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# β-1,3-Endoglucanase from Soybean Releases Elicitor-Active Carbohydrates from Fungus Cell Walls

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## ABSTRACT

**Two enzymes from soybean (*Glycine max* L. Merr. cv Harosoy 63) cotyledons released elicitor-active carbohydrates from cell walls of the phytopathogenic fungus *Phytophthora megasperma* f.sp. *glycinea*. They were identified as isoenzymes of β-1,3-endoglucanase (EC 3.2.1.39) with isoelectric points of pH 8.7 and 10.5. The pI 10.5 enzyme was extracted in the greatest amount and was isolated as a homogeneous protein of about 33,000 daltons as determined by gel filtration and sodium dodecyl sulfate-gel electrophoresis. The purified enzymes hydrolyzed several β-1,3-glucans in a strictly random manner, but degraded neither β-1,6- nor β-1,4-glucans.**

Plant pathogenic microorganisms contain factors, called elicitors, that stimulate the production of phytoalexins by plant tissues (8, 19). These have frequently been shown to be associated with the cell surface and/or cell wall. Yoshikawa *et al.* (21) observed that soybean cotyledons contained enzymic activity which released water-soluble elicitors from isolated cell walls of the phytopathogenic fungus Pmg.[1] In soybean tissues, the released factors were efficient elicitors of the phytoalexin glyceollin. In addition, it was recently shown that the soybean enzyme(s) released elicitor activity from living fungus hyphae (M. Yoshikawa, unpublished data). The consequent possibility that release of soluble elicitors from the fungus might play an important physiological role in the induction of plant defense reactions in response to fungal infection prompted us to purify the soybean enzyme(s) involved.

## MATERIALS AND METHODS

Soybean (*Glycine max* L. Merr. cv Harosoy 63) seedlings were grown in pots in a lighted growth chamber as previously described (9). Cotyledons were harvested for enzyme extraction, usually at 7 to 10 d after planting.

Mycolaminaran, a branched β-1,3 glucan of approximately 30 glucose residues, was prepared from various *Phytophthora* spp. according to the method of Wang and Bartnicki-Garcia (17). Radioactive [$^{14}$C]mycolaminaran from *Phytophthora palmivora* was a gift from M. C. Wang, and CM-pachyman and pustulan were generous gifts from B. A. Stone (20).

Protein was determined by the Lowry method using BSA as a standard or by measuring *A* at 280 nm.

**Enzyme Extraction.** Frozen soybean cotyledons (1 kg) were thawed and 100-g lots were extracted in an ice bath with 140 ml of 10 mM Tris-HCl (pH 7.3) in a 200-ml Omni-mixer cup at full speed for 1.5 min. The combined extracts were centrifuged at 15,000*g* for 10 min. The pigmented and cloudy supernatant fluids were dialyzed for 24 h against 20 L of 3 mM Tris-HCl (pH 7.3).

**Cetavlon Precipitation.** Solid cetavlon (hexadecyl trimethylammonium bromide, Sigma) was added to the dialyzed, crude enzyme to give a 0.5% (w/v) concentration, and the mixture was stirred on an ice bath for 3 h. Following centrifugation for 10 min at 15,000*g*, the supernatant fluids were dialyzed against several changes of 3 mM Tris-HCl (pH 7.3), for 48 to 72 h. The dialyzed fluids were again centrifuged to remove additional cloudy material, yielding clear, golden-colored supernatant solutions.

**DEAE-Cellulose Fractionation.** The cetavlon-treated enzyme preparation (pH 7.3) was pumped through a 2.7- × 20-cm column of DEAE-cellulose (DE 53, Whatman, in the chloride form) equilibrated and eluted with 3 mM Tris-HCl (pH 7.3). Cell wall carbohydrate-releasing activity was quantitatively recovered in the 280-nm absorbing fractions that passed through the column, but much of the applied protein and pigmented material adsorbed to the packing. The clear fractions absorbing at 280 nm were pooled.

**CM Bio-Gel Chromatography.** The enzyme from DEAE-cellulose was adjusted to pH 5.0 with HCl and pumped through a 2.7- × 15-cm column of CM Bio-gel A (Bio-Rad) equilibrated with 3 mM K-acetate (pH 5.0). Following application of the enzyme (approximately 2,000 ml), the column was washed with 150 ml of acetate buffer and eluted with 350 ml of the buffer containing 0.1 N NaCl. Finally, the column was washed with 250 ml of acetate buffer containing 0.4 M NaCl before reequilibration and reuse.

**Electrofocusing.** The CM fractionated enzyme was dialyzed against water and electrofocused using an LKB 8100 preparative column with pH 3 to 10 Ampholines (LKB) with sucrose as the density gradient medium. The cathode was at the top of the column and separations were performed at 400 v for 48 h at 1°C.

**Gel Filtration.** Dialyzed enzyme preparations from the CM Bio-gel or the electrofocusing column were concentrated in dialysis bags using Aquacide I (Calbiochem) and applied to 0.9- × 67-cm columns of Bio-gel P-150. For preparative work, enzyme from the CM Bio-gel column (1 kg of cotyledons) was applied in approximately 5 ml to a 2.5- × 83-cm column of Sephacryl S-300. All columns were equilibrated and eluted with 10 mM K-acetate (pH 5.3) containing 0.2 M NaCl. Columns were calibrated using blue dextran (Pharmacia) as a void volume marker and lysozyme, chymotrypsinogen, ovalbumin, and BSA as protein standards. Active fractions were dialyzed exhaustively against distilled $H_2O$ and lyophilized, yielding white amorphous powders.

**SDS-Gel Electrophoresis.** Slab gels (1 mm) were run on a LKB 2001 electrophoresis unit with 13% acrylamide separating and 1.3% stacking gels essentially according to Laemmli (11) and stained with Coomassie blue R-250 (18).

**Assays for Release of Elicitor-Active Carbohydrates from Fungus Cell Walls.** Lyophilized cell walls prepared as previously described from Pmg (3, 9) were suspended in 50 ml of water by blending for 30 s in a Sorvall Omni-mixer and sonicated for 1 min at 20 w to disperse wall aggregates. The cell walls were then

[1] Abbreviation: Pmg, *Phytophthora megasperma* f.sp. *glycinea*

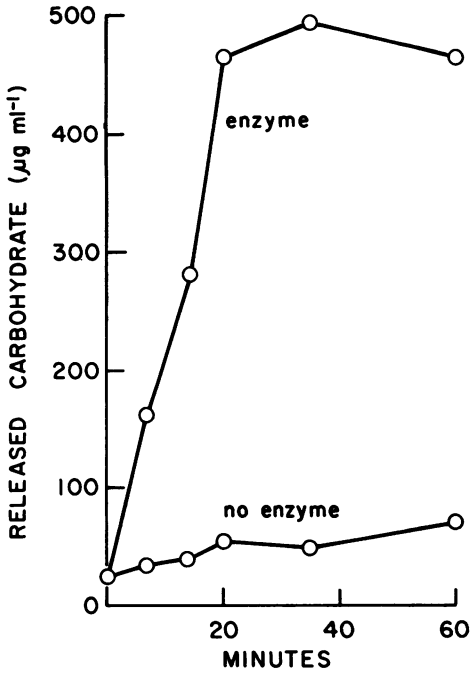


FIG. 1. Release of soluble carbohydrates from Pmg cell walls incubated with partially purified soybean enzymes (CM Bio-gel step) or water. The maximum release of carbohydrate represented about 5.6% of the fungus cell wall dry weight.

sedimented by centrifuging for 2 min at 1,500*g*, washed twice in water, and finally suspended in 20 mM K-acetate (pH 5.3) to give a 10 mg $ml^{-1}$ suspension. Enzyme solutions, generally in 10 mM Na-acetate (pH 5.2), were mixed with an equal volume of the cell wall suspension and incubated at 35°C.

In assays monitoring carbohydrate release, 2.0 ml of the buffered cell wall suspension and 2.0 ml of an appropriately diluted enzyme solution were mixed, and 0.5-ml aliquots were removed at intervals. The aliquots were passed through 0.45-μm Millipore filters. The collected filtrates were assayed for released carbohydrate by the anthrone method (2), using 0.1 ml of each aliquot per analysis. Activity of the released carbohydrates to elicit glyceollin accumulation in soybean cotyledons was determined as described in the accompanying paper (10).

Columns were monitored for activity by mixing 0.25 ml of the appropriately diluted column fractions with 0.25 ml of cell wall suspension and incubating for 15 min, and were assayed for soluble carbohydrate as above.

**β-1,3 Endoglucanase Assays.** Mycolaminaran or laminarin (Calbiochem) (1.8 ml for either) at 500 μg $ml^{-1}$ in 50 mM K-acetate (pH 5.3) were added to 0.6 ml of enzyme solution, and the mixture was incubated at 35°C. Aliquots (0.4 ml) were withdrawn at intervals and added to 0.4 ml of the Nelson alkaline copper reagent. All tubes were then assayed for reducing sugars by the Nelson method (2), and results expressed as μmol glucose equivalents released per min or per h per ml enzyme. Laminarin and mycolaminaran gave identical results with the soybean enzymes. However, since mycolaminaran is a relatively homogeneous, branched β-1,3-glucan of approximately 30 glucose residues with one reducing group (17), it was the preferred substrate when per cent substrate hydrolysis at the maximum was calculated and when reaction products were chromatographed on gel filtration columns.

Column fractions were assayed for glucanase activity by adding 0.1 ml of the suitably diluted fraction to 0.3 ml of the laminarin or mycolaminaran substrates above. Assay mixtures were incubated at 35°C for 10 min and assayed for reducing sugar content as above.

**Assays with Nitrophenyl Substrates.** Reaction mixtures con-

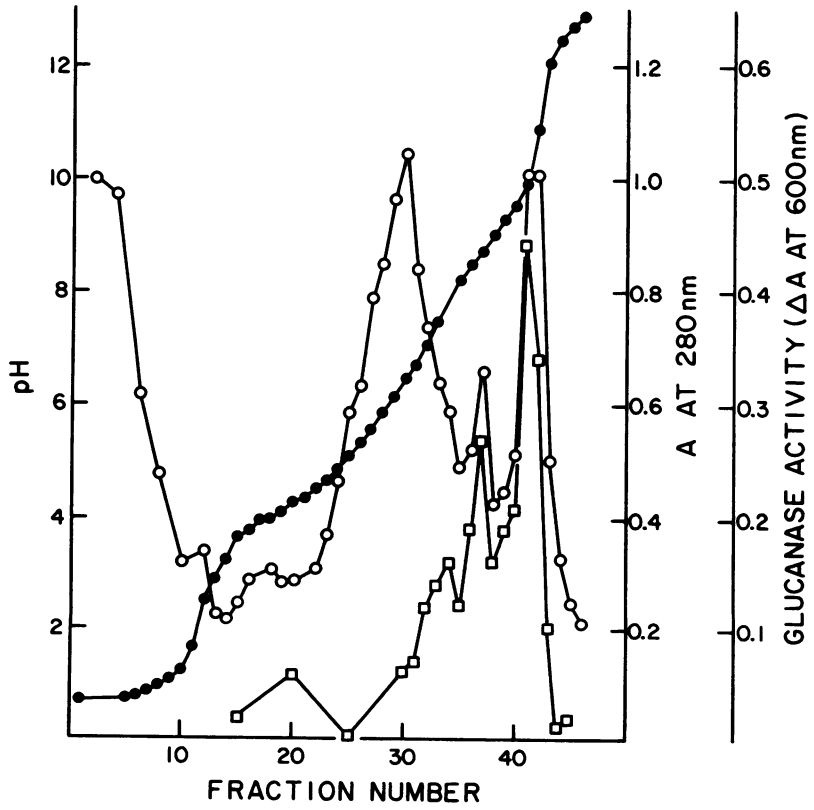


FIG. 2. Electrofocusing run of CM Bio-gel purified soybean glucanases. (●), pH of recovered fractions; (○), *A* at 280 nm; (□), activity against mycolaminaran.

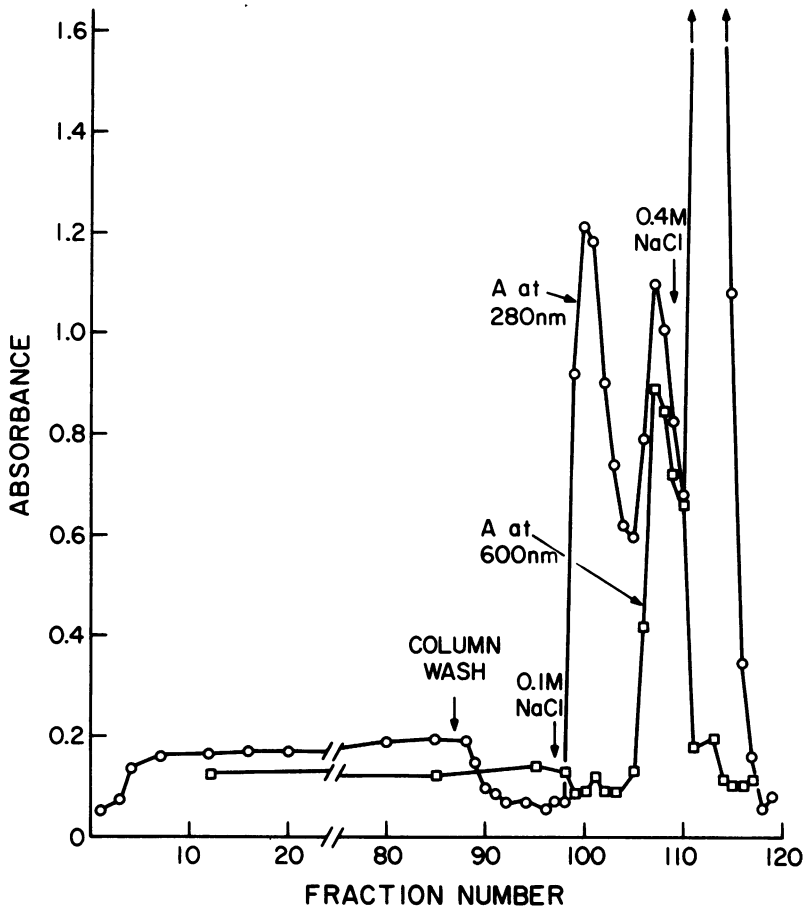


FIG. 3. CM Bio-gel chromatography. The enzyme preparation from the DEAE-cellulose step was pumped onto a 2.7- × 10-cm column, which was then washed with the column buffer followed by NaCl solutions at the points noted. The fractions (22 ml) were monitored for protein by determining their *A* at 280 nm (○) and glucanase activity (*A* at 600 nm in the Nelson assay) using laminarin as substrate (□).

Table I. *Summary of Purification of the pI 10.5 Endoglucanase from Soybean Cotyledons*

One kg of cotyledons was processed as described in "Materials and Methods."

| Step | Volume | Activity on Laminarin | Total Activity | Recovery of Activity | Protein | Total Protein | Specific Activity | Purification |
|---|---|---|---|---|---|---|---|---|
| | *ml* | *μmol glucose eq $h^{-1}$ $ml^{-1}$* | *× ml* | *%* | *$mg/ml^{-1}$* | *mg* | *μmol glucose eq $h^{-1}$ $mg^{-1}$ protein* | *-fold* |
| Crude, dialyzed | 2,190 | 3.11 | 6,800 | 100 | 8.11 | 17,800 | 0.38 | 1.0 |
| Cetavlon supernatant | 2,140 | 3.14 | 6,700 | 99 | 0.93 | 2,000 | 3.3 | 8.7 |
| DEAE-cellulose | 2,100 | 3.31 | 6,950 | 102 | 0.42 | 880 | 7.9 | 20.8 |
| CM Bio-gel | 50 | 119 | 5,950 | 87 | 2.60 | 130 | 45.8 | 121 |
| S-300 Sephacryl (3×) | 44 | 57 | 2,510 | 37 | 0.37 | 16 | 156 | 410 |

tained: 250 μl substrate (4 mM *p*-nitrophenyl derivative, all obtained from Sigma), 50 μl 0.5 M K-acetate (pH 5.3), and 200 μl enzyme. Tubes were incubated for 30 min at 35°C, 400 μl of 0.3 M $Na_2CO_3$ were added for color development, and *A* was read at 420 nm.

## RESULTS

**Purification and Identification of the Soybean Enzyme Activity.** Incubation of crude or purified soybean extracts with fungus cell walls led to the liberation of water-soluble, anthrone-reactive carbohydrates (Fig. 1). Similar curves were generated when the products were assayed for elicitor activity as previously reported (21). However, no protein was released from the walls as determined by monitoring with the Lowry reagent or measuring absorption of the reaction mixtures at 280 nm. Neither was detectable lipid or elicitor activity recovered in the organic fraction when reaction mixtures were extracted with chloroform. It was therefore tentatively concluded that the elicitor activity was associated with the released carbohydrates. Their partial characterization is a topic of the accompanying paper (10).

Inasmuch as the previous study (21) indicated that an enzyme was responsible for release of the elicitor active carbohydrates from fungus cell walls, attempts were made to identify it. Carbohydrate degrading enzymes were considered as the first candidates because the *Phytophthora* cell wall is composed primarily of carbohydrate polymers (3). Enzyme preparations partially purified through the DEAE-cellulose step did not exhibit detectable activity on the following *p*-nitrophenyl substrates: -β-glucoside, -α-mannoside, -β-mannoside, acetyl -β-D-*N*-glucosaminide, -β-D-gal-

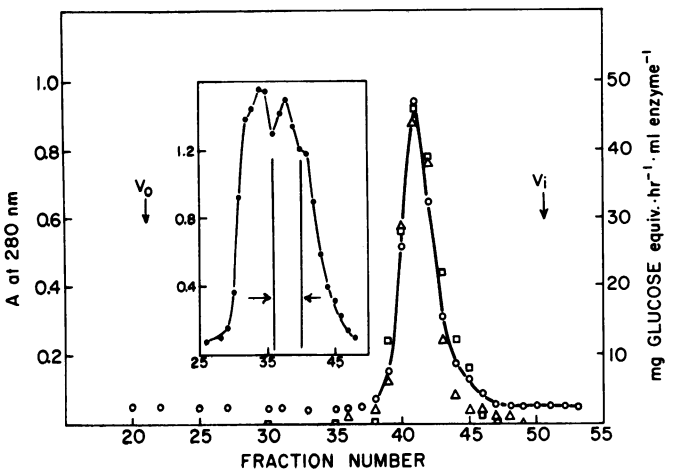


FIG. 4. Gel filtration chromatography on Sephacryl S-300. Inset shows the $A_{280}$ profile of CM Bio-gel enzyme run onto the column; the area denoted by the arrows contained the bulk of the glucanase activity, with less activity associated with the leading peak. The main figure was obtained when the shaded fractions of the inset were collected and rerun twice through the same column. (●), $A$ at 280 nm; (□), cell wall carbohydrate release as glucose equivalents; (△), reducing group liberation from laminarin. The $V_o$ was determined with blue dextran and the $V_i$ with glucose.

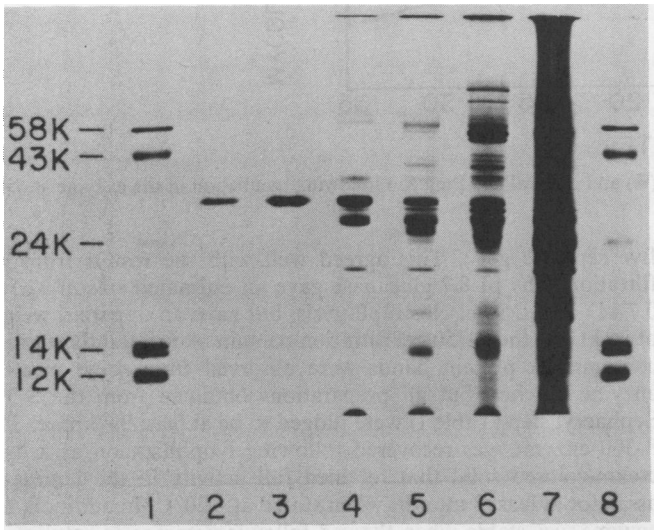


FIG. 5. SDS-gel electrophoresis of soybean endoglucanase at various steps in the purification regime. Slab gel wells were at the top of the photograph; lanes 1 and 8 contained standards of the designated mol wt (catalase, ovalbumin, trypsinogen, ribonuclease, Cyt *c*); lanes 2 and 3, two different batches of the pI 10.5 endoglucanase purified from a S-300 Sephacryl column; lane 4, CM Bio-gel step; lane 5, DEAE-cellulose step; lane 6, cetavlon-treated; and lane 7, crude enzyme.

acturonide, -sulfate, and -α-L-fucoside. The DEAE-cellulose preparations were active on the following *p*-nitrophenyl substrates: -acetate, -phosphonate, -phosphate, -β-D-galactoside, -α-D-glucoside, and -α-L-arabinoside. The activities against these substrates, however, did not coelute from a P-150 Bio-gel column with the cell wall degrading activity.

Failure to find a *p*-nitrophenyl substrate for the soybean enzyme(s) did not rule out the possibility that a carbohydrase was responsible for the release of cell wall carbohydrate if the enzyme had random specificity. Because other work in progress disclosed that soybean tissues contained substantial glucanase activity against mycolaminaran (N. T. Keen and M. Yoshikawa, unpublished), the possibility that such an enzyme might account for the release of fungal wall elicitors was tested.

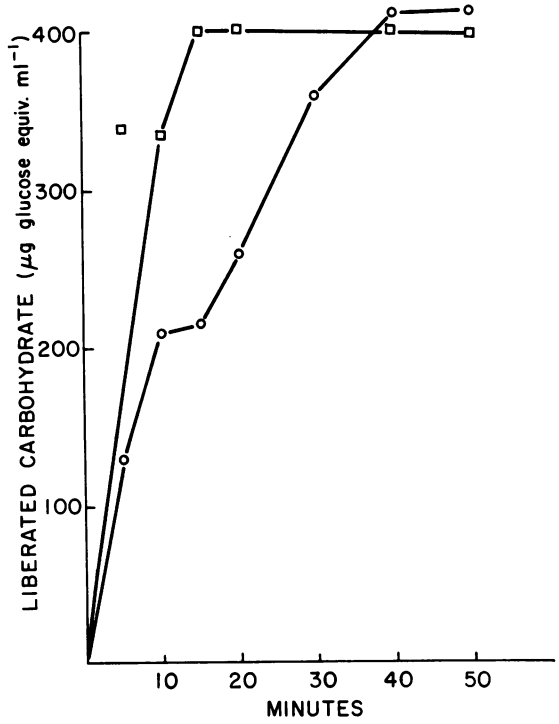


FIG. 6. Liberation of soluble carbohydrate from Pmg cell walls (9 mg $ml^{-1}$) by the pI 10.5 endoglucanase in the presence (○) or absence (□) of mycolaminaran (500 μg $ml^{-1}$). The control tubes without mycolaminaran contained 500 μg $ml^{-1}$ of glucose.

Enzyme purified through the CM Bio-gel step eluted from a Bio-gel P-150 column as a single peak of activity against fungus cell walls and mycolaminaran, but these did not exactly coincide, indicating that they were not associated with a single enzyme or that two closely related glucanases with slightly different substrate specificity were present. Electrofocusing of the CM Bio-gel enzyme indeed disclosed the presence of two major and one or more minor peaks of β-glucanase activity, with the major ones occurring at pH 8.7 and 10.5 (Fig. 2). The latter assignment was provisional since the enzyme occurred at the top end of the pH gradient where accurate determination of the pI was not possible. The use of ampholytes from Pharmacia and LKB with advertised upper limits of pH 10.5 and 11.0, respectively, did not improve resolution of the pI 10.5 glucanase. In most electrofocus runs, the pI 10.5 enzyme constituted the major peak, but considerable variation in relative activities of the various glucanases occurred between experiments. When the major glucanase peaks were recovered from the electrofocusing column and each was fractionated on the P-150 Bio-gel or S-300 Sephacryl columns, the glucanase and cell-wall carbohydrate releasing activities exactly coincided with single protein peaks (data not shown). These gave apparent mol wt of 36 and 33 kD, respectively, for the pH 8.7 and 10.5 glucanases, based on protein calibration standards.

Inspection of the purification outline (Table I) shows that relatively high recovery and purification of the glucanase activity was obtained. Adsorption to CM Bio-gel was a convenient method to concentrate the glucanase activity and also afforded additional purification (Fig. 3; Table I). In routine purification experiments, the CM Bio-gel preparations resulting from 1 kg of soybean cotyledons were applied to the S-300 Sephacryl column (Table I). Most of the glucanase activity was associated with a partially separated $A_{280}$ absorbing peak, and this could be purified by one or two additional passes through the column to yield a single symmetrical peak with coincident curves for protein and activity against laminarin and Pmg cell walls (Fig. 4). This purified glucanase consisted almost exclusively of the pI 10.5 enzyme upon

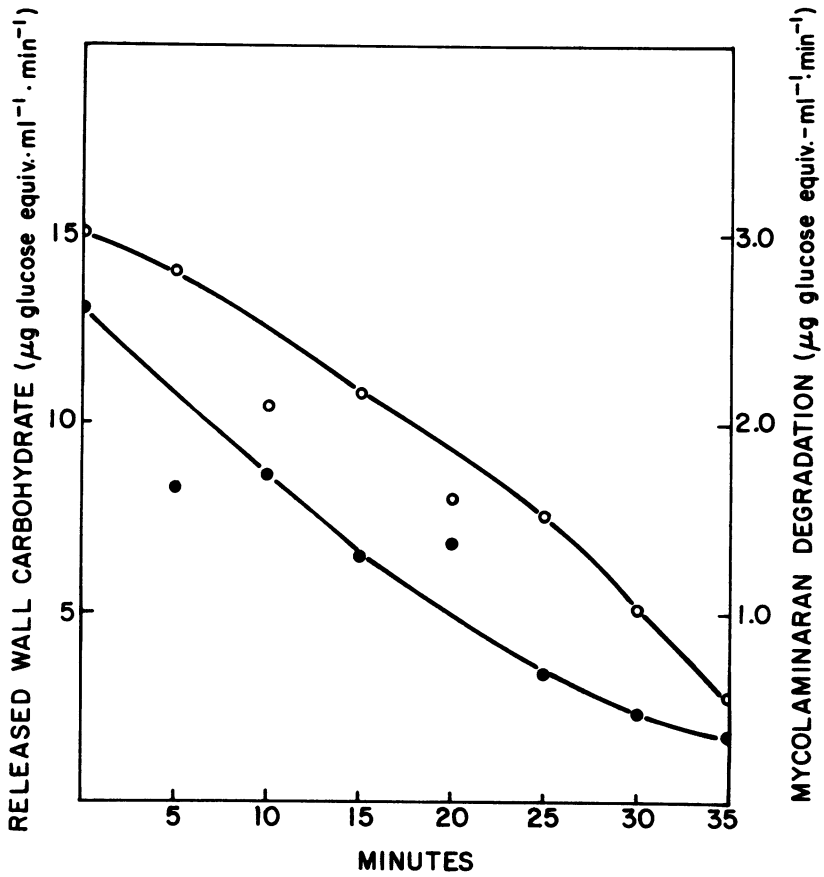


FIG. 7. Activity of purified pI 10.5 endoglucanase against mycolaminarin (●) and cell walls of Pmg (○) following incubation of the enzyme at 65°C for the periods of time noted.

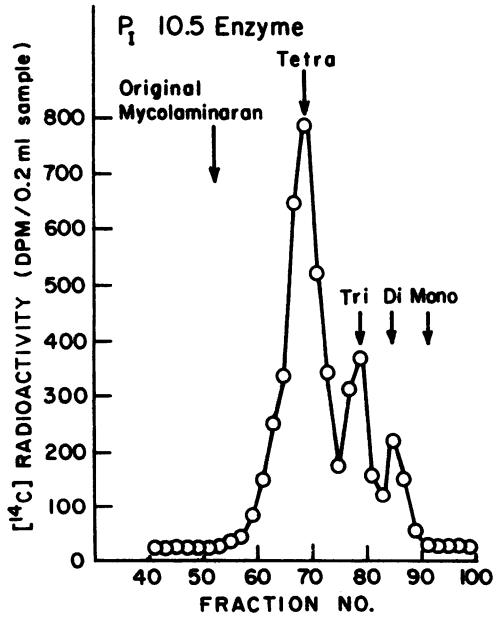


FIG. 8. Chromatography on a high-resolution Sephadex G-15 column of reaction products produced from [$^{14}$C]mycolaminaran by the pI 10.5 soybean endoglucanase purified by electrofocusing. The G-15 column was calibrated with authentic oligosaccharides and glucose. The elution position of the starting mycolaminaran preparation is noted by the arrow.

electrofocusing. The S-300 preparations also yielded a single intensely staining band on SDS-gel electrophoresis (Fig. 5), with an apparent mol wt of 33.5 ± 0.5 kD (mean ± SD obtained from five separate gels). This agreed well with the results from gel filtration. The pI 8.7 glucanase gave an estimated size of 32.5 ± 0.7 kD on SDS-gel electrophoresis, but gave an apparent weight of 36 kD on the P-150 gel filtration column. A few faintly staining contaminant protein bands were observed from some purified enzyme batches, but all preparations obtained from the S-300 Sephacryl step (Table I) were judged to be at least 98% pure. The S-300 enzyme was recovered following lyophilization as a light cream-colored solid that retained full activity in the laminarin assay for at least 3 months when stored at −20°C. In addition, the enzyme was stable when thawed following storage in solution at −20°C. Yield of the enzyme was high, varying from 13 to 28 mg in four experiments starting with 1 kg fresh weight of soybean cotyledons.

Both the endoglucanase and elicitor-active cell-wall carbohydrate-releasing activities were concluded to reside in single proteins based on the following tests with enzymes purified through the Sephacryl S-300 step or recovered from the electrofocusing column: (a) the pH optima of the pI 8.7 and 10.5 glucanases were indistinguishable, about 5.3 against cell walls and laminarin (data not shown); (b) addition of mycolaminaran to reaction mixtures containing isolated fungus cell walls competitively reduced the rate of carbohydrate release from the walls (Fig. 6); (c) inactivation curves run at 65°C resulted in simultaneous reduction in activity against both substrates (Fig. 7). These considerations strongly supported the interpretation that the β-glucanases accounted for activity against both laminarin and Pmg cell walls.

**Further Characterization of the Glucanases.** The pI 8.7 and 10.5 glucanase enzymes exhibited similar catalytic properties on defined substrates. Both enzymes catalyzed identical rates of reducing group liberation from mycolaminaran, laminarin, and chrysolaminarin, but did not attack carboxymethylcellulose,

starch, pectin, or yeast mannan. The enzymes were inactive against pustulan (only β-1,6-glucose linkages) but active on CM-pachyman (only β-1,3-glucose linkages; 20) (data not shown). Against mycolaminaran, reducing group liberation at the maximum accounted for approximately four glycosidic linkages hydrolyzed per native mycolaminaran molecule of average 30 glucose residues with one reducing end (17). This suggestion of random specificity was confirmed by product analysis of radioactive mycolaminaran reaction mixtures taken to saturation reducing group liberation (Fig. 8); the predominant products were tetrasaccharides, with small amounts of trimers and dimers, but no monomeric glucose was detected. The purified pI 8.7 and 10.5 enzymes were inactive against *p*-nitrophenyl-β-D-glucoside, even at 100 μg $ml^{-1}$ enzyme. The soybean glucanases were accordingly concluded to be strictly endoenzymes.

## DISCUSSION

The results of this and the accompanying paper (10) confirm the observation of Yoshikawa *et al.* (21) that soybean tissues contain enzymic activity capable of releasing phytoalexin elicitors from isolated cell walls of Pmg. The soybean enzymes attacking Pmg cell walls were concluded to be β-1,3-endoglucanases (EC 3.2.1.39) because: (a) enzyme preparations judged to be homogeneous by SDS-gel electrophoresis and gel filtration effected the release of elicitor-active carbohydrate from cell walls and attacked laminarin; (b) activities against both substrates copurified; (c) pH optima were the same against both substrates; (d) mycolaminaran competitively inhibited the release of carbohydrate from cell walls; and (e) both activities were inactivated in parallel at 65°C. Based on the relatively high recovery of activity through the purification scheme, all or most of the cell wall carbohydrate-releasing activity in crude soybean extracts was accounted for by the β-1,3-endoglucanase enzymes. Only a relatively small amount of exo-β-glucanase activity was present in the crude cotyledon extracts as determined by the *p*-nitrophenyl-β-D-glucoside assay, and this was entirely removed during purification of the endoglucanases. Cline and Albersheim (5, 6) recently isolated an exoglucanase enzyme from suspension-cultured soybean cell walls with high-salt solutions. The absence of significant activity of this enzyme in our preparations from cotyledons was probably due to use of the low-salt extraction buffer.

Release of the glucomannan phytoalexin elicitors from the fungus by the soybean glucanases may be important in the physiological interaction of fungus hyphae and soybean cells. Several authors have suggested that such enzymes may function as disease resistance factors by attacking the pathogen cell wall and inducing lysis or other inhibitory effects (12, 14, 15). Our results and those of Nichols *et al.* (13) suggest that the role of plant carbohydrases may be more subtle and involve the release of elicitor-active carbohydrates from the cell surface of the pathogen. Models for elicitor recognition by plant cells (8) as well as our unpublished direct evidence suggest that receptors for elicitor-active carbohydrates exist on plant plasma membranes. The enzyme-catalyzed solubilization of carbohydrate elicitors from advancing fungus hyphae could be important in facilitating their permeation of the plant cell wall to permit receptor binding at the plasma membrane surface. Significantly, the recent work of Tepfer and Taylor (16) has indicated that higher plant cell walls are permeable to proteins up to 60 kD in mol wt.

The soybean endoglucanases separated by electrofocusing are similar in mol wt and catalytic activity, and accordingly may represent isoenzymes. However, this has not been confirmed by amino acid analysis or sequence studies. The soybean enzymes, however, are similar to β-1,3-endoglucanases from certain other plants, sharing properties such as their mol wt, basic nature, acidic pH optimum, relatively high-temperature stability, and their strictly random attack on polymers containing repeating β-1,3-linked glucose residues (1, 4, 7).

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