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CHARACTERIZATION OF THE FRAGMENTS OBTAINED BY ENZYMIC AND ALKALINE DEGRADATION OF RICE-BRAN PROTEOGLYCANS*

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ABSTRACT

Repeated digestion of the proteoglycan B from rice bran with Pronase and hemicellulase yielded two types of glycopeptides. The first group, obtained from the third enzymic digest, was a type of low molecular-weight glycopeptide and was fractionated into two subfractions by paper electrophoresis. The second group, obtained from the fourth enzymic digest, was a mixture of glycopeptides of relatively high molecular-weight and was fractionated into three subfractions by gel filtration on Sephadex G-75. Alkaline degradation of these glycopeptides or the intact proteoglycan A yielded a sugar–amino acid compound, whose structure was established as *O*-α-L-arabinofuranosyl-hydroxyproline. These results indicate that the carbohydrate–protein linkage of the rice-bran proteoglycan is an *O*-glycosyl linkage between L-arabinose and hydroxyproline.

INTRODUCTION

Previous studies in this series have shown that the most probable carbohydrate–protein linkage in the proteoglycan of rice bran is an *O*-glycosyl linkage through hydroxyproline[1]. However, complete characterization of the linkage has not yet been achieved, as no information has yet been furnished as to which monosaccharide is involved in the linkage. In the present study, isolation and characterization of the fragment corresponding to the linkage region was attempted in order to establish definitively the structure of the carbohydrate–protein linkage of the rice-bran proteoglycan.

RESULTS AND DISCUSSION

Two types of glycopeptides were obtained by repeated digestion of the rice-bran proteoglycan B with Pronase and hemicellulase. One was recovered from the material absorbed on Dowex 50-W X-8 from the third enzymic digest (B-I) (Fig. 1) and was

*Studies on the proteoglycan from rice bran. Part II. For Part I, see ref. 1.

fractionated into two subfractions (B-I-1–2) by paper electrophoresis (Fig. 2). The other was obtained from the unabsorbed fraction of the fourth enzymic digest (B-II) and was fractionated into three subfractions (B-II-1–3) by gel filtration on Sephadex G-75 (Fig. 3). Elution patterns of these glycopeptides on gel filtration with Sephadex G-25 (Fig. 4, Fig. 5) showed that the former corresponded to a type of low molecular-weight glycopeptide, whereas the latter was a mixture of relatively high

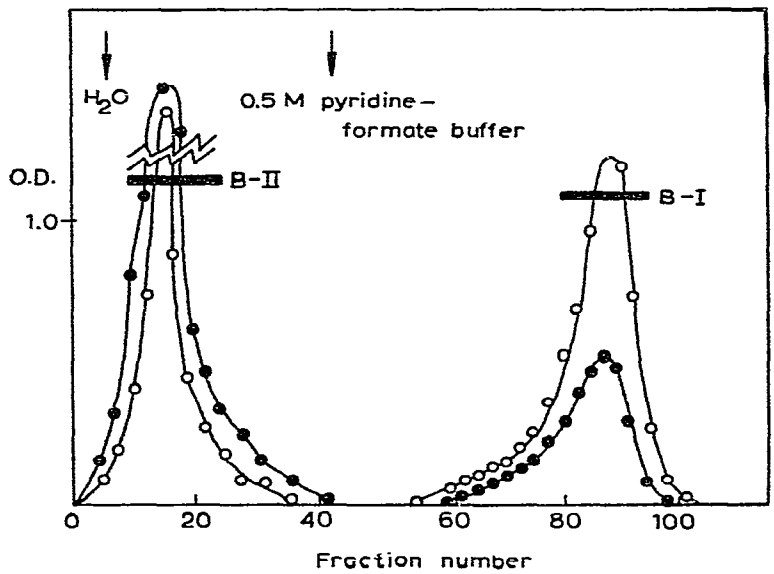

Fig. 1. Chromatography of the third enzymic digest on Dowex 50-W X-8 (H^+). ●——●, carbohydrate; ○——○, hydroxyproline.

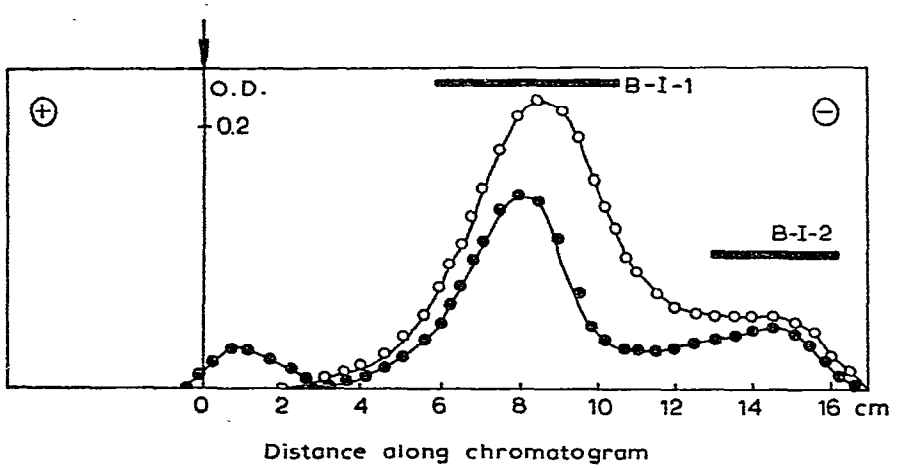

Fig. 2. Fractionation of B-I by paper electrophoresis. ●——●, carbohydrate; ○——○, hydroxyproline.

molecular-weight glycopeptides. The homogeneity of each subfraction (purified glycopeptide) was confirmed by paper electrophoresis.

Paper-chromatographic analysis (solvent system A) of the acid hydrolyzates of the purified glycopeptides showed that the low molecular-weight glycopeptides (B-I-1–2) was composed of arabinose and galactose, whereas the high molecular-

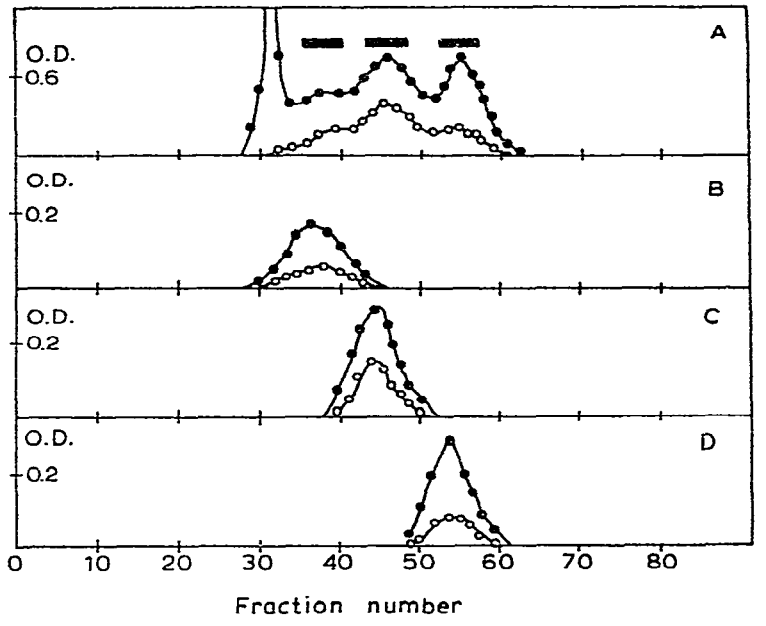

Fig. 3. Gel filtration on Sephadex G-75 of the fraction, unabsorbed on DEAE-Sephadex A-25, of the fourth enzymic digest. ●——●, carbohydrate; ○——○, hydroxyproline. A, Material unabsorbed on DEAE-Sephadex A-25. B, C, D. Fractions purified by gel filtration on Sephadex G-75. B, C and D are designated as glycopeptides B-II-1, B-II-2 and B-II-3.

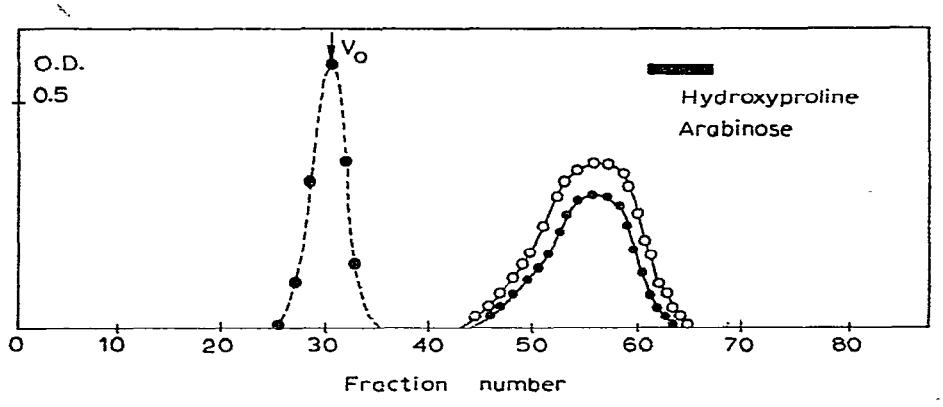

Fig. 4. Gel filtration on Sephadex G-25 of the fraction absorbed on Dowex 50-W X-8 from the third enzymic digest. ●——●, carbohydrate; ○——○, hydroxyproline; ●– –●, blue dextran.

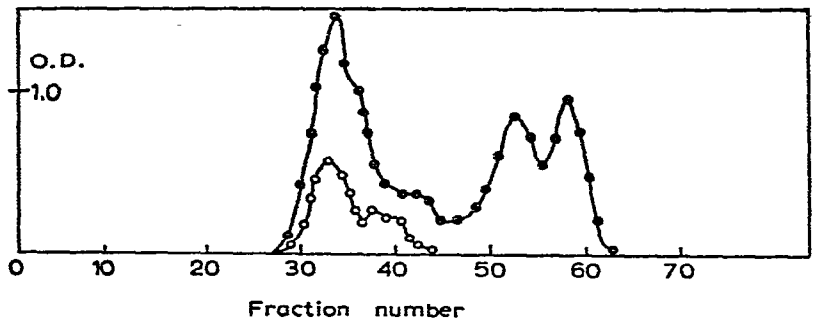

Fig. 5. Gel filtration on Sephadex G-25 of the fraction, unabsorbed on Dowex 50-W X-8, from the fourth enzymic digest. ●——●, carbohydrate; ○——○, hydroxyproline.

weight glycopeptides (B-II-1–3) contained a small proportion of xylose in addition to the main components (arabinose and galactose). Quantitative determination of the component sugars of the purified glycopeptides showed some differences in their composition. As may be seen from Table I, the proportions of arabinose in the low molecular-weight glycopeptides are larger than those of the high molecular-weight ones.

TABLE I

CARBOHYDRATE COMPOSITION OF GLYCOPEPTIDES OBTAINED BY SUCCESSIVE PRONASE-HEMICELLULASE DIGESTION OF RICE-BRAN PROTEOGLYCAN

Glycopeptides	*Composition (mol %)*		
	D-*Xyl*	L-*Ara*	D-*Gal*
B-I-1		66	34
B-I-2		72	28
B-II-1	9	31	60
B-II-2	4	45	51
B-II-3	3	54	43

Amino acid analysis of the purified glycopeptides showed that the content of hydroxyproline was almost half (two-thirds for the low molecular-weight glycopeptides) of the total amino acids (Table II). This result strongly supports the idea suggested in the previous paper[1] that the most probable bond between carbohydrate and protein of the rice-bran proteoglycan is an *O*-glycosyl linkage through hydroxyproline.

The previous paper[1] showed that the proteoglycan B was heterogeneous, and it was fractionated into five electrophoretically homogeneous subfractions. In the present study, five homogeneous glycopeptides were isolated following enzymic degradation of proteoglycan B. It is tempting to suggest that there may be some correlation between the glycopeptides and the subfractions of the original proteoglycan. However, more-detailed studies would be necessary to draw a definite

conclusion. Lamport[2] has also isolated five hydroxyproline-rich glycopeptides from the enzymic digest of suspension-cultured tomato cell-wall. However, the sugar and amino acid composition of these glycopeptides is considerably different from those in the present work.

TABLE II

AMINO ACID COMPOSITION[a] OF GLYCOPEPTIDES OBTAINED BY SUCCESSIVE PRONASE–HEMICELLULASE DIGESTION OF RICE-BRAN PROTEOGLYCAN

Amino acid	*Glycopeptides*				
	B-I-1	*B-I-2*	*B-II-1*	*B-II-2*	*B-II-3*
Lys	2	9	6.1	2.1	1.2
His			2.3	1.3	
Arg					
Hypro	65	72	43.1	45.4	42.2
Asp			0.7	1.0	2.3
Thr			5.3	5.2	5.3
Ser			10.5	11.1	13.4
Glu			3.9	3.9	4.9
Pro					
Gly			2.2	2.2	4.2
Ala	33	19	18.2	20.2	19.8
Val			5.6	5.5	4.1
Met			0.6	0.8	0.7
Ile			0.5	0.7	0.6
Leu			0.9	0.8	1.2
Tyr					
Phe					
½ Cys					

[a]Expressed as μmoles of amino acid per 100 μmoles of total amino acids.

Five homogeneous glycopeptides have been thus isolated from the proteoglycan B by exhaustive enzymic digestion. However, preparation of the fragment corresponding to the linkage region by the enzymic method was unsuccessful. For this purpose, glycopeptides from the proteoglycan B were further subjected to alkaline degradation. The alkali-degraded products were purified by absorption on a column of Dowex 50-W X-8, gel filtration through Sephadex G-25 and G-15, and finally by preparative paper-electrophoresis. The purified alkaline-degradation products of the low molecular-weight glycopeptides were separated into four fractions by ion-exchange chromatography on Aminex AG 50-W X-2 (H^+ form), whereas the degradation products of the high molecular-weight glycopeptides yielded single peak (Fig. 6). In each instance, the elution profile of carbohydrate coincided with that of hydroxyproline, and no ninhydrin-positive component was detected, suggesting that hydroxyproline was the sole amino acid component of these products. Paper chromatography and paper electrophoresis of each fraction revealed only one spot, indicating that these fractions were homogeneous.

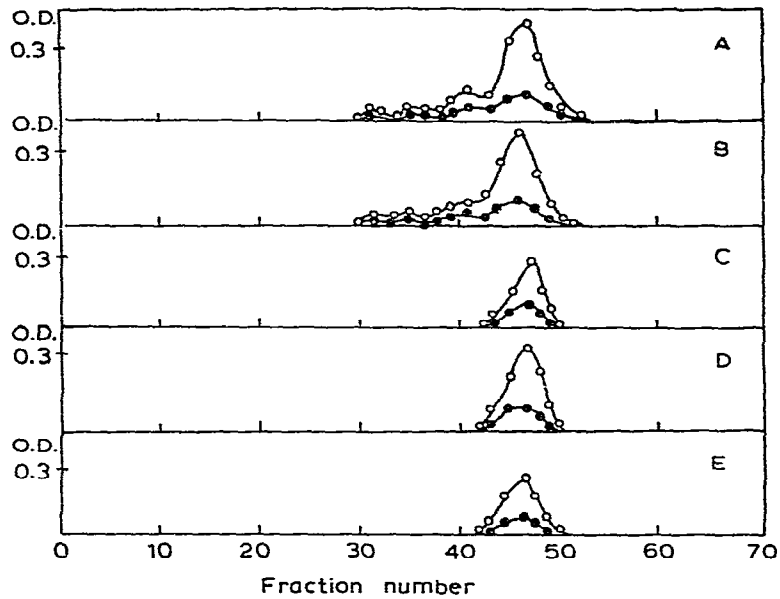

Fig. 6. Chromatography on Aminex resin (H^+) of the purified, alkaline-degradation products from the glycopeptides. ●——●, carbohydrate; ○——○, hydroxyproline. A, glycopeptide B-I-1. B, glycopeptide B-I-2. C, glycopeptide B-II-1. D, glycopeptide B-II-2. E, glycopeptide B-II-3.

TABLE III

ANALYSIS OF HYDROXYPROLINE GLYCOSIDES OBTAINED BY ALKALINE HYDROLYSIS OF GLYCOPEPTIDES

Fractions[a]	*Hydroxyproline*[b]	*Arabinose*[c]	*Reducing power*[d]	*Molar ratio of hydroxyproline to arabinose*
	μmoles/ml			
Fraction 44–48				
Unhydrolyzed	0.66	0.73	0.00	1.00:1.03
Hydrolyzed	0.69	0.70	0.71	
Fraction 39–41				
Unhydrolyzed	0.87	1.79	0.00	1.00:2.12
Hydrolyzed	0.82	1.74	1.81	
Fraction 34–37				
Unhydrolyzed	0.33	0.97	0.01	1.00:3.22
Hydrolyzed	0.32	0.93	1.01	
Fraction 30–33				
Unhydrolyzed	0.24	0.94	0.01	1.00:3.79
Hydrolyzed	0.24	0.90	0.91	

[a]These are the fractions from B-I-1. The same results were obtained with the other glycopeptides. [b]Determined by the method of Kivirikko. [c]Orcinol method. [d]Park–Johnson method.

The proteoglycan A, which had been shown to be less heterogeneous[1], was degraded directly with alkali, without any prior digestion by enzymes. The degradation product was purified by the foregoing procedures. In this case, ion-exchange chromatography gave only one peak, corresponding to that of the degradation product of the high molecular-weight glycopeptide from the proteoglycan B.

Acid hydrolysis of these sugar–amino acid compounds yielded arabinose and hydroxyproline, which were detected by paper chromatography. Quantitative determination of the hydrolysis products showed that these compounds were composed of arabinose and hydroxyproline in different ratios (Table III). As no reducing activity was observed, these compounds were considered to be a series of sugar–amino acid compounds in which arabinose or arabino-oligosaccharides (d.p. 2–4) are linked to hydroxyproline through an *O*-glycosyl linkage.

Firm identification of the smallest sugar–amino acid compound was based on the following evidence:

1. Quantitative determination of the hydrolysis product showed that the molar ratio of arabinose and hydroxyproline is unity.
2. The lability of the compound toward acid hydrolysis (Fig. 7) suggests that arabinose existed in the furanoid form.
3. Occurrence of glycerol and glycolaldehyde in the Smith-degradation product of the compound; this provided firm evidence for the arabinofuranose structure.
4. Susceptibility of this compound to α-L-arabinofuranosidase[3] showing that L-arabinose exists as the α-configuration.

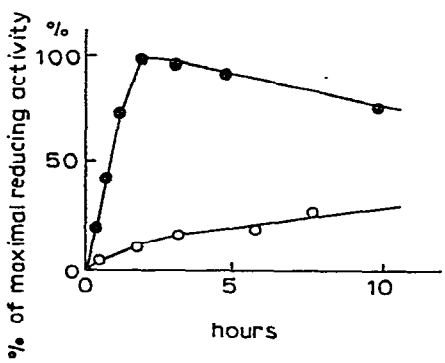

Fig. 7. Release of reducing activity during acid hydrolysis of arabinosylhydroxyproline (●——●) and methyl α-D-glucopyranoside (○——○) at pH 1.

Thus, the smallest sugar–amino acid fragment obtained by alkaline degradation of the rice-bran proteoglycan was characterized as *O*-α-L-arabinofuranosyl-hydroxyproline (Fig. 8). The isolation and characterization of this compound from the rice-bran proteoglycan now provides firm evidence that the carbohydrate–protein linkage is an *O*-glycosyl linkage between L-arabinose and hydroxyproline. *O*-L-Arabinosyl-hydroxyproline was isolated by Lamport[2] from the alkaline-degradation product of

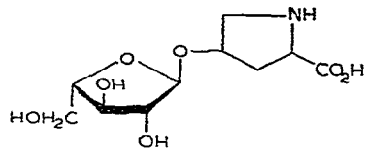

Fig. 8. Structure of *O*-α-L-arabinofuranosylhydroxyproline.

the glycopeptides obtained by enzymic digestion of the suspension-cultured, tomato cell-wall. Although lability of this compound toward acid suggests the arabinofuranosyl structure, fine-structural features have not yet been established. In the present study, the furanoid structure of the arabinose was firmly characterized by application of the Smith degradation, and the α-configuration of L-arabinose was established by the susceptibility of the product to α-L-arabinofuranosidase. The isolation and characterization of this sugar–amino acid compound from common plant tissue has not hitherto been reported, although Lamport[2] has reported its isolation from cultured cells. Recently, occurrence of an *O*-galactosyl linkage through hydroxyproline in the water-soluble arabinogalactan–peptide from wheat endosperm has been reported[4]. However, occurrence of this linkage in the rice-bran proteoglycan seems, from the foregoing results, to be unlikely.

EXPERIMENTAL

Materials. — The isolation and fractionation of the rice-bran proteoglycan are described in the previous paper[1]. The proteoglycans used in the present study were the crude fractions obtained by saturation with ammonium sulfate. Fraction A was from the precipitate and B from the supernatant. The enzymes (protease and hemicellulase) used for the degradation of the macromolecules were the same preparations described in the previous paper[1]. Purified α-L-arabinofuranosidase was a gift from Dr. Neukom (Swiss Federal Institute of Technology, Switzerland). The specificity of this enzyme has been established[3].

General methods. — All evaporations were conducted under diminished pressure below 40°. Paper chromatography was performed on Toyo No. 51A filter paper by the multiple ascending or descending method with the following solvent systems (v/v): A, 6:4:3 1-butanol–pyridine–water[5]; B, 15:2:2 2-propanol–formic acid–water[6]; C, 8:2:1 ethyl acetate–pyridine–water[7]; and D, 4:1:5 1-butanol–ethanol–water[8]. Aniline hydrogen phthalate[9] was used for detection of the neutral sugars, isatin–Ehrlich's reagent[10] for hydroxyproline, and tetrabase[8] for the periodate oxidation products. Paper electrophoresis was performed on Toyo No. 51A filter paper at 50 V/cm with 0.1M formic acid–acetic acid buffer (pH 2.1)[11] or 0.1M borate buffer[12] (pH 9.3) for qualitative purposes. Preparative, paper electrophoresis was conducted on Toyo No. 527 thick filter paper at 40 V/cm with the former buffer. Location of the glycopeptides was shown by dansylation of their terminal amino

groups[13]. Unless otherwise stated, carbohydrate was assayed by the phenol–sulfuric acid method[14]. Amino groups were assayed by the ninhydrin reaction according to Yemm and Cocking[15]. Hydroxyproline was assayed by the method of Kivirikko[16] after the samples had been hydrolyzed with 2.5M sodium hydroxide for 3.5 h at 90°.

Enzymic degradation of the proteoglycans. — For the preparation of glycopeptides, the proteoglycan B was digested with Pronase and hemicellulase as follows. The proteoglycan B (500 mg), fractionated by salting out with ammonium sulfate, was dissolved in 100 ml of 0.1M phosphate buffer (pH 7.8) and treated with 5 mg of Pronase for 48 h at 50°. The digest was then adjusted to pH 4.0 with 2M acetic acid and further treated with 2.5 mg of hemicellulase for 48 h at 45°. The digest was heated for 15 min in a boiling water-bath to inactivate the enzyme and placed on a column (20 × 3.6 cm) of Dowex 50-W X-8 (200–400 mesh, pyridine form). The column was eluted with deionized water (~500 ml) until the effluent became negative in the Molisch test for carbohydrate. The column was then eluted with 0.5M pyridine–formic acid buffer (pH 5.3) (~100 ml) until the effluent became negative toward the ninhydrin reagent. The aqueous effluent (unabsorbed material) was concentrated to low volume and subjected to gel filtration on a column (108 × 3.4 cm) of Sephadex G-25. The fractions emerging at the void volume were pooled and subjected to further digestion with Pronase and hemicellulase. The enzymic digestion, followed by purification of the digest by the foregoing procedure, was repeated three times. Until the second enzymic digestion, no significant amount of carbohydrate was detected in the fraction of the digest absorbed on Dowex 50-W X-8. However, a considerable amount of carbohydrate was detected, together with hydroxyproline, in the absorbed fraction of the third enzymic digest (Fig. 1). The absorbed material (B-I), eluted from the Dowex column, was then subjected to gel filtration on Sephadex G-25 (Fig. 4) to remove the residual, inactivated enzymes. The degradation product was further purified by gel filtration on Sephadex G-15, ion-exchange chromatography with Dowex 50-W X-8, and again by gel filtration on Sephadex G-15. By these steps, most of the ninhydrin-positive substance was removed. Final purification of the glycopeptides was achieved by paper electrophoresis. As shown in Fig. 2, paper electrophoresis of the partially purified glycopeptides revealed the presence of two components, a slow-moving major component (B-I-1) and a fast-moving minor one (B-I-2). These were separated by preparative paper-electrophoresis.

The unabsorbed material from the Dowex column of the third enzymic digest was purified by gel filtration on Sephadex G-25 (Fig. 5) and the effluent at the void volume was subjected to the fourth enzymic digestion with doubled amounts of the enzymes as before. The digest was passed through a Dowex column. Neither carbohydrate nor hydroxyproline was detected in the absorbed material from the fourth enzymic digest. As no significant degradation occurred during the fourth enzymic digestion, further enzymic treatment was not attempted. The product of the final enzymic degradation was applied to a column (21 × 4 cm) of DEAE-Sephadex A-25 that had been equilibrated with 50mM pyridine–formic acid buffer (pH 5.3). The column was eluted stepwise with deionized water (800 ml), 0.1M pyridine–formic acid

buffer (pH 5.3) (500 ml), 0.5M pyridine–formic acid buffer (pH 5.3) (600 ml), 3M ammonium hydroxide (800 ml), and 0.5M sodium hydroxide (400 ml). More than 90% of the total carbohydrate and hydroxyproline were recovered in the effluent with water. The water effluent (unabsorbed fraction) was concentrated to about 5 ml and applied to a column (60 × 3 cm) of Sephadex G-75. The column was eluted with deionized water and each 6 ml of the effluent was collected and examined for carbohydrate and hydroxyproline. As may be seen from Fig. 3, the final enzymic degradation-product was eluted in three peaks (B-II-1–3). In each peak, the elution pattern of the carbohydrate coincided with that of hydroxyproline. Each fraction was pooled, and purified by repeated gel-filtration on Sephadex G-75. Yields of the purified glycopeptides from 30 g of the proteoglycan B were as follows: B-I-1 103 mg, B-I-2 45 mg, B-II-1 125 mg, B-II-2 153 mg, and B-II-3 132 mg.

The scheme for the overall degradation procedure of the rice-bran proteoglycan is shown in Fig. 9.

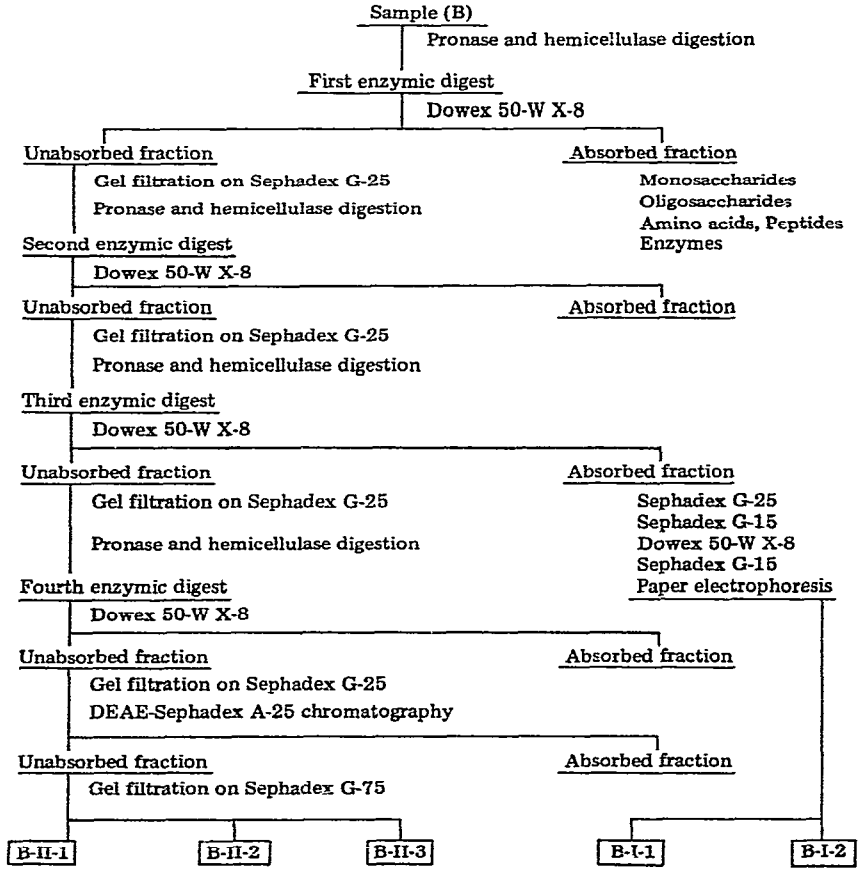

Fig. 9. Isolation and fractionation of glycopeptides from rice-bran proteoglycan.

Determination of the carbohydrate composition of the glycopeptides. — Carbohydrate analysis was performed paper chromatographically with solvent system C, after the samples had been hydrolyzed with 2M trifluoroacetic acid for 1 h at 100°. After development, guide strips were cut from both sides of the chromatogram and the positions of sugars located by aniline hydrogen phthalate. The zones corresponding to each sugar were cut out from the strip and eluted with boiling water. The eluted sugars were determined by the Park–Johnson method[17].

Amino acid analysis of the glycopeptides. — To determine the amino acid composition, the glycopeptides were hydrolyzed by the procedure described in the previous paper[1]. Amino acid analysis of the glycopeptides of high molecular-weight (B-II-1–3) was performed by means of an amino acid autoanalyzer. Amino acids of the glycopeptides of low molecular-weight (B-I-1–2) were determined after being separated by paper electrophoresis with formic acid–acetic acid buffer. The zones corresponding to each amino acid, as revealed by the aid of guide strips, were cut off from the strip and eluted with 10% acetic acid. The effluents were concentrated and amino acids determined by the foregoing methods.

Preparation of the sugar–amino acid compounds by alkaline degradation. — Each 100 mg of the glycopeptides (B-I-1–2 and B-II-1–3) or the intact proteoglycan A were hydrolyzed with 20 ml of a saturated barium hydroxide solution for 6 h at 105°. The hydrolyzates were neutralized with 2.5M sulfuric acid. After removal of barium sulfate by centrifugation, the supernatant was applied to a column (20 × 3.6 cm) of Dowex 50-W X-8 (200–400 mesh, H^+ form). The column was thoroughly washed with water and then eluted with 5–7 bed volumes of 0.25M aqueous ammonia. The eluate was concentrated and subjected to gel filtration on Sephadex G-25 and Sephadex G-15 to remove the free amino acids. The sugar–amino acid compounds were finally separated from the free amino acids by preparative paper-electrophoresis.

Fractionation of the sugar–amino acid compounds. — After removal of the free amino acids, the sugar–amino acid compounds were applied to a column (28 × 1 cm) of Aminex AG 50-W X-2 (H^+ form) and eluted with formic acid–acetic acid buffer, 8-ml fractions of the effluent being collected. Elution of carbohydrate and hydroxyproline was monitored by the foregoing methods.

Acid hydrolysis of O-*arabinosyl-hydroxyproline.* — Aliquots (0.7 ml) of the solution of *O*-arabinosyl-hydroxyproline (equivalent to ~0.08 μmoles) in 0.1M hydrochloric acid were heated at 100° in sealed tubes. After various periods of time, the hydrolyzates were slowly evaporated to dryness below 25°. To remove the residual hydrochloric acid, each residue was dissolved in a small amount of deionized water and again evaporated to dryness, the procedure being repeated three times. The hydrolyzates were analyzed for reducing activity by the method of Park and Johnson[17]. As the control, methyl α-D-glucoside was treated in the same manner.

Smith degradation of O-*arabinosyl-hydroxyproline.* — A solution of *O*-arabinosyl-hydroxyproline (equivalent to ~10 μmoles) in 0.1M acetate buffer (pH 4.4) was mixed with an equal volume of 0.2M sodium metaperiodate and the whole mixture was kept for 24 h at 4° in the dark. The mixture was adjusted to pH 8.0 with

0.1M sodium hydroxide and treated with an excess amount of sodium borohydride for 24 h in the cold. After the excess borohydride had been decomposed by adding a few drops of 4M acetic acid, the product was purified by passing it through a column (28 × 1 cm) of Dowex 50-W X-8 (200–400 mesh) resin. After the column had been thoroughly washed with deionized water, the periodate-oxidized and borohydride-reduced product was eluted from the column with 80 ml of 1.5M aqueous ammonia. The eluate was evaporated to dryness and the residual ammonia removed by repeated evaporation of deionized water from the residue. The periodate-oxidized and borohydride-reduced product was hydrolyzed with 3 ml of 0.1M hydrochloric acid for 24 h at 35°. The hydrolyzate was treated with 10 mg of hydroxylamine hydrochloride and heated in a sealed tube for 30 min at 80°. Detection of the Smith-degradation products was performed by paper chromatography with solvent system D. The location of the Smith-degradation products was achieved by spraying with tetrabase[8] reagent and also by a radioisotopic method. In the latter case, the periodate-oxidized product was reduced with [^{3}H]borohydride.

Digestion of O-*arabinosyl-hydroxyproline with purified* α-L-*arabinofuranosidase.* — *O*-Arabinosyl-hydroxyproline (~5 μmoles) was dissolved, together with 1 mg of α-L-arabinofuranosidase, in 1 ml of 0.2M acetate buffer (pH 4.0) and the mixture was incubated for 48 h at 45°. After the enzyme had been inactivated by heating for 5 min at 100°, the products were detected by paper chromatography with solvent systems A and B.

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