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ISOLATION AND PARTIAL CHARACTERIZATION OF PROTEOGLYCANS FROM RICE BRAN*

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ABSTRACT

Extraction of rice bran with hot water, followed by removal of protein and starch, yielded a proteoglycan. Successive fractionation of this proteoglycan by salting out with ammonium sulfate and by DEAE-Sephadex column chromatography yielded several fractions shown to be homogeneous by disc electrophoresis. Treatment of the fractions 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.

INTRODUCTION

Although the major polysaccharide of cereals is starch, the occurrence of a small proportion of non-amylaceous polysaccharide has been recognized. These minor polysaccharides are generally termed hemicelluloses. However, water-soluble, non-amylaceous polysaccharides are often called cereal gums. Among these polysaccharides, wheat-flour hemicellulose[1,2] and cereal gums of barley[3,4] and oats[5–9] have been quite extensively investigated, whereas little information is available on the non-amylaceous polysaccharides of rice[10–12]. Moreover, most studies on the minor polysaccharides of rice have been concerned with the water-insoluble hemicellulose, and little is known about the nature of the water-soluble, non-amylaceous polysaccharide. Thus, Gremli and Juliano[11] have reported that rice bran contains 0.1% of water-soluble and 1% of alkali-soluble hemicellulose. Although the latter was investigated in detail by these authors, nothing is known about the nature of the former.

The present study was conducted in order to elucidate the structural features of the water-soluble, non-amylaceous polysaccharide from rice bran.

*Studies on the Proteoglycan from Rice Bran. Part I.

RESULTS AND DISCUSSION

A water-soluble, non-amylaceous, polysaccharide preparation was obtained from a hot-water extract of rice bran, after removal of protein and starch. Salting out of this preparation with ammonium sulfate yielded two nitrogen-containing fractions. The fraction from the precipitate (A) was further fractionated into three subfractions by column chromatography on DEAE-Sephadex (Fig. 1), the main subfraction (A1) being proved homogeneous by disc electrophoresis (Fig. 4). The fraction from the supernatant (B) was further resolved by column chromatography into six subfractions (Fig. 2), five of which were homogeneous (Fig. 4). The homogeneity of the subfractions (purified fractions) was also suggested by their elution profiles on rechromatography (Fig. 3). On chromatography of these fractions, the elution patterns of carbohydrate coincided with those of protein, suggesting that protein might be an integral part of the non-amylaceous, polysaccharide preparation. Association of carbohydrate and protein in this polysaccharide preparation was also suggested by the coincidence of the staining of carbohydrate with that of protein on poly(acrylamide)-gel electrophoresis (Fig. 4).

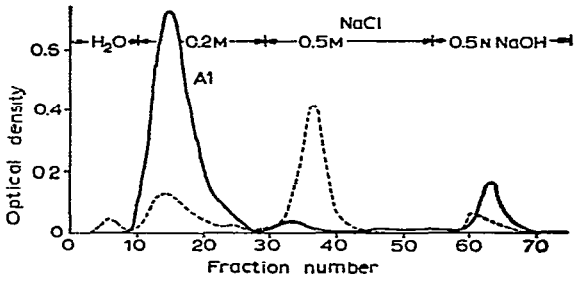

Fig. 1. Chromatography of A on DEAE-Sephadex (chloride form). ———, polysaccharide; — — —, protein (optical density at 280 nm).

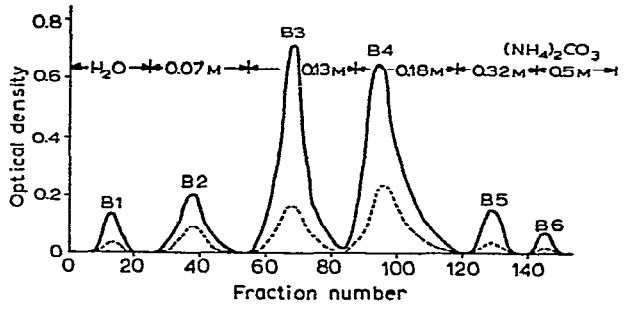

Fig. 2. Chromatography of B on DEAE-Sephadex (carbonate form). ———, polysaccharide; — — —, protein (optical density at 280 nm).

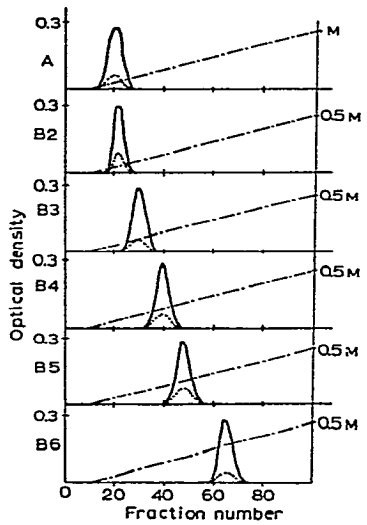

Fig. 3. Rechromatography of the purified fractions of the rice-bran proteoglycan on DEAE-Sephadex (carbonate form). ———, polysaccharide; — — —, protein (optical density at 280 nm); —·—·—, $(NH_4)_2CO_3$ concentration.

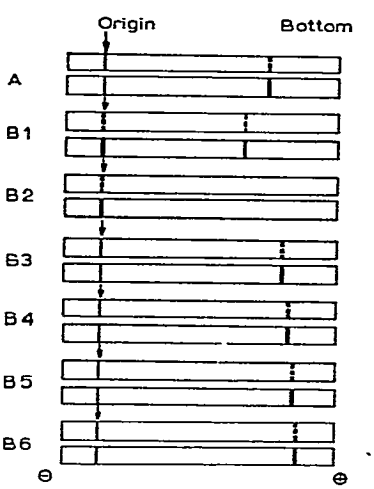

Fig. 4. Poly(acrylamide)-gel electrophoresis of A1 and the purified fractions of B.
▮, Periodate–Schiff staining, ▮, Amido black 10B staining.
The electrophoresis was conducted for 50 min in the cold (4°) at pH 8.9 with a current of 3 mA per gel column.

Each of the purified fractions gave a single peak on sedimentation analysis. Furthermore, not only the purified fractions, but also one of the original crude fractions (B) gave a single peak on sedimentation analysis. Moreover, the close similarity of the sedimentation patterns suggests that no significant difference of molecular size exists among the fractions. This view was confirmed by the sedimentation coefficients, which were 2.1S for A1 and 2.15S for B, respectively.

Paper-chromatographic analysis of the acid hydrolyzate of the crude fractions (A and B) showed that each fraction contains rhamnose, xylose, arabinose, glucose, and galactose. Interestingly, rhamnose was detected as a component monosaccharide of this proteoglycan, whereas no rhamnose has been detected in the acid hydrolyzates of either rice bran[11] or rice-endosperm hemicellulose[12]. Quantitative determination of the component monosaccharides in the purified fractions (A1 and B1–B6) shows dissimilarity in their sugar compositions (Table I). Whereas xylose and arabinose were the main sugar components in A1, arabinose and galactose were preponderant in B2–B6. It seems peculiar that B1, which was shown to be heterogeneous by disc electrophoresis, contains extraordinarily large amount of glucose (60%). It is not yet certain whether or not this glucose might have arisen from a residual fragment of the amylaceous polysaccharide.

TABLE I

CARBOHYDRATE COMPOSITION AND PROTEIN CONTENT OF THE PURIFIED FRACTIONS OF THE RICE-BRAN PROTEOGLYCAN

Fractions	*Composition (mol %)*					*Protein*[a]
	L-*Rha*	D-*Xyl*	L-*Ara*	D-*Glc*	D-*Gal*	(*w.* %)
A1	8	33	44	5	10	14.9
B1	9	4	7	60	20	12.5
B2	8	7	29	11	45	27.4
B3	9	9	31	11	40	13.8
B4	15	11	26	17	31	15.4
B5	22	14	17	17	30	17.9
B6	12	9	15	23	41	11.8

[a]Calculated on the basis of nitrogen content.

Gremli and Juliano[11] have reported that the alkali-soluble hemicellulose of rice bran comprised arabinose, xylose, and galactose, the first two being preponderant. The relative contents of arabinose and xylose in A1 seems to be somewhat similar to those of the rice-bran hemicellulose, although the latter was devoid of rhamnose and glucose. Recently, Pusztai and coworkers[13,14] have isolated a polysaccharide–protein complex from the leaves of *Vicia fava*. The main sugar components of the polysaccharide moiety of this complex were arabinose, galactose, and a uronic acid, accompanied by small proportions of xylose and rhamnose. There seems to be some similarity between the sugar compositions of the foregoing complex and those of

B2–B6, although no uronic acid was detected in the latter fractions. Another example of a cereal proteoglycan has been reported by Neukom *et al.*[15], who isolated a complex from wheat flour that comprised arabinose, xylose, and galactose together with a small proportion of protein. In any case, the structure of the polysaccharide moiety of the plant proteoglycan seems to be much more complicated than that of a simple arabinogalactan or arabinoxylan.

Paper-chromatographic analysis of the acid hydrolyzate of the crude fractions (A and B) of the rice-bran proteoglycan showed the presence of a small proportion of 2-amino-2-deoxy-D-glucose. Quantitative determination showed that A and B contained 0.3 and 0.4%, respectively, of amino sugar. This amino sugar is generally recognized as an essential component of the carbohydrate moiety of glycoproteins from animal tissue[16–18] or of plant seeds[19]. However, no amino sugar could be detected in acid hydrolyzates of the purified fractions (A1 and B2–B6). The 2-amino-2-deoxy-D-glucose in crude samples seems to be a contaminant arising from the rice-embryo gamma-globulin[20], which has been shown to contain hexosamine, or it may be produced in small quantity through low levels of fungal contamination of the seed.

As shown in Table I, the protein contents of the purified fractions were 12–18%, except for B2, which showed a relatively high protein content (27.4%). The lesser

TABLE II

AMINO ACID COMPOSITION[a] OF THE RICE-BRAN PROTEOGLYCAN

Amino acid	*Fractions*						
	A1	*B1*	*B2*	*B3*	*B4*	*B5*	*B6*
Lys	6.3	15.3	4.6	7.9	8.4	7.2	7.9
His	3.3	3.4	1.6	2.2	2.6	2.8	2.9
Arg	7.6	10.3	2.9	3.8	6.2	5.8	5.3
Hypro	9.5		13.5	8.9	4.6	+[b]	+[b]
Asp	9.2	7.1	5.4	6.1	8.7	10.9	13.5
Thr	5.1	5.3	6.5	6.0	4.6	6.2	3.5
Ser	5.8	8.9	13.4	15.1	12.1	9.5	8.1
Glu	15.3	14.4	8.5	9.3	15.9	20.7	26.9
Pro	4.9	3.7	4.5	4.2	3.5	4.4	3.5
Gly	11.8	14.9	9.5	11.7	13.5	12.7	12.9
Ala	6.9	9.7	18.9	15.7	11.2	9.0	7.6
Val	2.7	1.7	4.6	3.7	2.8	3.4	2.7
Met	1.5		0.6		0.6	0.4	0.5
Ile	1.6	1.6	1.1	1.4	1.2	1.5	1.1
Leu	3.3	2.8	2.9	3.1	3.0	3.2	2.7
Tyr	1.4	0.3	0.3		0.6	1.6	
Phe	1.2	0.5	0.9	1.1	0.5	1.6	0.8
½ Cys	2.7						

[a]Expressed as μmoles of amino acid per 100 μmoles of total amino acids. [b]Detected by two-dimensional chromatography on Toyo No. 51A paper with water-saturated phenol (first run) and (9:1:2.9) 1-butanol–acetic acid–water (second run). Hydroxyproline was stained by successive spraying with isatin and Ehrlich's reagent[34].

content of protein in the proteoglycan from rice bran, as well as those from other plant origins[13–15,21–23], is in marked contrast with the glycoproteins, in which the protein moiety is preponderant.

Amino acid analysis of the purified fractions showed that each fraction contained hydroxyproline as a characteristic component. However, the relative contents of hydroxyproline were substantially different from each other among the purified fractions (Table II). Whereas B2 showed extremely high contents of this imino acid, the same component was barely detectable by paper chromatography in B5 and B6. As compared with other rice proteins, the crude fractions (A and B) are rather similar in their amino acid composition to rice embryo γ-globulin[20], which is the only rice protein containing hydroxyproline. Hydroxyproline is best known as a component of collagen[24] or of a proteoglycan of plant seed-covering[25] such as corn pericarp[21]. This imino acid has also been found in the cell-wall protein of cultured plant-cells[26–28], in a complex from *Vicia fava* leaves[13,14], and, recently, in the cereal gum of wheat flour[29].

As already mentioned, the results from column chromatography and disc electrophoresis strongly suggest that polysaccharide and protein are covalently bound in the rice-bran proteoglycan. It has been generally accepted that determination of the linkage between polysaccharide and protein is of primary importance in

TABLE III

AMINO ACID COMPOSITION[a] OF THE RICE-BRAN PROTEOGLYCAN AFTER TREATMENT WITH ALKALI AND DIGESTION WITH ENZYMES[b]

Amino acid	*A1*			*B*		
	Control	*Alkali treated*	*Enzymic digest*	*Control*	*Alkali treated*	*Enzymic digest*
Lys	6.3	6.1	2.9	8.9	8.6	3.4
His	3.3	3.1	0.9	2.7	2.8	0.6
Arg	7.6	7.4	1.6	6.4	6.2	1.5
Hypro	9.5	10.6	19.1	5.0	5.3	14.4
Asp	9.2	9.2	10.4	7.9	8.0	7.2
Thr	5.1	5.0	8.7	5.2	5.1	7.2
Ser	5.8	5.4	11.6	12.3	12.0	12.8
Glu	15.3	15.9	9.3	14.1	14.3	6.8
Pro	4.9	4.9	3.6	3.7	3.6	8.7
Gly	11.8	10.8	14.7	12.6	12.8	7.4
Ala	6.9	6.6	7.2	12.0	12.1	23.0
Val	2.7	2.7	3.2	2.8	2 9	4.6
Met	1.5	1.5	1.4	0.3	0.3	
Ile	1.6	1.6	1.4	1.3	1.4	0.8
Leu	3.3	3.3	2.1	3.1	3.0	1.7
Tyr	1.4	1.4	1.2	0.9	0.8	
Phe	1.2	1.2	0.8	0.8	0.8	
½ Cys	2.7	0.9				

[a]Expressed as μmoles of amino acid per 100 μmoles of total amino acids. [b]Pronase and hemicellulase.

characterizing the structural features of a proteoglycan. The absence of hexosamine in acid hydrolyzates of the purified fractions from the crude, rice-bran proteoglycan suggests that the *N*-acylglycosylamine linkage through asparagine and 2-acetamido-2-deoxy-D-glucose does not occur in this proteoglycan. The possibility of an O-glycosidic linkage in which serine or threonine is involved is best recognized by methods based on the β-elimination[18,30] that occurs when the whole proteoglycan is subjected to mild treatment with alkali[31]. As shown in Table III, treatment of the purified fractions with alkali did not cause any notable decrease of the relative proportions of serine and/or threonine, suggesting that no β-elimination took place during this procedure. This view was further confirmed by reduction of the alkali-treated purified fractions with sodium [^{3}H]borohydride. The ratios of the specific radioactivities of the [^{3}H]borohydride-treated samples to those of the corresponding untreated samples were almost unity, suggesting that no cleavage of O-glycosidic linkages occured during the alkali treatment. These results indicate that O-glycosidic linkages through serine or threonine are most unlikely in the rice-bran proteoglycan.

In order to test for the last possible linkage, an O-glycosidic linkage through a hydroxyamino acid not having a β-hydroxyl group, enzymic degradation of the proteoglycan was performed. By conducting an amino acid analysis on an acid hydrolyzate of a proteoglycan before and after treatment with a proteolytic enzyme, the relative proportions of the amino acids involved in the carbohydrate–protein linkage or located in the vicinity of such a linkage must be significantly increased. After successive treatment of A1 and B with pronase and hemicellulase, the relative proportion of hydroxyproline apparently increased in each case, as shown in Table III. As the possibility of an O-glycosidic linkage through serine or threonine was excluded from the results of alkali treatment, the most probable mode of linkage between carbohydrate and protein is thus an O-glycosidic linkage through hydroxyproline. Amino acid analysis of the enzymic degradation-products of A1 and B also shows a marked increase in the relative proportions of alanine and serine, respectively. These results suggest that these amino acids may be located in the vicinity of the linkage.

As mentioned before, hydroxyproline is known to be abundant in some of the animal glycoproteins such as basement membrane[32] and collagen[24]. However, in these glycoproteins, hydroxylysine[32], and not hydroxyproline, is involved in the carbohydrate–protein linkage. Recent studies have shown the occurrence of this imino acid in the glycoprotein of the cultured tomato cell-wall[27,33], *Vicia fava* leaves[13,14], and maize seed[21]. However, no exact evidence for the involvement of this imino acid in the carbohydrate–protein linkage has been advanced, except in the glycoprotein from the cultured tomato cell-wall. Evidence for the involvement of hydroxyproline in this glycoprotein has been afforded by Lamport[33,34], who isolated *O*-L-arabinosylhydroxyproline from an enzymic digest.

Although the present results strongly support an O-glycosidic linkage through hydroxyproline, firm identification of the carbohydrate–protein linkage in the rice-bran proteoglycan is still lacking. Thus, no information is yet available as to which monosaccharide is involved in the linkage. The carbohydrate–protein linkage cannot

be firmly established unless the fragment corresponding to the linkage region is characterized. Further studies on this linkage will be reported later.

EXPERIMENTAL

Materials. — Rice bran was a residual product* of oil extracting. Prior to extraction of the polysaccharide, rice bran was twice extracted with three volumes of abs. ethanol for 48 h at room temperature to remove residual lipids.

The enzyme preparations were commercial products: alpha amylase from *Bacillus subtilis* (Type II A, 4× crystallized, Sigma Chemical Co.), glucoamylase from *Rhizopus niveus* (pure grade, 25.7 units/mg, Seikagaku Kogyo Co.), hemicellulase from *Rhizopus* species (grade II, Sigma Chemical Co.), and protease from *Streptomyces griseus* (Pronase, Kaken Kagaku Co.). In order to remove the extender, the crude hemicellulase preparation was dialyzed against deionized water for 4 days and insoluble material was removed by centrifugation. The supernatant liquor was concentrated by ultrafiltration and freeze dried. Thus, 640 mg of the purified enzyme was obtained from 25 g of crude preparation.

General methods. — All concentrations were conducted under diminished pressure below 45°. Nitrogen was determined by an automatic nitrogen analyzer. Paper chromatography was performed on Toyo No. 50 filter paper by the multiple ascending or descending method with solvent systems of: (*A*) 1-butanol–pyridine–water (6:4:3, v/v)[35], and/or (*B*) ethyl acetate–pyridine–water (8:2:1, v/v)[15]. Aniline hydrogen phthalate[36] was used for detection of neutral sugars and alkaline silver nitrate[37] or ninhydrin[38] for amino sugars. Gas–liquid chromatography was conducted under isothermal conditions (182°) on a Yanagimoto Type G-8 gas chromatography apparatus fitted with a flame-ionization detector. The glass column (3 m × 2 mm diam.) was packed with 1% ECNSS-M on Diasolid-L and was operated at a gas flow-rate of 30 ml of N_2 per min. Sedimentation analysis was performed with a Hitachi Model UCA-1 ultracentrifuge in a synthetic-boundary cell at 60,000 r.p.m. Samples were dissolved in 8M urea to a final concentration of 1% (w/v). Disc electrophoresis was effected on poly(acrylamide) gels at pH 8.9 according to the methods of Ornstein and Davis[39], with 0.1M Tris–borate buffer in place of 0.1M Tris–glycine buffer. Carbohydrate and protein bands were stained by periodate–Schiff reagent[40] and Amido Black 10B, respectively. Radioactivity was scanned in 10 ml of scintillation fluid (100 g of naphthalene and 7 g of phenylbiphenyl-1,3,4-oxadiazole in one liter of 1,4-dioxane) in a three-channel, Horiba–Hitachi liquid-scintillation spectrometer.

Extraction of non-amylaceous polysaccharide. — One kg of rice bran was extracted with a large excess of water for 10 h at 70°. Insoluble materials were filtered off through a coarse filter cloth and the filtrate was concentrated to about one tenth of the original volume. The extract was adjusted to pH 8.5 with M sodium hydroxide

*This was a generous gift from Sanwa Oil Manufacturing Co., Sendai.

and the precipitated protein was removed by centrifugation. The supernatant was then treated with trichloroacetic acid (pH 4.3) and the precipitate was again removed. The supernatant liquid was adjusted to pH 6.0 and dialyzed against deionized water. The dialyzate was concentrated to about half of the original volume and heated for 15 min at 100°. The coagulated protein was removed by centrifugation. The deproteinized extract was adjusted to pH 7.0 and treated with alpha amylase (100 mg per 100 ml) for 20 h at 68°. The digest was concentrated and again dialyzed. The dialyzate was adjusted to pH 4.5 and treated with glucoamylase (0.5 mg per 100 ml) for 24 h at 40°. The final digest was concentrated and dialyzed. Non-amylaceous polysaccharide was obtained by precipitation with methanol; yield 3 g. This polysaccharide preparation was not stained by iodine.

Fractionation of the non-amylaceous polysaccharide by saturation with ammonium sulfate. — The crude, non-amylaceous polysaccharide (3 g) was dissolved in 50 ml of water and saturated with ammonium sulfate. The precipitate was washed with saturated ammonium sulfate solution, dissolved in a small amount of water, and dialyzed against deionized water. The dialyzate was concentrated and freeze dried to yield fraction A; yield 1.2 g. The supernatant and the washings were combined and dialyzed. The dialyzate was concentrated and freeze dried to give fraction B; yield 1.5 g. These fractions (A and B) contained 3.60 and 1.64% of nitrogen, respectively.

Further fractionation of A by DEAE-Sephadex column chromatography. — DEAE-Sephadex A-50, activated by the conventional method and converted into the chloride form by treating with 0.5M hydrochloric acid, was packed into a column (21 × 4 cm). A solution of A (500 mg) in 6 ml of water was put onto the column. The column was eluted successively with various concentrations of aqueous sodium chloride (0, 0.2, and 0.5M) and finally with 0.5M sodium hydroxide at a velocity of 2.5 ml/min, each 20 ml being collected by a fraction collector. Elution of carbohydrate and protein was monitored by the phenol–sulfuric acid method[41] and the measurement of the absorbance at 280 nm, respectively. The main portion of A was eluted as a single peak with 0.2M sodium chloride, with 55% recovery.

Further fractionation of B by DEAE-Sephadex column chromatography. — DEAE-Sephadex A-50, activated by the conventional method and converted into the carbonate form by treating with 0.5M ammonium carbonate, was packed into a column (21 × 4 cm). A solution of B (500 mg) in 5 ml of water was applied to the column. Fractionation was effected by stepwise elution with water (400 ml), 0.07M (400 ml), 0.13M (500 ml), 0.18M (800 ml), 0.32M (400 ml), and 0.5M (200 ml) aqueous ammonium carbonate. Fraction B was fractionated into six subfractions. Each subfraction was dialyzed against deionized water and freeze-dried. Relative proportions (%) of the subfractions (B1–B6) were 9.3, 13.7, 25.5, 36.0, 12.1, and 3.4, respectively.

An outline of the isolation and fractionation of rice bran proteoglycan is illustrated in Fig. 5.

Rechromatography of the subfractions. — Each 10 mg of the subfractions (purified fractions) from A and B was rechromatographed on a DEAE-Sephadex

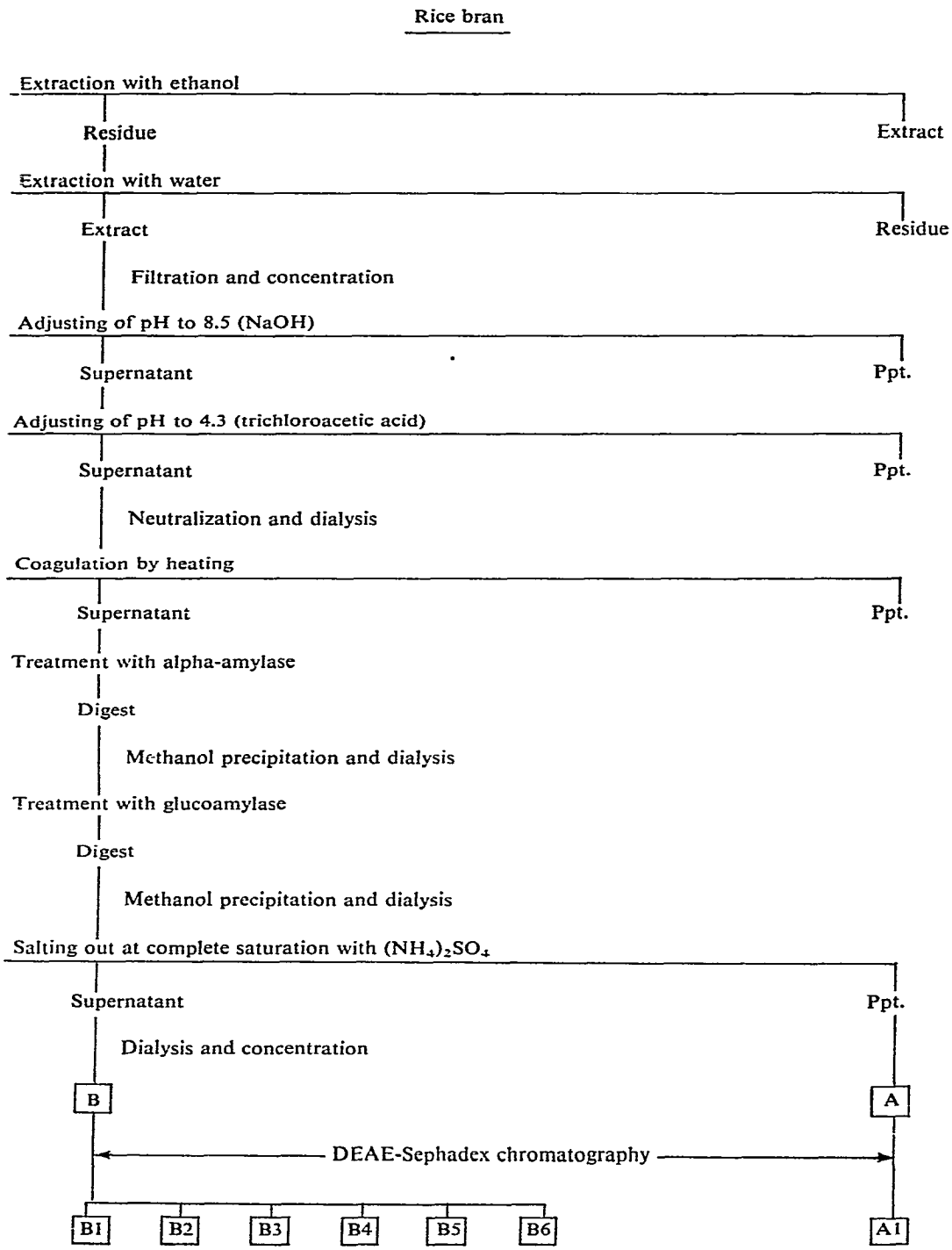

Fig. 5. Isolation and fractionation of rice-bran proteoglycan.

column (20 × 1 cm) by linear gradient-elution with aqueous ammonium carbonate. Each 2 ml was collected.

Detection and determination of neutral sugars. — For detection of the neutral sugars, samples were hydrolyzed with 0.75M sulfuric acid for 4 h at 105° in a sealed tube. The hydrolyzates were neutralized with barium carbonate and the filtrates treated with Amberlite IR-120 (H^+ form), concentrated, and submitted to paper chromatography with solvent systems *A* and *B*. For determination of the neutral sugars, samples were hydrolyzed with 2M trifluoroacetic acid for 1 h at 120° in sealed tubes. The hydrolyzates were evaporated to dryness and analyzed for neutral sugars by g.l.c. of their alditol acetates[42].

Amino sugar analysis. — Samples were hydrolyzed with 2M hydrochloric acid for 10 h at 100° in a sealed tube[21]. The hydrolyzates were concentrated and applied to a column (20 × 1 cm) of Dowex 50-W X-8 (H^+ form). After the neutral materials had been removed by elution with water, the column was eluted with 30 ml of 2M hydrochloric acid. The eluate was concentrated and submitted to paper chromatography with solvent system *A*. Amino sugars were determined by the modified Elson–Morgan reaction[43].

Amino acid analysis. — Samples were hydrolyzed with a large excess (2,000 volumes)[21] of 6M hydrochloric acid for 20 h at 105° in evacuated sealed tubes. The hydrolyzates were evaporated and analyzed by a Hitachi KLA-2 amino acid autoanalyzer.

Alkali treatment of the fractions. — The fractions A1 and B (100 mg of each) were treated with 1 ml of 0.5M sodium hydroxide for 24 h at 5° in evacuated, sealed tubes. The products were brought to pH 6.0 with dilute hydrochloric acid, concentrated, and desalted by gel filtration through Sephadex G-25. The desalted products were then subjected to amino acid analysis.

Alkali treatment and subsequent sodium [^3H]borohydride reduction. — Fractions A1 and B (10 mg of each) were dissolved in 0.5 ml of water or 0.5M sodium hydroxide and treated with an equal volume of 1% sodium [^{3}H]borohydride solution[44] (corresponding to 12.5 μCi) for 24 h at 4°. The products were treated with 2M acetic acid to remove residual [^{3}H]borohydride and concentrated. The boric acid was removed by repeated evaporation of methanol from the product, and the products were further purified by gel filtration on Sephadex G-25. The products were assayed for radioactivity.

Enzymic digestion of the proteoglycan. — Fractions A1 and B (100 mg of each) were dissolved in 50 ml of 0.05M phosphate buffer (pH 8.0) and treated with 5 mg of Pronase (45,000 p.u.k./g) for 48 h at 50°. The digest was then adjusted to pH 4.8 with 2M acetic acid and treated with 5 mg of hemicellulase for 48 h at 45°. The digest was adjusted to pH 6.0 and heated for 10 min in a boiling water-bath, during which time the enzymes were inactivated. Degradation products of the low molecular weight were removed by gel filtration through Sephadex G-25. Effluents at the void volume were collected and treated with Dowex 50-W X-8 (H^+ form) ion exchanger to remove the inactivated enzyme and other ninhydrin-positive substances. The purified

degradation-products were once again treated with the enzymes in the foregoing procedure, and the final degradation products were submitted to amino acid analysis.

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