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COMPOSITION OF CELL WALL PREPARATIONS OF RICE BRAN AND GERM*

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Key Word Index—*Oryza sativa*; Gramineae; rice bran; rice germ; cell walls; polysaccharides; solubility fractions.

Abstract—Cell walls of petrol-defatted non-waxy IR32 rice bran and germ were prepared by protein removal with 0.5% SDS–0.6% β-mercaptoethanol, heating the residue to 80°, and destarching with *Bacillus licheniformis* α-amylase. A waxy rice, IR29, had a similar cell wall composition as IR32. Principal wall sugars were arabinose, xylose, and glucose. The 0.5 M sodium or potassium hydroxide and 8 M urea preferentially extracted arabinose-, xylose- and uronic acid-rich polysaccharides but 6 M sodium hydroxide–0.81 M boric acid extracted mannose-rich polysaccharides. DEAE-cellulose BO_3^{3-} chromatography of the 0.5 M sodium hydroxide extracts gave fractions of similar arabinose–xylose ratios. Proteins in the cell wall preparations had only 0.4–1.6% hydroxyproline, and were bound mainly to polysaccharides, based on disc gel electrophoresis. The preparations were autofluorescent in UV and rich in phenols, mainly ferulic acid. The cell wall preparations and their 8 M urea fractions had a softening effect on defatted waxy starch aqueous gel at 0.2–2% of the starch.

INTRODUCTION

The cell walls of rice grain are important for maintaining the grain integrity during processing and cooking. They are thicker in the bran layers than in the starchy endosperm[1]. Considerable interest in the use of cereal bran to increase dietary fiber (DF) intake is evident in recent years[2, 3]. Substitution of milled rice by brown rice in the diet has been suggested as a means to increase nutrient intake because of the higher nutrient content, particularly B vitamins, of brown rice. In addition, rice cell wall constituents were recently shown to complex with starch and affect Amylograph viscosity[4, 5] and to effectively bind bile acids[6, 7].

Because of these and recent advances in methodology for polysaccharide characterization since our earlier study[8], the properties of bran and germ of IR32 brown rice and their cell wall preparations were studied. An aqueous method of cell wall preparation was adapted because it simulates the manner in which rice is prepared for consumption by boiling. A companion paper deals with the milled-rice fraction[9]. Because of differences in origin, bran and germ were analysed separately. The bran consists mainly of triploid aleurone layer (part of endosperm) plus maternal (diploid) tissues—pericarp, seed coat, and nucellus[1]. The germ consists mainly of the diploid embryo and the covering bran layers which are not separated during milling. A waxy rice, IR29, was thus analysed to determine if the *waxy* gene affects carbohydrate composition of the aleurone layer, in addition to amylose content and free sugars[10]. Bran has lower fat, protein, and free sugar content than germ, but higher levels of ash, lignin, and polysaccharides[11].

*Taken in part from 1981 M.S. thesis of C.C.M. from the University of the Philippines at Los Baños.

RESULTS AND DISCUSSION

Cell wall preparations

Because of the adverse effect of wet heating on rice protein solubility[12], protein extraction was done during the preparation of cell walls before gelatinizing the contaminant starch and α-amylolysis treatment. About seven extractions with 0.5% SDS–0.6% β-mercaptoethanol (ME) effectively removed the proteins of bran and germ. The preparation removed 85% of the protein, all the starch, and 90% of the ash from IR32 bran, and 96% of IR32 germ protein and ash. The preparations had 2.7% ash in the bran wall and 2.1% ash in the germ wall. *Ca* 40% of bran silicon and 30% of germ silicon were retained in the cell wall preparations, probably because of the reported localization of silicon in the cell walls[13]. Silicon contents were 0.8% in bran wall and 0.3% in germ wall. The removal of phosphorus, magnesium and potassium was consistent with the location of globoid (phytate) particles inside cellular aleurone protein bodies[14]. Calcium content was also higher in the cell walls (0.3% in bran and 0.4% in germ) than in the starting materials (0.2%). More dramatic was the sodium content of 0.1% in bran cell wall and 0.2% in germ cell wall as compared to 0.02% in the starting materials. Although the calcium content may be accounted for by retention alone, the results suggest binding of cations from the salts used during preparation, in conformity with the reported cation-exchange capacity of rice bran germ DF[15].

The actual recovery of IR32 cell wall preparations from bran (27%) and germ (11%) was closest to the neutral detergent fiber (27.7 and 11.0%), modified by adding an α-amylase treatment to reduce starch contamination[16]. The next closest was enzymic DF of 24.8 and 10.6%. Neutral detergent fiber was 32.4% for bran and 14.8% for germ; acid detergent fiber was 12.0% for bran and 4.1% for germ. Calculated hemicellulose content (neutral detergent fiber − acid detergent fiber) was 20.4% for bran and 10.7% for germ. Lignin content was 12.0% for bran wall and 7.2% for germ wall. Cellulose content (acid detergent fiber − lignin) was 8.9% for bran and 3.3% for germ. Crude fiber values were 9.5% for bran and 3.5% for germ. These values were similar to values reported for commercial rice bran (with germ) of 12% acid detergent fiber[15], 28.7–44.7% neutral detergent fiber[17], 22.6–24.4%[15], and 25.4%[18] modified neutral detergent fiber, 4.1–14.5%[19], 9.2–21.1%[17] and 8.8%[18] crude fiber, and 12–17% crude lignin[15].

Microscopic examination of the bran and germ cell wall preparations confirmed the absence of starch in the wall preparations. The bran preparation had contaminant sub-aleurone cell walls. The walls exhibited green autofluorescence at 340–380 nm with a 430 nm barrier filter and blue autofluorescence with the 460 nm barrier filter. With 450–490 nm excitation, the wall exhibited yellow autofluorescence with both barrier filters. Ferulic acid crystals showed similar colors. The total phenols were identified by Si gel TLC to be ferulic acid plus *p*-coumaric acid and two other unknown phenols, one of which was a major fraction. The minor and major unknown phenols had R_{ferulic} values of 0.53 and 0 in hexane–amyl alcohol–acetic acid and 0.84 and 0.58 in benzene–methanol–acetic acid, respectively. A ferulic acid-containing H_2O-soluble rice bran hemicellulose with MW $5–10 \times 10^4$ was recently isolated by Mod *et al.*[4]. Ferulic acid was also the major phenol of barley grain cell walls[20]. Phenolic content was 11.0% for bran wall and 7.4% for germ wall which approximated lignin contents of 12.0 and 7.2%, respectively.

Waxy IR29 and non-waxy IR32 cell wall preparations had a similar carbohydrate composition (Table 1), suggesting that the *waxy* gene had no dramatic effect on wall polysaccharide composition. Based on the composition of IR32 bran[11], polysaccharide retention in the cell wall preparation was 51% of hexosans, and 100% for pentosans and polyuronic acids. Values for the IR32 germ cell wall were 50% of hexosans, 76% of pentosans and 100% of polyuronic acids. These preparations were richer in hexosans and poorer in pentosans than the rice bran germ DF of Rasper[15] with 12–14% hexosans, 36–42% pentosans, 8% polyuronic acid and 27–33% crude cellulose.

Major neutral sugars for IR32 and IR29 bran and germ cell wall preparations were arabinose, glucose and xylose, together with galactose, mannose, fucose, and rhamnose (Table 1). The germ cell wall was richer in arabinose than xylose (arabinose–xylose ratio 1.7) than the bran cell wall (arabinose–xylose ratio 1.0). Comparison of the GC data with the colorimetric method of ref. [21] showed that the colorimetric method of correcting for mixtures of pentosans and hexosans over-estimated the pentosans content. Rhamnose was earlier reported present in rice bran hemicellulose[17]. Fucose had not been identified previously in rice bran cell walls but has been reported in milled-rice cell walls[22]. Spots with identical R_{glc} values as galacturonic acid (R_{glc} 0.29) and glucuronic acid (R_{glc} 0.38) were obtained by TLC with *iso*-propanol–acetone–1 M lactic acid[23], and also with *n*-butanol–pyridine–water.

Solubility fractionation of preparations

The complex nature of the water-insoluble cell wall preparations was evident from the solubility fractionation studies. Repeated extraction of the bran and germ cell wall preparations with 0.5 M sodium hydroxide and 0.013 M sodium borohydride under nitrogen according to ref. [8] preferentially extracted proteins, polyuronic acid and pentosans (hemicellulose) and the residue was richer in glucose and mannose (Table 1). This solvent was used by Gremli and Juliano[8] to extract rice bran hemicellulose. Mannose was also detected by these authors in the bran hemicellulose. The presence of glucose in the 0.5 M sodium hydroxide fraction was verified[15], in contrast to the earlier data of Gremli and Juliano[8] on IR8 bran. The present preparations also had a lower galactose content.

A more complicated scheme by Anderson and Stone[24] showed the 8 M urea extract to be rich in glucose (Table 1), probably mixed β-glucans, since the sample was essentially starch-free. The extremely high protein content may be due in part to urea contamination even after dialysis. The 0.5 M potassium hydroxide extract had a similar composition to the 0.5 M sodium hydroxide extract of ref. [8]. Mannose and glucose contaminations of the pentosans–polyuronic acid fractions were minimal. The 4.27 M potassium hydroxide solvent further extracted the pentosans with more glucose contamination. Mannose-rich polysaccharides were extracted by 6 M sodium hydroxide together with the other polysaccharides. The residues were mainly glucose and probably cellulose still containing contaminant polysaccharides. The polyuronic acid content of the 6 M sodium hydroxide extract and of the residue was very low. Galactose was present in all fractions.

Actual analysis of the 8 M urea extracts of bran and germ cell walls for mixed 1:3- and 1:4-β-glucans[25] revealed only 6% for bran and 4% for germ which represented 46 and 25% of the hexosan content of bran and germ walls, respectively (Table 1). By GC analysis, glucose represented 62% of hexoses in the 8 M urea extract of bran wall and 56% of hexoses in germ wall extract.

DEAE-cellulose BO_3^{3-} chromatography[8] of the 0.5 M sodium hydroxide fraction of IR32 bran and germ cell walls showed similar elution patterns, with the 0.01 and 0.1 M BO_3^{3-} fractions as the minor ones (10–13%) and the major fractions (30–47%) were those eluted with 0.3 and 0.5 M BO_3^{3-}. The major sugars of all fractions were 38–48% arabinose and 39–47% xylose in the bran wall and 40–50% arabinose and 34–42% xylose in the germ wall corresponding to arabinose–xylose ratios of 1.0–1.1 and 1.1–1.2, verifying the reported similarity of composition of the various fractions[8]. The other sugars were also detected. However, polyuronic acid content was

Table 1. Composition of whole cell wall preparations of IR29 and IR32 rice bran and germ and solubility fractions of IR32

Sample and solubility fraction*	Wt %	Protein	Total hexosans (% dry wt basis)	Total pentosans (% dry wt basis)	Total uronic acid poly-saccharides	Neutral sugar composition (% of total)							Ara–xyl ratio
						Rha	Fuc	Ara	Xyl	Man	Glc	Gal	
IR29 Bran cell wall		8	28	30	9	<1	<1	31	29	2	30	6	1.1
IR32 Bran cell wall	100	9	27	34	5	<1	<1	27	26	2	36	9	1.0
(A) Method of Gremli and Juliano[8]													
0.5 M NaOH (4 × 1 hr) extract	16	15	10	64	7	<1	5	44	35	1	7	7	1.2
0.5 M NaOH (2 × 14 hr more) extract	8	14	8	64	9	<1	2	47	41	<1	3	7	1.1
Residue	76	3	38	20	3	<1	2	19	12	7	52	6	1.6
(B) Method of Anderson and Stone[24]													
8 M urea extract	3	68	13	12	4	<1	3	19	15	10	41	11	1.2
0.5 M KOH extract	26	15	8	57	8	<1	4	49	35	<1	4	7	1.4
4.27 M KOH extract	20	2	10	20	3	1	3	35	28	2	21	10	1.2
6 M NaOH–0.81 M H_3BO_3 extract	18	3	3	5	1	1	3	40	13	16	18	9	3.0
Residue	32	2	46	10	1	<1	<1	9	6	3	78	3	1.6
IR29 Germ cell wall		7	27	28	6	<1	2	32	26	2	30	8	1.2
IR32 Germ cell wall	100	7	29	38	6	<1	4	31	19	4	33	8	1.7
(A) Method of Gremli and Juliano[8]													
0.5 M NaOH (4 × 1 hr) extract	17	13	14	67	7	<1	5	42	32	1	9	11	1.3
0.5 M NaOH (2 × 14 hr more) extract	13	8	12	69	10	1	5	50	29	<1	5	9	1.7
Residue	70	3	45	22	4	<1	1	19	14	5	53	7	1.4
(B) Method of Anderson and Stone[24]													
8 M urea extract	5	54	16	21	4	<1	8	27	19	4	30	12	1.4
0.5 M KOH extract	28	11	10	55	10	1	4	49	31	<1	4	10	1.6
4.27 M KOH extract	25	1	12	27	4	<1	2	39	26	2	20	11	1.5
6 M NaOH–0.81 M H_3BO_3 extract	17	1	4	3	<1	<1	2	28	11	17	25	17	2.6
Residue	25	2	52	9	1	<1	<1	8	3	11	75	2	2.4

*All solvents contained 0.013 M $NaBH_4$, except 8 M urea. Extractions done under N_2 atmosphere.

highest (9–12%) in the 0.3 and 0.5 M BO_3^{3-} fractions. All fractions showed absorbance at 280 nm, suggesting complexing of protein with polysaccharides. Phenolic binding with polysaccharides was probably labile in 0.5 M alkali[4].

Sepharose 4B-CL fractionation of the 0.5 and 4.27 M alkaline extracts of bran and germ cell walls revealed polydispersity of the polysaccharides in the 0.3% sodium chloride–0.05% sodium nitride eluant. The MW ranged from 7000 to 2×10^6. All fractions showed absorption at 280 nm. But a broad peak at MW 10000, which did not coincide with a polysaccharide peak, suggested low MW phenolics saponified during alkaline extraction[4] rather than protein since most of the proteins were bound to polysaccharides, as demonstrated by gel electrophoresis. They could also be low MW proteoglycans[26].

Protein characterization

Protein-rich fractions were mainly the urea and 0.5 M sodium or potassium hydroxide extracts (Table 1). Analytical polyacrylamide disc gel electrophoresis of the extracts showed two bands which stained for protein and carbohydrates for the 8 M urea extracts of both bran and germ walls and one band for the three alkaline extracts (Fig. 1). Carbohydrate staining was more intense than protein staining. Considerable staining was also shown in the stacking gel and immediately following the tracking dye. The protein staining at the tracking dye was greater than that of carbohydrate staining suggesting low MW bran proteoglycans, which are reported to be fast migrating on gel electrophoresis at pH 8.9[25]. Except for the urea extracts in which one or three protein bands were negative for carbohydrate, the proteins of the cell wall fractions were probably complexed with polysaccharides. Similar results were reported by Mod *et al.*[17, 27] for water- and alkali-soluble rice bran hemicelluloses but contrasted with earlier results of Gremli and Juliano[8] who reported the proteins to be a low MW contaminant not precipitated by TCA. None of the protein bands stained for lipids (Sudan IV), suggesting the absence of previously reported lipoprotein or proteolipid[28] in the preparations.

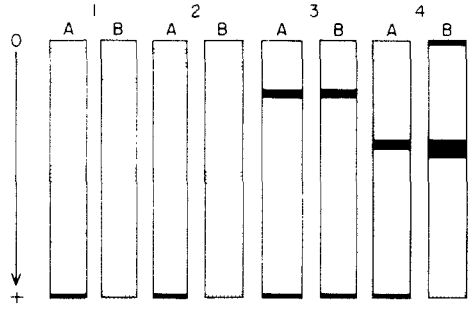

Fig. 1. Analytical disc gel electrophoregrams at pH 8.3 of proteins associated with solubility fractions of IR32 bran and germ cell walls (CW) stained with Coomassie Brilliant Blue G-250 (A) or basic fuchsin (B). 1: 8 M urea fraction of bran CW; 2: 8 M urea fraction of germ CW; 3: 0.5 M KOH fraction of bran or germ CW; and 4: 4.27 M KOH fraction of bran or germ CW.

Amino acid composition of IR32 bran and germ and their cell wall preparations and 0.5 M alkali fractions confirmed the higher lysine content of these outer tissues relative to milled rice and the poor content of hydroxyproline (Table 2)[29, 30]. Bran and germ showed similar patterns despite their differences in chromosome numbers, in support of the similarity in their morphology and function as lipid and phytin reserves for the embryo[1]. They were lower in sulphur-containing amino acids than milled-rice protein[29]. The proteins of cell wall preparations were richer in aspartic acid, hydroxyproline, leucine, phenylalanine, proline, threonine and valine than those of the starting tissues but were lower in arginine, glutamic acid and tryptophan. Most of the hydroxyproline was in the cell wall fraction. The proteins removed during cell wall preparation probably included most of the protein body proteins, particularly albumins and globulins[31].

The 0.5 M alkali fractions of bran and germ cell walls were similar in amino acid composition to the whole wall preparations (Table 2), except for lower cystine and glycine, and higher aspartic acid contents. Mod *et al.*[17] reported only 0.5–0.7% hydroxyproline in alkali-soluble rice bran hemicelluloses. By contrast, Yamagishi *et al.*[26] reported water-soluble rice bran proteoglycans with *ca* 10 mol% hydroxyproline.

Complexing with amylopectin

Addition of 0.2 or 0.4% IR32 or IR29 bran cell wall to an aqueous defatted IR29 waxy starch gel resulted in softening of the gel as reflected in longer gel length (Table 3). The wall preparations probably still contained components extracted in boiling water, as the samples were not heated above 80°. All IR32 bran cell wall fractions, particularly the urea-soluble fraction, showed this property. Among the corresponding germ cell wall fractions, only the 8 M urea fraction had the softening effect on IR29 amylopectin gel of the whole cell wall preparation.

The complexing ability of cell wall preparations has been attributed to lignin and polyuronic acid rather than to the neutral polysaccharide fractions[2, 3]. Uronic acid was present in the various fractions, particularly the 0.5 M potassium hydroxide fraction. The cold- and hot-water-soluble IR32 bran polysaccharides had been shown to harden, instead of to soften, the gel viscosity of IR29 waxy rice starch in water[32]. By contrast, current cell preparations softened the IR29 starch gels with the urea extract as the most reactive and the cellulose (residue) fraction as the least reactive (Table 3). Since water was the solvent, the minor water-soluble or hydratable fraction of the wall preparations must be very reactive in its complexing ability with amylopectin. Mod *et al.*[4] isolated a ferulate-containing hemicellulose fraction of water-soluble rice bran hemicellulose, which has varying effects on the Amylograph viscosity curves of milled-rice flours. The differences in response among rice varieties[4] may be related to differences in ratios of fractions which harden and those which soften starch gels. Other properties which have been studied include the binding of bile acids[6, 7] and water hydration and cation-exchange capacities[15].

Table 2. Amino acid composition of defatted IR32 rice bran and germ and their corresponding cell walls and 0.5 M alkali-soluble wall fractions (g/16.8 g N)

Amino acid	Bran	Bran cell wall			Germ	Germ cell wall			LSD† (5%)
		Whole	0.5 M NaOH fraction	0.5 M KOH fraction*		Whole	0.5 M NaOH fraction	0.5 M KOH fraction*	
Recovery (wt%)	100	27	4	7	100	11	2	3	
Ala	7.6	7.2	6	7	7.2	6.8	6	8	0.3
Arg	9.4	5.8	6	5	10.4	6.2	7	5	0.2
Asp	11.8	14.9	16	16	10.6	12.2	14	18	0.7
Cys	1.8	1.7	1	1	1.6	2.0	1	1	0.3
Glu	16.6	9.8	10	11	17.3	9.5	11	12	0.5
Gly	6.8	7.3	6	6	6.6	7.7	6	6	0.3
His	3.5	2.8	3	3	3.4	3.6	4	2	0.2
Hyp	0.1	0.4	<1	3	0.2	1.6	2	1	0.1
Ile	3.9	4.8	4	5	3.5	4.4	4	5	0.1
Leu	7.8	10.6	10	11	7.0	9.8	10	12	0.3
Lys	5.7	5.4	5	5	6.2	6.0	6	4	0.1
Met	1.3	1.0	2	1	1.4	1.2	2	2	0.5
Phe	5.3	9.2	7	8	4.5	7.4	7	10	0.4
Pro	4.9	7.7	8	11	4.3	7.6	8	10	0.5
Ser	5.0	5.2	5	4	4.8	5.3	5	4	0.1
Thr	4.4	5.0	5	6	4.2	6.0	6	5	0.3
Trp	0.6	0.2	<1	<1	1.0	0.5	<1	2	0.1
Tyr	3.6	3.1	4	6	3.7	4.4	4	4	0.4
Val	6.0	7.2	10	8	5.6	6.6	7	8	0.3
Amm	1.8	2.2	3	2	1.8	2.2	2	2	0.3

*After extraction with 8 M urea[24].

†For bran and germ and their cell walls.

Table 3. Effect of addition of IR32 rice bran and germ cell walls and their fractions on the gel consistency of 100 mg defatted IR29 starch in 1.6 ml water

Additive and weight added	Gel consistency* (mm)	
	Bran cell wall	Germ cell wall
None (control)	56	68
Whole wall, 0.2 mg/0.4 mg	100/100	89/87
8 M urea fraction, 1 mg/2 mg	100/100	100/100
0.5 M KOH fraction†, 1 mg/2 mg	66/79	62/61
4.27 M KOH fraction†, 1 mg/2 mg	67/74	70/51
6.0 M NaOH fraction†, 1 mg/2 mg	64/68	61/55
Residue, 1 mg/2 mg	75/57	65/59

*In 100 × 11 mm i.d. test tube.
†Solvent contained 0.013 M $NaBH_4$.

EXPERIMENTAL

Materials. Rough-rice samples of non-waxy IR32 rice and waxy IR29 rice were taken from the 1976 crops of the Experimental Farm of the Institute. The IR32 rough rice was dehulled in a Satake Type SB-2B one-pass pearler–dehuller. The broken grains of the resulting brown rice were removed using a Satake Type RG-C6A rice grader. The head (whole grain) brown rice was undermilled using the Satake rice milling machine to yield undermilled rice and 5.0% by wt crude bran. For the IR29 rough rice, the dehuller used was a Satake THU-35A testing husker and head brown rice was obtained using the Lewis Grant sizer. Undermilling was done on a Satake Type MC-250 one-pass rice whitening machine to obtain 6.2% by wt crude bran. The wt% bran removal was calculated by comparing the wts of triplicate 100 whole grains each of brown rice and undermilled rice. The crude bran was successively passed through 12-, 20-, 30- and 40-mesh sieves to remove contaminant broken grains and hull fragments. The fraction that passed through the 40-mesh sieve was termed bran. The germ fraction, together with broken grains and hull fragments, was retained in the 20-mesh sieve. These contaminants were removed by winnowing and the germ was collected by flotation in a $CHCl_3$–95% EtOH (2:1) medium in 3.2% yield from IR32 brown rice and 1.8% yield from IR29 brown rice. The bran and germ samples were defatted by refluxing petrol for 48 hr and the lipid extracts were dried under red. pres. while the defatted samples were air-dried in the hood for 4 days. The defatted bran and germ samples for chemical analyses were ground in a Udy cyclone mill with either a 40- or 60-mesh sieve.

Cell wall preparation. A sample wt (25 g) was soaked in 200 ml H_2O for 1 hr and then homogenized for 10 min in a Waring blender. The aq. suspension was passed through a 40-mesh sieve, then centrifuged at 4000 *g* for 15 min at 25° and the supernatant was decanted. The homogenization step in the Waring blender was done twice on the residue. The residue after 3 H_2O extractions was then extracted (×8) by 200 ml 0.5% SDS–0.6% β-ME as in ref. [33] and then washed with H_2O until the washing was negative for SDS and β-ME. The washed residue was suspended in 250 ml 0.05 M NaOAc buffer pH 5.6 containing 90 ppm Ca^{2+} heated to 80° and maintained for 10 min at that temp. Additional 250 ml NaOAc buffer containing Ca^{2+} was added to the heated sample and the diluted suspension was equilibrated at 60–70°. A 5-ml *Bacillus licheniformis* α-amylase enzyme soln (NOVO, Termamyl 60L) was added to 45 ml NaOAc buffer containing Ca^{2+} and the β-glucanase contaminant was inactivated according to ref. [34] by maintaining the enzyme soln in an 80° water bath for 5 min. Afterwards, a 10-ml aliquot of the enzyme soln was added to the sample suspension and the sample–enzyme mixture was incubated at 60–70° for 3 hr[33]. After complete starch hydrolysis as verified by staining an aliquot of the suspension with I_2–KI reagent, the suspension was centrifuged at 4000 *g* for 15 min, washed with H_2O by repeated suspension and centrifugation, dried by solvent exchange using absolute EtOH, and then air-dried in the hood for 4 days[35]. Wt recoveries of cell walls were 27% for IR32 bran, 34% for IR29 bran, 11% for IR32 germ and 19% for IR29 germ.

Microscopy. The bran and germ samples were examined by light microscopy using Sudan III and erosin–methylene blue dyes. Starch in the cell wall preparations was investigated by microscopy under ordinary and polarized light before and after staining with I_2–KI reagent. The cell walls and cell wall fractions were mounted directly on the slide and examined by fluorescence microscopy[20]. Exciter filters which provided excitation in the UV (340–380 nm) and blue (450–490 nm) regions of the spectrum were used. The samples were viewed either with the 430 or the 460 nm barrier filters. Ferulic acid was used as reference material.

Analytical methods. Moisture content was determined as loss of wt for 1 hr at 130° according to ref. [36]. The sample was ashed in a furnace at 490° for 4 hr and K, Mg, Ca, and Na were determined on the ash by atomic absorption spectrophotometry and P by colorimetry[37]. Si was determined by wet digestion described in ref. [37] followed by colorimetry of the digest[38]. Free sugars were extracted by hot 80% EtOH[10] and the extract was assayed for total sugars by the PhOH–H_2SO_4 reagent[39] using glucose as standard.

Protein content was analysed by the standard micro-Kjeldahl digestion followed by colorimetric assay of NH_3 in the digest[37] and using the factor 5.95. Protein was hydrolysed with 6 M HCl for 23 hr at 110° under N_2 in sealed tubes and the amino acids of the hydrolysate were analysed with a Model 120 Beckman Amino Acid Analyser with AA-15 and PA-35 resins[40]. Hydroxyproline was determined in the physiological fluid run using M-72 resin[41]. Cys and Met were assayed as cysteic acid and methionine sulfone, respectively, on sample subjected to performic

acid oxidation before hydrolysis[42]. Tryptophan was determined by $Ba(OH)_2$ hydrolysis and short PA-35 CC[43]. Polyacrylamide disc gel electrophoresis of the protein was done according to ref. [44] using Coomassie Brilliant Blue G-250, basic fuchsin, and Sudan IV dyes as stains.

Crude fiber was determined by the method of ref. [36]. The procedure of ref. [45] was used in analysing for acid detergent fiber and lignin. Determination of neutral detergent fiber was carried out according to ref. [46]. To obtain the modified neutral detergent fiber[16], amylase treatment of the neutral DF residue at 60–70° for 3 hr was done using *Bacillus licheniformis* α-amylase. The enzymic dietary fiber was analysed by the procedure of ref. [47] using pepsin (Wako Pure Chemical Industries, Ltd) and pancreatin from porcine pancreas (Sigma).

Associated lipids of the cell walls were extracted with $CHCl_3$–MeOH (2:1) for 8 hr followed by H_2O-satd *n*-BuOH for 23 hr as in ref. [48]. After lipid extraction, the residue was saponified by 0.5 M KOH at 60° for 90 min under N_2 atmosphere[49]. The extracted phenolics were examined by TLC using Si gel F-254 plate and solvent systems hexane–amyl alcohol–HOAc (100:16:0.25) and C_6H_6–MeOH–HOAc (90:16:8)[50, 51]. After each run, the spots were observed under UV light before and after exposure to NH_3 vapor.

The cell wall preparations were extracted (×4) at 25° with 25 vol. 0.5 M NaOH + 0.013 M $NaBH_4$ under N_2 for 1 hr according to ref. [8]. The residue was further extracted (twice) for 14 hr under the same conditions. The polysaccharide of the 4 × 1 hr extract was fractionated on a column (2.6 × 37 cm) of DEAE-cellulose (Cellex D, 0.76 meq/g, Bio-Rad Laboratories) in BO_3^{3-} form[8]. The carbohydrates were eluted with 0.01, 0.1, 0.3, and 0.5 M Na–BO_3^{3-} (pH 9.2). Serial extraction of the bran and germ cell wall preparations was done using 8 M urea, 0.5 M KOH + 0.013 M $NaBH_4$, 4.27 M KOH + 0.013 M $NaBH_4$ and 6 M NaOH + 0.81 M H_3BO_3 + 0.013 M $NaBH_4$ as employed in ref. [24]. Cell walls (500 mg) were extracted (×3) with 25 ml extractant for 16 hr at 25° with constant stirring under N_2. After the third extraction the residue was washed thoroughly with H_2O. Combined extracts and washings were dialysed against H_2O and freeze-dried.

Samples (25 mg) were dispersed by wetting with 72% H_2SO_4 (6.83 g) and left for 3 hr at 25°[52]. H_2O was added to the sample dispersion to make 50 ml of a 2 N H_2SO_4 slurry. This slurry was filtered through a coarse sintered glass filter and aliquots were analysed for uronic acid content by the procedure in ref. [21] using glucuronic acid as standard. Another set of samples was dispersed in 72% H_2SO_4, left for 3 hr at 25°, diluted to make a 2 N H_2SO_4 slurry, and heated at 100° for 2.5 hr [52]. Aliquots of the filtered hydrolysate were analysed for total sugar[39], total hexosan[21] (glucose × 0.90) and total pentosan[21] (arabinose × 0.88). Mutual interference effects of hexoses, pentoses, and uronic acids were corrected using the correction factors suggested in ref. [21]. The total polyuronic acid was estimated by multiplying the total uronic acid (as glucuronic acid) by 0.91. The identity of the uronic acid constituent was checked in the hot 0.25% $(NH_4)_2C_2O_4$[22] extract of the cell wall preparations, after hydrolysis at 100° for 2.5 hr[52], by TLC using Si gel 60 plates and solvent systems *n*-BuOH–C_5H_5N–H_2O (10:3:3) and *iso*-PrOH–Me_2CO–1 M lactic acid (4:4:2)[23].

Aliquots of the hydrolysates[52] of the cell walls and their fractions were neutralized with 0.25 M $Ba(OH)_2$ to pH 5.5–6.5, allowed to stand for 1 hr and the $BaSO_4$ ppt was filtered through Whatman 42 filter paper. The filtrate was freeze-dried and the sugar monosaccharides were transformed into aldononitrile acetate derivatives[53, 54]. Separation of aldononitrile acetates was done using a gas chromatograph equipped with H_2 FID and a 122 cm × 2 mm glass column packed with 3% OV 225/2.5% tetramethylcyclobutanediol succinate on 80–100 mesh Supelcoport[53]. The run was temp. programmed from 190 to 226° at 4°/min. The carrier gas flow rate was set at 30 ml/min. Injector port temp. and detector temp. were adjusted to 230° and 250°, respectively. Quantification was done by the peak-height method and was calculated as % total area of identified sugars after normalization for differences in response among standard sugars.

Gel filtration chromatography of selected cell wall fractions was done on a column (2.6 × 90 cm) of Sepharose 4B-CL (Pharmacia Fine Chemicals) using 0.3% NaCl containing 0.05% NaN_3 as eluant[55]. Elution flow rate was maintained at 24 ml/hr and 4-ml fractions were collected. The A_{280} of the eluate was monitored and the fractions were assayed for total sugar[39]. The column was calibrated using standard Dextran T fractions from Pharmacia.

The content of mixed β-glucan was determined on selected samples using purified β-glucanase from *Bacillus amyloliquefaciens* [25].

Complexing tests with starch were performed according to ref. [32]. Cell wall preparation or fraction—0.2, 0.4, 1, or 2 mg—was added to 100 mg IR29 waxy rice starch (defatted with H_2O-satd *n*-BuOH at 25°) in 100 × 11 mm i.d. test tubes. The sample was wetted with 0.2 ml 95% EtOH, 1.5–1.6 ml H_2O was added mixing with a Vortex Genie mixer with the speed set at 6 and one 4-mesh alumina granule (Hengar) added. The tube was covered with a glass marble and placed for 15 min in a covered vigorously boiling water bath. The tubes were cooled to 25° within 25 min and then placed horizontally for 1 hr before reading gel length. The control sample should have a reading of 60–70 mm.

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