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XYLOGLUCAN IN CELL WALLS OF RICE HULL*

TOSHIYUKI WATANABE, MARIKO SHIDA, TETSUYA MURAYAMA, YOSHIHIRO FURUYAMA, TASUKU NAKAJIMA, KAZUO MATSUDA,

Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai 980 (Japan)

AND KEIJI KAINUMA

National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Kannondai, Yatabe, Tsukuba, Ibaraki 305 (Japan)

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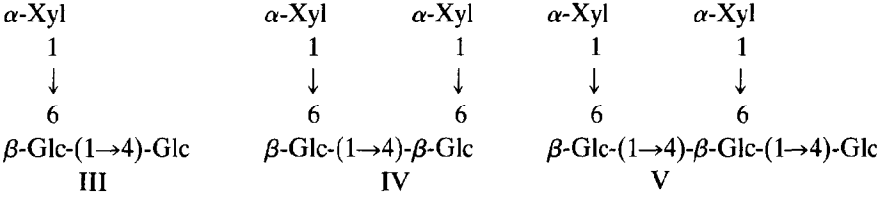
ABSTRACT

Rice hull was successively extracted with hot water (giving PS-I), 0.5% ammonium oxalate (PS-II), 4% potassium hydroxide (HC-I), and 24% potassium hydroxide (HC-II). By neutralization of the second alkaline extract, fraction HC-II was fractionated into subfractions HC-IIA (precipitated) and HC-IIB (not precipitated). To remove a xylan-like polysaccharide that contaminated HC-IIB, the fraction was treated twice with *Streptomyces* sp. endo-(1→4)-β-D-xylanase. Treatment of the xyloglucan-rich fraction from rice hull with partially purified *Trichoderma viride* cellulase, followed by gel filtration on Bio-Gel P-2, yielded seven saccharide fractions, I–VII (V_o). Fractions III, IV, and V, the main oligosaccharides derived from the xyloglucan, were purified by repeated gel-filtration on Bio-Gel P-2, and preparative paper chromatography. The structures of III, IV, and V were examined by determination of the molar ratio of component monosaccharides and of the degree of polymerization, methylation analysis, and hydrolysis with partially purified β-D-glucosidase from *Aspergillus oryzae* and purified α-D-xylosidase from *Aspergillus niger*. The structures of III, IV, and V were characterized as follows.



The structure of the xyloglucan of rice hulls was shown to be similar to those of xyloglucans in the cell walls of rice seedlings and barley seedlings.

*Cell-wall Polysaccharides of Rice Hull, Part II. For Part I, see ref. 1.

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INTRODUCTION

In a previous paper[1], we reported on the structural features of a rice-hull arabinoxylan in the hemicellulose I fraction, which was extracted from the cell walls with 4% potassium hydroxide.

The present study was conducted to elucidate structural features of a xyloglucan in the hemicellulose II fraction, which was obtained from the cell walls by extraction with 24% potassium hydroxide.

EXPERIMENTAL

Materials. — Unhulled rice (Sasanishiki, harvested in Miyagi Prefecture in 1980) was threshed, and the rice hull liberated was ground with a ball mill. The powdered rice-hull was extracted twice with 1:2 ethanol–benzene for 8 h at 80°, cooled, dried, and used in this study.

Enzymes. — *Aspergillus oryzae* enzyme preparation (Sanzyme 1000) was a generous gift of Dr. A. Endo, Tokyo University of Agriculture and Technology. Cellulase was partially purified from "Meicellase P" from *Trichoderma viride* by column chromatography on DEAE-Sephadex A-50 and Sephadex G-100. Endo-(1→4)-β-D-xylanase from *Streptomyces* sp. KT-23 was partially purified by column chromatography on DEAE-cellulose. β-D-Glucosidase was a partially purified preparation from "Sanzyme 1000". α-D-Xylosidase was a preparation from the culture filtrate[2] of *Aspergillus niger* (AMS 4111).

General methods. — All evaporations were conducted under diminished pressure below 40°. Paper chromatography was performed on Toyo No. 50 filter paper by the multiple-ascending method, with a solvent system of 6:4:3 (v/v) 1-butanol–pyridine–water. The silver nitrate dip method was used for the detection of sugars. Gas–liquid chromatography (g.l.c.) was performed with a Yanagimoto Model G-80 chromatograph fitted with a flame-ionization detector. Total carbohydrate was determined by the phenol–sulfuric acid method[3]; xyloglucan, by the method of Kooiman[4]; and glucose, by the Glucostat method[5].

Complete hydrolysis of the polysaccharides with acid. — Complete hydrolysis of the polysaccharide was achieved by heating a sample (1–2 mg) with 2M trifluoroacetic acid (TFA) for 6 h at 100°. The hydrolyzate was cooled and evaporated to dryness.

With fraction HC-IA or HC-IIA, a sample (1–2 mg) was heated with 90% formic acid (0.5 mL) for 30 min at 100°. After removal of formic acid by evaporation, 2M TFA (1 mL) was added to the residue. The mixture was heated for 6 h at 100°, cooled, and evaporated to dryness.

Complete hydrolysis of the oligosaccharides with acid. — Complete hydrolysis of the oligosaccharides with acid was achieved by heating a sample (1–2 mg) with 2M TFA (0.5–1.0 mL) for 6 h at 100°. The hydrolyzates were cooled, and examined by paper chromatography and g.l.c.

Neutral sugar analysis of the polysaccharides and oligosaccharides. — After complete acid hydrolysis of the polysaccharides and oligosaccharides, the monosaccharides in the hydrolyzates were converted into the corresponding alditol trifluoroacetates[6], which were analyzed by g.l.c. Conditions for g.l.c. were those reported previously[1].

Determination of the molecular weight of the xyloglucan fraction by gel filtration on Sepharose CL-6B. — The xyloglucan fraction (5 mg) was dissolved in 0.1M sodium hydroxide solution (0.5 mL), and the solution was applied to a column (1.5 × 96 cm) of Sepharose CL-6B. The column was eluted with 0.1M sodium hydroxide solution, and the eluate was collected in tubes containing 1.0 mL each. The xyloglucan content of each fraction was determined by the method of Kooiman[4]. The column was calibrated with standard dextrans (T-250, T-110, T-40, and T-10).

Methylation analysis of oligosaccharides. — In order to examine the linkages of the glycosyl residues and of the glycose residue at the reducing end, fractions III, IV, and V, both before and after reduction, were methylated by the method of Hakomori[7]. The methylated oligosaccharides were hydrolyzed by the method of Garegg and Lindberg[8]. The sugars in the hydrolyzate were converted into the corresponding alditol acetates, and these were analyzed by g.l.c.

Enzymic hydrolysis of the oligosaccharides. — To obtain further information about the structure of the oligosaccharides derived from rice-hull xyloglucan, an enzymic-degradation study of fragment oligosaccharides was performed as follows.

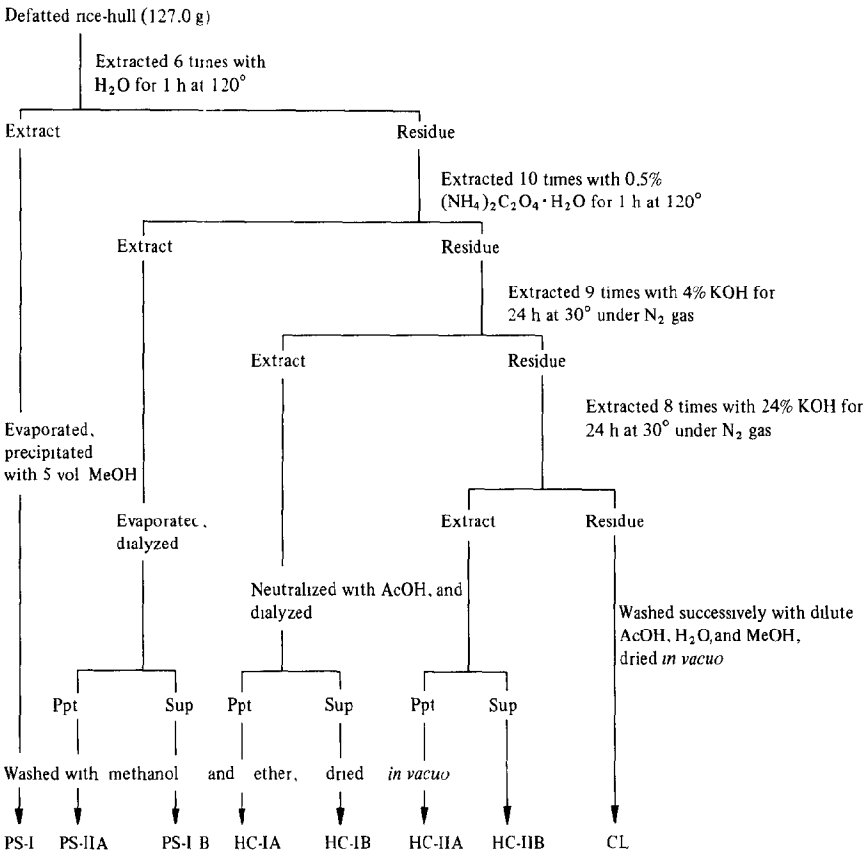
(1) Hydrolysis with β-D-glucosidase from Aspergillus oryzae. — 0.2M Acetate buffer, pH 5.0 (100 μL) and the enzyme solution (50 μL) were added to 50 μL of the sample (100–200 μg). The mixture was incubated for 6 h at 45°, and the enzyme reaction was stopped by heating in a boiling-water bath for 5 min. The glucose produced was determined by the Glucostat method.

(2) Successive treatment with β-D-glucosidase and α-D-xylosidase. — 0.2M Acetate buffer, pH 3.5 (100 μL) containing α-D-xylosidase solution (50 μL) was added to 50 μL of the β-D-glucosidase digest (100–200 μg) of oligosaccharide. The mixture was incubated for 2 h at 45°, and the enzyme reaction was stopped by heating in a boiling-water bath for 5 min. The glucose produced was determined by the Glucostat method.

RESULTS

Extraction and fractionation of the polysaccharides in rice hull. — The polysaccharides in defatted rice-hull (127 g) were extracted and fractionated by the conventional method. The flowsheet of the fractionation of the polysaccharides in rice hull is shown in Scheme 1. The carbohydrate content of each fraction was determined by the phenol–sulfuric acid method.

Fraction PS-II was fractionated into subfractions PS-IIA (insoluble fraction after dialysis) and PS-IIB (soluble fraction after dialysis). By neutralization of the 4% alkali solution, fraction HC-I was fractionated into subfractions HC-IA (pre-



Scheme 1 Flow-chart for fractionation of the polysaccharides of rice hull. (Sup. = supernatant liquor.)

TABLE I

YIELDS, TOTAL SUGAR CONTENT, AND NEUTRAL SUGAR COMPOSITION (MOL %) OF THE FRACTIONS OBTAINED FROM RICE HULL

Fraction	*Yield (g)*	*Total sugar content (g)*	*Rha*	*Ara*	*Xyl*	*Man*	*Glc*	*Gal*
PS-I	3.8	1.8	2.6	10.7	17.2	—	59 0	10.5
PS-IIA	0.4	0.1	2.2	13.1	60.2	1 0	14.4	9.1
PS-IIB	2.4	0.9	2.2	16.1	54.6	—	12.5	14.6
HC-IA	25.0	9.0	—	5.3	94.7	—	—	—
HC-IB	8.9	6.7	—	18.7	73.7	—	2.1	5.5
HC-IIA	3.0	2.1	—	—	98.2	—	1.8	—
HC-IIB	2.9	1.8	—	9.1	75.2	—	11.4	4.3
CL	66.5	24.6	2.4	6.7	42.1	—	44.9	3.9

cipitated) and HC-IB (not precipitated). Similarly, fraction HC-II was fractionated into subfractions HC-IIA and HC-IIB. The neutral-sugar composition of rice hull is shown in Table I.

Examination of xyloglucan in each polysaccharide fraction. — Each polysaccharide fraction was treated with enzyme preparation from *Aspergillus oryzae* (Sanzyme 1000), and the digest was examined by paper chromatography. Isoprimeverose (6-*O*-α-D-xylopyranosyl-D-glucose) was detected in fractions HC-IIA (small amount) and HC-IIB.

Purification of xyloglucan. — To remove xylan-like polysaccharides, fraction HC-IIB was treated with the endo-(1→4)-β-D-xylanase. Xyloglucan was not hydrolyzed with this endo-xylanase. Fraction HC-IIB (1 g) was dissolved in 200 mL of 0.1M acetate buffer (pH 5.5) containing xylanase (3 units). The mixture was incubated for 40 h at 40°. The enzyme reaction was stopped by heating in a boiling-water bath for 10 min. After removal of the precipitated material by centrifugation at 8,400*g* for 20 min, methanol (5 vol.) was added to the supernatant liquor, the mixture was allowed to stand overnight, and the precipitate was collected by centrifugation; total carbohydrate 180 mg, xyloglucan 67 mg. This precipitate was dissolved in 50 mL of 0.1M acetate buffer, pH 5.5, containing xylanase (2 units). The mixture was incubated for 36 h at 40°, and the enzyme reaction was stopped by

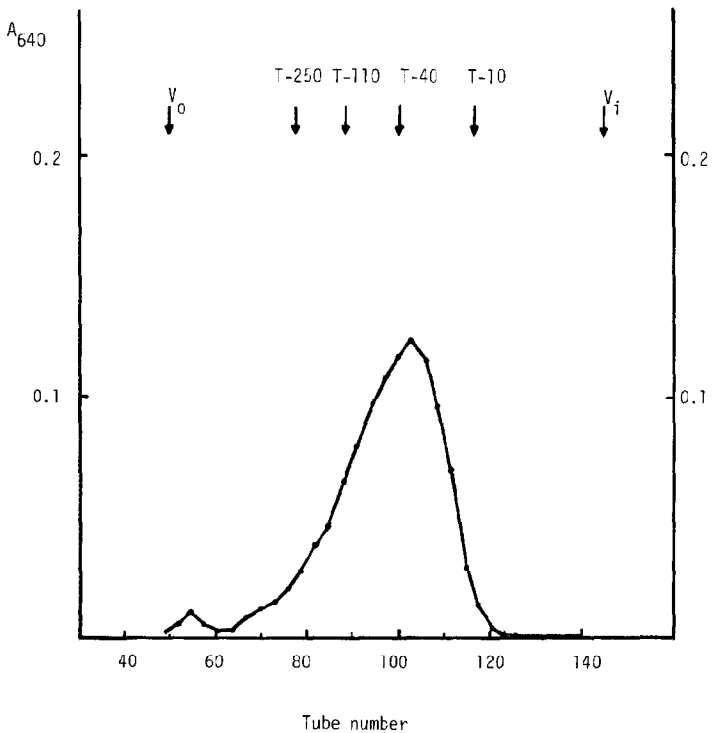


Fig. 1. Gel filtration of rice-hull xyloglucan on Sepharose CL-6B.

heating in a boiling-water bath for 10 min. The xylanase digest was dialyzed against running water overnight, and then centrifuged at 8,400*g* for 20 min. The dialyzate was lyophilized to give xyloglucan (60 mg).

Molecular weight of the xyloglucan fraction. — The molecular weight of the xyloglucan fraction was determined by gel filtration on Sepharose CL-6B. As shown in Fig. 1, the molecular weight of the xyloglucan fraction was estimated to be ~30,000.

Treatment of the xyloglucan fraction with partially purified cellulase from Trichoderma viride. — The xyloglucan fraction (175 mg) was dissolved in 0.02M acetate buffer, pH 5.0 (100 mL) containing partially purified cellulase from *Trichoderma viride* (0.8 unit), toluene (6 mL) was added, and then the solution was incubated for 20 h at 45°. The enzyme reaction was stopped by heating in a boiling-water bath for 10 min.

The cellulase digest was applied to a column (3.0 × 130 cm) of Bio-Gel P-2. The column was eluted with de-ionized water, and the eluate was collected in 10-mL fractions. The carbohydrate content in each fraction was determined by the phenol–sulfuric acid method. As may be seen from the elution profile (see Fig. 2), the cellulase digest of the xyloglucan fraction was separated into seven saccharide fractions [fractions I–VII (V_o)]. The sugar composition of I, II, III, IV, V, VI, and VII (V_o) was examined by paper chromatography and g.l.c. The neutral-sugar composition of each fraction is shown in Table II.

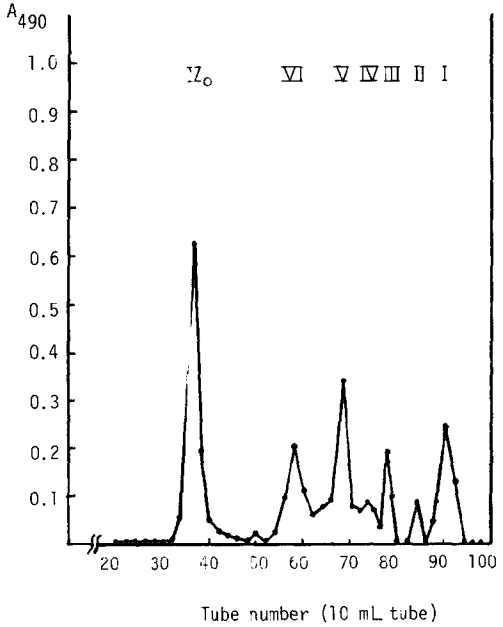


Fig. 2. Fractionation of enzymic-degradation products of rice-hull xyloglucan.

TABLE II

YIELDS AND NEUTRAL-SUGAR COMPOSITION (MOL %) OF THE FRACTIONS FROM CELLULASE DIGEST OF PURIFIED FRACTION HC-IIB

Fraction	*Yield (mg)*	*Ara*	*Xyl*	*Glc*	*Gal*
I	13.0	—	35.0	50.2	14.8
II[a]	1.0				
III	6.2	—	38.0	62.0	—
IV	6.7	—	57.0	43.0	—
V	17.5	—	41.3	58.7	—
VI	16.3	10.3	60.7	16.0	13.0
VII	70.0	29.5	50.8	3.4	16.3

[a]Neutral-sugar analysis of fraction II was not performed.

As shown in Table II, fractions III, IV, and V were composed of D-glucose and D-xylose and did not contain any other sugars. Therefore, these fractions were presumed to be derived from the xyloglucan. Fractions III, IV, and V were purified by repeated gel filtration on Bio-Gel P-2, and preparative paper-chromatography.

Structural analysis of fractions III, IV, and V. — The structural analysis of fractions III, IV, and V was performed by determination of the molar ratio of component monosaccharides, determination of the degree of polymerization (d.p.) by gel filtration on Bio-Gel P-2, methylation analysis, hydrolysis with partially purified β-D-glucosidase from *Aspergillus oryzae*, and hydrolysis with purified α-D-xylosidase from *Aspergillus niger*.

(1) D.p. and neutral-sugar composition. The yields, R_{Glc} values, d.p. by gel filtration on Bio-Gel P-2, and neutral-sugar composition of fractions III, IV, and V are shown in Table III.

(2) Methylation analysis of oligosaccharides. The methylation analysis of oligosaccharides III, IV, and V, before and after reduction, was performed. The results are shown in Table IV.

TABLE III

YIELDS, R_{Glc} VALUES, DEGREES OF POLYMERIZATION, AND SUGAR COMPOSITION OF FRAGMENT OLIGOSACCHARIDES OBTAINED FROM RICE-HULL XYLOGLUCAN

Oligosaccharide	*Yield (mg)*	R_{Glc}	*D.p.*	*Neutral sugar composition (mol %)*	
				Glucose	*Xylose*
III	5.5	0.75	3	67.0	33.0
IV	5.9	0.59	4	47.4	52.6
V	16.9	0.47	5	63.0	37.0

TABLE IV

METHYLATION ANALYSIS OF FRAGMENT OLIGOSACCHARIDES OBTAINED BY ENZYMIC DEGRADATION OF RICE-HULL XYLOGLUCAN

Methylated sugar[a]	T^b	*Relative mol %*					
	Alditol acetate	*III*	*R-III*	*IV*	*R-IV*	*V*	*R-V*
2,3,4,6-Me_4-Glc	**1.00**						
2,3,4-Me_3-Xyl	0.69	37.6	30.5	49.9	46.7	38.6	40.9
2,3,4-Me_3-Glc	2.40	62.4	40.7	25.0	30.8	39.7	24.8
2,3,6-Me_3-Glc	2.40						
2,3-Me_2-Glc	4.64			25.1		21.7	23.0
1,2,3,5-Me_4-Glucitol	0.88				22.5		
1,2,3,5,6-Me_5-Glucitol	0.29		28.8				11.3

[a]2,3,4,6-Me_4-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, and so on. [b]Retention times of the corresponding alditol acetates on an OV-210 column, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as unity.

(3) Enzymic hydrolysis. The hydrolyzates of fractions III, IV, and V with β-D-glucosidase, and the hydrolyzates of fractions III, IV, and V with β-D-glucosidase followed by α-D-xylosidase, were examined by paper chromatography. The results are shown in Fig. 3.

Structure of fraction III. — As may be seen from Table IV, the molar ratio of 2,3,4-Me_3-Xyl to 2,3,4-Me_3-Glc plus 2,3,6-Me_3-Glc is ~1:2 for fraction III. The molar ratios of 2,3,4-Me_3-Xyl, 2,3,4-Me_3-Glc, and 1,2,3,5,6-Me_5-Glucitol are ~1:1:1 for reduced fraction III. Glucose and isoprimeverose were liberated by the action of β-D-glucosidase on fraction III. Degradation of fraction III with β-D-

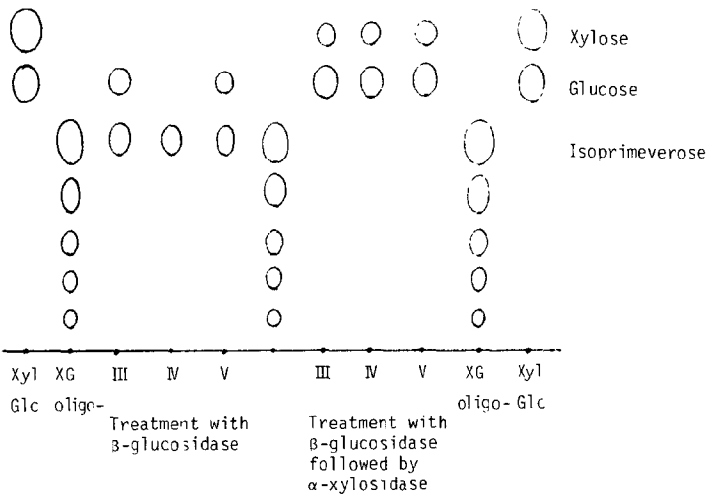


Fig. 3. Paper chromatogram of enzymic digest of oligosaccharides III, IV, and V.

TABLE V

DETERMINATION OF GLUCOSE RELEASED FROM FRAGMENT OLIGOSACCHARIDES III, IV, AND V AFTER TREATMENT WITH *Aspergillus oryzae* β-D-GLUCOSIDASE, AND *Aspergillus oryzae* β-D-GLUCOSIDASE FOLLOWED BY *Aspergillus niger* α-D-XYLOSIDASE

Oligosaccharide	*Glucose produced (μg)*		
	Treatment with β-D-glucosidase	*Ratio*	*Treatment with β-D-glucosidase followed by α-D-xylosidase*
III	57.9	1:2	111.1
IV	0		79.3
V	34.3	1:3	102.3

glucosidase followed by α-D-xylosidase gave rise to xylose and glucose. The glucose content in the digest of fraction III, obtained by successive treatment with β-D-glucosidase and α-D-xylosidase, was about twice that of the digest with β-D-glucosidase alone (see Table V).

From these results, the structure of fraction III was identified as

α-Xyl
1
↓
6
β-Glc-(1→4)-Glc.

Structure of fraction IV. — As may be seen from Table IV, the molar ratios of 2,3,4-Me_3-Xyl, 2,3,4-Me_3-Glc, and 2,3-Me_2-Glc are ~2:1:1 for fraction IV, and those of 2,3,4-Me_3-Xyl, 2,3,4-Me_3-Glc, and 1,2,3,5-Me_4-Glucitol are ~2:1:1 for reduced fraction IV.

Degradation of fraction IV with β-D-glucosidase gave only isoprimeverose. Fraction IV was converted into xylose and glucose by β-D-glucosidase followed by α-D-xylosidase.

From these results, the structure of fraction IV was identified as

α-Xyl α-Xyl
1 1
↓ ↓
6 6
β-Glc-(1→4)-Glc.

Structure of fraction V. — As may be seen from Table IV, the molar ratios of 2,3,4-Me_3-Xyl, 2,3,4-Me_3-Glc plus 2,3,6-Me_3-Glc, and 2,3-Me_2-Glc are ~2:2:1 for fraction V. The molar ratios of 2,3,4-Me_3-Xyl, 2,3,4-Me_3-Glc plus 2,3,6-Me_3-Glc, 2,3-Me_2-Glc, and 1,2,3,5,6-Me_5-Glucitol are ~2:1:1:0.5 for reduced fraction V.

The proportion of 1,2,3,5,6-Me_5-Glucitol is low, possibly due to its volatility.

Glucose and isoprimeverose were liberated by the action of β-D-glucosidase on Fraction V. Degradation of fraction V by successive treatment with β-D-glucosidase and α-D-xylosidase gave rise to xylose and glucose. The glucose content in the digest of fraction V by successive treatment with β-D-glucosidase and α-D-xylosidase was about three times that of the digest with β-D-glucosidase alone (see Table V).

From these results, the structure of fraction V was identified as

α-Xyl α-Xyl
1 1
↓ ↓
6 6
β-Glc-(1→4)-β-Glc-(1→4)-Glc.

DISCUSSION

Two types of xyloglucan are thus far known. The first type includes those obtained from the seeds of some plants, such as *Tamarindus indica*[9-11], *Tropeoleum majus*[12], *Annona muricata* L.[13], *Brassica campestris*[14], and white mustard[15]. These polysaccharides, often called amyloids because of their amylose-like iodine-staining, are present in rather large proportions and are generally regarded as reserve materials. The component sugars of these xyloglucans are glucose, xylose, and galactose (see Table VI).

Another type of xyloglucan has been isolated from the suspension-cultured cells of sycamore and red kidney-bean[16], rapeseed meal[17], rapeseed hulls[17], hypocotyls of mung bean[18] and soybean[19], suspension-cultured soybean cells[20], jojoba seed[21], and tobacco leaves[22]. This type of xyloglucan is recognized as a cell-wall component. These xyloglucans contain fucose, in addition to glucose, xylose, and galactose (see Table VII and Fig. 4). Whereas the structures of the xyloglucans of dicotyledons have been investigated in some detail[18,19,21], xyloglucans of monocotyledons have been less extensively studied.

TABLE VI

SUGAR COMPOSITION OF SEED AMYLOIDS

Amyloid	*Extractant*	*Neutral-sugar composition*			
		Glc	*Xyl*	*Gal*	*Ara*
Tamarindus indica seeds	hot water	4	2.99	1.31	
Tamarindus indica kernel	hot water	8	4	2	1
Tropeoleum majus seeds	hot water	3	2	1	
Brassica campestris seeds	hot water	16	6	3	
Annona muricata seeds	2MNaOH	4	1	1	
White-mustard seeds	hot EDTA	+	+	+	

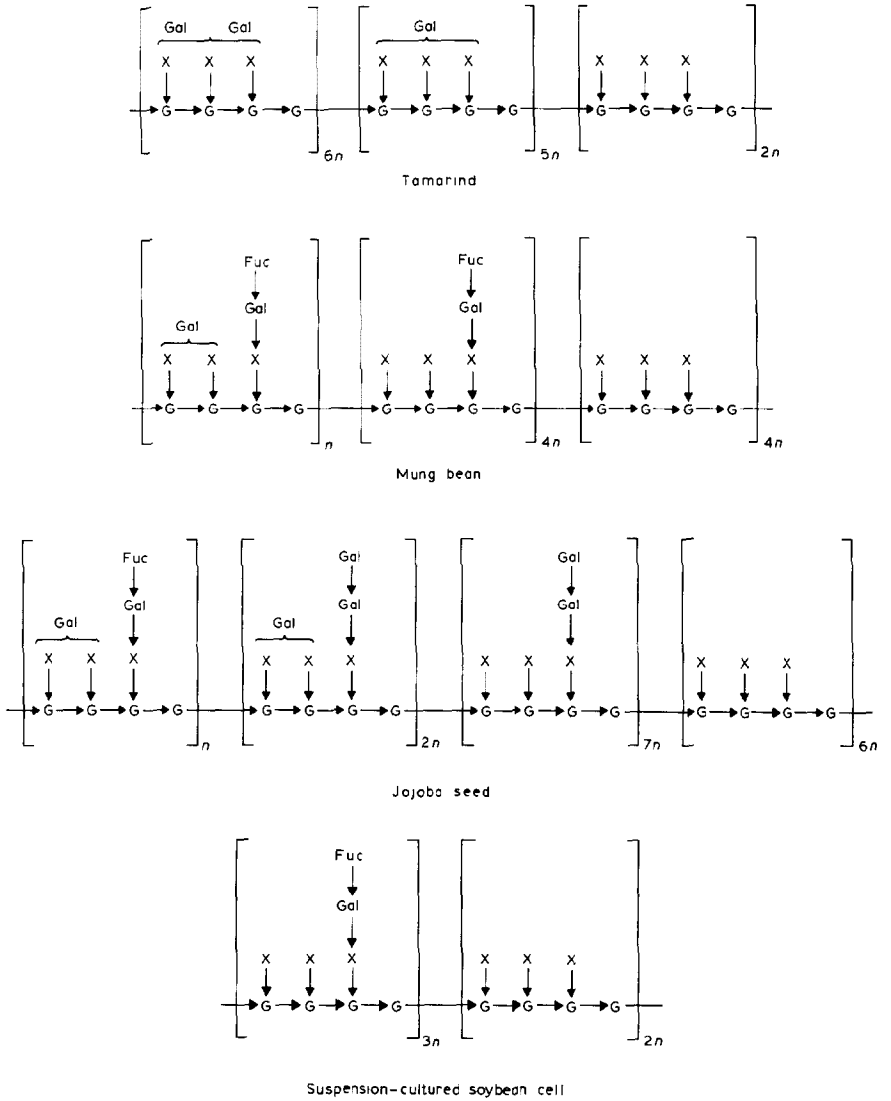


Fig. 4. Various kinds of xyloglucan.

Shibuya and Misaki[23] reported that the xyloglucan in rice-endosperm cell-walls consists of a β-D-(1→4)-linked D-glucan backbone and side chains of single D-xylosyl or galactosylxylosyl groups, both attached to O-6 of the D-glucosyl residues.

Kato *et al.* obtained xyloglucans from immature Gramineae plants (barley[24], rice[25], and bamboo[26]), and proposed their possible structures.

Although the main structural feature of such a xyloglucan is a backbone of β-D-(1→4)-linked D-glucosyl residues and side chains of single D-xylosyl groups branched at O-6 of the backbone D-glucosyl residues, the fine structures of xyloglu-

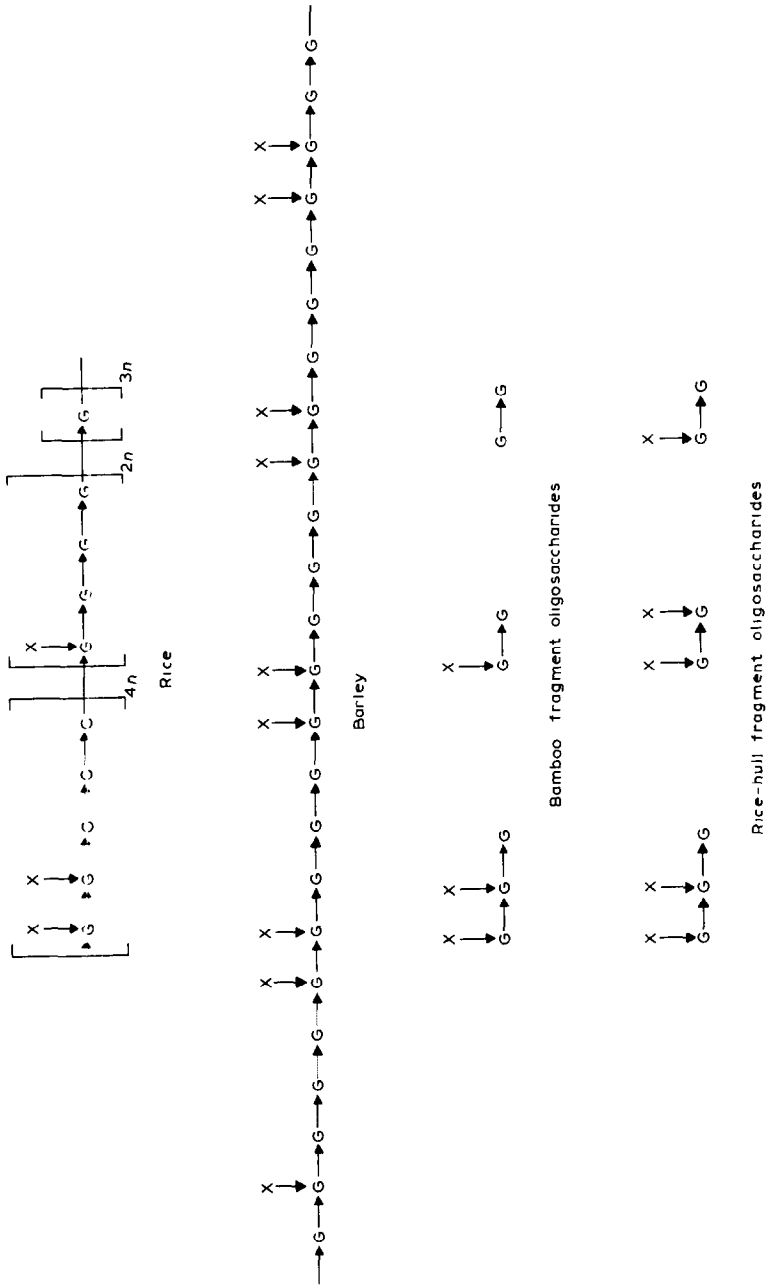


Fig. 5. Various kinds of xyloglucan.

TABLE VII

SUGAR COMPOSITION OF XYLOGLUCANS CONTAINING FUCOSE

Source	*Neutral-sugar composition*			
	Glc	*Xyl*	*Gal*	*Fuc*
Phaseolus aureus etiolated hypocotyls	10	7.0	2.5	1.0
Glycine max etiolated hypocotyls	10	6.0	4.0	1.0
Vigna sesquipedalis etiolated hypocotyls	10	7.0	3.0	1.0
Sycamore extracellular polysaccharide	40	36	8	5
	(10)	(9)	(2)	(1.25)
Sycamore extracellular polysaccharide	53	29	13	5
	(10)	(5.47)	(2.45)	(0.94)
Rapeseed meal	56	30	9	8
	(10)	(5.35)	(1.60)	(1.42)
Rapeseed hull	53	28	12	6
	(10)	(5.28)	(2.26)	(1.13)
Jojoba seeds	10	6.3	4.5	0.3

cans may be somewhat different from each other. The most distinct characteristic of monocotyledon xyloglucan is the lack of component sugars other than D-glucose and D-xylose (see Fig. 5). Moreover, the content of D-xylose is much lower in monocotyledon xyloglucan than in dicotyledon xyloglucan. Thus, a heptasaccharide (glucose:xylose = 4:3), corresponding to the most fundamental, structural unit of dicotyledon xyloglucan, has not been obtained as a fragment oligosaccharide from monocotyledon xyloglucan.

Isolation of a trisaccharide [α-D-xylosyl-(1→6)-β-D-glucosyl-(1→4)-D-glucose], a tetrasaccharide {α-D-xylosyl-(1→6)-β-D-glucosyl-(1→4)-[α-D-xylosyl-(1→6)]-β-D-glucose}, and a pentasaccharide {α-D-xylosyl-(1→6)-β-D-glucosyl-(1→4)-[α-D-xylosyl-(1→6)]-β-D-glucosyl-(1→4)-D-glucose} from the cellulase digest of rice-hull xyloglucan suggests that rice-hull xyloglucan has a structure fundamentally similar to the proposed structures of the xyloglucans from immature Gramineae plants (barley, rice, and bamboo).

ACKNOWLEDGMENTS

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