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PHENOLIC ACIDS AND THEIR CARBOHYDRATE ESTERS IN RICE ENDOSPERM CELL WALLS

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Key Word Index—*Oriza sativa*, Gramineae, endosperm, cell wall, phenolic acids, ferulic acid, diferulic acid

Abstract—Ferulic acid, *p*-coumaric acid and diferulic acid were detected in the alkaline extract of rice endosperm cell walls The amount of each component was estimated as 9 1, 2 5 and 0 56 mg/g cell wall, respectively. Several phenolic–carbohydrate esters were isolated from the enzymatic digest of this cell wall, which included a series of ferulic acid esters of arabinoxylan fragments and also some fractions containing a high proportion of diferulic acid

INTRODUCTION

Ferulic acid and some other phenolic acids have been found to be cell wall components of various monocots [1–4] and also some dicots [5, 6] The acids are considered to be present as phenolic–carbohydrate esters because they are released by alkaline solvents Since diferulic acid, a dimeric product of ferulic acid produced by oxidative coupling, was isolated from the cell walls of wheat germ [7], *Lolium multiflorum* [8] and some other plant source [4, 6], it has been suggested that it might be cross-linking the matrix polysaccharides of these cell walls, thus insolubilizing them [5] However, direct evidence for this hypothesis has not been obtained Indeed the locations of these phenolic acids in the cell wall polysaccharides are not fully known

In this paper, I describe the type and amount of phenolic acids present in rice endosperm cell walls and the isolation of their carbohydrate esters obtained by enzymatic degradation of the cell walls

RESULTS AND DISCUSSION

A crude phenolic acid preparation extracted from rice endosperm cell walls had an absorption spectrum very similar to that of ferulic acid The amount of total phenolic acid, expressed in terms of ferulic acid, was calculated as 12 1 mg/g cell walls, based on the A_{320nm} value of the extract

An HPLC system for the simultaneous analysis of phenolic acids was developed, in which seven phenolic acids (protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, *p*-coumaric, ferulic and diferulic acids) could be analysed quantitatively within 40 min Less than 100 ng of the phenolic acids were readily detectable in this system Figure 1 shows the elution profile of the phenolic acid preparation extracted from rice endosperm cell walls The trace confirms the presence of ferulic acid and *p*-coumaric acid, and also a small amount of diferulic acid, which was collected and further verified by TLC, UV and MS Diferulic acid from the endosperm cell walls showed two peaks The main peak coincided with authentic *trans*, *trans*-diferulic acid and the other peak coincided with the peak which appeared after the treatment of *trans,trans*-diferulic acid with UV light This peak is considered to be *cis,trans*-diferulic acid [8] Ferulic acid and *p*-coumaric acid were mainly present as their *trans* form and the peaks coincident with the authentic *trans* isomers were used for the calculations of the relative amounts The amounts of these phenolic acids calculated using their relative amounts estimated by HPLC and the total amount (12 1 mg/g) of phenolic acids present in the cell wall were 9 1, 2 5 and 0 56 mg/g cell wall for ferulic, *p*-coumaric and diferulic acids, respectively These values are slightly higher than those obtained for the cell walls of *L*

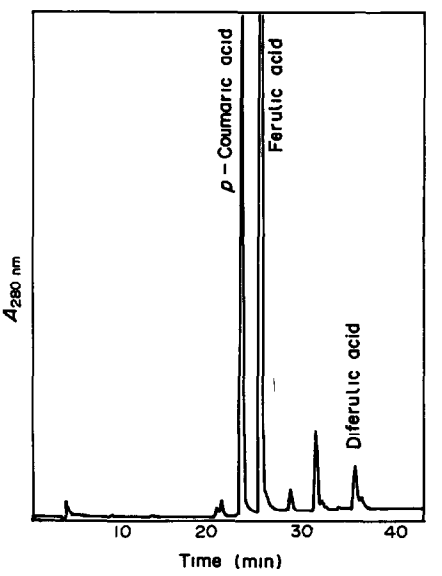

Fig. 1 HPLC analysis of phenolic acids extracted from rice endosperm cell walls

multiflorum by Hartley and Jones (6 5, 0 9 and 0 2 mg of each compound/g cell wall, respectively) [8] However, it should be noted that the amount of each phenolic acid calculated by this method could be somewhat overestimated because of the presence of unidentified phenolic compounds in the extract

On enzymatic digestion of the endosperm cell walls with a cellulase preparation of *Irpex lacteus*, over 40% of the cell wall was solubilized Gel filtration of the soluble products on Biogel P-2 (Fig 2) gave low- and high-Mr fractions, both of which appeared to contain phenolic–carbohydrate complexes, judging from their carbohydrate content and UV absorption The sugar composition of these fractions (Table 1) suggested that the high-Mr fraction contained arabinoxylan [9] and pectic polysaccharides [10], and the low-Mr fraction contained oligosaccharides derived from glucan-type polysaccharides, such as cellulose, β-(1, 3),(1, 4)-glucan or xyloglucan [11], and also arabinoxylan in addition to free sugars

The low-Mr fraction was further fractionated by HPLC (Fig 3) Fractions 1–11 showed completely different UV spectra (λ_{max} 250–270 nm) compared to ferulic or *p*-coumaric acid and were not further examined Fractions 12–17 showed absorption spectra similar to these phenolic acids and fraction 16 and 17 were identified as free *p*-coumaric acid and ferulic acid from their elution

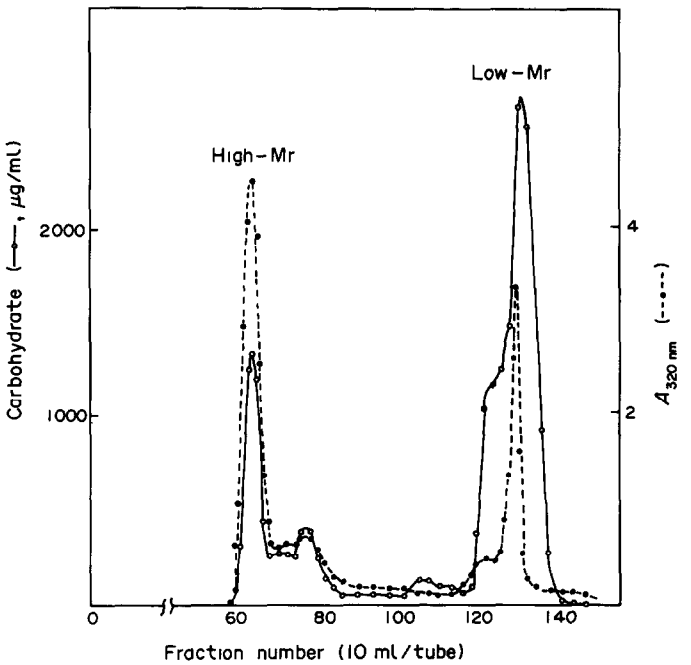

Fig 2 Gel filtration of enzymatic hydrolyzate of rice endosperm cell walls

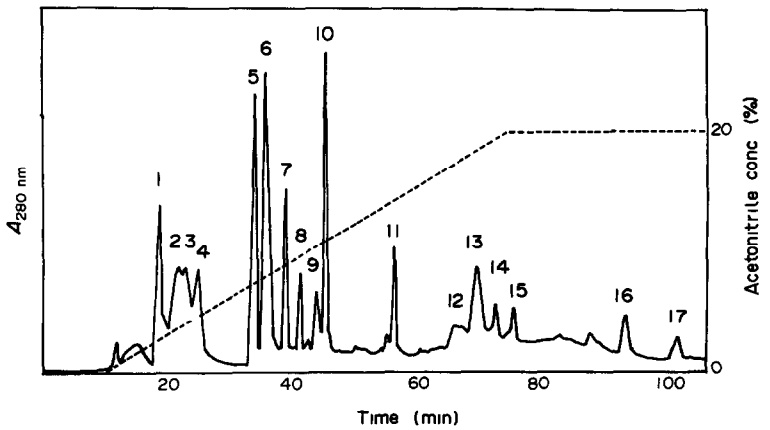

Fig 3 HPLC analysis of enzymatic hydrolyzate of rice endosperm cell walls Low Mr fraction numbers 115–135 (Fig 2) were treated with Amberlite CG-120 (H^+ form) and concentrated to *ca* 1–1 5 ml Aliquots of this concentrate (each 0 3–0 4 ml) were applied to a preparative HPLC column (a 4 × 250 mm and a 8 × 250 mm columns in series) and eluted with a linear gradient of acetonitrile (0–20%) buffered with 0 05 M sodium acetate (pH 4 5) Flow rate, 1 ml/min

volumes Fractions 12–15 contained carbohydrate in addition to phenolic acids and were re-chromatographed on the same column to give fractions low-Mr 1–8 (Fig 4) These fractions gave arabinose, xylose and a small amount of glucose in different ratios after acid hydrolysis and also gave ferulic acid and a small amount of *p*-coumaric acid by saponification (Table 1), indicating that they were mainly composed of the ferulic acid (partly *p*-coumaric acid) esters of arabinoxylan fragments Oligomeric products which consisted of arabinose, xylose, glucose and esterified ferulic acid were also detected in the enzymatic digests of the cell walls of *L multiflorum* [12] and *L perenne* [13] Recently, Kato *et al* [14] isolated *O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1,3)-*O*-β-D-xylopyranosyl-(1,4)-D-xylopyranose from the enzymatic digest of bagasse lignin–carbohydrate complex (LCC) Fry [15] also isolated 4-*O*-(6-*O*-feruloyl-β-D-galactopyranosyl)-D-galactose and 3-*O*-(3-*O*-feruloyl-α-L-arabinopyranosyl)-L-arabinose from the enzymatic digest of spinach cell walls Although the structures of low-Mr 1–8 could not be determined, these substances seem to be related to the one obtained from the bagasse LCC If so, the different chromatographic behaviour of these fractions might come from the differences in the DP and structure of the oligosaccharides and the positions to which the phenolic acids are linked

The high-Mr fraction was further degraded enzymatically using purified α-L-arabinofuranosidase of *Rhodotorula flava* and endoxylanase of *Streptomyces sp E*-86 twice Figure 5 shows the result of gel filtration of the product using Sephadex G-25 Several oligomeric products were obtained which showed absorption spectra similar to ferulic acid and also contained carbohydrate (high-Mr 2–4) However, a large portion of the original high-Mr fraction still remained at the void volume (high-Mr 1) Table 1 shows the sugar composition of these fractions and also the phenolic acids liberated from them by saponification High-Mr 1 and 2 contained 7–13% of rhamnose, indicating the presence of pectic polysac-

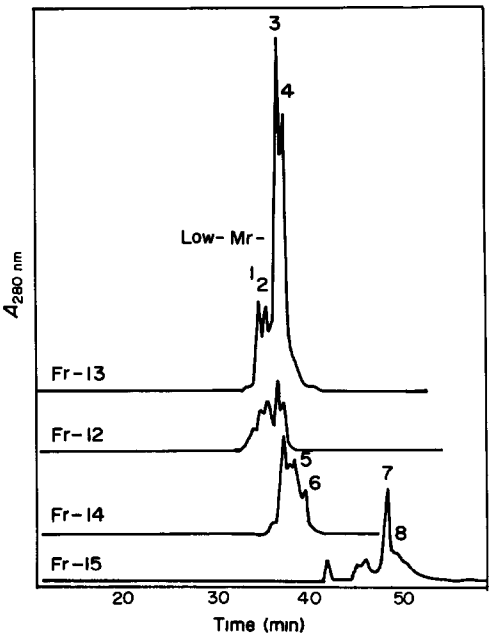

Fig 4 Rechromatography of phenolic–carbohydrate ester fractions Each of fractions 12–15 (Fig 3) were concentrated in a similar manner to that described in the legend to Fig 3 and rechromatographed using the same column system (8 × 250 mm) and same eluting conditions

Table 1 Composition of phenolic–carbohydrate esters obtained by the enzymatic degradation of rice endosperm cell walls

	Sugar composition (wt % of total)*						Phenolic acid content (wt % of carbohydrate)†	Relative amount of phenolic acids (wt % of total)‡		
	Rham	Ara	Xyl	Man	Gal	Glc		*p*-Coumaric	Ferulic	Diferulic
High-Mr	7	43	38	tr	6	6	38		(n d)§	
Low-Mr	—	9	9	—	5	77	14		(n d)	
High-Mr fractions										
1	13	26	37	3	14	8	25	21	68	11
2	7	27	64	—	tr	3	28	16	74	10
3	—	23	46	—	—	32	95	17	35	48
4	—	82	18	—	—	tr	61		(n d)	
Low-Mr fractions										
1	—	18	67	—	tr	14	(n d)	18	82	—
2			(n d)				(n d)	20	80	—
3	—	26	74	—	tr	tr	(n d)	21	79	—
4	—	27	66	—	tr	7	(n d)	21	79	—
5	—	28	62	—	tr	10	(n d)	17	83	—
6	—	29	58	—	tr	13	(n d)	18	82	—
7	—	28	67	—	tr	5	(n d)	17	83	—
8	—	30	55	—	tr	16	(n d)	14	86	—

*Estimated by GC of alditol acetates
†Estimated from the $A_{320\,nm}$ value in terms of ferulic acid
‡Estimated by HPLC
§n d, Not determined

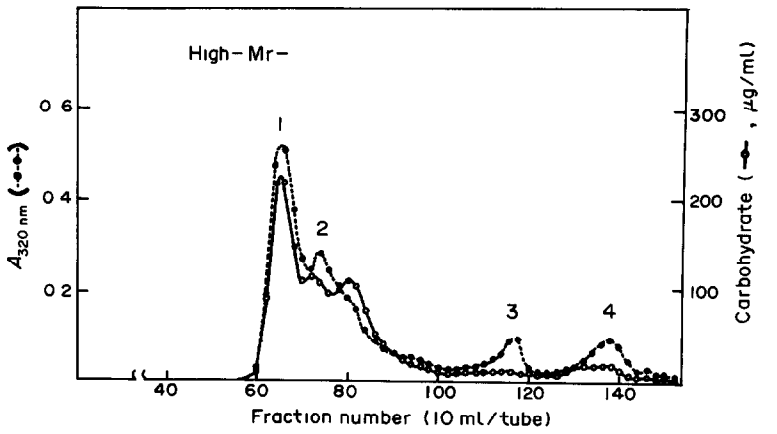

Fig 5 Gel filtration of enzymatic digest of high-Mr fraction on sephadex G-25

charides in addition to arabinoxylan They also contained a fairly high proportion of diferulic acid High-Mr 4 contained free arabinose which was liberated by α-L-arabinofuranosidase High-Mr 3 was especially interesting because it contained the highest concentration of phenolic acid, and the main component was diferulic acid It also contained a high proportion of glucose in addition to arabinose and xylose However, further purification and detailed structural analysis of this fraction could not be done, because of the small amount It is noteworthy in relation to the possible cross-linkage formations through diferulic acid that the high-Mr fractions contained higher levels of phenolic acids, especially higher proportions of diferulic acid and were highly resistant to the cellulase and hemicellulase

The occurrence of the high diferulic acid levels in rice endosperm cell walls (*ca* one diferulic acid/2600 sugar residues of arabinoxylans, based on the assumption that the arabinoxylan content of this cell wall is 50%, since arabinose plus xylose accounted for some 50–55% of sugars released by acid hydrolysis of this cell wall) correspond to the levels which are necessary for the gelation of arabinoxylans (one diferuloyl bridge/3000 sugar residues) [5, 16], and also the detection of various types of ferulic acid esters of arabinoxylan fragments in the enzymatic digest, especially that containing diferulic acid, are consistent with the hypothesis that diferuloyl bridges are cross-linking the matrix polysaccharides in this cell wall To prove this hypothesis ultimately, however, the isolation and the structural elucidation of the oligomers which are derived from the vicinity of the cross-linking portion will be necessary

EXPERIMENTAL

Material Rice (*Oriza sativa*, cultivar Norin 29) was harvested in Ibaraki prefecture, Japan A crude cellulase preparation of *Irpex lacteus* was purchased from Kyowa Hakko Co, Tokyo, Japan α-L-Arabinofuranosidase was purified from the culture filtrate of *Rhodotorula flava* by the method of Uesaka *et al* [17] Purified endoxylanase of *Streptomyces sp E*-86 was provided by Dr I Kusakabe and Prof T Yasui of Tsukuba University [18] Diferulic acid was synthesized from vanillin by the methods of Richtzenhein [19] and Baumgartner [20], and was also supplied by Prof H Neukom of the Swiss Federal Institute of Technology

General procedure All the extracts and eluates were concd under red pres using a rotary evaporator at 35–40°

Preparation of endosperm cell walls and extraction of phenolic acids Rice endosperm cell wall was prepared from milled rice flour using bacterial α-amylase and fungal protease as previously reported [21] The cell wall preparation (300 mg) was extracted with 0 5 M NaOH (30 ml) at 60° for 90 min under a stream of N_2 The extract was acidified to pH 3 5 (6 M HCl) and then extracted with *n*-BuOH (× 3) The amount of phenolic acids extracted was determined from the A_{320nm} value of the extract using ferulic acid as a standard material (yield 3 64 mg)

Enzymatic degradation of endosperm cell wall and fractionation of phenolic–carbohydrate esters Endosperm cell wall (500 mg) was incubated with the cellulase preparation of *I lacteus* (200 mg) in 0 1 M NaOAc buffer (200 ml), pH 4 5, at 30° for 19 hr After heat inactivation of the enzyme (100°, 10 min) and centrifugation, the reaction mixture was concd to *ca* 20 ml and applied to a column of Biogel P-2 (200–400 mesh, two 2 5 × 85 cm columns in series) and eluted with H_2O (20 ml/hr) Low-Mr fractions were further fractionated using prep HPLC High-Mr fractions from Biogel P-2 (fraction number 57–85) were combined and concd to a syrup, dissolved with 30 ml of 0 5 M NaOAc buffer, pH 4 0, and incubated with the purified α-L-arabinofuranosidase of *R flava* (0 5 mg, 48 units) at 30° for 24 hr Then, the pH was adjusted to 5 8 (1 M NaOH) and further incubated with the purified endoxylanase of *St E*-86 (0 5 mg, 200–250 units) at 30° for another 24 hr After heat inactivation (100°, 10 min) and centrifugation, the reaction mixture was concd to *ca* 20 ml and applied to a column of Sephadex G-25 (fine grade, 5 × 78 cm) and eluted with H_2O (38 ml/hr) The high-Mr fractions so obtained (fraction number 60–100) were concd to a syrup, dissolved with 5 ml 0 5 M NaOAc, pH 4 0, and digested again with the same enzyme system The reaction temp was changed to 50° for both enzymes and the reaction time was 4 5 hr for the first step and 18 hr for the second step The reaction product was fractionated similarly using Sephadex G-25 column Phenolic acids in these fractions were released by treatment with 0 5 M NaOH overnight at room temp, under N_2 atmosphere, and were extracted with EtOAc after acidification and analysed by HPLC

Phenolic acid analysis TLC of phenolic acids was performed on silica gel plates (Merck) with the solvent system C_6H_6–dioxane–HOAc (90 25 4) Spots were detected under UV illumination HPLC was carried out with a Hitachi model 635A liquid chromatograph equipped with a UV detector Stainless steel columns (4 × 250 or 8 × 250 mm) were packed with Unisil

C-18 (5 μm, Gasukuro-Kogyo Co) and used for analytical purposes and also for preparation Phenolic acids were analysed with a linear gradient of MeCN (5–25%) in 0 05 M NaOAc buffer, pH 4 0, and detected by absorption at 280 nm Flow rate was 1 ml/min Quantitative analysis was performed using the peak height method Mass spectra were recorded with a Hitachi model M-80 mass spectrometer by the direct inlet methods using electron impact ionization

Carbohydrate analysis The total carbohydrate content was determined by the phenol–H_2SO_4 method [22] For analysis of component sugars, samples were hydrolysed by heating with 2 M trifluoroacetic acid at 121° for 1 hr The neutral sugars in the hydrolysate were converted into their corresponding alditol acetates and analysed by GLC using a column of 3% ECNSS-M on Gas Chrom Q (0 3 × 200 cm) [23]

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