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Cell Wall Cementing Materials of Grass Leaves

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ABSTRACT

Treatment of grass leaves with either a purified pectin lyase of *Aspergillus japonicus* or a purified xylanase of *Trichoderma viride* could lead to the isolation of some single leaf cells. However, a mixture of pectin lyase and xylanase brought about more rapid isolation of single cells than did either of the two enzymes alone, indicating a synergistic effect. Analysis of the components released from oat cell walls by the enzymes indicated that both homogalacturonans with a high degree of esterification and a kind of glucuronoarabinoxylan with ferulic acid ester may play a role in cell wall cementing in grass leaves.

The enzymic maceration of plant tissues, in which cells are freed from one another, is the first reaction step of enzymic protoplast isolation. Breakdown of intercellular cementing materials leads to separation of individual cells. Endo-types of PG[1] (2, 3, 25), PL (5, 16, 22), and pectate lyase (8, 10, 20), which randomly cleave the α-1,4-galacturonide linkages in pectic polysaccharides, are found to be the primary enzymes responsible for tissue maceration. These results and other evidence lead to the well known conclusion that pectic polysaccharides are the principal binding agents between higher plant cells (7).

This is true in the tissues of dicots and some monocots such as Liliaceae and Araceae (12, 14), but not always true in the case of graminaceous tissues. It has been demonstrated in many species that pectic polysaccharides are very minor components in cell walls of Gramineae, suggesting that they do not play as important a role in the cell walls as they do in cell walls of dicots (9). No one has yet succeeded in isolating single cells from graminaceous tissues by these purified pectolytic enzymes. Thus, it is not known what kinds of cell wall components play a role in cell wall cementing in the Gramineae.

Recently, Ishii and Mogi (15) demonstrated that cellulase C_1, cellulase Cx, xylanase, and PL were the enzymes that are effective for isolating protoplasts from grass leaves. The present paper describes the isolation of single cells from grass leaves by purified xylanase and PL and the characterization of components released from the cell walls by the action of these enzymes.

MATERIALS AND METHODS

Plant Materials. Seedlings of oat (*Avena sativa*) were grown in vermiculite in controlled environment chambers at 25°C (16-h photoperiod of low light intensity, 5000 lux). The first leaves of 7-d-old seedlings were used for the isolation of single cells. Seedlings of barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), and corn (*Zea mays*) were grown under the same conditions and their first leaves were used to obtain single cells. For oat cell wall preparations the first leaves (17.2 g) were boiled in 500 ml of 80% ethanol for 10 min to kill the cells. After cooling, the boiled leaves were ground in ice-cold 50 mM Tris buffer (pH 7.5) with a cold mortar and pestle. The fragmented tissues were filtered on a Büchner funnel, washed successively with cold 50 mM Tris buffer (pH 7.5), 50% ethanol, 95% ethanol and ether, and dried *in vacuo* at room temperature. The yield of cell wall preparation (438 mg) represented 2.5% of the fresh weight of the leaves.

Purified Enzymes. Purified PG and PL were obtained from the culture medium of *Aspergillus japonicus* by the methods described previously (17, 18). Xylanase (fraction P-4) was purified from a cellulase preparation of *Trichoderma viride* by the method described in a previous paper (15).

Assay of Macerating Activity. The lower epidermis of oat leaves was removed, and the leaves (140 mg) were placed in a 50-ml Erlenmeyer flask containing 0.8 M mannitol, 20 mM Mes buffer (pH 5.5), 10 mM cysteine, enzymes, and water to a final volume of 5.0 ml. Mannitol was added as an osmotic stabilizer. The flasks were shaken on a rotary shaker at 120 rpm and 25°C. After 2 h the mixture was filtered through Miracloth, and the OD of the filtrate was measured at 660 nm. A linear relationship exists between the OD at 660 nm and the number of single cells counted in a hemocytometer.

Treatment of Oat Cell Walls by Enzyme. The cell walls (100 mg) were placed in 150-ml Erlenmeyer flasks containing 50 ml of 0.1 M ammonium acetate buffer (pH 5.5) and 370 μg of PL or 1.65 mg of xylanase. The flasks were shaken on a rotary shaker at 100 rpm and 30°C. After 3 h the reaction mixtures were filtered through Toyo No. 5C paper and the residues were repeatedly washed with 0.1 M ammonium acetate buffer (pH 5.5). The filtrate and washings were combined and lyophilized.

Gel Filtration. Gel filtration was carried out on a Sephadex G-100 column (1.9 × 100 cm) equilibrated with 0.1 M ammonium acetate buffer (pH 5.5). Samples were applied in 2.0 ml to the column, and the elution was carried out with 0.1 M ammonium acetate buffer (pH 5.5) at a flow rate of 20 ml/h.

Analysis of Sugars. The uronide content was estimated by the *m*-hydroxydiphenyl method using galacturonic acid as a standard (4). Total uronides of oat cell walls were determined by the method of McCready and McComb (19). Neutral sugar was calculated as the difference between the total sugar estimated by the phenol-H_2SO_4 method (11) and galacturonic acid estimated by the *m*-hydroxydiphenyl method, and was expressed as μmol of xylose. After hydrolysis at 121°C with 2 M TFA in sealed tubes for 1 h, neutral sugar composition was determined as alditol acetates by GLC according to the method of Albersheim *et al.* (1). Identification of uronides in the hydrolysate was carried out by TLC using ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol) and the organic phase from a mixture of 100 g of phenol, 100 ml of water, and 1 ml of 85% HCOOH (23) as solvent systems. Galacturonic acid, methyl-esterified galacturonic acid, glucuronic acid, and 4-*O*-methyl glucuronic acid were used as authentic standard samples.

Analysis of Phenolic Acids. Phenolic acid content was measured at 320 nm (24). Soluble products released from cell walls

[1] Abbreviations: PG, polygalacturonase; PL, pectin lyase.

by xylanase were saponified with 0.5 N NaOH at 25°C for 16 h under N_2 gas. The treated and nontreated solutions were individually acidified with 1 N HCl to pH 2.5 and extracted with ether. The ethereal extract was dried over dry Na_2SO_4, evaporated to dryness, dissolved in 80% ethanol, and then measured at 320 nm.

RESULTS AND DISCUSSION

Isolation of Single Cells from Grass Leaves by Purified Enzymes. Both PL and xylanase, by themselves, could isolate single cells from oat leaves. A marked synergistic action between the two was found in the maceration, however (Table I). In contrast with dicot tissues PG, which is specific for nonesterified galacturonide linkages in pectic polysaccharides, had a little ability to isolate single cells from the leaves even when a high concentration of PG was used. Purified PG of *A. japonicus*, as well as PL, could easily macerate radish root and potato tuber (12) and tobacco leaf tissues (21) at a low concentration of enzyme. In 0.8 M mannitol solution single cells isolated were plasmolyzed (Fig. 1). Cells damaged during maceration were frequently observed in single cells isolated by PL and/or xylanase. Leaf tissues of barley, wheat, and corn were also macerated by PL and xylanase, but with lower efficiency than for oat.

Analysis of Components Released from Oat Cell Walls by PL and Xylanase. Soluble products released from 100 mg of oat cell walls by the enzymes were concentrated by lyophilization and applied on a Sephadex G-100 column. Almost all the components released by PL were oligogalacturonides (Fig. 2). Inasmuch as PL is specific for methyl-esterified galacturonide linkages, galacturonides of the cell walls attacked by the enzyme may have a high degree of esterification. PL solubilized 46.5% of the total uronides from cell walls which contained 5.3% by weight uronides. Assuming that two-thirds of the total uronides were galacturonic acid, as demonstrated by Ray and Rottenberg (23) in oat coleoptile cell wall, it was calculated that about 70% of galacturonides were released from the cell walls by PL. Little neutral sugar was solubilized from the cell walls by the action of PL. The result was quite different from those obtained in the cell walls of potato tuber (13) and onion bulb (14). In these cases, large amounts of neutral sugars which are covalently linked to galacturonans were released from the cell walls by PL in addition to oligogalacturonides.

Table I. *Macerating Activity of PG, PL, and Xylanase (X) on Oat Leaves*

Enzymes	Addition	Activity
	μg	A_{660}
PG	440	0.029
PL	37	0.312
	74	0.438
X	165	0.282
	330	0.352
PL + X	37 + 165	1.260

On the other hand, xylanase could solubilize both uronides (1.7 mg) and neutral sugars (8.7 mg as xylan) from 100 mg of oat cell walls. The elution pattern on Sephadex G-100 (Fig. 2B) showed that about 80% of neutral sugars solubilized were low mol wt products. However, the soluble products also contained some high mol wt neutral sugars and uronides. The eluant was divided into three fractions with respect to mol wt, concentrated by lyophilization, and analyzed (Table II). After hydrolysis, uronides of fractions 2 and 3 were identified as glucuronic acid and 4-*O*-methyl-glucuronic acid on TLC. Most of the uronides in fraction 1, however, were detected as galacturonic acid, suggesting that pectic polysaccharides may be released from the cell walls by xylanase as high mol wt substances.

Xylose and arabinose were the major neutral sugar components in all fractions. This indicated that the soluble products were mainly composed of glucuronoarabinoxylans and their degradation products, which are the quantitatively dominant components of Gramineae cell walls (9). Fraction 1 was characterized by the presence of high galactose and arabinose contents. It is possible that a part of these neutral sugars may be derived from pectic galactan or arabinogalactan, since a trace amount of rhamnose was detected only in this fraction on GLC. A detailed structural analysis is necessary to clarify this point.

The three fractions also contained phenolic acid which was released as free acid by saponification. The acid in 80% ethanol was tentatively identified as ferulic acid by comparing its UV absorption spectrum with the authentic sample. Ferulic acid may be bounded to arabinosyl residues of glucuronoarabinoxylan through ester linkages (24).

These results may indicate that the xylanase degrades β-1,4-xyloside linkages in glucuronoarabinoxylan rich in ferulic acid ester in cell walls to release single cells from oat leaves.

The fact that this xylanase solubilized a limited amount of arabinoxylan component during maceration and another endo-xylanase of *T. viride* (fraction P-6 in the previous paper [15]), was not effective for isolating single cells from oat leaves, although it could attack arabinoxylans in the cell walls (S. Ishii, unpublished data), suggests that a special kind of arabinoxylan may play a role as cell wall cementing in grass leaves. Carpita (6) demonstrated that at least three kinds of arabinoxylan components were present in the primary cell walls of *Zea mays* coleoptiles.

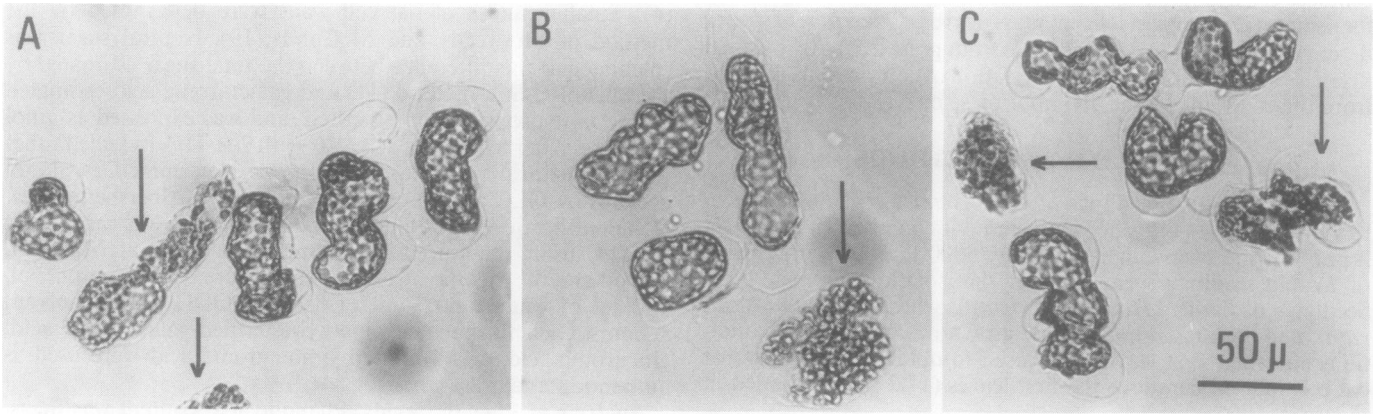

FIG. 1. Single cells released from oat leaves by pectin lyase (A), xylanase (B), and pectin lyase plus xylanase (C). Arrow, damaged cells.

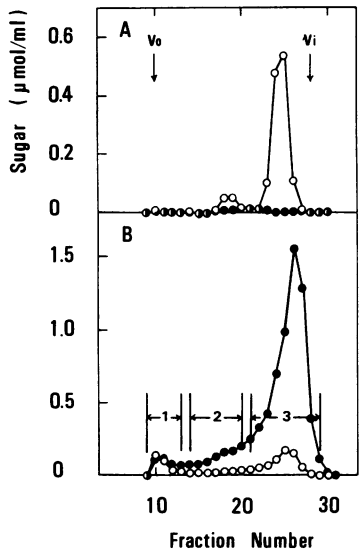

FIG. 2. Gel filtration patterns of soluble products from oat cell walls by pectin lyase (A) and xylanase (B) on Sephadex G-100. The lyophilized sample was dissolved in 2 ml of 0.1 M ammonium acetate buffer (pH 5.5), and the solution was applied on a Sephadex G-100 column (1.9 × 100 cm). 10 ml fractions were collected and each fraction was assayed for uronides (O) and neutral sugars (●).

Table II. *Compositions of Fractions 1, 2, and 3 Isolated by Gel Filtration from Soluble Products Released from Oat Cell Walls by Xylanase*

Fraction[a]	Total Amounts of Components			Neutral Sugar Composition				
	Uronate	Neutral sugar	Phenolic acid	Ara	Xyl	Man	Gal	Glc
	μmol		A_{320}	*mol %*				
1	2.49	2.80	1.82	38.7	32.0	0.0	27.6	1.7
2	1.55	8.07	5.98	33.2	53.7	4.0	5.9	3.2
3	5.66	47.45	37.70	20.5	65.9	0.0	1.8	11.8

[a] Fraction 1 was No. 9–13 as designated in Figure 2B, fraction 2 was No. 14–20, and fraction 3 was No. 21–29.

From the results obtained in this paper, we concluded that both homogalacturonans with a high degree of esterification and a particular glucuronoarabinoxylan with ferulic acid ester may be cell wall cementing materials of grass leaves. The degradation of each of the components by enzyme resulted in tissue maceration of grass leaves, but that of both components brought about more rapid maceration than did individual degradation.

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