400 ml/min; N_2: 10 ml/min; temperature was programmed: 120–190 °C (10 °C/min) to 240 °C (3 °C/min). High-purity helium was used as the carrier gas. The products were identified by their characteristic retention times. The percentage of monosaccharide in the sample was calculated from the peak areas using response factors.

2.8. ^1H, ^{13}C NMR

^1H, ^{13}C NMR spectra were recorded in D_2O with a Buker Avance 500 NMR spectrometer. The chemical shifts were expressed in ppm relative to the response of the internal standard, 3-trimethylsily-1-propaneulfoic acid (sodium salt).

2.9. Anti-complementary activity

The in vitro anti-complementary activity was determined by the complement fixation test based on complementary consumption and degree of red blood cell lysis by the residual complement (Meyer, 1961). Sheep erythrocytes were washed twice with 150 mM NaCl and once with gelatin veronal buffer (pH 7.4, containing 0.1% gelatin, 141 μM NaCl, 500 μM $MgCl_2$, 150 μM $CaCl_2$, and 1.8 mM sodium barbital [GVB^{2+}]) and sensitized with rabbit anti-sheep erythrocyte antibodies. The cells were incubated at 37 °C for 30 min on a shaker, and then washed as described above. A 1% cell suspension in GVB^{2+} was prepared and stored at 4 °C until use the same day.

In the test for anti-complementary activity the polysaccharide sample was incubated with human serum that contains intact complement proteins. The serum used was diluted to a concentration giving 50% lysis of the indicator cells, which were antibody-sensitized sheep erythrocytes. Various dilutions of the sample in water (50 μg) were mixed with equal volume of normal human serum (NHS) and GVB^{2+}. The mixtures were incubated at 37 °C for 30 min and the residual total complement hemolysis (TCH_{50}) was determined by a method using IgM-hemolysin-sensitized sheep erythrocytes at 1×10^8 cells/ml. The NHS was incubated with distilled water and GVB^{2+} to provide a control ($A_{control}$). The anti-complementary activity (%) is calculated from the formula:

$$\left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100\%$$

2.10. Data handling

Data were expressed as means ± standard deviations of three replicated determinations. Originpro (Version 7.5, Origin Lab Inc.) statistical computer software was used for data analysis.

3. Results and discussion

3.1. Separation and purification of polysaccharides

The result showed that separation of the water extract from defatted rice bran with anion-exchange chromatography on Q-Sepharose big beads yielded four sub-fractions RBPS1, RBPS2, RBPS3 and RBPS4 (Fig. 1). RBPS2 fraction demonstrated higher activity in anti-complementary test than other fractions (Fig. 2). Therefore, further purification was performed by applying RBPS2 to gel filtration on Sepharose CL-6B to obtain two sub-fractions, RBPS2a and RBPS2b (Fig. 3). The former sub-fraction showed highest activity in anti-complementary test (Fig. 4), Hence, RBPS2a was collected for further identification of structure.

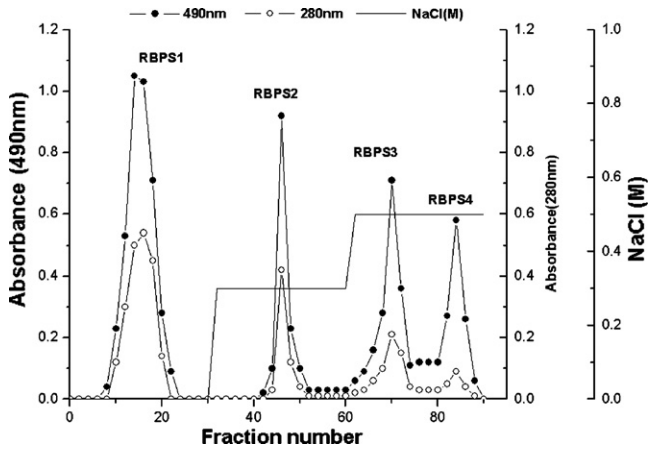

Fig. 1. Anion-exchange chromatography of RBPS on Q-Sepharose big beads. The column was eluted stepwise with H_2O, 0.3 and 0.5 M NaCl solutions.

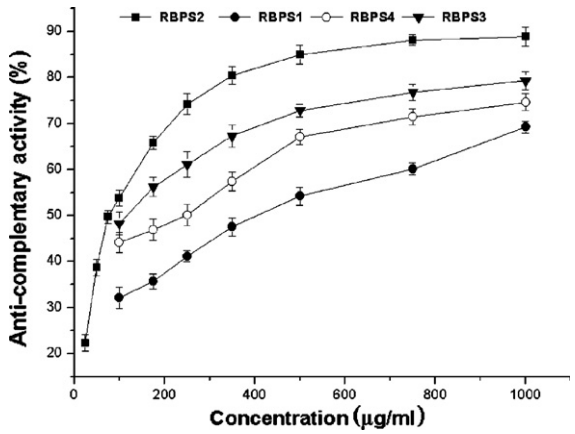

Fig. 2. The anti-complementary activity of polysaccharide fractions RBPS1, RBPS2, RBPS3 and RBPS4 obtained after ion exchange chromatography.

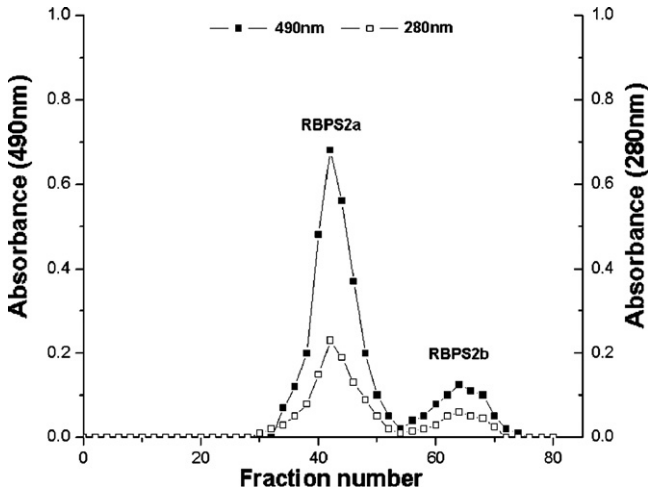

Fig. 3. Gel filtration anion-exchange chromatography of RBPS2 on Sepharose CL-6B column by elution with 0.05 M phosphate buffer.

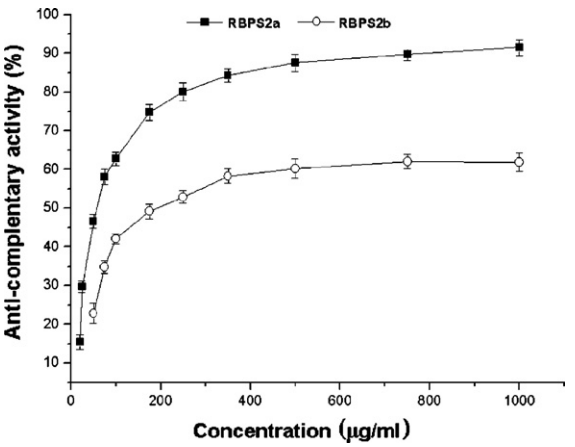

Fig. 4. The anti-complementary activity of polysaccharide fractions RBPS2a and RBPS2b obtained after gel chromatography of RBPS2.

3.2. Identification of the purified polysaccharide

The molecular weight of the polysaccharide was determined by HPGPC. RBPS2a could be eluted as a single peak and its apparent molecular weight was estimated as 90,000 Da. Furthermore, this result revealed that RBPS2a might be a homogeneous polysaccharide on the basis of its molecular weight and polarity. RBPS2a contained approximated 86.7% polysaccharide according to phenol-sulphuric acid method, 2.3% uronic acid residue and 7.8% protein conforming that this was a hetero-polysaccharide.

The composition and relative percentages of hydrolyzed amino acids is shown in Table 1. RBPS2a was rich in glutamic acid, arginine, aspartic acid, lysine and alanine. The molar content of the above five amino acids constituted 59.31% of the total amino acids. Besides these amino acids, the molar ration of leucine and histidine was also fairly high, having 4.86% and 4.75%, respectively. GC analysis

Table 1
Amino acid composition of RBPS2a

Amino acid	Composition (mol%)
Aspartic acid	8.17
Glutamic acid	25.90
Serine	4.29
Histidine	4.75
Glycine	4.3
Threonine	4.45
Alanine	5.11
Arginine	14.8
Tyrosine	2.66
Cystine	3.60
Valine	3.50
Methionine	2.03
Phenylalanine	1.96
Isoleucine	1.67
Leucine	4.86
Lysine	5.33
Proline	2.67

showed that RBPS2a was composed of arabinose, xylose, glucose and galactose in the molar ration of 4:2:1:4.

To determine the functional groups of the purified heteropolysaccharide RBPS2a, FT-IR was used. The IR spectra of RBPS2a were shown in Fig. 5. A broad stretching intense characteristic peak at approximately the region of 3420 cm^{-1} and a weak C–H stretching band at around 2937 cm^{-1} are suggested the hydroxyl group (Santhiya, Subramanian, & Natarajan, 2002), C═O vibration in the carbonyl group at 1650 cm^{-1} was hydrogen bonded. The bands approximately in the regain of 3400, 2930 and 1650 cm^{-1} are characteristic of a carbohydrate ring (Gi, Hyung, Byong, Sang, & Jae, 2003). The absorption of 860 cm^{-1} indicated the α-glycosidic linkages of the glycosyl residues (Tsumuraya & Misaki, 1979). Two stretching peaks at 1076 and 1162 cm^{-1} indicated the presence of C–O bonds. Protein structures could also be represented by the absorption at 1258 cm^{-1}, N–H vibration generally expected at 3400 cm^{-1} could be overlapped by OH stretch vibration at 3420 cm^{-1}. It was suggested that the purified fraction RBPS2a was proteoglycan.

The ^{13}C NMR spectrum of RBPS2a and its chemical shift data (Table 2) showed that it contained carbohydrate and protein, with one major and five minor C-1 signals, a CO2-6 signal at 177.7 ppm. The resonances in the region of 96–110.7 ppm were attributed to the anomeric carbon atoms of arabinopyranose (Ara*p*), galactopyranose (Gla*p*), glucopyranose (Glc*p*) and xylpyranose (Xyl*p*), respectively. In the low field region (178 ppm), minor typical signal was observed for the carboxyl group of the uronic acid units, which was in agreement with the result from *m*-hydroxydiphenyl colorimetric method. The anomeric carbon sign of both α and β configurations were detected at 100 and 104 ppm, respectively. The broad C-3 signal in the region at 81.4 ppm could be ascribed to the presence in the polysaccharide of linear D-(1,3) and terminal D-residues (Gi et al., 2003). In the proton spectrum, the four signals in the region of 4.91–5.76 ppm were assigned to anomeric protons of α-D-xyl*p*, α-D-Ara*p*, α-D-Glc*p* and β-D-Gal*p*

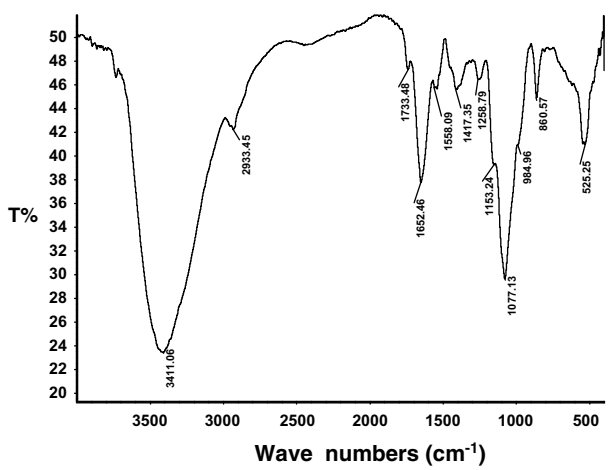

Fig. 5. Fourier-transform infrared spectra of RBPS2a.

Table 2
^{13}C NMR chemical shifts of RBPS2a

Residue	C-1	C-2	C-3	C-4	C-5	C-6
→3)→β-D-Gal-(1→	110.2	83.7	86.8	71.7	79.7	64.9
→4)→α-D-Ara-(1→	106.3	81.0	78.2	85.2	64.7	_
→4)→α-D-Ara-(1→	106.0	81.1	77.9	85.0	65.0	_
α-D-Xyl-(1→	104.7	80.3	76.1	71.2	64.1	_
α-D-Glc-(1→	110.7	74.0	78.5	70.6	78.8	63.7

residues. The anomeric region of the ^{1}H spectrum (4.9–5.8 ppm) was consistent with the RBPS2a, which had a hexose – repeating unit. Based on above results, it could be concluded that the backbone of RBPS2a contained β-(1→3)-linked D-galacopyranosyl residues substituted at O-2 with glycosyl residues composed of α-D-xylose-(1→4)-α-D-arabinose-(1→ and α-D-glucose-(1→4)-α-D-arabinose-(1→ linked residues.

In conclusion, the structural features of RBPS2a were different from those of the polysaccharides that had been isolated from rice bran. Rice bran polysaccharide fractions treated with alkali and a proteolytic enzyme has shown that the polysaccharide and protein of the proteoglycan are most probably linked through an *O*-glycosyl linkage through hydroxyproline (Tatsunori, Kazuo, & Toshiyuki, 1975). MGN-3 is a denatured hemicellulose which is obtained by reacting rice bran hemicellulose with multiple carbohydrate hydrolyzing enzymes from Shiitake mushrooms. The main chemical structure of MGN-3 is an arabinoxylan, with a xylose in its main chain and an arabinose polymer in its side chain (Ghoneum, 1998). Another kind of rice bran hemicellulose consisted mainly of highly branched arabinoxylan and xyloglucan, It was fractionated by ammonium sulphate precipitation, ion-exchange chromatography and enzymatic techniques (Naoto & Tetsuya, 1985). This was also in accordance with the results that structures of the polysaccharides varied between the extraction conditions and the purification procedures.

3.3. Anti-complementary activity of different polysaccharide fractions

Fig. 2 shows that different polysaccharide fractions extracted and purified from defatted rice bran have a dose-dependent anti-complementary activity. The anti-complementary activity enhanced with increased concentration. Fraction RBPS2 was the most active fraction obtained after ion exchange chromatography. The activities of fractions RBPS2a, RBPS2b obtained by further fractionation of RBPS2 by gel chromatography are given in Fig. 4. The heteropolysaccharide RBPS2a purified by anion-exchange column and gel-permeation column exhibited the highest anti-complementary activity.

Studies indicated that many kinds biologically active polysaccharides extracted from rice bran had shown significant anti-tumour and immune modulating activities. Most

reports conformed that rice bran polysaccharides exerted their anti-tumour action via activation of the immune response of the host organism. Rice bran polysaccharides from different breed or different separation conditions have different immune modulating activities, probably depending on their monosaccharide composition, protein contents, molecular mass, and chain conformation.

4. Conclusions

A purified biologically active heteropolysaccharide RBPS2a from defatted rice bran was obtained using Q-Sepharose big beads anion-exchange chromatography and Sepharose CL-6B gel chromatography. RBPS2a was a novel heteropolysaccharide, with a molecular weight of 90,000 Da and the main chain contained β-(1→3)-linked D-galacopyranosyl. Activity tests showed that RBPS2a had the highest anti-complementary activity in vitro. Further study should be carried out to elucidate the relationship between structural features of different rice bran polysaccharide fractions and their bioactivity in our lab.

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