NOTE

Potent Natural Immunomodulator, Rice Water-Soluble Polysaccharide Fractions with Anticomplementary Activity[1]

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ABSTRACT

Water-soluble polysaccharide fractions, fractionated with ammonium sulfate from the hot-water-extract of rice bran and endosperm, showed a potent anticomplementary activity. As compared with water-soluble polysaccharide isolated from *Angelica acutiloba*, which is a well-known medicinal herb, the rice fractions showed similar or higher potency. Protease digestion, periodate oxidation, and hydroxylamine treatments indicated that anticomplementary activity is due to polysaccharide moiety rather than protein moiety and the polyphenol moieties, ferulic acid, being an integral component in rice bran proteoglycan. These results suggest that a water-soluble proteoglycan and a polysaccharide in rice modulate complement activity. This is a new example of natural biological response modifiers in food.

Cereal Chem. 80(1):5–8

The study of foods has been mostly focused on nutritional and sensory aspects in relation to functional properties such as color, flavor, texture, emulsification, foaming, gelation, etc. (Kinsella 1981; Yamagishi et al 1987). In recent years, there has been considerable interest in the physiological effects of foods on health—from cancer prevention and lowering of cholesterol to prevention of various gastrointestinal and vascular diseases. Research has focused on the biological effects of low molecular weight dietary compounds, such as polyphenols and phytochemicals. Dietary polyphenol compounds may function as natural biological response modifiers by protecting cells or tissues against injuries, especially those caused by lipid peroxidation or enzyme-mediated oxidation, and also as preventive factors against dental caries (Hattori et al 1990), hypercholesterolemia (Fukuyo et al 1986), and hypertension (Hara et al 1987).

The physiological effects of macromolecules such as dietary fiber and its components are also of interest. Of particular interest is the water-soluble fiber fraction. Normand et al (1987) suggested that a water-soluble polysaccharide fraction in rice bran is an active ingredient and, through its ability to bind bile, may play a role in lowering cholesterol and phospholipid contents in blood serum. This could contribute positively to the health of 1.6 billion Asian people for whom rice provides half of the diet.

Previous studies have shown that the water-soluble, nonstarchy polysaccharides from rice bran are conjugated carbohydrates (proteoglycan) (Yamagishi et al 1975, 1976). Rice bran proteoglycan has scarcely been studied regarding its effects on immunological reactions. Human immunological reaction is classified by humoral and cellular immunity. Anticomplementary activity discussed here belongs to humoral immunity, which is responsible for >99% of the human immunological defense system. The human complement system plays an important role in the host defense against foreign invasive organisms such as bacteria, fungi, and viruses. On the other hand, aberrant activation of the system may contribute to or evoke pathologic reactions in a variety of inflammatory and degenerative diseases (Vogt 1985), for example various hemolytic anemia (Henry and Lim 1990), dermatological diseases, rheumatoid arthritis, gout, and microbial infections. Therefore, modulation of complementary activity can be important (Walport 1993).

When normal human serum is incubated with some complement modulators, and the remaining complement titer is measured by hemolysis of sensitized sheep erythrocytes as an antigen-antibody complex, the modulating substances for the hemolytic activity of complement are referred to as "anticomplementary substances". When a complement modulator activates the complement system, the complement components constituting the complement system are consumed. As a result of measurement of the remaining complement titer, activators result in the decrease of hemolysis due to the reduced complement titer by the activation of the complement system. The inhibitory substances for hemolysis also refer to anticomplementary substances. Therefore, anticomplementary activity includes both activation and inhibition of the complement system. Our objective was to evaluate the effects of rice bran proteoglycans and endosperm polysaccharide in an in vitro complement system. Beneficial roles of the macromolecules from rice bran and endosperm as natural biological response modifiers were also considered.

MATERIALS AND METHODS

Materials

Rice (*Oryza sativa* var. 'Hitomebore') was harvested in Miyagi prefecture Japan. Alpha amylase of *Bacillus subtilis* (3× crystallized) and glucoamylase of *Rhizopus niveus* (fine grade) were purchased from Seikagaku Kougyo Co., Tokyo. Protease (Pronase-P, 45,000 proteolytic units/g) was obtained from Kaken Kagaku Co. Ltd. Arabinogalactan from the root of medicinal herb, *Angelica acutiloba* Kitagawa (Japanese name = Yamamoto Tohki) (Yamada et al 1984) was used as a positive control and was a gift from H. Yamada. Other positive controls included antitumor glucans, Shizophylan (Komatsu et al 1969), which were gifts from T. Nakajima, and heparin (Klerx et al 1985). Sephadex G-25, and DEAE-Sepharose were obtained from Pharmacia Co. Ltd.

Methods

Total carbohydrate content was determined by the phenol-sulfuric acid method (Dubois et al 1956). Uronic acid content was determined by the carbazole-sulfuric acid method (McComb and McCready 1952), corrected for neutral sugar. The analysis of the sugar composition was performed as described earlier (Yamagishi et al 1975). Dialysis was performed for 24 hr at 6°C using cellulose tube (Viskase Co. Ltd.) with a molecular range of 12,000–14,000.

[1] A part of this work was presented at the Annual Meeting of the Japan Society for Bioscience and Biotechnology and Agrochemistry, Tokyo, April 1999.
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Publication no. C-2002-1202-02R.

Rice Endosperm Polysaccharide and Bran Proteoglycan

Brown rice was milled with a testing mill (Satake Engineering Co., Tokyo; milling yield, 80% on a bran rice basis) and then ground to pass through a 50-mesh sieve. The milled rice flour was extracted three times with 1:1 ethanol and ether. The defatted flour was suspended in a tenfold volume of distilled water which contained α-amylase (80 units/mL) and 0.002*M* of calcium chloride. After adjusting to pH 6.0 with sodium hydroxide, the suspension was heated to 90°C for 60 min. The supernatant was further digested with glucoamylase (6 units/mL, pH 5.0, 50°C, 5 hr) to convert this dextrin into glucose, dialyzed against distilled water, and lyophilized. This water-soluble fraction was designated as rice endosperm polysaccharide.

Crude rice proteoglycans were prepared as described previously (Yamagishi et al 1975). Salting out with saturated ammonium sulfate gave two fractions. The precipitate fraction was applied to DEAE-Sepharose (chloride form) and eluted with 0.2M NaCl (designated as A1). Supernatant was also absorbed to DEAE-Sepharose (carbonate form) and eluted with 0.5*M* aqueous ammonium carbonate (designated as Bm). Electrophoretic gels of bran proteoglycan A1 and Bm, and endosperm polysaccharide were stained for both carbohydrate and protein. A band for carbohydrate had the same mobility as that of protein in the each sample, which showed the presence of both components.

Preparation of Ferulic-Acid-Free Proteoglycan A1

Proteoglycan A1 was treated with aqueous 0.5*M* NaOH at 60°C for 90 min under a N_2 stream (Yamagishi et al 1984). The alkaline solution was acidified to pH 2.0 and extracted with ethyl ether. The solvent extract was used to detect ferulic acid and the aqueous part was neutralized, concentrated, and subjected to gel filtration on Sephadex G-25. The void volume fractions were concentrated and lyophilized.

Proteoglycan A1 was treated with alkaline hydroxylamine at 40°C for 90 min under a N_2 stream (Yamagishi et al 1984). Hydroxylamine-treated proteoglycan A1 was applied to a Sephadex G-25 column. Void volume fractions were concentrated and lyophilized, and total volume fractions were used to detect ferulic acid hydroxamate. The resulting ferulic acid and its hydroxamate were identified as described previously (Yamagishi et al 1984).

Anticomplementary Activity

Gelatin-veronal-buffered saline (pH 7.2) containing 500 M Mg^{++} and 150 μM Ca^{++} (GVB^{++}) was prepared according to Mayer (1966). Normal human serum (NHS) was obtained from a healthy adult. Various dilutions of proteoglycan in phosphate buffered saline (PBS) (pH 7.4) (50 μL) were incubated with 50 μL of NHS and 50 μL of GVB^{++}. The mixtures were incubated at 37°C for 30 min and the residual total hemolytic complement (TCH_{50}) was determined by a method using IgM hemolysin-sensitized sheep erythrocytes (EA) at 1×10^8 cells/mL (Mayer 1966). NHS was incubated with PBS and GVB++ to provide a control. The anticomplementary activity of the proteoglycan fractions was expressed as percent inhibition from TCH_{50} of the control.

Pronase Digestion of the Fractions

A sample (200 mg) was dissolved in 50 mL of 10 m*M* Tris-HCl, pH 7.9, containing 10 m*M* $CaCl_2$, and then 50 mg of Pronase-P was added. The reaction mixture was incubated at 37°C for 48 hr and layered with a few drops of toluene (Yamada et al 1984). The digestion was terminated by boiling the mixtures for 5 min. The sample were then dialyzed against H_2O for two days, and the nondialyzable portion (an internal portion and a soluble portion) was lyophilized to obtain the proteoglycan pronase digest.

Periodate-Oxidation of Fractions

A sample (50 mg) was dissolved in 30 mL of 50 m*M* acetate buffer, pH 4.5, and then 50 m*M* $NaIO_4$ (10 mL) was added. The reaction mixture was incubated at 4°C in the dark for three days. Ethylene glycol (5 mL) was added to destroy the excess periodate and the mixture was dialyzed against H_2O for two days. The nondialyzable solution (an internal part of membrane) was concentrated to ≈20 mL, and 20 mg of $NaBH_4$ was added to the concentrates while being continuously stirred for 12 hr at room temperature. After neutralization of the mixture with acetic acid, the H_3BO_3 in the sample was removed by repeated additions and evaporation with methanol. The oxidized proteoglycan fractions were obtained as a lyophilizate after dialysis.

Statistics

The results are presented as mean values ± standard deviation of at least four experiments. Paired *t*-test (levels of significance, 0.01 or 0.05) were used to evaluate the statistical significance of differences. Differences with $P < 0.01$ or 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Endosperm polysaccharide contained 85% carbohydrates and 5.2% protein. The carbohydrate composition was galactose (24.1 mol%), mannose (18.3%), arabinose (15.1%), glucose (11.2%) and xylose (8.1%), and uronic acid (14.9%, estimated in terms of galacturonic acid), together with lesser quantities of fucose (3.8%) and rhamnose (4.5%). Bran proteoglycan showed a sugar composition similar to that observed previously (Yamagishi et al 1975).

Concentrations of bran proteoglycans A1 and Bm causing 50% inhibition of complementary activity (IC_{50}) were 7.5 μg/mL and 52 μg/mL, respectively, whereas that of endosperm polysaccharide

TABLE I
Anticomplementary Activity of Rice Bran Proteoglycan and Rice Endosperm Polysaccharide With and Without Chemical Modification[a]

Fractions	IC_{50} (g/mL)[b]	PD[c]	PO[d]	HT[e]
Bran proteoglycan				
Bm	52.6 ± 13.9	51.2 ± 17.9	251 ± 25.8	nd[f]
A1	7.5 ± 1.9	7.1 ± 2.2	165 ± 29.1	8.1 ± 1.7
Endosperm polysaccharide	78.0 ± 10.2	77.5 ± 9.9	608 ± 42.8	nd
Positive controls				
Angelica acutiloba polysaccharide	81.0 ± 9.2			
Schizophyllan	1,753 ± 68.8			
Heparin	2,015 ± 75.5			

[a] Mean values ± SD from four to six determinations.
[b] Concentration causing 50% inhibition of complement activity.
[c] Protease digestion.
[d] Periodate oxidation.
[e] Hydroxylamine treatment.
[f] Not determined.

was 78 μg/mL. Proteoglycan A1 had significantly higher ($P < 0.01$) anticomplementary activity compared with that of arabinogalactan from *A. acutiloba* Kitagawa (Table I), but bran proteoglycans Bm and endosperm polysaccharide were similar in activity to *A. acutiloba* Kitagawa.

In a previous study (Yamagishi et al.1984), ferulic acid was demonstrated to be an integral component of bran proteoglycan A1. Nakagami et al (1995) discussed that flavonoids could efficiently exert protective and prophylactic effects in atherogenesis, thrombosis, and other chronic, inflammatory diseases because they show anticomplementary and antioxidant properties. Ferulic acid has not only an antioxidant function but also anticomplementary properties (Georgieva et al 1997). Table I shows that hydroxylamine treatment of bran proteoglycan A1 did not result in significant change in the anticomplementary activity compared with no treatment ($P > 0.05$). It is not likely that ferulic acid in proteoglycan A1 contributes to the anticomplementary activity. Treatment with hydroxylamine of endosperm polysaccharide did not give its hydroxamate, therefore it does not seem that the polysaccharide contains phenolic compounds.

Bran proteoglycans A1 and Bm, and endosperm polysaccharide, showed anticomplementary activity that was significantly reduced after periodate oxidation. On the other hand, Table I shows that protease digestion did not significantly change ($P > 0.05$) their anticomplementary activity. These results suggest that the carbohydrate moiety and not the protein moiety contribute to the activity. Bran proteoglycans A1 and Bm, and endosperm polysaccharide, have dissimilarity in their sugar compositions. Whereas xylose and arabinose were the main sugar components in A1, arabinose and galactose were predominant in Bm. The endosperm polysaccharide contains mannose, fructose, and uronic acid in addition to sugar component of bran proteoglycans. The anticomplementary polysaccharide isolated from the root of *A. acutiloba* Kitagawa, as a positive control, contained arabinogalactan moiety (Yamada et al 1984). Also, a considerable number of Chinese medicinal herbs have high anticomplementary activity in their extracts. It has been suggested that the active principle is related to a kind of polysaccharide molecule containing the arabinogalactan moiety. Bran proteoglycan Bm contains remarkable amounts of arabinose and galactose, and the molar ratio is similar to that of the positive control. The carbohydrate moiety of bran proteoglycan Bm may be an arabinogalactan (Fincher and Stone 1983) and participate in the anticomplementary activity. Proteoglycan A1 contains arabinose content similar to Bm but the former seems to be an arabionxylan. It is interesting to note the difference in their activities (Table I). Several acid (Yamada 1994; Yamada and Kiyohara 1999) and neutral (Yamada and Kiyohara 1999) polysaccharides possess anticomplementary activity. Yamada and Kiyohara (1999) pointed out that the heterogeneity of structures makes it difficult to establish a relationship between structure and anticomplementary activity. Because the activation of the complement requires steric recognition, the geometry of the molecules should play an important role.

Some polysaccharides with anticomplementary activity have already been isolated from bacteria (Sung-Gu 1998), fungi (Son et al 1998), and medicinal plants but not from cereal plants. Aoe et al (1993) reported that the water-soluble hemicellulose in rice bran had a preventive effect on 1,2-dimethylhydrazine-induced colon carcinogenesis. The effect of hemicellulose B on the tumor seems to not be related to immunological function, but to alteration of fecal microflora and significant decrease in fecal pH. Ghoneum (1998) reported that MGN-3 (an arabioxylan enzymatically treated with an extract from *Hyphomecetes mycelia* and commercially known as arabinoxylan) supplementing foods in the United States enhanced human natural killer cell activity, which is related to cellular immunity. Our water-soluble polysaccharide fractions were extracted by water alone, and so were different from the hemicellulose fraction used by Aoe et al (1993), which was extracted by alkali following water, MGN-3, and the fraction by Ghoneum (1998), which was obtained by digestion following extraction by alkali. It is unclear whether Ghoneum's sample was intact because no evidence was provided for its chemical property and fine structure. Rice bran proteoglycans and endosperm polysaccharide had more or similar anticomplementary activity when compared with the positive controls (Table I). Ghoneum's polysaccharide (MGN-3) seems to be responsible for cellular immunity for two kinds of human immunological reaction. On the other hand, rice bran proteoglycans and endosperm polysaccharide are involved in humoral immunity, which is responsible for >99% of human immunological defense system. These findings suggest that the modulation of complement activity by a water-soluble proteoglycan and a polysaccharide in rice is a new example of natural biological response modifiers in food. Further investigations on the relationship between structure and activity of these components are in progress.

ACKNOWLEDGMENTS

We thank H. Yamada and H. Kiyohara from the Oriental Medicine Research Center of the Kitasato Institute for providing arabinogalactan and for valuable advice. In addition, we also thank T. Nakajima, Tohoku University, and T. Narumi, Liberal Art and Engineering Science, Hachinohe National College of Technology, for statistical analysis.

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[Received May 18, 2002. Accepted July 11, 2002.]