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PURIFICATION AND PROPERTIES OF AN ENDOGLUCANASE ISOLATED FROM THE CELL WALLS OF *Zea mays* SEEDLINGS*

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ABSTRACT

An endoglucanase liberated from *Zea mays* seedling cell-walls by LiCl was purified by using SP-Sephadex, CM-Sephadex, and gel filtration, resulting in a 98-fold increase in specific activity. It has a pH optimum of 4.5–5.0 and is heat stable up to 40–45°. Compounds that interact with sulfhydryl groups did not inhibit the activity of the enzyme, nor did EDTA, suggesting that the enzyme does not require free sulfhydryl groups or metal ions for activity. The endoglucanase has an apparent molecular weight of 20–25,000 and an isoelectric point of ≥9. Hydrolytic activity against (1→3),(1→4)-β-D-glucans is restricted to isolated sites, with the release of high-molecular-weight products (10–15,000). Action on isolated, inactivated *Zea* cell-walls caused the release of approximately the same products as observed when the enzyme was incubated with soluble (1→3),(1→4)-β-D-glucans.

INTRODUCTION

The (1→3),(1→4)-β-D-glucans are cell-wall components of many grasses[1]. The role of this polysaccharide in the primary plant cell-wall is not understood, although certain evidence would suggest a structural role[2]. Such a role is supported by the observation that modification of (1→3),(1→4)-β-D-glucan occurs during cell elongation. Loescher and Nevins demonstrated loss of glucose from hemicellulosic glucan during indole-3-acetic acid (IAA)-induced cell elongation in *Avena sativa* coleoptile segments[3]. Similar modifications of hemicellulosic glucan have been reported during IAA-induced growth in *Oryza sativa*[4] and *Hordeum vulgare*[5] coleoptiles. In addition, Luttenegger and Nevins have shown that the (1→3),(1→4)-β-D-glucan content of coleoptile cell-walls of *Zea* generally ranges from 1 to 14%, depending on the developmental stage of the coleoptile[6]. The highest levels were present during the rapid-elongation phase of *Zea* coleoptile growth. Although these

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observations suggest a potential role of (1→3),(1→4)-β-D-glucan in cell elongation, the enzymes leading to modifications during growth have not been isolated. Potential enzymes that could lead to such modifications during growth may be the same or similar to those involved in plant cell-wall autolysis[7–10].

Autolytic activity of isolated *Zea* coleoptile cell-walls results in specific release of D-glucose from the (1→3),(1→4)-β-D-glucan component of these walls. Recently, Huber and Nevins identified two enzyme activities from *Zea* cell-wall extracts that appeared to be responsible for the autolytic release of glucose[11]. These two enzymes were partially purified and identified as an exoglucanase and an endoglucanase. The action of the endoglucanase appeared to be restricted to the hydrolysis of mixed linked (1→3),(1→4)-β-D-glucans in the cell wall. This conclusion was supported by the observation that Hg^{2+} had little effect on the total autolytic release of carbohydrate, but caused a shift from monosaccharide to products of higher molecular weight[12]. Further analysis of these higher-molecular-weight products indicated that they were derived from the (1→3),(1→4)-β-D-glucan cell-wall component. The addition of Hg^{2+} to active cell walls effectively inhibited a wall-bound exoglucanase, but not the endoglucanase. The authors postulated that these two enzymes may operate in conjunction, to account for the autohydrolytic release of glucose from *Zea* cell-walls. The work described here was undertaken to purify and characterize the properties of the endoglucanase from *Zea* cell-wall extracts. Such information would be useful in assessing the potential role of the endoglucanase during modifications of (1→3),(1→4)-β-D-glucan accompanying growth.

RESULTS AND DISCUSSION

Purification of the endoglucanase. — Cell-wall proteins were extracted from walls of *Zea mays* seedlings using the procedure of Huber and Nevins[11], with slight modifications as described here (Experimental). The resulting protein extract was dialyzed against citrate–phosphate buffer (20mM, pH 5.6) containing 200mM NaCl to remove the LiCl. Insoluble material precipitated during dialysis was removed by centrifugation at 10,000*g* and the supernatant solution was concentrated by ultrafiltration (membrane Diaflo PM-10). After concentration to 30 mL, the protein sample was dialyzed against acetate buffer (20mM, pH 5.0) containing 20mM NaCl. A second centrifugation was used if further precipitates formed.

The crude protein mixture was initially fractionated on a cation-exchange column (SP-Sephadex) equilibrated with acetate buffer (20mM, pH 5.0) containing 20mM NaCl. Bound proteins were eluted with a NaCl gradient from 20 to 500mM in the same acetate buffer, resulting in the separation of the crude mixture into several subfractions (Fig. 1). The SP-Sephadex column was a particularly useful first step in purification of the endoglucanase. An exoglucanase that also hydrolyzes the (1→3),(1→4)-β-D-glucan molecule was separated clearly from the endoglucanase. A small amount of hydrolytic activity eluted in the void fraction of this

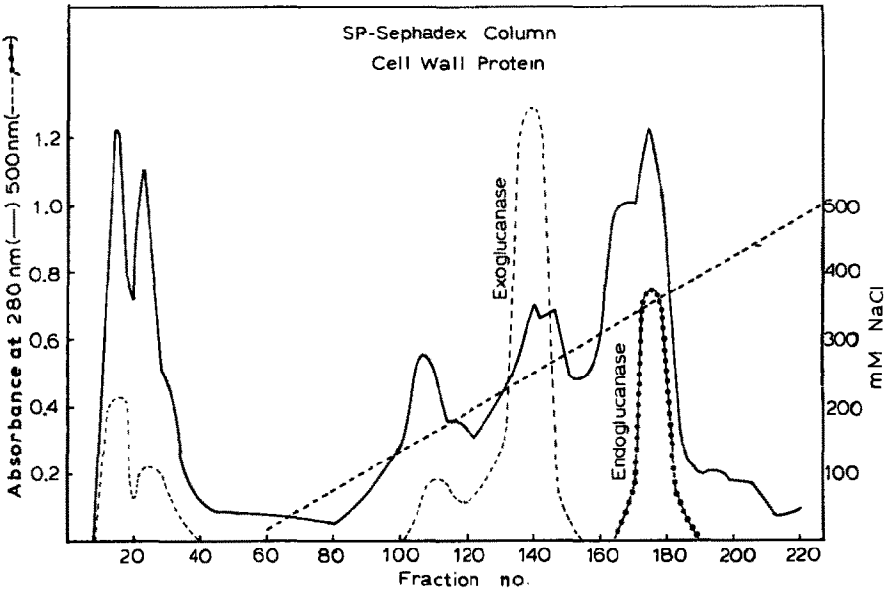

Fig. 1. Cation-exchange chromatography of *Zea* endoglucanase on SP-Sephadex (see Experimental). Fractions (3 mL) were assayed for protein at 280 nm (——) and hydrolytic activity (------) as described in the Experimental section.

column (fractions 1–40, Fig. 1). This activity was similar to that of the bound exoglucanase, and may be the same enzyme that has been slightly modified during extraction, altering its charge properties. This activity was not caused by overloading of the column, as columns loaded with half the amount of total protein still showed activity in these fractions. No endoglucanase activity was observed in the void fractions.

Column fractions containing endoglucanase activity were pooled, concentrated, and dialyzed against acetate buffer (20mM pH 5.6) containing 20mM NaCl. The concentrated endo-fraction was loaded on a cation-exchange column of CM-Sephadex. Elution of the bound proteins occurred by imposing a gradient from 325 to 450mM NaCl (Fig. 2). This resulted in a broad peak of endoglucanase activity, with the main activity eluting at 380–410mM NaCl. Fractions containing the major activity were pooled and concentrated as before.

The concentrated fractions from the CM-Sephadex column were dialyzed against citrate–phosphate buffer (pH 5.6) containing 200mM NaCl. After dialysis, the endoglucanase fraction was further concentrated to 1.5–2.0 mL and loaded onto a Bio-Gel P-150 size-exclusion column equilibrated with the same citrate–phosphate buffer. Endoglucanase activity eluted in a fraction corresponding to an apparent molecular weight of ~20–25,000, based on the elution profile of protein standards (Fig. 3). Active fractions were pooled, concentrated, and subjected to a second fractionation on the same column. This resulted in a single peak of protein and corresponding activity.

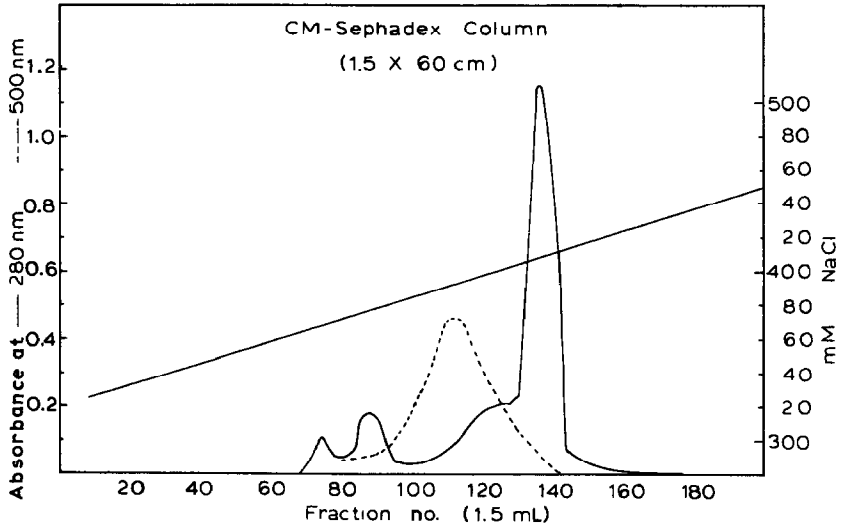

Fig. 2. Cation-exchange chromatography of endoglucanase on CM-Sephadex (see Experimental). Fractions (1.5 mL) were assayed for protein at 280 nm (——) and endoglucanase activity (------).

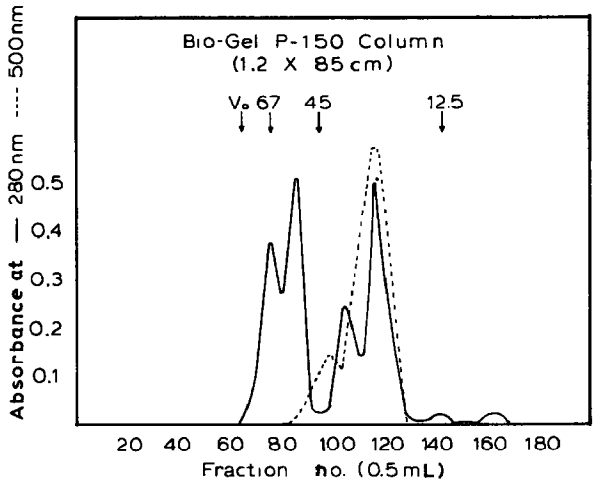

Fig. 3. Gel filtration of endoglucanase on Bio-Gel P-150 (see Experimental). Fractions (0.5 mL) were assayed for protein at 280 nm (——) and endoglucanase activity (------). Arrows denote the elution profile of molecular-weight marker proteins consisting of bovine-liver catalase, mol.wt. 240,000, bovine albumin, mol.wt. 67,000, egg albumin, mol.wt. 45,000 and cytochrome C, mol.wt. 12,500.

The chromatographic purification-scheme described here resulted in a 98-fold increase in specific activity (Table I) on the basis of hydrolysis of oat glucan, as assessed by change in viscosity. Polyacrylamide-gel electrophoresis (PAGE) of protein samples from individual steps of this purification scheme was used to monitor and assess the effectiveness of each step. There was specific enrichment of a single band corresponding to an apparent molecular weight of 30,000. The

TABLE I

PURIFICATION OF *Zea* CELL-WALL ENDOGLUCANASE

Step in purification process	*Specific activity*[a] *(units/mg)*	*Purification*
Crude protein[b]	0.33	1.0
SP-Sephadex	0.82	2.5
CM-Sephadex	3.70	11.2
Bio-Gel P-150	32.00	98.5

[a]Specific activity is defined as change in units of activity as measured by viscosity per mg of protein. See Experimental, enzyme purification, for an explanation of units of activity. [b]LiCl extract of cell walls.

estimate of molecular weight by PAGE was slightly higher than that by gel filtration, possibly because of conformational changes caused by sodium dodecyl sulfate. The purified endoglucanase was subjected also to isoelectric focusing electrophoresis on a wide-range gel (pH 3–9) to determine the isoelectric point. Best estimates of the pI would be pH 9 or higher, as the protein banded at or very near the high-pH side of the gel.

Endoglucanase properties. — The endoglucanase was stable for >12 months when stored at −20° in either acetate buffer (20mM pH 5.0, 20mM NaCl) or citrate–phosphate buffer (pH 5.6, 200mM NaCl). However, it was found that activity slowly decreased (15%) with repeated freeze–thaw cycles. Individual aliquots of the enzyme were therefore stored for use in subsequent assays.

Temperature–activity relationships and thermal-stability properties. — The activity of the endoglucanase increased in a linear fashion over the temperature range of 25–40° (Fig. 4). At temperatures >40°, the increase in activity declines and approaches a plateau. The plateau in enzyme activity may be accounted for by the

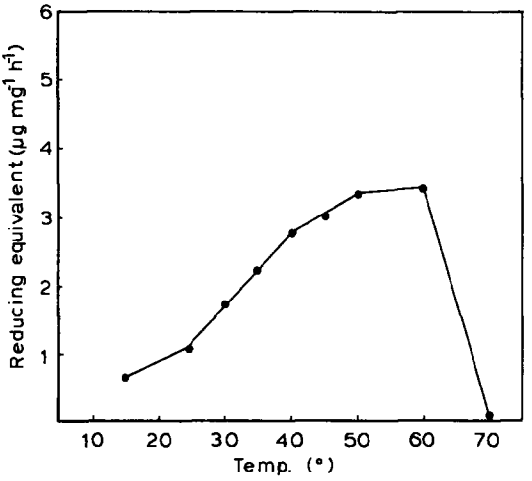

Fig. 4. Effect of reaction temperature upon the activity of the endoglucanase (see Experimental).

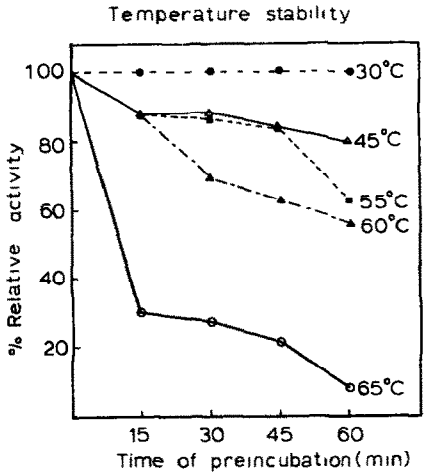

Fig. 5. Effect of increasing the length of exposure to preincubation temperature on stability of the endoglucanase activity (see Experimental).

thermal instability of this enzyme (Fig. 5). From these results, the endoglucanase is relatively stable to increasing temperature up to 45°. At temperatures above 55°, there is a sharp decrease in activity with increased incubation time; ~40% of the activity is lost after 1 h of preincubation at 60°. The plateau in activity therefore may be attributable to the kinetic increase in activity at higher temperatures, coupled with a net decline of activity as the enzyme is exposed to the higher temperature for increasing lengths of time. At temperatures above 65°, virtually all of the catalytic capacity is lost.

pH–Activity relationships. — The endoglucanase exhibits a sharp pH-activity maximum at 4.5–5.0, with activity decreasing rapidly at higher and lower pH values

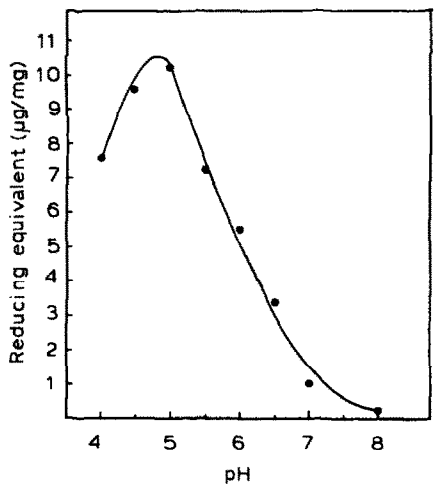

Fig. 6. Effect of pH on the activity of the endoglucanase (see Experimental).

TABLE II

EFFECT OF INHIBITORS AND Ca^{2+} IONS ON *Zea* ENDOGLUCANASE ACTIVITY

Treatment	*Concentration*	*Relative activity (%)*
EDTA[a]	10mM	100.0
EDTA	100mM	100.0
Ca^{2+}	1mM	104.0
Ca^{2+}	10mM	107.0
Ca^{2+}	50mM	100.0
Hg^{2+}	50μM	100.0
Hg^{2+}	100μM	100.0
PCMB[b]	50μM	98.5
PCMB[b]	100μM	86.0
Nojirimycin	10μM	98.9
Nojirimycin	100μM	103.9
D-Glucono-1,5-lactone	100μM	90.2
D-Glucono-1,5-lactone	1000μM	101.7

[a]Ethylenedinitrilo(tetraacetic acid) (disodium salt). [b]*p*-(Hydroxymercuri)benzoic acid.

(Fig. 6). Such a sharp optimum may suggest a possible means of *in vivo* regulation of activity. Acidification of cell-wall free space has been observed during rapidly growing phases of the coleoptile[13], and in response to the plant hormone auxin (indole-3-acetic acid, IAA), which induces elongation of this tissue[14]. Such acidification may alter the pH of the wall to values of 4.5–4.75. It is yet to be demonstrated, however, that the endoglucanase plays a direct role during these rapid growth inductions. The shift in wall pH could enhance the activity of this enzyme against the native *Zea* (1→3),(1→4)-β-D-glucan.

Effects of inhibitors and Ca^{2+} ions. — Table II summarizes the effects of various inhibitors and Ca^{2+} ions. The data from the treatment with EDTA indicate that this enzyme does not in general require divalent metal ions for activity. Furthermore Ca^{2+} ions did not stimulate activity of the endoglucanase. Manners and Marshall found that Ca^{2+} stimulated the hydrolytic activity of the (1→3)-β-D-endoglucanase of malted barley, but did not affect the (1→4)-β-D-endoglucanase[15]. Work by Woodward and Fincher showed that the presence of EDTA or the addition of metal ions had no effect on the β-D-endoglucanase of barley[16].

The *Zea* endoglucanase is not inhibited by Hg^{2+} or *p*-(hydroxymercuri)benzoic acid, indicating that it does not contain free sulfhydryl groups whose modification by Hg^{2+} or *p*-(hydroxymercuri)benzoic acid would have caused loss of enzyme activity. Such inhibitors as nojirimycin and D-glucono-1,5-lactone did not inhibit the activity of the endoglucanase. This would be expected if the enzyme has a true endo-hydrolytic pattern of activity. These inhibitors appear to be specific to the inhibition of glucosidases and exoglucanases[17].

Enzyme kinetics. — The K_M and V_{max} values were determined at pH 5.0 and 30° with three related substrates. Substrates constituting (1→3),(1→4)-β-D-glucans

TABLE III

KINETIC PROPERTIES OF THE *Zea* ENDOGLUCANASE

Substrate	K_m *(mg/mL)*	V_{max} *(μg/h/μg)*[a]
Avena β-D-glucan	0.33	2.75
Hordeum β-D-glucan	0.33	1.68
Lichenan	0.34	1.51

[a]Reducing equivalents (μg) produced from each substrate per h of incubation per μg of endoglucanase added.

from *Hordeum, Avena,* and *Cetraria islandica* (lichenan) were employed. The K_m values were close to each other (Table III), indicating a similar relative affinity of the endoglucanase for each of the substrates. Although it cannot be said that the enzyme has a strong affinity for the substrates, it appears that the endoglucanase is saturated at a relatively low concentration. Compared with the K_m values for the (1→3),(1→4)-β-D-glucanase from *Bacillus subtilis* (3.4 mg/mL)[18] and *Hordeum vulgare* (3.0, 3.4 mg/mL)[16], the value for the *Zea* endoglucanase is 10 times lower, indicating a possible higher affinity for the (1→3),(1→4)-β-D-glucan molecule. The K_m and V_{max} values may indicate something about the potential of the endo-

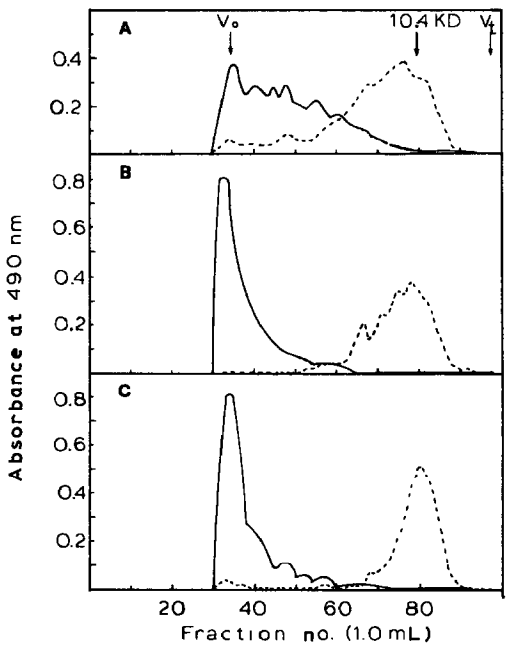

Fig. 7. Chromatographic profile of hydrolytic products released from (1→3),(1→4)-β-D-glucans on a column of Bio-Gel A 1.5M. A, lichenan; B, *Hordeum* β-D-glucan; C, *Avena* β-D-glucan. (——) before endoglucanase treatment (------) after endoglucanase treatment. The elution profile of a molecular-weight series of dextrans (Blue 2000, 500,000, 73,000, 10,400 avg. mol.wt.) and glucose was used to calibrate the column.

glucanase to hydrolyze mixed-linkage substrates, but does not necessarily reflect the *in vivo* activity of the enzyme. *In vivo*, the wall matrix may restrict the accessibility of the enzyme to the hydrolytic sites on the substrate.

Hydrolytic activity. — The hydrolytic activity against such model substrates as the (1→3),(1→4)-β-D-glucan from *Avena, Hordeum,* and *Cetraria islandica* suggest that the endoglucanase hydrolyzes unique sites within these molecules. This observation is based on the release of limit products (molecular weight 10–15,000) from each of the substrates (Fig. 7). All three substrates afford similar product-profiles. There are small differences in the product profiles in that hydrolysis of *Avena* (1→3),(1→4)-β-D-glucan resulted in a more-refined distribution of products around the average molecular weight of 10–15,000 as compared to *Cetraria islandica* lichenan. This result suggests that these two molecules differ in the uniformity of distribution of the unique hydrolytic sites. It appears that lichenan from *Cetraria islandica* does not have the same molecular-weight distribution as *Hordeum* and *Avena* (1→3),(1→4)-β-D-glucan prior to hydrolysis by the endoglucanase. These smaller molecular-weight substrates are hydrolyzed to approximately the same 10–15,000 molecular-weight products. Increased incubation time with the endoglucanase, or reincubation of the products with fresh enzyme, does not result in a decrease in the average molecular weight of the products.

When the *Zea* endoglucanase is added to isolated *Zea* cell walls that had been boiled to inactivate wall-bound enzymes, products of similar molecular weight are released (Fig. 8). If this released carbohydrate is hydrolyzed with the endoglucanase (EC 3.2.1.73) from *Bacillus subtilis,* the resulting products are oligosaccharides having 3 and 4 sugar residues (Fig. 8). When these products are resolved into separate fractions upon a Bio-Gel P-2 column (400 mesh) and subjected to methylation analysis, the trisaccharide yields a molar ratio of 1:1:1 for terminal Glc, (1→3)-Glc, and (1→4)-Glc. The tetrasaccharide yields a molar ratio of 1:1:2 for terminal Glc, (1→3)-Glc, and (1→4)-Glc. These results, and the specificity of

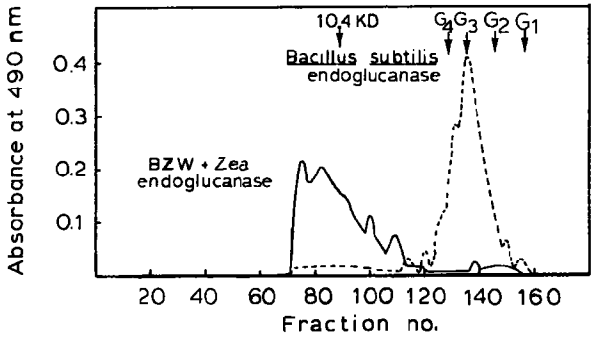

Fig. 8. Chromatographic profile on Fractogel TSK 50SFW of products released from inactivated *Zea* cell-walls (BZW) treated with the endoglucanase (——). Treatment of the released carbohydrate with *Bacillus subtilis* endoglucanase (------). The elution profile of dextran (73,000 and 10,400 mol.wt.), tetra- and tri-saccharides released from *Hordeum* (1→3),(1→4)-β-D-glucan by *Bacillus* endoglucanase, cellobiose, and glucose were used to calibrate the column.

the *Bacillus* endoglucanase[19,20], indicate that the oligosaccharides are 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose, which are the major structural units that make up (1→3),(1→4)-β-D-glucans. The uniformity of the products from all tested substrates suggest that the *Zea* endoglucanase is hydrolyzing unique sites that exist within the (1→3),(1→4)-β-D-glucans.

Bathgate and Palmer[21] observed that a bacterial (1→3)-β-D-endoglucanase and a (1→3)-β-D-endoglucanase from *Hordeum vulgare* hydrolyzed a (1→3),(1→4)-β-D-glucan extracted from *Hordeum* in a similar manner. The molecular weight of the products released by action of the bacterial enzyme was ~10,000. They claimed to have identified regions within the *Hordeum* (1→3),(1→4)-β-D-glucan molecule that contained more than one adjacent β-D-(1→3) linkage. Such regions would provide potential hydrolytic sites for the (1→3)-β-D-endoglucanases. More-recent work by Woodward *et al.*[22] indicated that soluble *Hordeum* (1→3),(1→4)-β-D-glucan contained extended regions of adjacent β-D-(1→4) linkages[5–11], but limited or no regions of adjacent β-D-(1→3) linkages. In both cases, evidence for or against contiguous (1→3) linkages was based upon periodate oxidation, which may lack sufficient sensitivity. Perhaps a stepwise enzymic degradation such as that employed by Kato and Nevins[23] would be a more-sensitive method of determining the presence or absence of sequences of (1→3)-linked residues.

Kato and Nevins[23] determined by enzymic methods that the *Zea* (1→3),(1→4)-β-D-glucan molecule resembled the soluble (1→3),(1→4)-β-D-glucan described by Woodward *et al.*[22] in that both contained extended regions of (1→4)-linked residues. In addition, contiguous (1→3) linkages were detected in *Zea* walls. It seems likely that the *Zea* endoglucanase recognizes these extended regions of (1→4)-linked residues and possibly runs of (1→3)-linked residues, and hydrolyzes an associated linkage. The relative abundance of these regions supports this assumption, resulting in the release of products of 10–15,000 molecular weight. The possibility cannot be ruled out that the enzyme recognizes regions of extended β-D-(1→4) linkages but hydrolyzes either a β-D-(1→4) or a β-D-(1→3) linkage. Conversely, the endoglucanase may recognize extended regions of β-D-(1→3) linkages but hydrolyze a β-D-(1→4) linkage.

As the *Zea* endoglucanase can hydrolyze (1→3),(1→4)-β-D-glucans from *Hordeum*, and *Zea* producing similar products, the hydrolytic site must be a common structural feature of these molecules. Based on the detailed structural analysis of *Hordeum*[21] and *Zea*[23] (1→3),(1→4)-β-D-glucan, a common structural feature of these two molecules includes extended regions of (1→4)-linked residues. It is not yet possible to say whether or not the molecules also have regions of more than one contiguous (1→3) linkage in common. Preliminary evidence indicates that *Avena* (1→3),(1→4)-β-D-glucan, extracted from oat bran, contains regions of extended, (1→4)-linked residues as well as regions of more than one contiguous (1→3) linkage, based on enzymic methods and methylation analysis (unpublished results). The *Zea* endoglucanase cannot hydrolyze laminaran, but shows limited hydrolytic

activity against *O*-(carboxymethyl)cellulose (unpublished data). When these pieces of information are analyzed together, they suggest that the extended regions of β-D-(1→4) linkages are of primary importance to enzyme activity. The *Zea* endoglucanase differs in its hydrolytic action pattern from the barley β-D-endoglucanase[16] and the *Bacillus subtilis* endoglucanase[18–20], which release tri- and tetrasaccharides as limit products from (1→3),(1→4)-β-D-glucan molecules.

EXPERIMENTAL

Enzyme. — The endoglucanase was extracted from *Zea mays* (B73 X Mol7) seedling cell-walls by a modification of the procedure of Huber and Nevins[11], *Zea* seedlings (500–600 g) were routinely homogenized in 1.1–1.5 L of 50mM NaCl (4°) containing 200–300 g of ice (distilled H_2O). The homogenate was filtered through Miracloth (Calbiochem) with the aid of suction and washed extensively with a sequence of 50mM NaCl (1–2 L, 4°), acetone (4 L, −20°), and 50mM NaCl (1–2 L, 4°). The isolated cell-walls were suspended in 300–400 mL of 3M LiCl and stirred slowly for 48 h at 4°, after which time the mixture was filtered through Miracloth to obtain the LiCl-extracted proteins.

Substrates. — *Hordeum* (1→3),(1→4)-β-D-glucan was obtained from Biocon (U.S.) Inc. (Lexington, KY), *Avena* (1→3),(1→4)-β-D-glucan from Quaker Oats, and lichenan *(Cetraria islandica)* from Sigma.

Enzyme purification. — (*a*) *Chromatography on SP-Sephadex.* A column (2.3 × 30 cm) of SP-Sephadex (SP-C-50-120) was equilibrated with 20mM acetate buffer (pH 5.0) containing 20mM NaCl. The dialyzed, concentrated, crude cell-wall extract (15–20 mL) was applied to the column at a flow rate of 8 mL/h. The endoglucanase remained bound to the column and was eluted with a NaCl gradient (20–500mM). The single peak of activity eluted between NaCl concentrations of 350 and 400mM. Activity of column fractions was determined by measuring the rate of change in viscosity of a 1% *Avena* (1→3),(1→4)-β-D-glucan solution in 20mM acetate buffer (pH 5.0). An aliquot fraction (50 μL) was added to 350 μL of *Avena* (1→3),(1→4)-β-D-glucan solution for each fraction assayed. The change in viscosity was monitored by measuring the drainage time from a 0.2-mL pipette. A plot of 1/T *vs.* incubation time, where T = drainage time, gives a straight line in which the slope is the relative change in viscosity (slope = $\Delta 1/T\ min^{-1}$). Units of activity are therefore defined in terms of slope of the plotted line (one unit = 0.001 $\Delta 1/T\ min^{-1}$).

An alternative method was to incubate an aliquot (100 μL) of selected column fractions with 400 μL of a solution of *Avena* ((1→3),(1→4)-β-D-glucan (1 mg/mL in acetate buffer, pH 5.0) in which Hg^{2+} was added to a final concentration of 100μM. The addition of Hg^{2+} effectively inhibited the exoglucanase that also degrades (1→3),(1→4)-β-D-glucans. Enzyme activity was assessed by measuring the increase in reducing-sugar equivalents after incubation for 12–18 h. Both methods identified identical peaks of activity.

The activity of the exoglucanase was monitored in these fractions by using laminaran as the substrate. A 100-μL aliquot of selected fractions was added to 400 μL of a laminaran solution (1 mg/mL) in acetate buffer (pH 5.0). After incubation for 10 min at 30°, the reaction was stopped by adding 500 μL of the copper reagent for the reducing-sugar assay. Relative activity was determined by measuring the absorbance at 500 nm.

(*b*) *Chromatography on CM-Sephadex.* A column (1.5 × 60 cm) of CM-Sephadex (C-50-120) was equilibrated with 20mM acetate buffer (pH 5.6 containing 20mM NaCl). The pooled, active fractions from the SP-Sephadex column were dialyzed against the column-equilibration buffer and concentrated to a final volume of 3–5 mL with an Amicon ultrafiltration cell Model 52 (PM-10). The concentrate was loaded on the column with a flow rate of 10 mL/h. The bound activity was eluted with a NaCl gradient (325–450mM) with 1.5-mL fractions being collected. The endoglucanase activity in the column profile was assayed as described for the SP-Sephadex column, using the reducing-sugar method.

(*c*) *Chromatography on Bio-Gel P-150.* A column (1.2 × 80 cm) of Bio-gel P-150 (100–200 mesh), was equilibrated with citrate–phosphate buffer (pH 5.6) containing 200mM NaCl. The pooled endoglucanase fractions from the CM-Sephadex column were dialyzed overnight against the equilibration buffer. After concentrating to 1–2 mL with an Amicon cell Model 12 (PM-10), the protein was applied to the column and eluted with the same buffer at a flow rate of 3 mL/h. Fractions of 0.5 mL were collected. The active fractions were pooled and re-chromatographed on the same column under the same conditions.

(*d*) *Electrophoresis and isoelectric focusing.* SDS–polyacrylamide-gel electrophoresis[24] was performed by using a 12.5% gel at pH 8.8. Protein bands were made visible by staining with Coomassie Brillant Blue R-250 (Bio-Rad). Mobility of the endoglucanase was compared to standards of low molecular-weight proteins (SDS kit, Sigma).

Isoelectric focusing was performed by using an LKB 2117 Multiphor apparatus. Preformed polyacrylamide gels (LKB) were used that had a pH range 3–9. The established pH gradient was determined after focusing with a flat-surface electrode.

Enzyme properties. — (*a*) *Molecular weight and isoelectric points.* The molecular weight of the endoglucanase was estimated from the elution profile on a Bio-Gel P-150 size-exclusion column by comparison with the profile of protein standards. This molecular weight was compared to the value obtained from mobility in a 12.5% SDS–PAGE gel. Estimation of molecular weight was by comparison to the mobility of molecular-weight standards (SDS Kit, Sigma). The isoelectric point was determined by isoelectric focusing in a wide-range gel (pH 3–9).

(*b*) *pH optimum.* The endoglucanase activity was determined using *Hordeum* (1→3),(1→4)-β-D-glucan (2 mg/mL) as the substrate in citrate–phosphate buffer at different pH values. Enzyme activity was assessed by measuring the increase in reducing-sugar equivalents in 1 mL of substrate after incubation for 3 h at 30° with 2.5 μg of enzyme.

(*c*) *Temperature optimum*. The endoglucanase activity was measured at various incubation-temperatures by determining the increase in reducing-sugar equivalents produced after 2 h from a 1-mL solution of *Hordeum* (1→3),(1→4)-β-D-glucan (2 mg/mL). The β-D-glucan was dissolved in 20mM acetate buffer (20mM NaCl, pH 5.0). Endoglucanase was added to each temperature trial at a concentration of 2.5 μg/mL.

(*d*) *Temperature stability*. The endoglucanase was preincubated at various temperatures. Sufficient enzyme was placed in each temperature regime to allow aliquots to be removed at 15-min intervals over a 1-h preincubation period. Enzyme activity was assayed as described in the section on optimum temperature.

(*e*) *Effect of inhibitors and Ca^{2+} ions*. Endoglucanase (2.5 μg/mL) was added to a solution of *Hordeum* (1→3),(1→4)-β-D-glucan (2 mg/mL) in acetate buffer (pH 5.0). The activity was measured by the increase in reducing equivalents. Various inhibitors or ions were added in a concentrated solution to give the indicated resulting concentrations: Ca^{2+}, 1 10, and 50mM; EDTA, 10 and 100mM; Hg^{2+}, 50 and 100μM; *p*-(hydroxymercuri)benzoic acid, 50 and 100μm; nojirimycin, 10 and 100μM; and D-glucono-1,5-lactone 100 and 1000μM.

(*f*) *Kinetic parameters*. The activity of the endoglucanase was measured with variation of the concentration of each substrate tested from 0.312 to 5.00 mg/mL. The rate of the reaction was monitored by removing a small aliquot (200 μL) of the total mixture at various time-periods (0, 0.5, 1, 2, and 4 h) and assaying it for the presence of reducing sugars. Enzyme was added to the initial mixture to give a final concentration of 5μg/mL. The K_m and V_{max} values for each substrate was determined from Lineweaver–Burk plots.

Hydrolytic activity. — Endoglucanase (2.5 μg/mL) was incubated with various (1→3),(1→4)-β-D-glucan substrates (2 mg/mL) for 12–18 h. After incubation, the mixtures were heated for 10 min in boiling water to inactivate the enzyme. The mixture was applied to a column (1.5 × 60 cm) of Bio-Gel A 1.5M equilibrated with 20mM acetate buffer (pH 5.0) containing 20mM NaCl and eluted with the same buffer. The buffer was allowed to elute the carbohydrate from the column at a flow rate of 15 mL/h and 1.0-mL fractions were collected. The column profile was determined by measuring the total sugar in even-numbered fractions.

To determine the activity against native *Zea* (1→3),(1→4)-β-D-glucan, *Zea* coleoptile walls were isolated as described before except that instead of LiCl treatment the walls were subjected to boiling water for 20 min. The boiling step was repeated at least once and the wall residue filtered over glass filters with the aid of suction after the final heating. The inactivated walls were resuspended in 20mM acetate buffer (pH 5.0) and divided into roughly equal aliquots. The final concentration of dried walls was routinely between 1.5 and 2.5 mg per treatment sample. Endoglucanase was added to give a final concentration of 1 μg/mL. Boiled walls without enzyme were used as a control. Samples were incubated for 48 h, filtered, and the supernatant solution treated in boiling water for 10 min. A portion of the supernatant (0.5–1 mg total sugar) was fractionated on a Fractogel TSK 50SFW

column. A second portion (1 mg) of the released carbohydrate was treated with the endoglucanase from *Bacillus subtilis* (EC 3.2.1.73). The resulting hydrolytic products were fractionated on the same Fractogel column under the same conditions. For some samples, fractions corresponding to d.p. 3 and 4 were further resolved by liquid chromatography on a column of Bio-Gel P-2 (1.5 × 150 cm, 400 mesh) and subjected to methylation analysis.

General methods. — Reducing-sugar assays were performed by the method of Nelson[25] as modified by Somogyi[26]. Total-sugar assays were determined by the phenol–sulfuric method[27]. Protein contained in column-effluent fractions was monitored at 280 nm. Total protein in pooled fractions was estimated by using the Bio-Rad Protein assay with bovine serum albumin (Sigma) as a standard. Methylation analysis was performed according to the procedure of Harris *et al.*[28]. Partially methylated alditol acetates were analyzed by g.l.c. on a 30-m DB-1 fused-silica capillary column with a temperature program from 150–230° (4°/min) and a split ratio of 50:1.

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