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The isolation and characterization of cell wall proteins from Zea mays seedlings

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THE ISOLATION AND CHARACTERIZATION OF CELL WALL PROTEINS FROM ZEA MAYS SEEDLINGS

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The isolation and characterization of cell wall proteins from *Zea mays* seedlings

by

Ronald D. Hatfield

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ABBREVIATIONS

B-D-glucan	(1→3),(1→4)-β-D-glucan
D.P.	degree of polymerization
GLC	gas-liquid chromatography
IAA	indole-3-acetic acid
IEF	Isoelectric focusing
kD	kilodaltons
PI	isoelectric point
RS	reducing sugar
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid
TS	total sugar

GENERAL INTRODUCTION

Cell walls are dynamic structural components of plants. Cell walls must act as barriers, rigid enough to resist the outward force exerted by large turgor pressures that exist in the protoplast (35). Yet, walls must be amenable to change in order for cell enlargement and, consequently, for plant growth in general to occur. In plants such as the cereals cell enlargement may be as much as 100-200 fold at a rate of 1 mm h^{-1} in tissues such as the epidermis of the coleoptile (29). Cell enlargement is restricted virtually to longitudinal elongation with little lateral expansion. A similar growth pattern occurs in Nitella. Initial internode cells enlarge from 20 μm in length to elongated cyclinders 6 cm long and 0.5 mm in diameter (35). Cell enlargement in both cases occurs by a process of surface growth (33). By this process, the cell wall expands throughout the entire longitudinal surface, which involves extension of wall already present and synthesis of new wall material. The mechanism regulating cell wall extension has been investigated intensively since the early 1900s.

Heyn was the first to demonstrate that the addition of the plant hormone, auxin, to Avena coleoptiles altered the mechanical properties of the cell wall, increasing extensibility (7). In general, wall extensibility can be defined as the ability of cell walls to undergo irreversible extension under the driving force of turgor pressure (3,35). Since the early work of Heyn, several

other plant tissues have been investigated in terms of auxin induced alteration of cell wall mechanical properties (see references in 3). It has been found that if auxin stimulates plant growth, it also increases wall extensibility (3). For this to be accomplished load-bearing bonds within the cell wall must be cleaved allowing a partial relaxation of the wall matrix (wall loosening) which in the presence of turgor pressure leads to irreversible extension. For controlled cell expansion to occur there must be a regulation of the biochemical events leading to wall loosening.

Several models for cell wall loosening have been proposed. These include breaking hydrogen bonds between xyloglucan and cellulose microfibrils by acidic conditions (16), release of calcium from cell walls by auxin, decreasing Ca^{+2}-pectic chain cross-links (3), cleavage of specific load-bearing polysaccharides by acidic conditons (31), and enzymatic hydrolysis of specific polysaccharides (1,4). The enzyme regulated mechanism of wall loosening appears to offer the most feasible explanation since cell extension requires respiratory metabolism (2,30), protein synthesis (28), non-denatured cell wall proteins (32), and displays a relatively high Q_{10} (32).

A transglycosylase has been proposed as a possible model wall loosening enzyme (3). Such an enzyme would cleave specific glycosyl linkages and reform new ones after a slight displacement due to turgor proessure. Yamamoto and Nevins identified a (1→3)-

β-glucanase from Sclerotinia libertiania that had transglucosylase activity (39) and could cause a limited growth simulation in Avena coleoptiles (25,37). To date the only report of a transglycosylase in higher plants is the reversible endo-dextranase associated with cell walls from Avena coleoptiles (9). However, the native substrate of this enzyme in the cell wall matrix has not been identified.

An alternative wall loosening enzyme would be one that results in the irreversible hydrolysis of load-bearing bonds. Several cell wall hydrolases have been identified that increase in activity when the tissues containing them are treated with auxin. Fan and Maclachlan demonstrated that cellulase activity in Pisum stems increases several fold in response to auxin (6). However, the time course for increased activity lags significantly behind the onset of auxin induced growth (5). Auxin can stimulate the activity of a (1→3)-β-glucanase in Hordeum coleoptiles (36) and Avena coleoptiles (24) after a lag of only 10 min, placing it more in line with the commencement of auxin induced growth. Though the exact role of this enzyme has not been defined, the observation that nojirimycin, which inhibits exo-glucanase activity, also inhibits growth suggests a potential role in cell wall loosening (26).

A role for hydrolases in cell wall loosening is supported by the observation that a turnover of specific polysaccharides occurs during auxin induced growth. Loescher and Nevins described a

release of glucose from auxin treated Avena coleoptiles (21). The release of glucose, from hemicellulosic glucan, occurred even when turgor pressure was reduced to the point that cell elongation was inhibited (22). Similar turnover of hemicellulosic glucan has been described for Oryza coleoptiles (40) and Hordeum coleoptiles (34). In these grasses it appears that the loss of glucose is from the specific solubilization of the (1→3),(1→4)-β-D-glucan molecule. Although dicots do not contain this mixed linked glucan, auxin stimulates a turnover of other polysaccharides within the wall matrix. For Pisum sativum epicotyls (18) and Vigna angularis epicotyls (27) the major polysaccharide solubilized during auxin induced growth is xyloglucan. Although the two systems (grasses and dicots) are quite different, the evidence clearly indicates that cell wall matrix modification must occur during auxin stimulated growth. Furthermore, the release of defined carbohydrate fragments indicates specific enzymic activity. The specific enzymes responsible for the polysaccharide solubilization during growth have not been isolated for either the grass or dicot system.

In the case of grasses, a possible enzyme system has been identified in Zea coleoptiles. Lee et al. were the first to describe autolysis in higher plants (20). They isolated and purified cell walls from Zea coleoptiles which, when resuspended in buffer, released glucose from the cell wall matrix. Kivilaan et al. tentatively identified the wall component being solubilized

as a molecule similar to the mixed linked glucan, lichenan (17). Later work by Huber and Nevins confirmed that the polysaccharide solubilized during autolysis was (1→3),(1→4)-β-D-glucan (10). They were able to identify and partially purify an exo- and endo-β-D-glucanase that appeared to be responsible for the autolytic release of glucose (11). These enzymes were associated tightly with the wall matrix. Extraction of the the enzymes with 3M LiCl resulted in a loss of autohydrolytic activity in isolated cell walls (10).

Similar authydrolytic activities have been described in isolated cell walls of Avena (15) and Oryza (19). Although these systems have not been investigated as throughly as Zea, the autolytic activity appears to involve the solubilization of (1→3),(1→4)-β-D-glucan. Importantly, autolysis activity parallels the auxin induced B-D-glucan solubilization during growth for each system investigated in monocots. The direct evidence has not been provided for both processes being mediated by the same enzymes.

The functional role of the (1→3),(1→4)-β-D-glucan molecule in the cell wall matrix of Avena, Zea, and Oryza coleoptiles has not been defined. There is a strong correlation with growth as indicated by turnover during auxin induced growth. In addition, cell wall analysis of many grasses and cereals reveals that the (1→3),(1→4)-β-D-glucan content is high in immature tissues undergoing rapid growth but absent in mature tissues in which growth potential is lost (38). An exception to this general

observation is the large ammounts found in mature endosperm cell walls of many cereals (38). Luttenegger and Nevins clearly documented the transient nature of (1→3),(1→4)-β-D-glucan in Zea coleoptiles (23). They were able to show that the B-D-glucan content varied from near 0 to 10% of the total wall matrix. The high concentration of β-D-glucan was found to occur in walls just prior to and during the most rapid growth stage. As growth of the coleoptile declined and stopped there was a rapid solubilization of the molecule out of the wall matrix. These observations suggest a role of β-D-glucan turnover in cell wall extension.

The work described here was undertaken to characterize specific cell wall proteins in an attempt to elucidate their potential role in cell wall extension. Initial work focused on the characterization of a cell wall-protein fraction containing endoglucanase activity against model substrates such as Avena endosperm β-D-glucan. Antibodies raised against cell wall protein fractions indicated that certain proteins elicited antibodies that could inhibit auxin induced growth (12). Preliminary characterization of this fraction indicated that it contained endoglucanase activity. This suggested that the endoglucanase may be involved directly in the depolymerization of β-D-glucan during wall extension and that antibodies to it resulted in inhibited growth.

The major objective of this work was to characterize specific cell wall proteins and attempt to elucidate their metabolic role

in cell wall modifications during growth. Three subobjectives were identified:

1 Isolate and characterize the protein(s) responsible for eliciting antibodies that could inhibit auxin induced growth

2 Isolate and determine the properties of an endoglucanase that had been identified in previous work

3 Characterize the specific hydrolytic activity of the endoglucanse.

The following papers describe the characterization of specific cell wall proteins identifed in the above objectives.

PAPER I. PLANT CELL WALL PROTEINS: PRELIMINARY CHARACTERIZATION OF A FRACTION FROM CORN SEEDLINGS WITH A PUTATIVE ROLE IN AUXIN INDUCED-INDUCED GROWTH

ABSTRACT

Antibodies raised against cell wall proteins inhibit auxin-induced growth of Zea mays coleoptile segments. The inhibition of growth was attributed to a specific antibody-antigen interaction within the cell wall matrix. This conclusion was based on the observations that only serum containing antibodies against cell-wall proteins inhibited growth, that gamma globulins purified from active serum inhibited growth, and that a specific subfraction of isolated cell wall proteins could precipitate the growth inhibiting antibody. A bioassay was developed to evaluate cell wall-protein fractions for their ability to precipitate the growth inhibiting antibody. The total complement of proteins isolated from the cell walls of Zea mays seedlings was fractionated by cation exchange and gel filtration chromatography. The antigens responsible for the anti-growth antibodies were identified as an acidic group of proteins with apparent molecular weights in a range of 20-25,000. This subfraction of cell wall proteins was not effective in hydrolyzing cell wall polysaccharides.

INTRODUCTION

The cell wall of higher plants resists turgor induced expansion of the protoplast. Therefore, cell elongation is limited by expansion of the existing cell wall matrix (3,26). The relationship between auxin (IAA) induced cell elongation and cell wall metabolism has long been sought. Heyn demonstrated that IAA could increase the extensibility of coleoptile segment cell walls (9) suggesting that wall loosening events occur in response to applied IAA. This controlled wall loosening may involve the breaking of load bearing bonds within the wall matrix to allow molecular realignments and hence expansion under the driving force of turgor pressure (26).

Studies of cell wall matrices in relation to growth have indicated metabolic turnover of specific polysaccharides (5,14,15,17,21,28,30). Loescher and Nevins demonstrated a loss of glucose from the non-cellulosic portion of the walls of Avena coleoptile segments in response to IAA treatment (17). Similar observations have been made with rice coleoptile tissue (30). In both cases, the most predominant polysaccharide turnover appears to be restricted to the hemicellulosic glucan. In contrast, pea tissue walls exhibit a specific turnover of xyloglucan in response to applied auxin (14). Although the major polysaccharides involved in the two systems are different, both tissues share specific polysaccharide turnover associated with auxin-induced growth.

Cell wall hydrolases have been identified that increase in activity in response to the addition of auxin and may have a potential role in specific polysaccharide modifications during auxin induced cell elongation (9,18,27,28). However, the overall mechanism regulating auxin-induced wall loosening remains to be clarified. The use of inhibitors has provided additional information but, in many cases, because of the lack of inhibitor specificity the results are difficult to interpret (1,4,20,22). Huber and Nevins reported on a potentially specific inhibitor of cell elongation. They found that antibodies raised against wall proteins isolated from corn seedlings could inhibit auxin induced growth (11).

The work reported here was undertaken to characterize the inhibition of auxin-induced growth by antibodies. A bioassay procedure was developed for initial isolation and characterization of the protein(s) responsible for eliciting the anti-growth antibodies.

MATERIALS AND METHODS

Protein extraction and fractionation

Proteins were extracted from the shoot cell walls of 3 to 3.5 d old corn seedlings (Zea mays L. Mol7 X B73) with 3 M LiCl according to the procedure of Huber and Nevins (11). The procedure was modified slightly by continuous stirring of the extraction mixture for 48 h at 4 C. The suspension (250-300 ml) was filtered through Miracloth and the filtrate dialyzed against 4 L of citrate-phosphate buffer (10 mM citric acid, 426 ml/L + 20 mM Na_2HPO_4, 574 ml/L final pH 5.6) containing 200 mM NaCl at 4 C. The buffer was changed twice over a 48 h period. After dialysis, the protein preparation was centrifuged at 10,000 xg for 20 min to remove insoluble material and concentrated with an Amicon Ultrafiltration cell (PM-10 membrane) to a final volume of 20-25 ml. The concentrated mixture represented the total cell-wall crude-protein extract.

Initial fractionation of total cell wall protein was performed on a SP-Sephadex (SP-50-120) cation exchange column (2.3 X 30 cm). The concentrated crude protein was dialyzed against 20 mM acetate buffer (20 mM acetic acid + 5 M NaOH to pH 5.0) containing 20 mM NaCl, applied to the column equilibrated with the same buffer, and eluted at a flow rate of 8 ml h^{-1}. Unbound proteins were eluted from the column with 20 mM acetate buffer. Bound proteins were eluted with a linear gradient consisting of 20 to 500 mM NaCl in acetate buffer with a total elution volume of

400 ml. Three ml fractions were collected and assayed for protein (280 nm), exo-(1→3)-β-D-glucanase and endoglucanase activity. Selected, pooled fractions were tested by bioassay to determine if individual fractions were responsible for eliciting antibodies that inhibited auxin-induced growth. Active fractions were pooled, dialyzed against citrate-phosphate buffer (pH 5.6, 200 mM NaCl), and concentrated to about 2 ml. The active fraction was subjected to gel filtration on a Bio-Gel P-150 (100-200 mesh) column (1.2 X 80 cm). Fractions (0.5 ml) were assayed for protein (280 nm) and individual peaks were isolated, concentrated with an Amicon Ultrafiltration Cell (YM-5 membrane) and rechromatographed on the same column. The fractions comprising the majority (80%) of each protein peak after rechromatography were pooled and used in the antibody bioassay.

Antibody bioassay

A bioassay procedure was developed to test cell wall proteins for the ability to elicite anti-growth antibodies. Initially, antibodies were raised against the total crude cell wall extract and tested for the ability to inhibit auxin-induced growth. This complex complement of antibodies formed the base bioassay solution with which all subsequent antibody solutions were compared. The basic procedure for the bioassay is summarized in Figure 1.

All protein fractions and antibody solutions were dialyzed initially against Na-phosphate buffer (10 mM NaH_2PO_4 + 5 M NaOH

to pH 7.0) containing 1% NaCl. The bioassay consisted of adding 250 uL of antibody, cell wall protein (20-40 ug total protein), and buffer to give a total volume of 1 ml. The antibody-antigen complexes were incubated at room temperature for 30 min before transferring to 4 C for an additional 24-30 h. The tubes were then centrifuged for 10 min at 2000 xg (4 C). The supernatant was removed carefully from the pelleted antibody-antigen complex and transferred to dialysis tubing. After dialysis overnight against 5 mM citrate-KOH buffer (5 mM citric acid + 2 M KOH to pH 5.5) the unprecipitated-antibody solution was used as preincubation medium for coleoptile segments used in growth assays.

Coleoptiles used for growth experiments were prepared from corn seedlings grown under continous red light (0.1 uW m^{-1} integrated over the range 600-725 nm) for 72 h. Each coleoptile was abraded with silicon carbide (320 mesh), the primary leaves removed, and floated on a 5 mM citrate-KOH buffer until used. When sufficient coleoptiles had been harvested for a single experiment (1-1.5 h), 10 uniform coleoptiles were selected and single 10 mm segments were cut from each. The 10 segments were transferred to a 5 ml beaker containing the dialyzed antibody solution that comprized the individual antigen treatments. Beakers were placed on a waterbath shaker (24-25 C) and gently agitated for the 2 h preincubation period. The coleoptile segments then were removed, washed once with citrate-KOH buffer and 5mm sub-segments were cut from the middle portion of each.

Individual subsegments were quickly measured using a binocular microscope equipped with an ocular micrometer and placed in 5ml of citrate-KOH buffer (pH 5.5) containing 32 µM IAA. The individual treatment containers were gently agitated and maintained at a temperature of 24-25 C for the 6 h duration of the experiment. At 2 h intervals the segments were measured.

Three controls were used for each experiment. The antibody control consisted of coleoptiles pretreated with a solution containing the total complement of antibodies. A blank control consisted of coleoptile segments pretreated in citrate-KOH buffer without antibody and transferred to buffer without IAA. The IAA control segments were treated as the blank control except for being transferred to buffer containing 32 µM IAA. Active fractions or samples were defined as those antigens (cell wall proteins) that could precipitate the antibodies responsible for inhibiting IAA-induced growth. The supernatant, though containing other antibodies, would not inhibit growth.

Antibody preparation

Antibodies generated in response to total cell wall proteins were prepared as described by Huber and Nevins (11). New Zealand white rabbits were administered weekly intramuscular injections. The initial injection contained 20 µg of protein and the amount was increased each week by 20 µg to a total of 100 ug per injection. This concentration was maintained in subsequent injections. After 8 weeks, rabbits were bled via cardiac puncture

at two week intervals.

The blood from each rabbit (20-25 ml) was maintained at room temperature for 30 min then stored at 4 C overnight in 50 ml centrifuge tubes. The clot which formed was removed and the serum centrifuged at 750 xg for 20 min. Clarified serum was removed with a pasteur pipette and stored in 15 ml serum vials at -20 C. Serum was purified to the IgG fraction using a Protein A-Sepharose column (1 X 8 cm). Bound IgG proteins were eluted with 5% acetic acid containing 1% NaCl. The IgG component eluted as a single peak (280 nm). Fractions containing the highest concentration of protein were neutralized with 5 M NaOH and stored in capped serum vials at -20 C until used.

Hydrolytic activity against isolated Zea cell walls

Zea seedling cell walls (50-60 g) were isolated as described except that rather than being resuspended in LiCl the walls were resuspended in acetate buffer (pH 5.0). A sample (5-6 ml) of the suspension medium was removed and placed in a test tube to assess autolytic activity. The remaining cell walls were heated in a boiling waterbath for 1 h with stirring. Aliquots of the inactivated cell walls were distributed in test tubes and various protein samples were introduced to each (25 ug total protein). One tube contained inactivated cell walls with no added protein (blank control). All incubation mixtures were placed in a waterbath (30 C) for 48 h. After incubation the insoluble

portion was retained on a pre-weighed glass mat by filtration with the aid of suction. The wall residue on the glass filters was dried over P_2O_5 until a constant weight was obtained. The supernatant from each treatment was measured for total volume, total sugar content, neutral sugar composition, and molecular weight distribution of released carbohydrate.

Electrophoresis and isoelectric focusing

SDS-polyacrylamide gel electrophoresis was performed using the method of Delepelaire and Chua (6) with a 12.5% gel at pH 8.8. Protein blands were visualized by staining with Coomassie Brillant Blue R-250 (Bio-Rad). Mobilities of fractionated proteins were compared to low molecular weight-protein standards (SDS kit, Sigma).

Isoelectric focusing was preformed using an LKB 2117 Multiphor apparatus. Preformed polyacrylamide gels (LKB) were used which developed a pH range of 3 to 9. The pH gradient was determined after focusing with a flat surface pH electrode. Protein bands were visualized with Coomassie Brilliant Blue R-250.

General methods

Fractions containing cell wall proteins were assayed for exo-(1→3)-β-D-glucanase and endoglucanase activity. To assay for exo-(1→3)-β-D-glucanase activity 0.5 ml of laminarin (1 mg ml^{-1}) citrate-phosphate buffer (pH 5.6) was incubated with 0.1 ml of cell wall protein for 10 min. Activity was measured as an

increase in reducing equivalents using the method of Nelson (19) as modified by Somogyi (24). Activity of the endoglucanase in column fractions was determined by measuring the reducing equivalents produced from Avena β-D-glucan solution (1 mg ml^{-1}) in acetate buffer. The column fraction sample (0.1 ml) was incubated with 0.5 ml of the β-D-glucan solution for 24 h at 30 C in the presence of 100 uM Hg^{+2}. The Hg^{+2} inhibits the exo-(1→3)-β-D-glucanase but not the endoglucanase (10).

Total sugar assays were performed using the phenol-H_2SO_4 method (7). Neutral sugar analysis was carried out on 100-250 ug (as glucose equivalents) samples of carbohydrate. Samples were hydrolyzed with 2N TFA for 1 h at 121 C. For carbohydrate associated with protein, the samples were hydrolyzed with 1N TFA for 7 h at 100 C. After drying under a stream of filtered air, the residue was dissolved in distilled H_2O and passed through a column of Dowex H^+ (50W-X8 20-50 mesh) usually 2.5-3 cm in a pasteur pipette. Neutral sugars were eluted with distilled H_2O, converted to corresponding alditol acetates and analyzed by using GLC on a glass column (0.2 X 190 cm) packed with 3% SP-2340 (Supelco) at 210 C with a helium flow rate of 35 ml/min.

RESULTS AND DISCUSSION

Fractionation of crude cell wall protein

Fractionation of crude cell wall protein on SP-Sephadex resulted in a broad distribution of proteins (Fig. 2). Even numbered column fractions were assayed for exoglucanase and endoglucanase activity. The major exo-(1→3)-β-D-glucanase and endoglucanase activities were bound to the column but were eluted with a linear NaCl gradient of 20 mM to 500 mM. A small amount of exoglucanase activity eluted in the void fraction of this column. Samples from each of the pooled major fractions (Vo,A,B,C,& D) were subjected to the antibody bioassay procedure to determine which fractions were active (precipitated the growth inhibiting antibody). Only the void fraction (Vo) effectively precipitated the growth inhibition component indicating that the protein or proteins responsible for eliciting this antibody class were contained in this fraction.

The void fraction was further resolved on a Bio-Gel P-150 column into five peaks (Fig. 3). Even numbered column fractions were assayed for exo-(1→3)-β-D-glucanase and endoglucanase activity. No endoglucanase activity was identified but profile fractions 1,2, and 3 (Fig. 3) contained exo-(1→3)-β-D-glucanase activity. This exoglucanase activity probably represents a portion of the wall solubilized exo-(1→3)-β-D-glucanase that was not bound to the SP-Sephadex column in the initial fractionation step. The enzyme may have become associated with proteins that

neutralized its binding to the cation exchange column or perhaps slight modifications during the extraction procedure may have altered the net charge sufficiently to prevent binding. Preliminary characterization of the activity indicated properties similar with the bound exo-(1→3)-β-D-glucanase. Further characterization was not carried out in this investigation since the P-150 fractions 1,2 and 3 were not active in the antibody bioassay.

Each of the Bio-Gel P-150 fractions were pooled and rechromatographed on the same column to reduce cross contamination before testing with the antibody bioassay. As shown in Figure 4 only P-150 fraction #5 was found to be active. This fraction would appear to have contained the protein or proteins responsible for eliciting the anti-growth antibody. All other fractions resulted in growth responses that were essentially the same as the growth inhibiting antibody without added antigen. In addition, neutral serum taken from the rabbits before immunization with cell wall proteins resulted in no inhibition of IAA induced growth. These observations suggest that a specific antibody-antigen interaction occurs in Zea coleoptile cell walls which leads to the growth inhibition observed.

Preliminary characterization of the active cell wall protein

Molecular weight distribution Bioassays suggest that the active fraction (the isolated cell wall protein(s) responsible for

eliciting the antigrowth antibodies) is restricted to 20-25 kD. This estimate of the apparent molecular weight is based on the elution profile on a Bio-Gel P-150 column (Fig. 4). An estimate of the apparent molecular weight based on SDS PAGE places the protein(s) in a range of 20-30 kD. On an SDS gel (12.5 %) there was one major band that migrated at approximately 30 kD and 4-5 minor bands with slightly faster migrations.

Assessment of potential cell wall hydrolyase activity In an attempt to determine if P-150 #5 fraction contained cell wall hydrolase activity a sample was incubated with isolated, inactivated corn cell walls. As a comparison, aliquots from the other P-150 fractions (1,2,3 & 4) were added to isolated cell wall samples. The results of such experiments are summarized in Table I. Fractions 2,3, and 4 resulted in substantial release of carbohydrate from the cell wall matrix as compared to the blank control. Neutral sugar analysis revealed that 90 to 96% of the released carbohydrate for each treatment was glucose. The Bio-Gel P-2 elution profile of the carbohydrate released by each treatment indicated that the majority of the sugars present were monosaccharides. This would suggest that the release of carbohydrate by treatment with fractions 2 and 3 is due to exoglucanase activity identified in the Bio-Gel P-150 elution profile (Fig. 3). The carbohydrate released by fraction 4 may be due to some other glucanase, as no exo-(1→3)-β-D-glucanase activity was detected in this fraction.

The release of a small amount of carbohydrate by fraction #5 is most likely due to a non-specific solubilization of wall polymers as opposed to any specific hydrolytic activity. The total amount of carbohydrate released was only slightly higher than the boiled control (Table I) but less than that released from active cell walls. The elution profile on Bio-Gel P-2 indicated that the major portion of the released sugars was contained in higher molecular weight fragments (D.P.>10). A similar elution profile was observed for the boiled control.

This does not rule out the possibility that the antibody binds to a specific cell wall hydrolase in coleoptile segments leading to inhibition of IAA induced growth. Such an enzyme may have been partially denatured during isolation resulting in a loss of hydrolytic activity while antigenic recognition sites were not affected. One possibility is that the active protein(s) is not a hydrolyase but functions in a role as second messenger, regulatory molecule or as a structural component of the cell wall. The specific antigen-antibody binding of such molecules may be sufficient to disrupt the normal sequence of events leading to wall loosening.

Isoelectric points of the proteins in the active fraction

The active fraction (P-150 #5) contained one major protein and 4 to 5 minor proteins based on SDS-PAGE. The isoelectric points of the proteins contained in this fraction were determined by gel

electrophoresis to assess the potential diversity of proteins. As illustrated in Figure 5, this fraction contains several distinct proteins banding between pH 6 and 3. There is a prominent protein that bands at a pH of 3.5-3.7, however, it is not known if this is the major protein responsible for eliciting the anti-growth antibody. The complexity of this fraction may reflect a diversity of proteins and functions or a family of proteins with similar functions.

Characterization of the associated carbohydrate The active fraction (Bio-Gel P-150 #5) contained approximately 10-15 % carbohydrate. The possibility exists that the protein or proteins of interest are glycosylated which may have functions as wall structural components. Kauss and Glasser have suggested that cell wall extension may involve non-covalent bonds between polysaccharides and lectins (13). Alternatively the carbohydrate may be associated with this fraction but not covalently linked to protein. It is possible therefore that antibodies may be elicited against specific polysaccharides (2). Such antibodies may bind to the polysaccharide within the wall matrix inhibiting auxin-induced extension.

Initial analysis of this fraction indicated a composition primarily of arabinose, xylose and galactose. This fraction was passed through a DEAE-Sephadex column in an attempt to determine if the carbohydrate was bound to protein or merely associated with this fraction. Approximately 1/3 of the total carbohydrate

remained unbound and eluted in the void volume of the column (Fig. 6). The balance of carbohydrate bound to the column and was eluted when a NaCl gradient (0-500 mM) was applied. Relatively small amounts of the total protein loaded on the column co-eluted with the carbohydrate fractions.

Kato and Nevins studied a glucuronoarabinoxylan that was dissociated enzymatically from Zea seedling cell walls by purified endo-(1→4)-β-xylanase from Bacillus subtilis (12). A comparison of the neutral sugar composition of fraction F-2 with the fractions described by Kato and Nevins would suggest that it is predominantly glucuronoarabinoxylan. Although there appears to be a distinct population of proteins associated with F-2 as indicated by IEF gels (Fig. 7), it is not known if any of these are covalently linked to the polysaccharide components.

Fraction F-1 contains a neutral sugar composition of nearly equal proportions of arabinose and galactose (Table II). Examination of F-1 by IEF reveals a number of distinct protein bands. The neutral sugar composition suggests some similarities with the arabinogalactan glycoproteins (AGPs) (16,23). Such proteins are wide spread in plants, but to date, are poorly defined and specific roles have not been elucidated (14).

An alternative explanation of F-1 is the possibility of co-extraction and co-elution of polysaccharides with the cell wall proteins without covalent linkages between them. Based on the sugar composition the major polysaccharide could be an

arabinogalactan with other minor polysaccharides such as arabinoxylan and β-D-glucan associated with it. Arabinogalactans have been isolated from several grasses (29).

Preliminary analysis of DEAE-Sephadex subfractions (F-1, F-2, F-3, F-4) with the antibody bioassay indicated that all the fractions partially reverse the antibody mediated growth inhibition. This may indicate that there are several active proteins with similar functions, possibly as structural components of the cell wall matrix. Binding of the antibodies to specific cell wall components results in a restiction of their normal functional role leading to inhibited cell wall extension.

Summary

Evidence is presented for a specific antibody-antigen interaction in Zea coleoptile cell walls that results in inhibition of IAA induced growth. This conclusion is based on the following observations of coleoptile growth responses:

1) there is no growth inhibition upon pretreatment with neutral serum;
2) growth inhibition results from serum raised against total cell-wall complement of proteins or specific subfractions (SP-Sephadex void fraction);
3) growth inhibition occurs with whole active serum or active serum purified to the IgG complement;
4) growth inhibition can be removed by precipitation of active

antibodies with specific subfractions of cell wall proteins. The subfraction of isolated cell wall proteins that is responsible for eliciting growth inhibitory antibodies appears to belong to an acidic group of proteins (PI 3.5-6.0) with apparent molecular weight of 20-25 kD. There appears to be no hydrolytic activity associated with this subfraction at least after extraction from the cell wall matrix. The carbohydrate associated with this subfraction (10-15 %) would appear to be composed mainly of glucuronoarabinoxylan and possibly arabinogalactan.

LITERATURE CITED

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Table I. Total sugar released from isolated cell walls of Zea seedlings

Treatments consisted of the addition of cell wall protein from fractions resolved on a Bio-Gel P-150 column, BCW= boiled cell wall, CW= active cell wall. Numbers refer to the appropriate Bio-Gel P-150 protein fraction (see Fig. 4) added to inactivated cell walls (BCW). A total of 25 ug of protein was added to each sample and incubated 48 h at 30 C.

	Protein fractions added to isolated cell walls						
	BCW + #1	BCW + #2	BCW + #3	BCW + #4	BCW + #5	BCW	CW
total sugar released µg/mg[a]	13.9	38.0	41.5	32.0	14.7	11.3	68.2

[a] µg of sugar released as glucose equivalents per mg of total cell wall material.

Table II. Neutral sugar analysis of the major carbohydrate fractions resolved by DEAE-Sephadex chromatography

Neutral sugar analysis was used to determine if similarities exisited between the sugar composition of the carbohydrate fractions separated by DEAE-Sephadex chromatography and sugar composition of known glycoproteins.

	mol %[a]	
	DEAE-Sephadex Fraction	
Sugar	F-1	F-2
fucose	1.80	--
arabinose	31.04	32.56
xylose	14.85	47.96
mannose	5.54	--
galactose	29.08	15.10
glucose	17.67	4.38

[a]mol % of each sugar component based on the total carbohydrate composition.

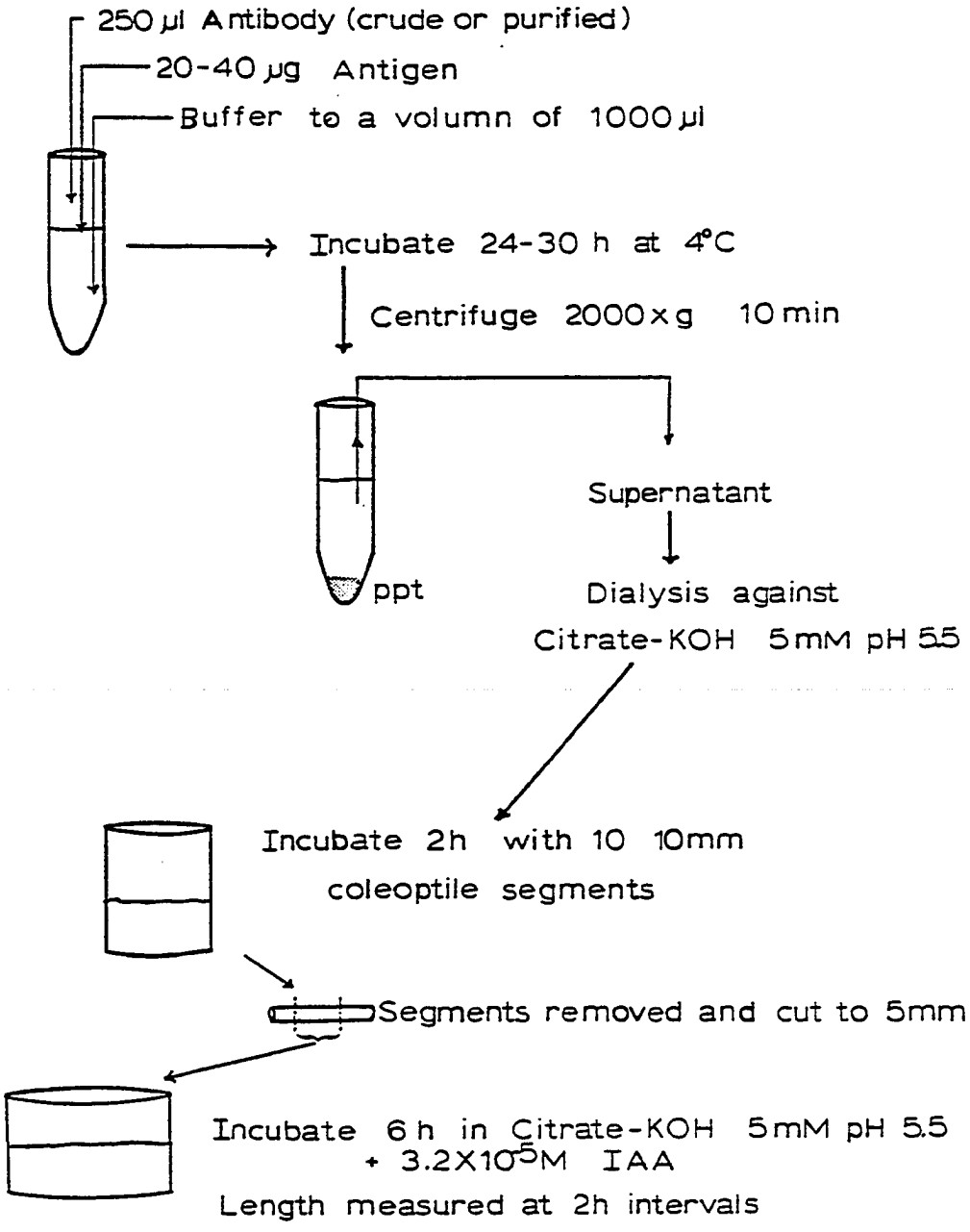
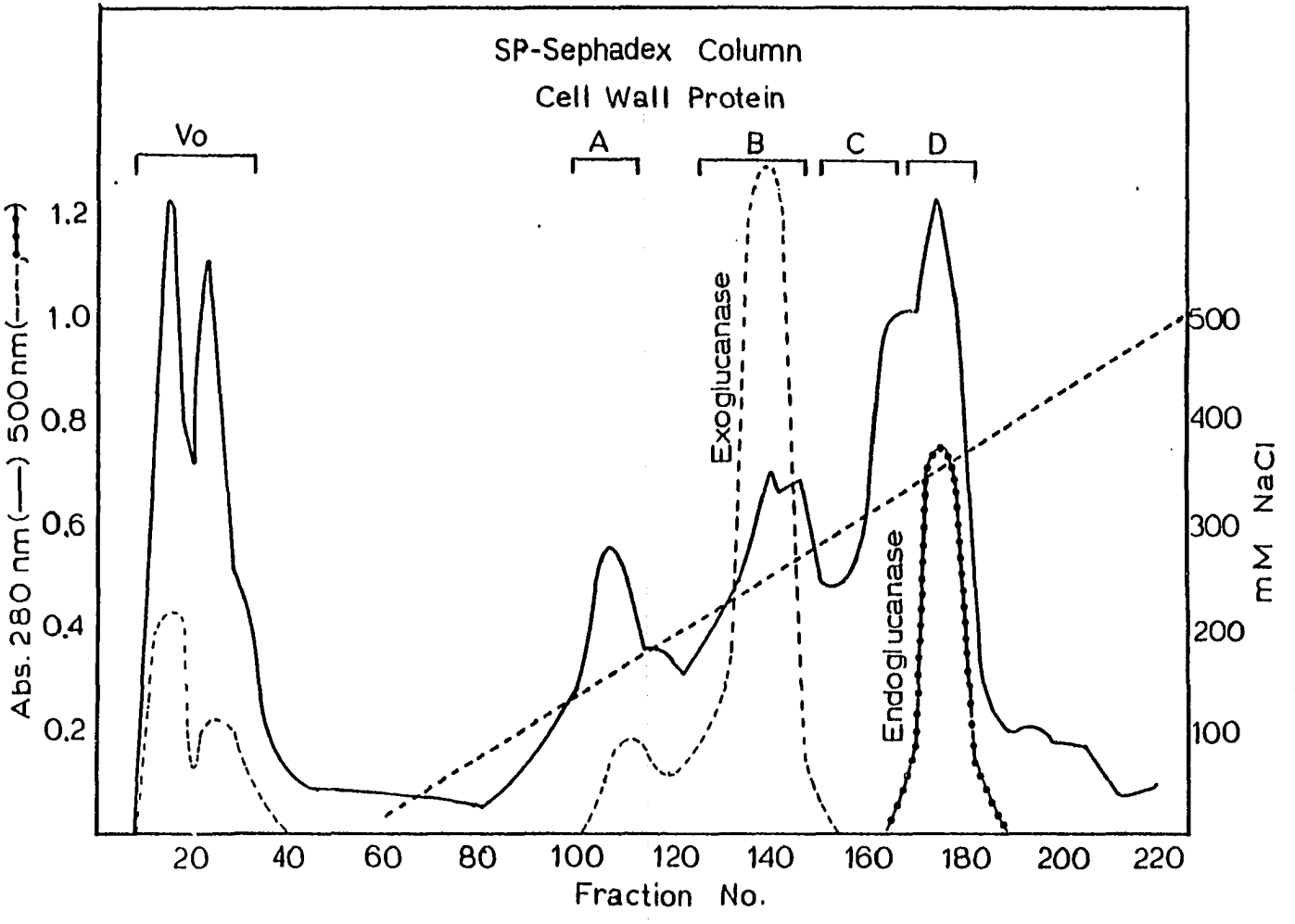


Fig. 1 Flow diagram summarizing the major steps of the antibody bioassay system

Fig. 2 Sp-Sephadex chromatography of total cell wall protein extract. The eluted proteins were pooled into five major fractions as indicated by brackets and letters. Each fraction was assayed with the antibody assay


SP-Sephadex Column
Cell Wall Protein
Vo
A
B
C
D
Exoglucanase
Endoglucanase
Abs. 280 nm(—) 500nm(----)
1.2
1.0
0.8
0.6
0.4
0.2
500
400
300
200
100
mM NaCl
20
40
60
80
100
120
140
160
180
200
220
Fraction No.

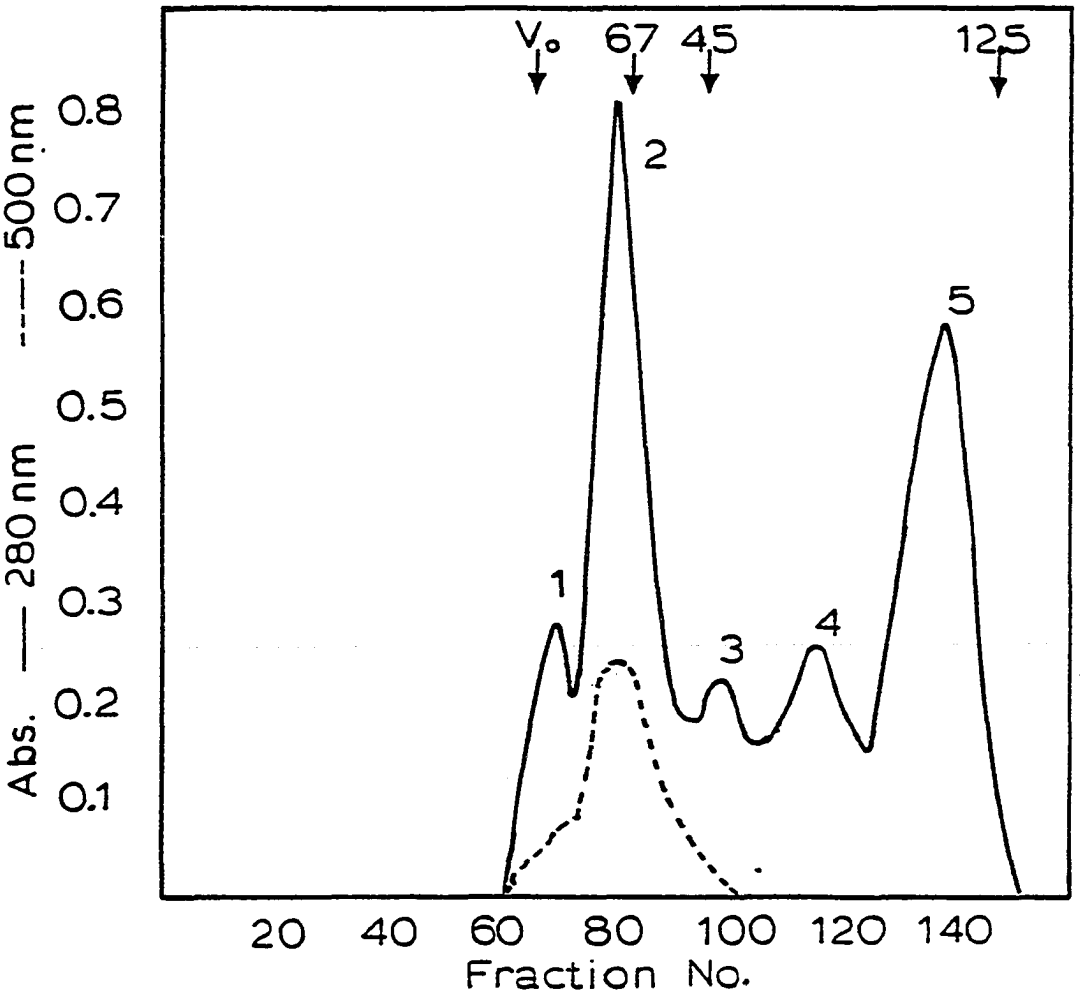


Fig. 3 Gel filtration pattern of Sp-Sephadex void fraction (Vo) on Bio-Gel P-150. Even numbered column fractions (0.5 ml) were assayed for total protein (280 nm), exo-(1--3)-β-glucanase (----) and endoglucanase activity. Arrows represent the elution position of molecular weight standards; bovine liver catalase (240 kD), bovine albumin (67 kD), egg albumin (45 kD), and cytochrome C (12.5 kD)

Fig. 4 Growth responses of corn coleoptiles treated with antiserum in the antibody bioassay. Individual treatments consisted of coleoptile segments treated with buffer (O—O -IAA), buffer + 32 µM IAA (O——O IAA), buffer + 32 µM IAA + total antiserum (O---O), and buffer + 32 µM IAA + antiserum treated with proteins fractionated on a Bio-Gel P-150 column. The number represents the P-150 pooled fractions (Fig. 3) added to the antiserum in the antibody bioassay procedure. All points represent the means of three separate experiments in which 10 individual segments were measured for each treatment. The standard error of the mean (SEM) was calculated for each of the individual points of a given treatment. The SEM listed here represents the maximum error of all the points. All other SEM values were smaller. (SEM $=\pm 0.04$)

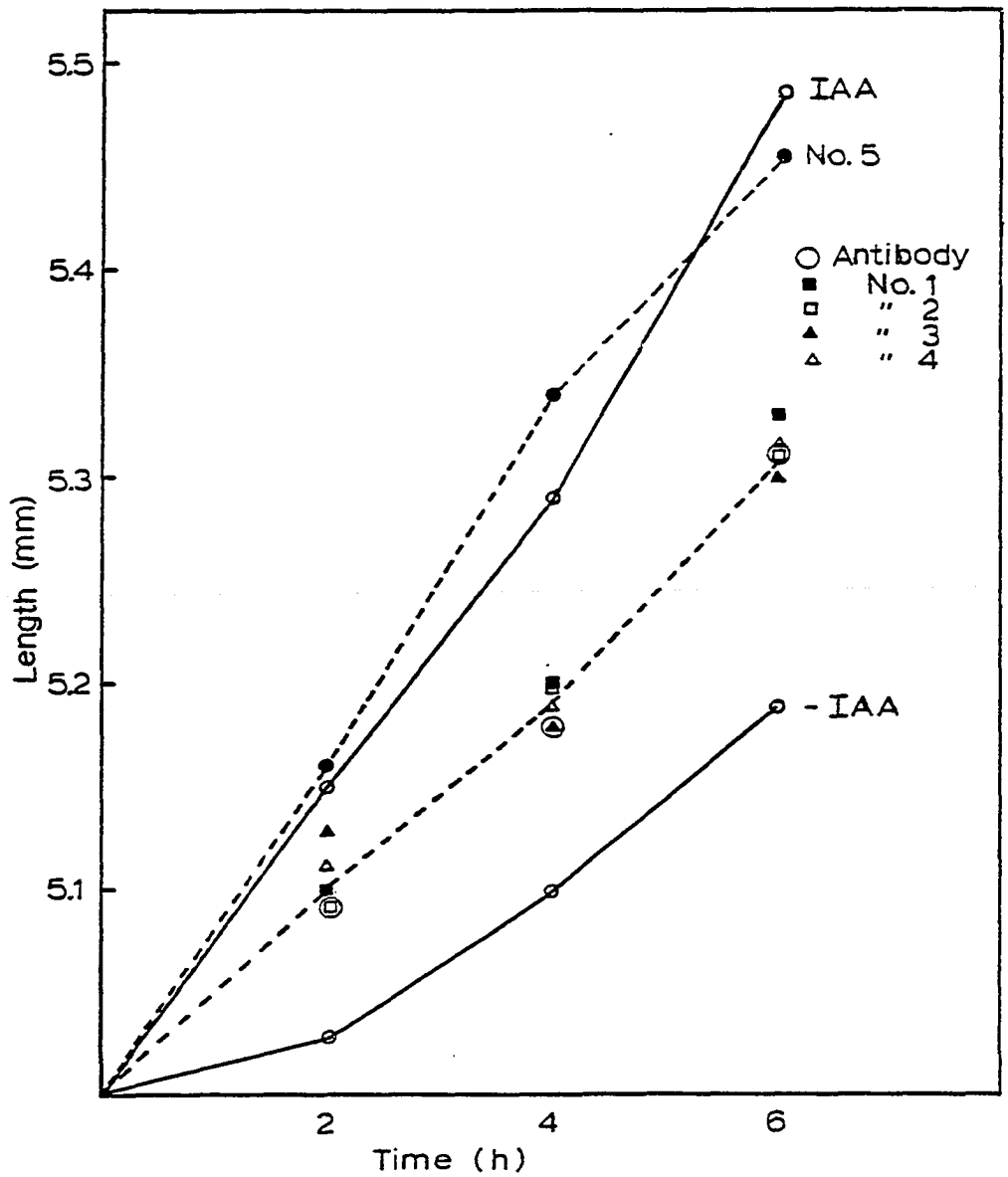

5.5
5.4
5.3
5.2
5.1
Length (mm)
2
4
6
Time (h)
IAA
No. 5
Antibody
No. 1
" 2
" 3
" 4
- IAA

Fig. 5 Isoelectric focusing gel electrophoretograms of cell wall proteins resolved on a Bio-Gel P-150 column. Proteins were visualized by Coomassie Brillant Blue 250-R staining. The pH gradient developed during focusing was noted at the left side of the gel. Numbers on the top of lanes represent the respective Bio-Gel P-150 fractions (see Fig. 3)

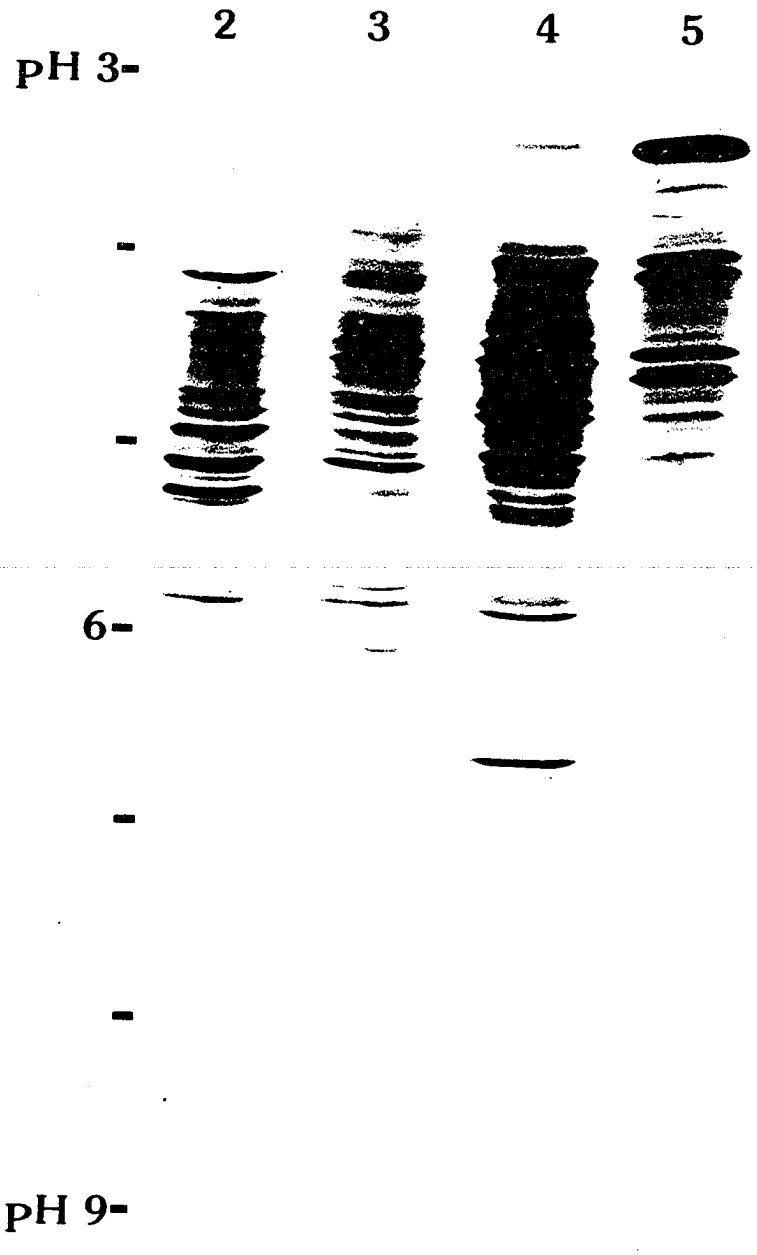

2
3
4
5
pH 3-
6-
pH 9-

Fig. 6 DEAE-Sephadex chromatography of active fraction #5 from the Bio-Gel P-150 column. The active fraction #5 was fractionated on a DEAE-Sephadex (A-50-120) column (1 X 25 cm) equilibrated with 20mM acetate buffer (pH 6.0). Two mg of total protein was loaded on the column at a flow rate of 8 ml h^{-1}. Bound proteins were eluted with a linear NaCl gradient (0-500mM) and collected in 1 ml fractions. Fractions (even numbered) were assayed for total protein (280 nm) and total sugars (490 nm)

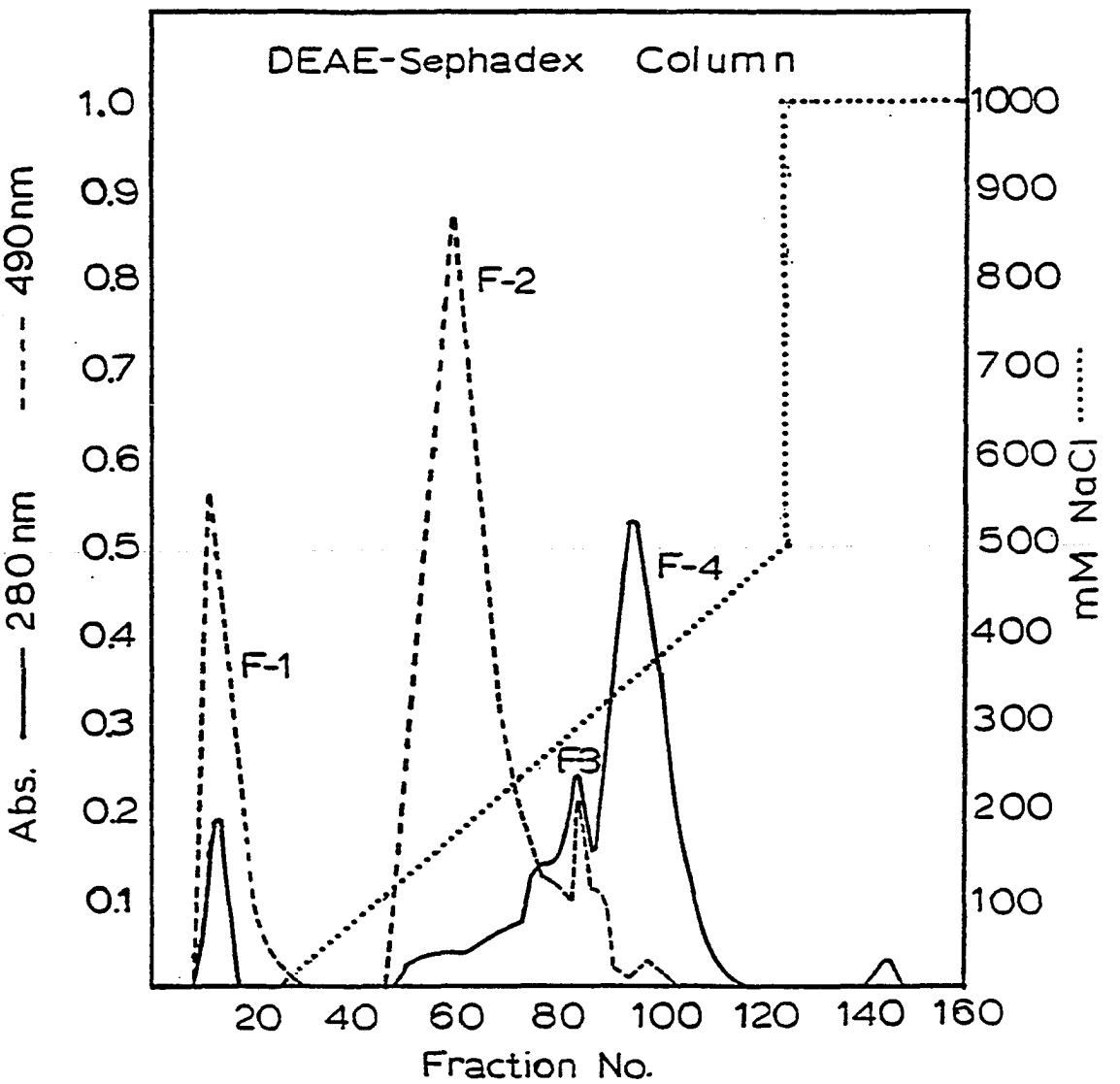

DEAE-Sephadex Column
Abs. —— 280nm ---- 490nm
mM NaCl
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1000
900
800
700
600
500
400
300
200
100
F-1
F-2
T3
F-4
20
40
60
80
100
120
140
160
Fraction No.

Fig. 7 Isoelectric focusing gels of fractions resolved by DEAE-Sephadex chromatography. Protein bands were visualized by Coomassie Brillant Blue 250-R staining. The pH gradient developed during focusing was noted at the left side of the gel. Numbers at the top of lanes represent the respective DEAE-Sephadex protein-carbohydrate fractions (see Fig. 6)


F-1
F-2
F-3
F-4
pH 3
6
pH 9

PAPER II. PURIFICATION AND PROPERTIES OF AN ENDOGLUCANASE ISOLATED FROM THE CELL WALLS OF *ZEA MAYS* SEEDLINGS

ABSTRACT

An endoglucanase has been liberated from Zea mays seedling cell walls by using LiCl. Purification of the enzyme by using SP-Sephadex, CM-Sephadex, and gel filtration resulted in a 98 fold increase in specific activity. The endoglucanase has a pH optimum of 4.5-5.0 and is heat stable up to temperatures of 40-45 C. Compounds that interact with sulfhydryl groups did not inhibit the activity of the enzyme nor did EDTA. This would suggest that sulfhydryl groups are not directly involved at the active site. It would also appear that the endoglucanase does not have a requirement for metal ions. The endoglucanase has an apparent molecular weight of 20-25,000 and an isoelectric point of $\geq$9. Hydrolytic activity against (1→3),(1→4)-β-D-glucans is restricted to isolated sites resulting in the release of high molecular weight products (10-15,000). Activity against isolated, inactivated corn cell walls resulted in the release of approximately the same products as was 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 found as cell wall components of many grasses (18). The role of this polysaccharide in the primary plant cell wall is not understood though certain evidence would suggest a structural role (4). The β-D-glucan content of coleoptile cell walls of corn generally ranges from 1% to 14% depending on the developmental stage of the coleoptile (10). However, this polysaccharide can constitute up to 19% of the endosperm cell walls of barley grains (18) and as high as 49% of the walls in cultured Lolium endosperm cells (16).

Few enzymes from higher plants have been identified that specifically hydrolyze the β-D-glucan molecule. Certainly the best characterized system involves the depolymerization of β-D-glucan during the germination and the malting process of brley grains (1,11,12,19). From germinating barley grains, three types of endohydrolases have been identified that will hydrolyze this molecule. These include (1→4)-β-D-glucan 4-glucanohydrolase (cellulase EC 3.2.1.4) (11), (1→3),(1→4)-β-D-glucan 4-glucanohydrolase (lichenase or endo-barley-β-D-glucanase EC 3.2.1.73) (1) and (1→3)-β-D-glucan glucanohydrolase (endo 1,3-glucanase EC 3.2.1.39) (1). Of these three, only the endo-barley-β-D-glucanase was determined to play a major role due to its increase and abundance during germination (1).

Two isozymes of the endo-barley-β-D-glucanase were detected,

isolated, and characterized by Woodward and Fincher (19) in germinating barley seeds. The hydrolytic activity of the two enzymes were very similar producing the same type of products, 3-O-β-cellobiosyl-D-glucose and 3-O-β-cellotriosyl-D-glucose. They were able to detect differences in the rates of reaction, isoelectric points, molecular weights, and slight differences in heat stability.

Recently, Huber and Nevins identified an endoglucanase from corn cell wall extracts that would hydrolyze mixed linked (1→3), (1→4)-β-D-glucans (8). They postulated that this enzyme may operate in conjunction with an exoglucanse also found in the same cell wall extract. This cooperative hydrolytic activity appears to account for the autohydrolytic release of glucose from corn cell walls (7). The work described here was undertaken to purify and characterize the properties of the endoglucanase from corn cell wall extracts.

EXPERIMENTAL METHODS

Enzyme

The endoglucanase was extracted from Zea mays (B73 X Mo17) seedling cell walls using the procedure of Huber and Nevins (8) with modifications. Routinely 500-600 g of corn seedlings were homogenized in 1-1.5L of 50 mM NaCl (4 C) containing 200-300g of ice (distilled H_2O). The homogenate was filtered through miracloth with aid of suction and washed extensively with a sequence of 50 mM NaCl (1-2L, 4 C), acetone (4L, -20 C), and 50 mM NaCl (1-2L, 4 C). The isolated cell walls were suspended in 300-400mL of 3M LiCl and stirred slowly for 48h at 4 C. After which the mixture was filtered through miracloth to obtain the LiCl extracted proteins.

Substrates

Hordeum β-D-glucan was obtained from Calbiochem, Avena β-D-glucan from Quaker Oats, and lichenan (Cetraria islandica) from Sigma.

Enzyme purification

Chromatography on Sp-Sephadex A column (2.3 X 30 cm) of Sp-Sephadex (SP-C50-120) was equilibrated with 20 mM acetate buffer (pH 5.0) containing 20 mM NaCl. The dialyzed concentrated crude cell wall extract (15-20 mL) was applied to the column at a flow rate of 8 mL h^{-1}. The endoglucanase remained bound to the column and was eluted with a NaCl gradient (20 mM to 500 mM). The

single peak of activity eluted between the NaCl concentrations of 350 and 400 mM. Activity of column fractions was determined by measuring the rate of change in viscosity of a 1% Avena β-D-glucan solution in 20 mM acetate buffer pH 5.0. A fraction aliquot (50 µL) was added to 350 µL of oat 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= $1/T\ min^{-1}$). Therefore units of activity are defined in terms of slope of the plotted line.

An alternative method was to incubate an aliquot (100 µL) of selected column fractions with 400 µL of an Avena β-D-glucan solution (1mg mL^{-1} in acetate buffer, pH 5.0) in which Hg^{+2} was added to a final concentration of 100 µM. The addition of Hg^{+2} effectively inhibited the exoglucanase enzyme which 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 using laminarin as a substrate. A 100 µL aliquot of selected fractions was added to 400 µL of a laminarin solution (1 mg mL^{-1}) in acetate buffer (pH 5.0). After incubation for 10 minutes at 30 C the reaction was stopped by adding 500 µL of the

copper reagent of the reducing sugar assay. Relative activity was determined by measuring the absorbance at 500nm.

Chromatography on CM-Sephadex A column (1.5 X 60 cm) of CM-Sephadex (C-50-120) was equilibrated with 20 mM acetate buffer (pH 5.6 containing 20 mM 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 to 450 mM) 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.

Chromatography on Bio-Gel P-150 A column (1.2 X 80 cm) of Bio-gel P-150 mesh 100-200, was equilibrated with citrate phosphate buffer (5.6 pH) containing 200 mM NaCl. Tne pooled endoglucanase fractions from the CM-Sephadex column was 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^{-1}. Fractions of 0.5 mL were collected. The active fractions were pooled and rechromatographed on the same column under the same conditions.

Electrophoresis and isoelectric focusing SDS-polyacrylamide gel electrophoresis (5) was performed by using a

12.5% gel at pH 8.8. Protein bands were visualized by staining with Coomassie Brillant Blue R-250 (Bio-Rad). Mobility of the endoglucanase was compared to low molecular weight protein standards (SDS kit Sigma).

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

Enzyme properties

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 as compared to the profile of protein standards. This molecular weight was compared to the value obtained from mobility in an SDS-PAGE 12.5% gel. Estimation of molecular weight was by comparison to the mobility of molecular standards (SDS Kit Sigma). The isoelectric point was determined by using isoelectric focusing in a wide range gel pH 3 to 9.

pH optimum The endoglucanase activity was determined using Hordeum β-D-glucan (2mg mL^{-1}) as a 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 a 3 h incubation (30 C) with 2.5 µg of enzyme.

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 β-D-glucan (2mg mL^{-1}). The β-D-glucan was dissolved in 20 mM acetate buffer (20 mM NaCl, pH 5.0). Endoglucanase was added to each temperature trial at a concentration of 2.5 µg mL^{-1}.

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 minute intervals over a 1 h preincubation period. Enzyme activity was assayed as described in the optimum temperature section.

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

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^{-1}. The rate of the reaction was monitored by removing a small aliquot (200 µL) of the total reaction mixture at various time periods (0, 0.5,1,2,and 4 h) and assayed for the presence of increased reducing sugars. Enzyme was

added to the initial mixture to give a final concentration of 5 ug mL^{-1}. The K_m and V_{max} values for each substrate was determined from Lineweaver-Burke plots.

Hydrolytic activity

Endoglucanase (2.5 µg mL^{-1}) was incubated with various mixed linked (1--3),(1--4)-β-D-glucan substrates (2 mg mL^{-1}) for 12-18 h. After incubation, the reaction mixtures were heated for 10 minutes in boiling water to inactivate the enzyme. The mixture was applied to a Bio-Gel A1.5M column (1.5 X 60 cm) equilibrated with 20 mM acetate buffer (pH 5.0) containing 20 mM 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^{-1} 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 corn β-D-glucan, corn 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 boiling. The inactivated walls were resuspended in 20 mM acetate buffer (pH 5.0) and divided into roughly equal aliquots. 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^{-1} Boiled walls without enzyme were used as a control. Samples

were incubated 48 h, filtered, and the supernatant 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.

General methods

All reducing sugar assays were performed using the method of Nelson (14) as modified by Somogyi (17). Total sugar assays were determined by the phenol-H_2SO_4 method (6). Protein contained in column effluent fractions was monitored at 280 nm. Total protein in pooled fractions was estimated using the Bio-Rad Protein assay with bovine serum albumin (Sigma) as a standard.

RESULTS AND DISCUSSION

Purification of the endoglucanase

Cell wall proteins were extracted from Zea mays seedling walls by using the procedure of Huber and Nevins (8) with slight modifications as described in the experimental methods. The resulting protein extract was dialyzed against citrate-phosphate buffer (20 mM, pH 5.6) containing 200 mM NaCl to remove the LiCl. Insoluble material precipitated during dialysis was removed by centrifugation at 10,000 xg and the supernatant concentrated using an Amicon Ultrafiltration Cell Model 402 (membrane Diaflo PM-10). After concentration to 30 ml, the protein sample was dialyzed against acetate buffer (20 mM, pH 5.0) containing 20 mM NaCl. A second centrifugation was used if further precipitates formed.

The crude protein mixture was initially fractionated on an SP-Sephadex cation exchange column equilibrated with acetate buffer (20 mM, pH 5.0) containing 20 mM NaCl. Bound proteins were eluted with a NaCl gradient from 20 mM to 500 mM in the same acetate buffer. This resulted 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 which also hydrolyzes the β-D-glucan molecule was separated clearly from the endoglucanase.

Column fractions containing endoglucanase activity were pooled, concentrated, and dialyzed against acetate buffer (20 mM pH 5.6) containing 20 mM NaCl. The concentrated endo-fraction was

loaded on a CM-Sephadex cation exchange column. Elution of the bound proteins occurred by imposing a gradient form 325 mM to 450 mM NaCl (Fig 2). This resulted in a broad peak of endoglucanase activity, with the main activity eluting at 380 to 410 mM 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 200 mM NaCl. After dialysis, the endoglucanase fraction was further concentrated to 1.5-2.0 mL and loaded on 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 approximately 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.

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 electrophoreis of protein samples from individual steps of this purification scheme was used to monitor and assess the effectiveness of each step. There was a specific enrichment of a single band corresponding to an apparent molecular weight of 30,000. The molecular weight estimate by PAGE was slightly higher than by gel filtration possibly due to

conformational changes caused by the SDS. The purified endoglucanase was subjected also to IEF electrophoreosis on a wide range gel (pH 3-9) to determine the isoelectric point. Best estimates of the PI would be pH 9 or higher since the protein banded at or very near the high pH side of the gel.

Endoglucanase properties

The endoglucanase was stable for over 12 months when stored at -20 C in either acetate buffer (20 mM pH 5.0, 20 mM NaCl) or citrate-phosphate buffer (pH 5.6, 200 mM NaCl). However, it was found that activity slowly decreased (<15%) with repeated freeze-thaw cycles. Therefore individual aliquots of the enzyme were stored for use in subsequent assays.

Optimum temperature

The activity of the endoglucanase increased in a linear fashion in the temperature range of 25 to 40 C (Fig. 4). It would appear that the enzyme has a wide optimum temperature range from 45 to 60 C. The wide optimum temperature range may be accounted for by the thermal stability of this enzyme (Fig. 5). From these results, the endoglucanase is relatively stable to increasing temperature up to 45 C. At high temperatures (> 55 C), there is a sharp decrease in activity with increased incubation time. Approximately 40% of the activity is lost after 1h of preincubation at a temperature of 60 C. The broad optimum therefore may be due to a kinetic increase in activity from higher

temperatures coupled with a net decline of activity as the enzyme is exposed to the higher temperature for increasing lengths of time. In some experiments in which the increase in reducing equivalents from endoglucanase activity, was measured every 0.5 h as a function of temperature, the higher temperatures of 50-60 C resulted in sharp increases during the first 0.5 h of incubation. After this initial surge of activity, the rate of increase in reducing equivalents decreased 2-3 fold (data not shown). This would support the thermal stability data suggesting that the enzyme is not stable for prolonged periods at increased temperatures (50-60 C). At temperatures above 65 C virtually all of the catalytic capacity is lost.

Optimum pH

The endoglucanase exhibits a sharp pH optimum at 4.5-5.0, with activity dropping rapidly at higher and lower pH values (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 to the plant hormone auxin (indole-3-acetic acid, IAA), which induces elongation of this tissue (3). Such acidification may alter the pH of the wall to values of 4.5 to 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 corn β-D-glucan.

Effects of inhibitors and Ca^{+2} Ions

Table II summarizes the results of various inhibitors and Ca^{+2} ions. The data from the EDTA treatments indicate that this enzyme in general does not require divalent metal ions for activity. Also Ca^{+2} ions did not stimulate activity of the endoglucanase. Manners and Marshall (11) found that Ca^{+2} stimulated the hydrolytic activity of the (1→3)-β-D-endoglucanase of malted barley but did not effect the (1→4)-β-D-endoglucanase. Work by Woodward and Fincher showed that the presence of EDTA or the addition of metal ions had no effect on the endo-barley-β-D-glucanase (19).

The corn endoglucanase is not inhibited by Hg^{+2} or PCMB. This would indicate that the endoglucanase does not employ sulfide bonds directly at the active site. This observation was reported ealier by Huber and Nevins (8) working with partially purified fractions. They had observed that Hg^{+2} would effectively inhibit the wall bound exoglucanase but would not interfere with the autohydrolytic release of carbohydrate from active isolated walls. They concluded that the endoglucanase must be present in the walls and work in conjunction with the exoglucanase to hydrolyze the β-D-glucan molecule during autohydrolysis.

Inhibitors such as nojirimycin and gluco-lactone did not inhibit the activity of the endoglucanase (data not shown). This would be expected if the enzyme has a true endo-hydolytic pattern of activity. These inhibitors appear to be specific to

the inhibition of glucosidases and exoglucanase enzymes (15).

Enzyme kinetics

The K_m and V_{max} were determined at pH 5.0 and 30 C using three related substrates. Substrates representing (1→3),(1→4)-β-D-glucans 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 one cannot say that the enzyme has a strong affinity for the substrates it would appear that the endoglucanase is saturated at a relative low concentration. The K_m and V_{max} may indicate something about the potential of the endoglucanase to hydrolyze mixed linked substrates but does not reflect necessarily the in vivo activity of the enzyme. In vivo the wall matrix may restrict the assessability of the enzyme to the hydrolytic sites on the substrate.

Hydrolytic activity

The hydrolytic activity against model substrates such 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 result in similar product profiles. However, it would appear that lichenan from Cetraria islandica does not have the same molecular weight distribution as Hordeum and Avena β-D-glucan prior to hydrolysis

by the endoglucanase. These smaller molecular weight substrates are hydrolyzed to the same uniform 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 corn endoglucanase is added to isolated corn cell walls that have been boiled to inactivate wall bound enzymes similar molecular weight products 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 3-O-β-cellobiosyl-D-glucose and 3-O-β-cellotriosyl-D-glucose (Fig. 8). The uniformity of the products from all tested substrates suggest that the corn endoglucanase is hydrolyzing unique sites that exist within the mixed linked glucans.

Bathgate and Palmer (2) observed that a bacterial (1→3)-β-D-endoglucanase and a (1→3)-β-D-endoglucanase from barley hydrolzed the β-D-glucan in a similar manner. The molecular weight of the products released from the bacterial enzyme action was approximately 10,000. They claimed to have identified regions within the barley β-D-glucan molecule that were composed of more than one adjacent (1→3)-β linkage. However, more recent work by Woodward et al. (20) indicated that barley β-D-glucan contained regions rich in adjacent (1→4)-β linkages (5-11) but limited or no regions of adjacent (1→3)-β linkages. Kato and Nevins (9) determined that the corn β-D-glucan molecule contained regions

rich in (1→4)-β and (1→3)-β linkages. Similar regions appear to be present in the β-D-glucan extracted from oat bran (data not shown). It would seem likely that the corn endoglucanase recognizes these enriched regions and hydrolyzes an associated linkage. The relative abundance of these regions would support this assumption, resulting in the release of products of 10-15,000 molecular weight. One cannot rule out the possiblity that the enzyme recognizes regions rich in (1→4)-β linkages but hydrolyzes either a (1→4)-β or a (1→3)-β linkage. The Zea endoglucanase differs from the endo-barley-β-D-glucanase which releases tri- and tetrasaccharides as limit products.

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Table I. Purification of Zea cell wall endoglucanase

Step in Purification process	Specific Activity[a] Units/mg	Purification
Crude protein[b]	3.3×10^{-4}	1.0
SP-Sephadex	8.2×10^{-4}	2.5
CM-Sephadex	3.7×10^{-3}	11.2
Bio-Gel P-150	3.2×10^{-2}	98.5

[a]Specific activity is defined as change in units of activity as mesured by viscosity per mg of protein. See Experimental Methods, enzyme purification (Pp. 49) for an explanation of units of activity.

[b]LiCl extract of cell walls.

Table II. Effect of inhibitors and Ca^{+2} ions on Zea endoglucanase activity

Treatment	Concentration	% of relative activity
EDTA[a]	10 mM	100.0
EDTA	100 mM	100.0
Ca^{+2}	1 mM	104.0
Ca^{+2}	10 mM	107.0
Ca^{+2}	50 mM	100.0
Hg^{+2}	50 µM	100.0
Hg^{+2}	100 µM	100.0
PCMB[b]	50 µM	98.5
PCMB	100 µM	86.0

[a]Ethylenediaminetetra-acetic acid (disodium salt).

[b]p-(hydroxymercuri)-benzoic acid.

Table III. Kinetic properties of the *Zea* endoglucanase

Substrate	K_m (mg/mL)	V_{max} (µg/h/µg)[a]
Avena β-D-glucan	0.333	2.75
Hordeum β-D-glucan	0.326	1.68
lichenan	0.339	1.51

[a] µg of reducing equivalents produced from each substrate per hour of incubation per µg of endoglucanase added.

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

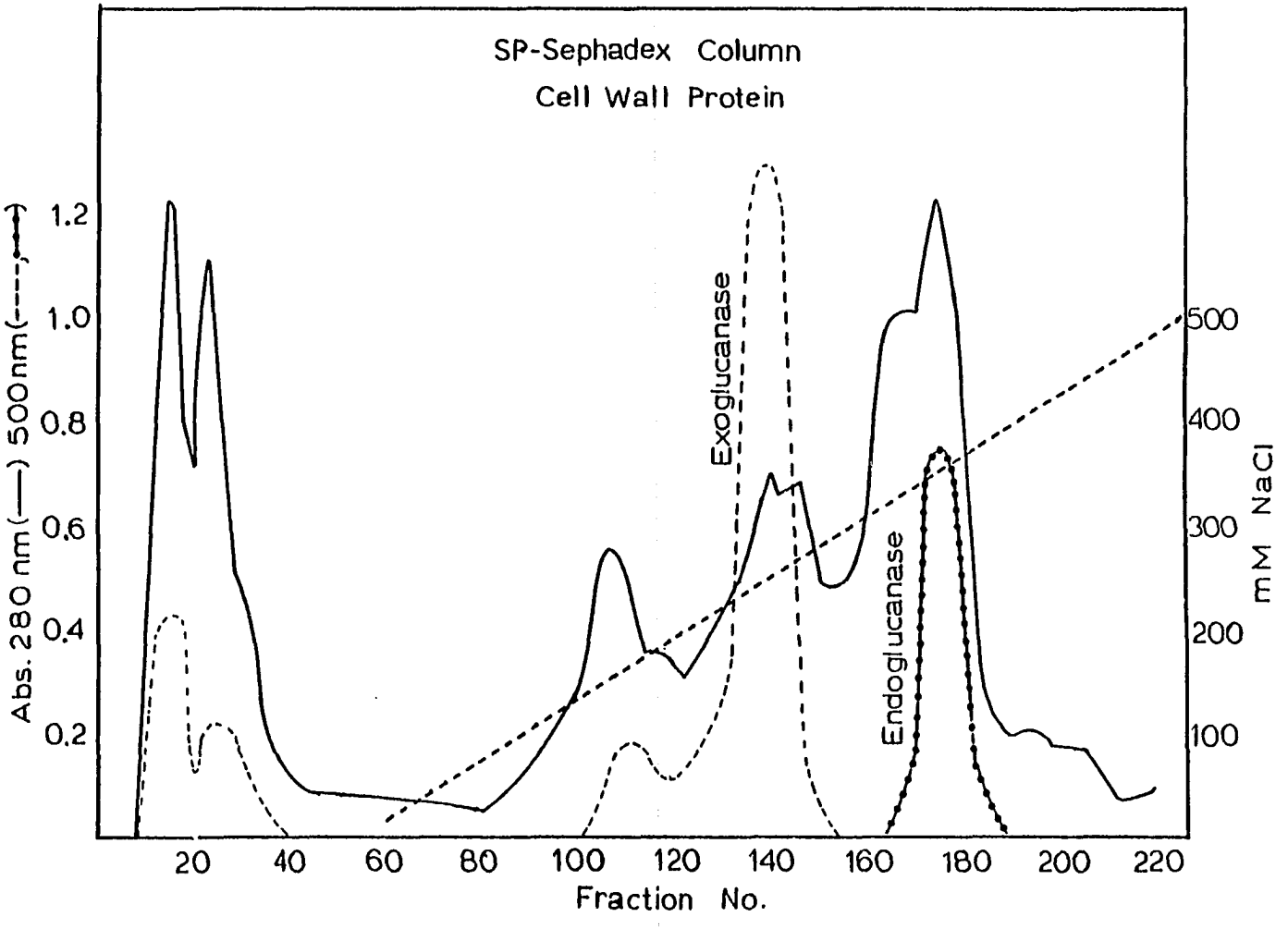
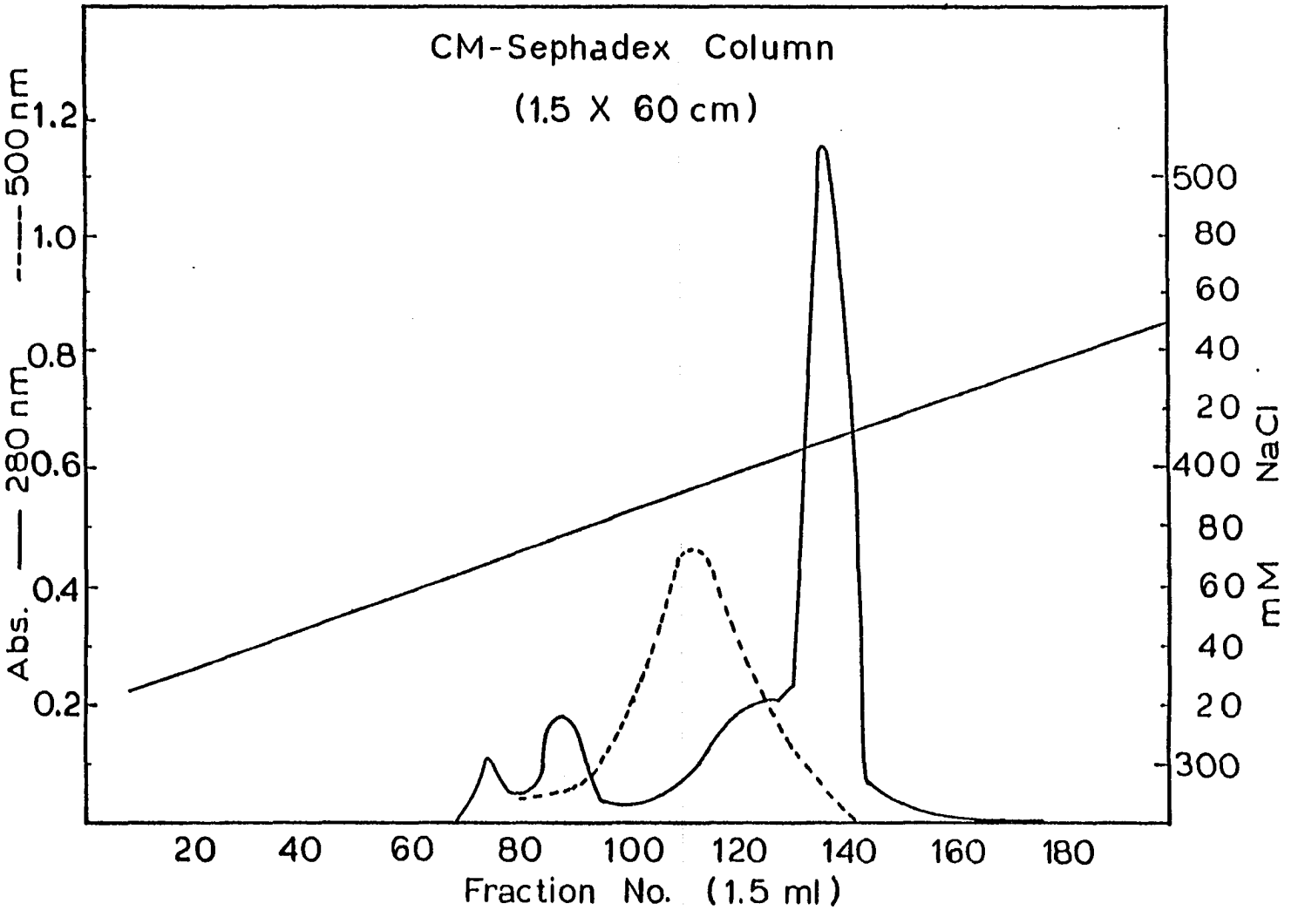

SP-Sephadex Column
Cell Wall Protein
Abs. 280 nm (——) 500nm (- - - -)
1.2
1.0
0.8
0.6
0.4
0.2
Exoglucanase
Endoglucanase
500
400
300
200
100
mM NaCl
20
40
60
80
100
120
140
160
180
200
220
Fraction No.

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


CM-Sephadex Column
(1.5 X 60 cm)
Abs. — 280 nm ---- 500 nm
1.2
1.0
0.8
0.6
0.4
0.2
500
80
60
40
20
400
80
60
40
20
300
NaCl
mM
20
40
60
80
100
120
140
160
180
Fraction No. (1.5 ml)

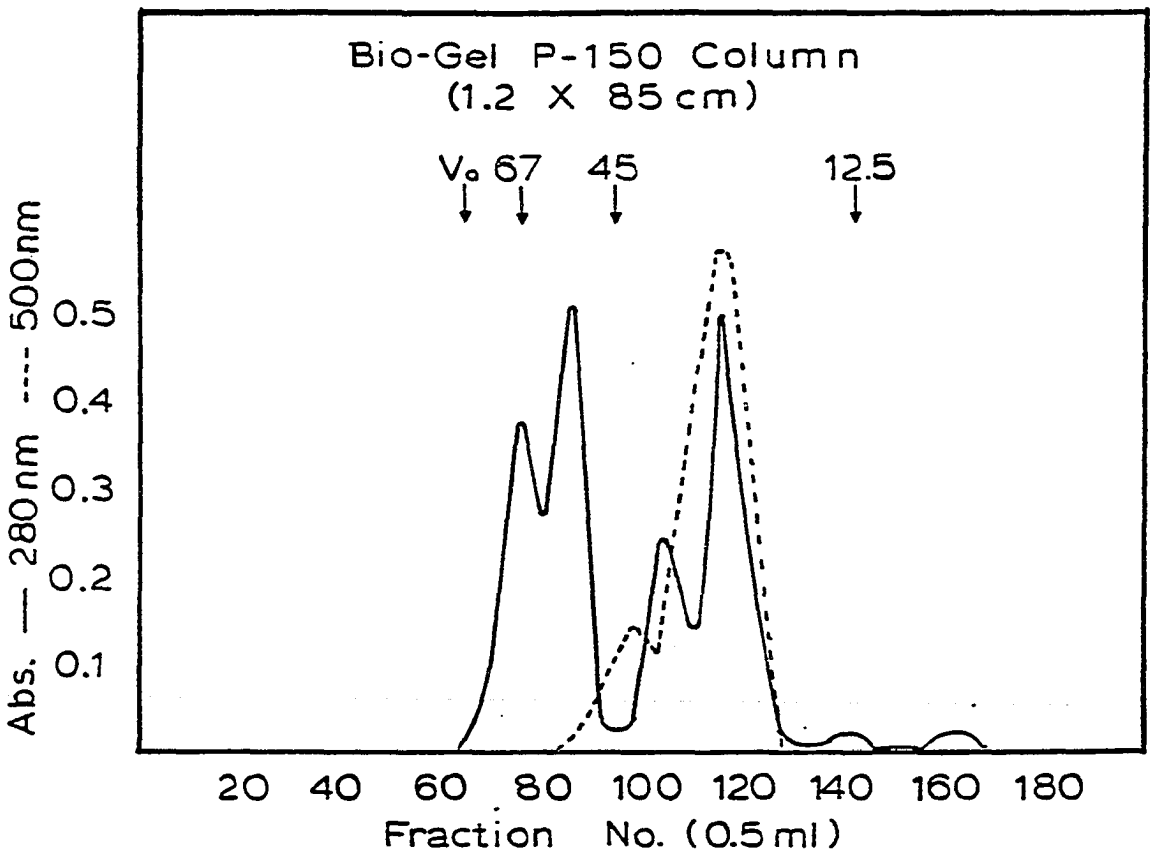


Fig. 3 Gel filtration of endoglucanase on Bio-Gel P-150 (see experimental methods. Fractions (0.5 mL) were assayed for protein at 280 nm (——) and endoglucanse activity (----). Arrows represent the elution profile of molecular weight marker proteins which consisted of bovine liver catalase 240,000 mol.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

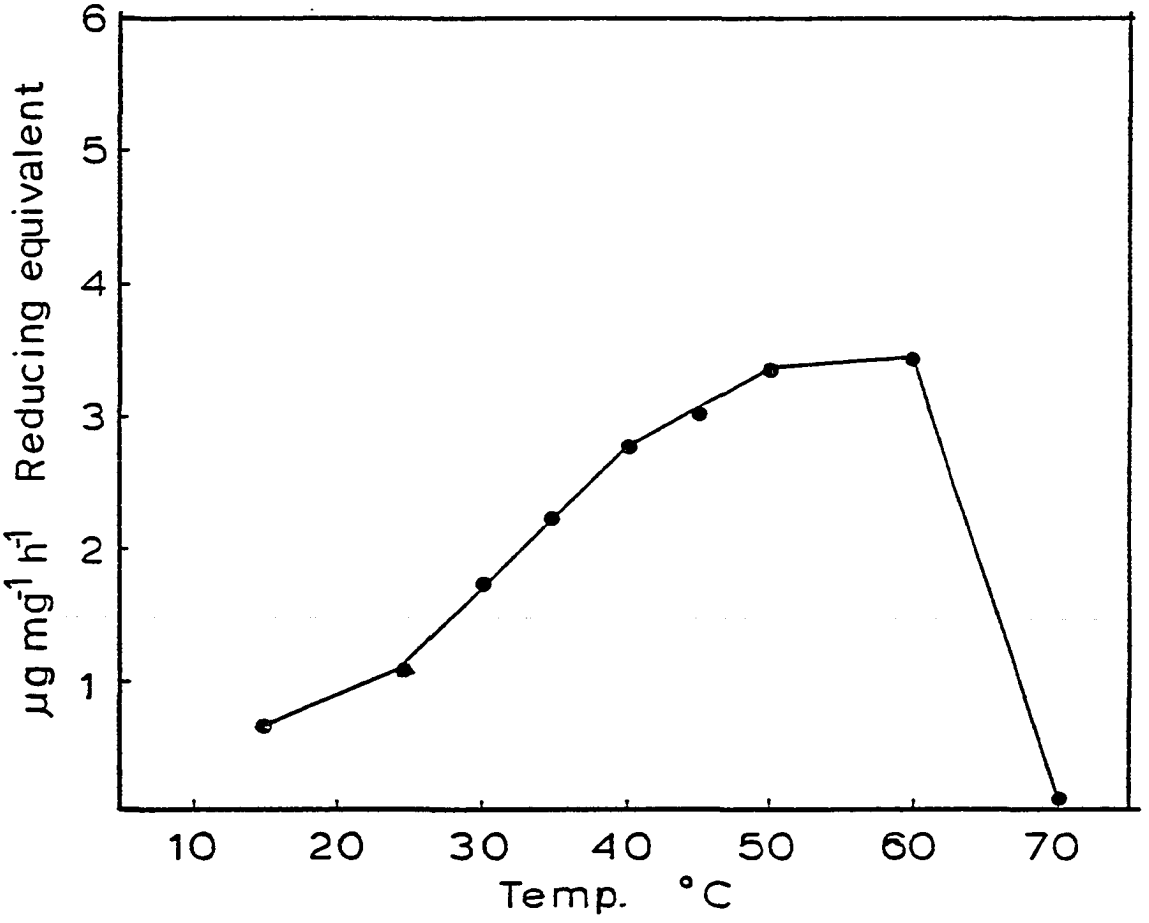


Fig. 4 Effect of reaction temperature upon the activity of the endoglucanase (see experimental methods)

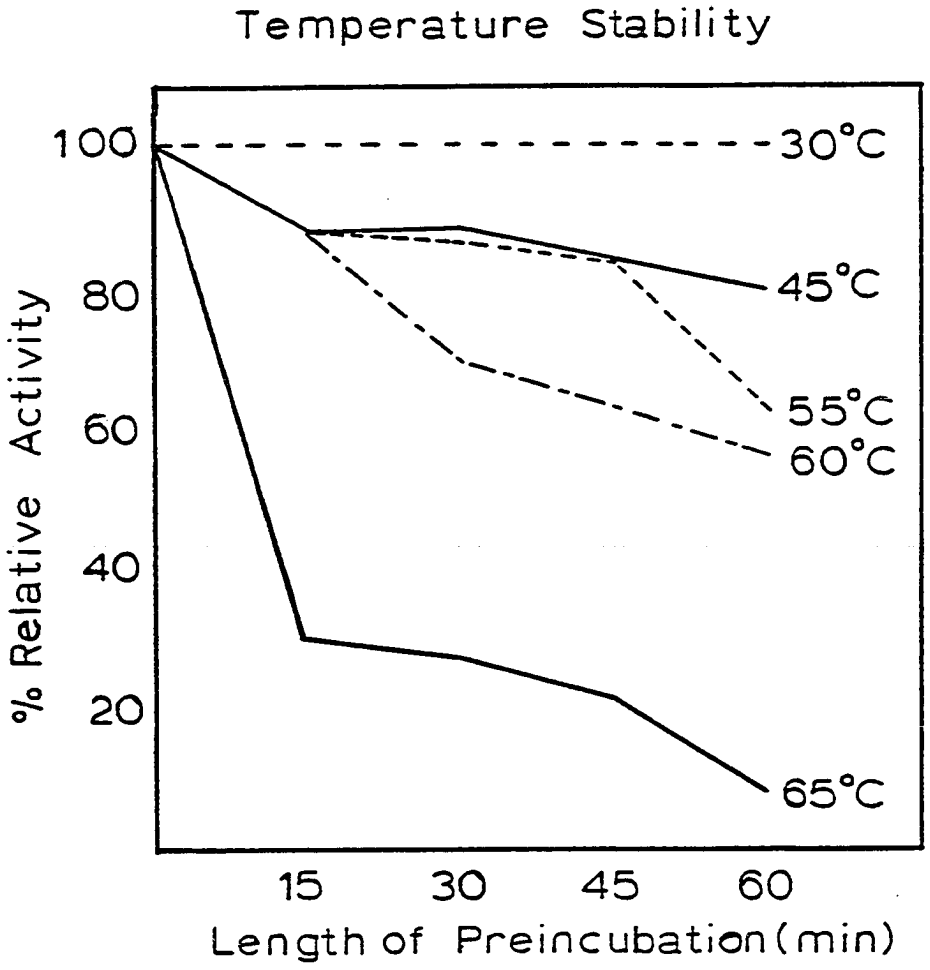


Fig. 5 Effect of increasing preincubation temperature upon stability of the endoglucanase activity (see experimental methods)

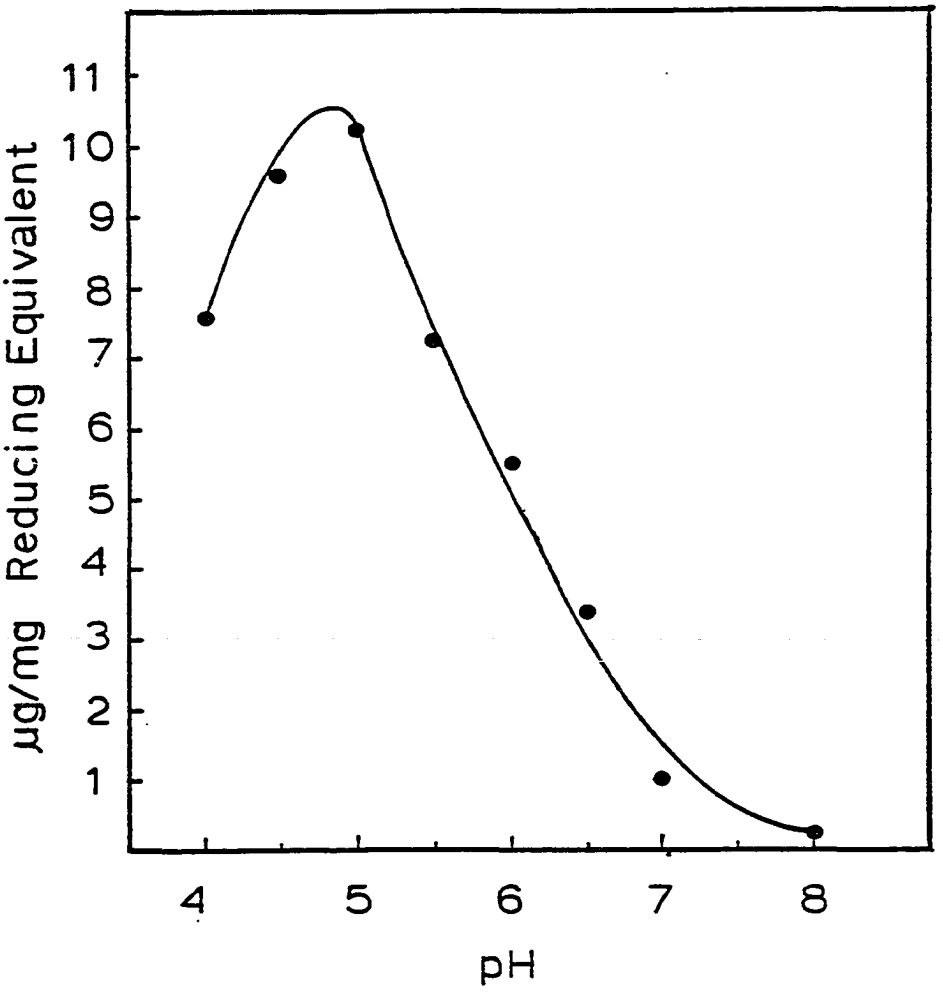
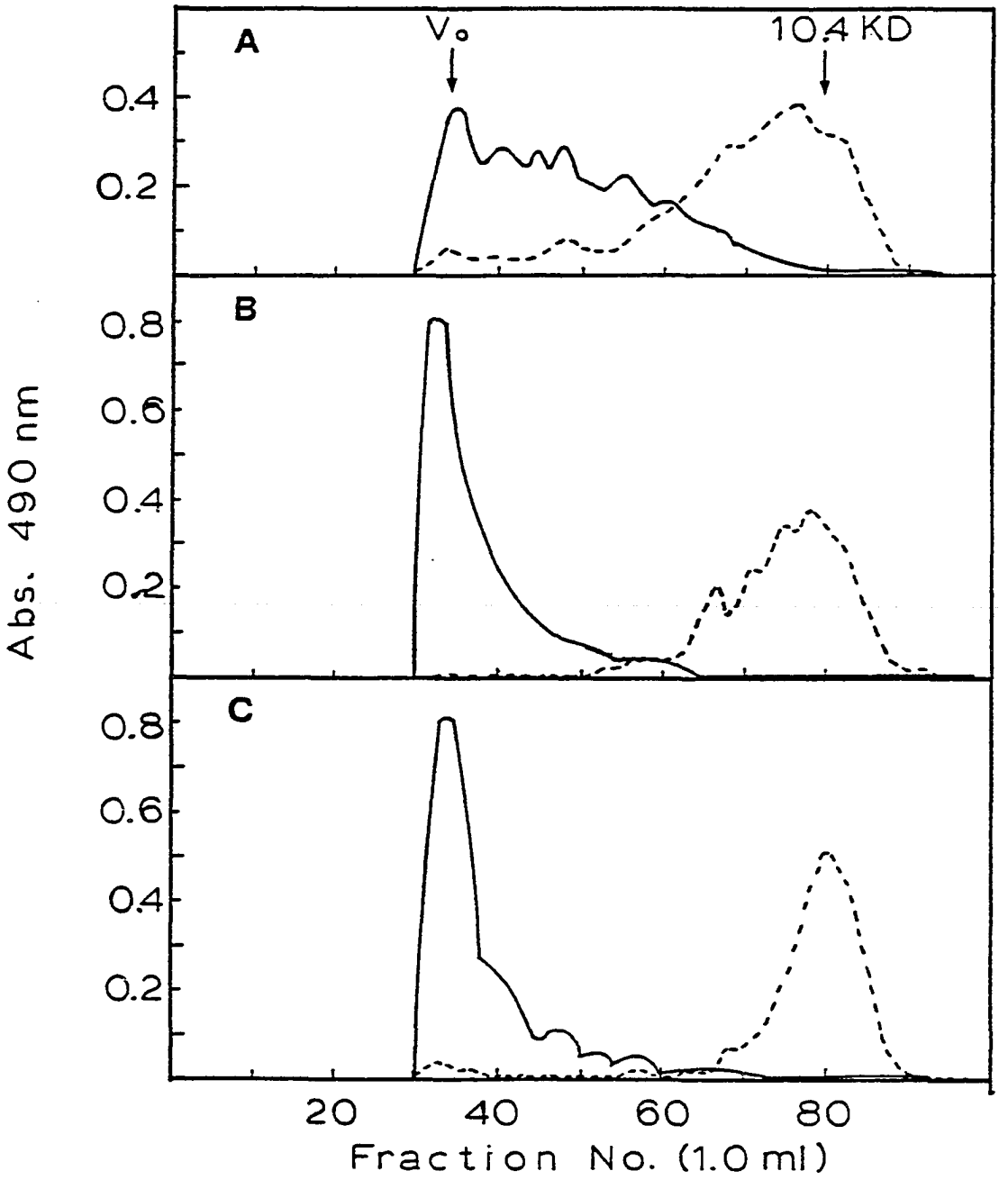


Fig. 6 Effect of pH on the activity of the endoglucanase (see experimental methods)

Fig. 7 Chromatographic profile of hydrolytic products released from (1→3),(1→4)-β-D-glucans on a Bio-Gel A1.5M column. A, lichenan; B, *Hordeum* β-D-glucan; C, *Avena* β-D-glucan. (———) before endoglucanase treatment (----) after endoglucanase treatment


A
B
C
Vo
10.4 KD
0.4
0.2
0.8
0.6
0.4
0.2
0.8
0.6
0.4
0.2
Abs. 490 nm
20
40
60
80
Fraction No. (1.0 ml)

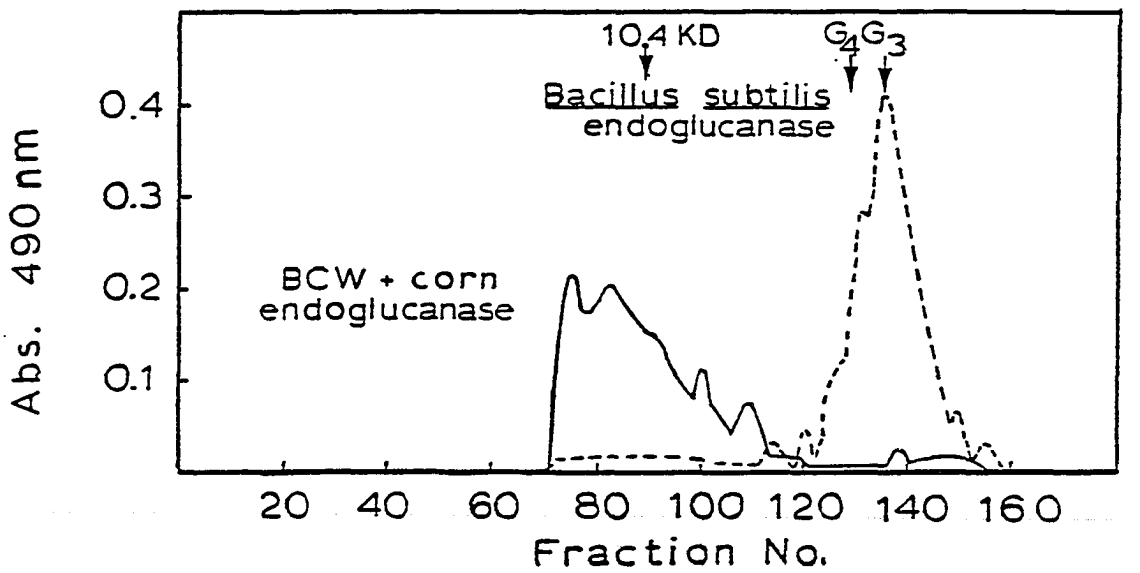


Fig. 8 Chromatographic profile on Fractogel TSK 50SFW of products released from inactivated corn cell walls treated with the endoglucanase (———). Treatment of the released carbohydrate with Bacillus subtilis endoglucanase (----)

PAPER III. HYDROLYTIC ACTIVITY AND SUBSTRATE SPECIFICITY OF AN ENDOGLUCANASE FROM *ZEA MAYS* SEEDLING CELL WALLS

ABSTRACT

An endoglucanase was isolated from cell walls of Zea mays seedlings. Characterization of the hydrolytic activity of this glucanase using model substrates indicated a high specificity for molecules containing intramolecular (1→3),(1→4)-β-D-glucosyl sequences. Substrates with (1→4)-β-glucosyl linkages, such as carboxymethylcellulose and xyloglucan were, degraded to a limited extent by the enzyme, whereas (1→3)-β-glucans such as laminarin were not hydrolyzed. When (1→3),(1→4)-β-D-glucan from Avena endosperm was used as a model substrate a rapid decrease in viscosity was observed concomitant with the formation of a glucosyl polymer (molecular weight of 1-1.5 X 10^4). Activity against a water soluble (1→3),(1→4)-β-D-glucan extracted from Zea seedling cell walls revealed the same depolymerization pattern. The size of the limit products would indicate that a unique recognition site exists at regular intervals within the β-D-glucan molecule. Unique oligosaccharides isolated from the Zea (1→3),(1→4)-β-D-glucan that contained blocks of (1→4) linkages and/or more than a single contiguous (1→3) linkage were hydrolyzed by the endoglucanase. The unique regions of the (1→3),(1→4)-β-D-glucan may be the recognition-hydrolytic site of the Zea endoglucanase. The activity of this enzyme on native cell walls could be important in the turnover of the β-D-glucan molecule during growth.

INTRODUCTION

The cell walls of immature vegetative tissues of gramineous plants contain variable amounts of (1→3),(1→4)-β-D-glucan (β-D-glucan) (18,23). In plants such as Hordeum and Avena, the β-D-glucan is a major component of the hemicellulosic fraction of endosperm cell walls as well as rapidly elongating coleoptile tissues (23). Recently, Luttenegger and Nevins demonstrated that the content of β-D-glucan in coleoptile cell walls of Zea mays is correlated closely with the growth pattern of the coleoptile (16). The highest β-D-glucan content was found to be present in coleoptiles during the most rapidly elongating phase. When coleoptile elongation decreased or stopped the content of β-D-glucan decreased significantly. These observatons suggest a close relationship of coleoptile elongation with β-D-glucan turnover.

An autohydrolytic degradation of β-D-glucan was first described by Lee et al. (14) and Kivilaan et al. (12) in isolated cell walls of Zea coleoptiles. Huber and Nevins confirmed that the polysaccharide solubilized during autolysis was derived from the hemicellulosic (1→3)(1→4)-β-D-glucan (8). In addition, they identified an exo-β-D-glucanase and an endo-β-D-glucanase tightly associated with the Zea cell wall matrix that appeared to be responsible for the autohydrolysis. Other reports of similar autolytic systems have been made for Avena coleoptiles (11), Oryza coleoptiles (13), and Phaselous hypocotyls (5). However, these systems have not been as well characterized in terms of specific

polysaccharides and hydrolases involved in the autolytic activity.

A direct relationship between autolysis and β-D-glucan turnover during cell elongation has not been established at this time. Loescher and Nevins demonstrated a loss of glucose from hemicellulosic glucan of Avena coleoptile segments in response to IAA induced elongation (15). Similar hemicellulosic glucan turnover responses have been observed in IAA induced growth of Oryza (28) and Hordeum (19) coleoptiles. These results suggest the existence of specific hydrolyases within the cell wall that lead to the solubilization of β-D-glucan in response to IAA-induced elongation. An enzyme system, such as that partially purified by Huber and Nevins which accounts for autolytic solubilization of β-D-glucan (9) may also account for β-D-glucan turnover during growth.

To evaluate whether the same enzymes are involved in growth-associated β-D-glucan turnover, the hydrolytic specificity of each enzyme must be investigated. The present work was undertaken to determine substrate specificity and hydrolytic activity of the endo-β-D-glucanase associated with Zea seedling cell walls.

MATERIALS AND METHODS

Enzyme preparation

Zea endoglucanase was extracted from the cell walls of 3 to 3.5 d old seedlings and purified as described previously (7). The purified endoglucanase was stable in 20 mM acetate buffer (5.0 pH, 20 mM NaCl) for several months when stored at -20 C. Smaller aliquots of the endoglucanase were prepared and frozen for subsequent use in substrate specificity and hydrolytic activity studies to avoid possible loss of activity from repeated freezing and thawing cycles.

Carbohydrates evaluated as potential substrates

The following polysaccharides were used to evaluate the hydrolytic specificity of the Zea endoglucanase: lichenan, Cetraria islandica and laminarin, Laminaria digitata from Sigma Chemical Co.; carboxymethylcellulose (CMC 7M F) from Hercules Co.; hydroxyethylcellulose and hydroxypropylcellulose from Polysciences; swollen cellulose prepared from Avicel PH-101 (FMC Corporation) according to the method of Wood (24); β-D-glucan Avena endosperm from Quaker Oats Co.; β-D-glucan Hordeum endosperm from Calbiochem; xyloglucan, soybean hypocotyl, was prepared by Dr. Y Kato; pneumococcal polysaccharide RS III was generously provided by Professor B.A. Stone (Department of Biochemistry, LaTrobe University); laminarin containing β-(1--6) linkages was prepared from Eisenia bicyclis and pachyman from Porin cocos.

Soluble β-D-glucan was extracted from Zea seedling cell walls

by the following procedure. Approximately 500-600 g (wet weight) of LiCl extracted cell walls were boiled in distilled H_2O for 1 h (5:1, vol:wt). The mixture was filtered through miracloth and the supernatant adjusted to pH 7.0 with 0.1 M Na-Phosphate buffer and 1 N NaOH, the final concentration of Na-phosphate buffer was 10 mM. The supernatant was treated with α-amylase (Type 1-A PMSF, porcine pancreas, Sigma Chemical Co.) at 15 units of activity per 300 grams of original cell wall weight for 48 h at 30 C. A few drops of toluene were added to suppress microbial activity. The supernatant was fractionated with 20% $(NH_4)_2SO_4$ followed by 40% $(NH_4)_2SO_4$. The precipitate formed after each fractionation was collected by centrifugation at 5000 Xg. The 40% $(NH_4)_2SO_4$ precipitate contained the highest concentration of β-D-glucan. This precipitate was dissolved in distilled H_2O (50-75 ml) and dialyzed against distilled H_2O (4 L, 3-4 changes) for 24 h at 4 C. The precipitate that formed (β-D-glucan) was removed by centrifugation (5000 xg), dissolved in hot distilled H_2O and freeze dried. The resulting β-D-glucan preparation had an average molecular weight of approximately 7.0×10^4 based on gel filtration.

The procedure of Kato and Nevins (10) was used to isolate β-D-glucan oligosaccharides which contained more than 3 contiguous (1→4) linkages and/or two or more contiguous (1→3) linkages. These oligosaccharides were liberated from Zea cell walls using purified Bacillus subtilis (1→3),(1→4)-β-D-glucan 4-

glucanohydrolase. The released oligosaccharides were concentrated by rotary evaporation under diminished pressure at 30-35 C. Concentrated oligosaccharides were loaded on a Bio-Gel P-2 column (3.5 X 50 cm) and eluted with water. Oligosaccharides of D.P. > 4 were pooled, concentrated and further resolved on a Bio-Gel P-2 column (1.5 X 150 cm) using distilled H_2O at 50 C as elutant. One ml fractions were collected and analyzed for total sugar content. Individual peaks ranging from D.P. 4 to D.P. 8 were collected and stored at -20 C until used for analysis.

Determination of endoglucanase substrate specificity

Individual substrates were prepared in 20 mM acetate buffer (pH 5.0) at a final concentration of 1 mg ml^{-1}. For substrates that were not soluble (avicel and pachyman), suspensions were made in acetate buffer at an approximate concentration of 1 mg ml^{-1}. Purified endoglucanase was added to 1 ml of each substrate to a final concentration of 2.5 µg ml^{-1}. Enzyme and substrate were incubated 24 h at 30 C. Activity was assessed by determining the increase in reducing equivalents produced as compared to a control with no enzyme.

Assessment of hydrolytic action pattern

Avena β-D-glucan was used as a model substrate to determine the hydrolytic pattern of the Zea endoglucanase. Avena β-D-glucan was dissolved in 20 mM acetate buffer (pH 5.0) to give a concentration of 10 mg ml^{-1}. The β-D-glucan solution (total

volume 5.5 ml) was added to a Cannon-Manning Semi-Micro Viscometer (size 300) and placed in a water bath maintained at 30 C. After temperature equilibrium had been reached, endoglucanase was added (3 µg ml^{-1} final concentration) with thorough mixing. The viscosity of the mixture was determined immediately and a 0.2 ml aliquot was removed for reducing sugar analysis. Viscosity measurements were taken every 10 min for the first 90 min followed by readings at 2,4,8, and 24 h. Samples were withdrawn and analyzed for reducing equivalents at 0.5,1,2,4,8,12,24,36,and 48 h.

Hydrolytic activity against cellodextrins

Cellodextrins were prepared using the procedure described by Yamamoto and Nevins (27). The cellodextrins were fractionated into appropriate oligosaccharide groups (D.P. 2-6) by eluting through a Bio-Gel P-2 column (1.5 X 150 cm) with water at 50 C. Fractions with the same D.P. were rechromatographed on the same column. Aliquots of individual stock solutions (1mg ml^{-1}) of cellodextrins (D.P. 3-6) were added to 20 mM acetate buffer containing endoglucanase (6 ug total protein) to give a final concentration of 100 µg ml^{-1} in 1.25 ml of reaction mixture. Samples (200 µL) were removed and assayed for reducing equivalents at 0, 0.5,1,2, and 4 h.

Hydrolytic activity against β-D-glucan oligosaccharides

Higher molecular weight oligosaccharides (D.P. 4) released from Zea cell walls by Bacillus subtilis endoglucanase were

prepared in 20 mM acetate buffer (100 µg ml^{-1}). One ml of the oligosaccharide mixture was incubated with 2.5 µg of Zea endoglucanase for 12 h at 30 C. The total activity was assessed by comparing the increase in reducing equivalents over a control with no enzyme.

In other experiments, the oligosaccharide mixture was fractionated into equivalent D.P. groups before reaction with the Zea endoglucanase. After the 12 h reaction time, the individual oligosaccharide groups were freeze dried and analyzed by methylation analysis. A comparison of the linkage ratio (T-Glc: 3-Glc:4-Glc) present in the enzyme treated samples with the blank control was made to determine the type of linkage hydrolyzed.

General methods

Reducing equivalents were determined by the procedure of Nelson (17) as modified by Somogyi (21), with glucose as a standard. Total sugars were determined by the phenol-sulfuric method (3) using glucose as a standard. Protein concentrations were estimated using the Bio-Rad Protein assay with bovine serum albumin (Sigma) as a standard.

Methylation analyses of carbohydrate samples were performed according to the procedure of Harris et al. (6). The partially methylated alditol acetates were analyzed by GLC on a 30 m DB-1 fused silica capillary column with a tempeature program from 150-230 C (4 c min^{-1}) with a split ratio of 50:1.

RESULTS AND DISCUSSION

Substrate specificity

The hydrolytic activity of the Zea endoglucanase against different β-glucans was investigated to evaluate the recognition site specificity. Three distinct groups of β-glucans, differing in the type of predominant glucosyl linkage, were used to test this specificity (Table I). The group of substrates that was depolymerized most extensively were those containing intramolecular (1→3) and (1→4) linkages (Table I). One exception within this group was the pneumococcal polysaccharide RS III. In the reduced form, this polysaccharide consists of alternating (1→3) and (1→4) linkages (1). The lack of activity against this molecule would suggest that the Zea endoglucanase does not have a recognition site consisting of a single (1→4) linkage, a single (1→3) linkage, or alternating (1→3),(1→4) linkages. These observations would also eliminate a possible recognition-hydrolytic site for the (1→3),(1→4)-β-D-glucan 4-glucanohydrolase of Bacillus subtilis (1) and the (1→3),(1→4)-β-D-glucan endo-hydrolyase from germinating Hordeum (25). Both of these enzymes have similar substrate specificities and hydrolyze the reduced pneumococcal polysaccharide RS III to laminaribiose. The hydrolytic pattern of these enzymes has been found to specifically cleave a (1→4) linkage that follows on the reducing side of a (1→3) linkage (1,25). Although the Zea endoglucanase appears to have a high specificity for the mixed linked glucans

its hydrolytic recognition site would appear to be different than that of the Bacillus subtilis and Hordeum endoglucanases.

Glucans containing predominantly β-(1→4) linkages were hydrolyzed to a limited extent. Of the five substrates in this group only xyloglucan and carboxymethylcellulose (CMC) were depolymerized. Both substrates have a high degree of substitution of the (1→4)-β-D-glucan backbone resulting in high solubility. The relatively lower hydrolytic activity against xyloglucan, as compared to CMC, most likely reflects a more restricted number of potential recognition-hydrolytic sites.

The modified (1→4)-β-D-glucans hydroxyethylcellulose and hydroxypropylcellulose are not hydrolyzed by the endoglucanase. Both substrates are as soluble as CMC. The lack of activity may be due to the bulkiness of the substituted groups on the individual glucose molecules. Although a hydroxyethyl group by itself would not appear to be any larger than a carboxymethyl group, the process of generating such substitutents on the glucan polymer may lead to dihydroxyethyl side chains. Such side chains may be large enough to inhibit enzyme binding. The insoluble (1→4)-β-D-glucan, avicel, was not hydrolyzed suggesting that the endoglucanase activity is restricted to soluble substrates. Glucans containing β-(1→3) linkages were not hydrolyzed by the Zea endoglucanase indicating that the recognition site was not contiguous (1→3) linkages.

Determination of the hydrolytic action pattern

The Zea endoglucanase exhibits a characteristic endo-hydrolase action-pattern on the (1→3),(1→4)-β-D-glucan from Avena endosperm (Fig. 1). During the first hour of incubation, there is a rapid decrease in viscosity. Additional incubation of the reaction mixture from 2 to 4 h results in a minimal decrease in viscosity. There is a linear increase in reducing equivalents produced during the first 4 h of incubation (Fig. 1). Increased incubation time results in a slow increase in reducing equivalents (see inset Fig. 1) for the first 24 h, and an additional 24 h of reaction time results in a slight increase. The low production of reducing equivalents combined with the rapid decrease in viscosity indicates that the endoglucanase hydrolyzes widely separated glucosyl bonds. The reducing equivalents produced after 48 h indicates that the products released have an average D.P. of 60 to 70.

To better assess the action pattern of the Zea endoglucanase aliquots of the reaction mixture were removed at various times and chromatographed on a Bio-Gel A-1.5M column. This column has a theoretical exclusion limit of 1.5×10^6 molecular weight. At the start of incubation the Avena β-D-glucan elutes in the void volume of this column (Fig. 2). After 1 h there is a shift to lower molecular weight products. This shift continues with increased incubation time up to approximately 24 h. Further incubation for an additional 24 h or adding fresh enzyme (25 ug) did not result

in a continued shift to lower molecular weight products. The estimated molecular weight (1.0-1.5 X 10^4) of the final products corresponds with the estimated D.P. based on the ratio of TS to RS.

These observations suggest that the Zea endoglucanase can rapidly depolymerize (1→3),(1→4)-β-D-glucans (Fig. 1) but that there is a limited number of recognition sites within the molecules, limiting depolymerization. Other model substrates such as lichenan, Hordeum β-D-glucan, and soluble Zea β-D-glucan are hydrolyzed to limit products that fall within this molecular weight range of 1.0-1.5 X 10^4 (data not shown). In addition, inactivated cell walls isolated form Zea seedlings treated with the endoglucanase release polymeric carbohydrate that have a molecular weight of 1.0-1.5 X 10^4. The similarity of the products released by the endoglucanase indicates that not only is there a limited number of recognition sites, but the distribution of such sites must be relatively uniform. A random distribution of sites would lead to a more heterogenous distribution of products in terms of molecular weight. Based on the molecular weight estimate for the released products each recognition site would be roughly 60 to 70 glucosyl residues apart.

The observation that the Zea endoglucanase depolymerizes the β-D-glucan molecules to the same limit products suggests that each has a similar recognition site. A possible recognition-hydrolytic site in Zea β-D-glucan maybe the regions rich in (1→4)

and (1→3) linkages described by Kato and Nevins. They identified oligosaccharides that contained blocks of more than three contiguous (1→4) linkages and more than a single contiguous (1→3) linkage. Such a site would have to be distributed evenly throughout the molecule, giving rise to the release of uniform products when hydrolyzed by the endoglucanase (Fig.3).

Similar unique regions could exist in the other β-D-glucans depolymerized by Zea endoglucanase. Previous reports had proposed regions rich in (1→3) linkages for Hordeum endosperm β-D-glucan (2,4). Woodward et al. identified regions in Hordeum β-D-glucan which contained extended blocks of 4 to 10 (1→4) linkages but did not identify regions containing more than a single (1→3)-linkage (26). The differences between these results may be due to the use of different chemical means used to assess the presence of adjacent (1→3)-linkages. It has also been reported that the Avena β-D-glucan contains unique oligosaccharides rich in (1→4) and/or (1→3) linkages (20). Unpublished results from this lab using the Bacillus subtilis endoglucanase to solubilize Avena β-D-glucan from oat bran resulted in the production of oligosaccharides with D.P. > 4. Preliminary analysis of these oligosaccharides indicated an enrichment in (1→3) linkages and blocks of more than 4 contiguous (1→4) linkages.

Hydrolytic site assessment

The activity of Zea endoglucanase against cellodextrins and laminaridextrins was used to determine the type of glucosyl

linkage that potentially would be hydrolyzed within the proposed recognition site (Fig. 3) of the Zea β-D-glucan molecule. Cellodextrins were hydrolyzed by the endoglucanase (Table II). Based on the reducing equivalents produced, there appears to be a minimum sequence (D.P. 4) that is hydrolyzed by the enzyme. Although D.P. 4 is hydrolyzed, the rate was 5 to 6 times less than D.P. 5 or 6 indicating an enzyme preference for longer sequences of (1→4) linkages. Oligosaccharides containing only (1→3) linkages are not hydrolyzed by the endoglucanase.

In addition, higher molecular weight oligosaccharides (D.P.> 4) released from oat bran or inactivated Zea cell walls by the Bacillus subtilis endoglucanase were hydrolysed by the Zea endoglucanase (data not shown). The activity of the Zea endoglucanase resulted in approximately 30% increase in reducing equivalents. This would indicate that these unique oligosaccharides could be the recognition-hydrolytic sites for the Zea endoglucanase.

Activity of the endoglucanase against cellodextrins suggests that one of the (1→4) linkages (Fig. 3) of the proposed recognition site would be hydrolyzed. However, one cannot rule out the possibility that the endoglucanase has a binding preference for (1→4) linkages but could hydrolyze an adjacent (1→4) or (1→3) linkage. In an attempt to resolve this question, the hydrolytic activity was measured against oligosaccharides from Zea β-D-glucan containing the propsed recognition sites. The

unique oligosaccharides were fractionated into size groups ranging from D.P. 3 to D.P. 8. The oligosaccharides were not resolved to homogenity. Therefore, each D.P. fraction could be composed of oligosaccharides containing variable ratios of (1→3) and (1→4) linkages within a given D.P. fraction. Selected D.P. fractions were analyzed by methylation analysis after treatment with Zea endoglucanase or with buffer. The results are summarized in Table III. Of the D.P. fractions tested, only the larger fragments (D.P. > 4) were hydrolyzed. Methylation analysis of the hydrolyzed fractions revealed that apparently only (1→4) linkages were cleaved. This is based on the relative decrease in (1→4) linked glucose.

From the results presented here, the unique regions of Zea β-D-glucan molecule described by Kato and Nevins (10) appear to be the recognition sites of the Zea endoglucanase. Furthermore, based on the hydrolytic activity against cellodextrins and isolated oligosaccharides, the enzyme hydrolyzes a (1→4) linkage. The distribution of the unique regions must be relatively uniform throughout the β-D-glucan molecule. Kato and Nevins proposed that regions with contiguous (1→3) linkages conform to a folded molecular structure (10). Such a structure accommodates an antiparallel intramolecular arrangement, such as proposed by Tvaroska et al. for crystallized Hordeum β-D-glucan based on x-ray diffraction and conformational analysis (22). The even distribution of regions that have the potential to impart a folded

conformation on the β-D-glucan molecule may be important in terms of cell wall matrix interactions and function.

Huber and Nevins (9) proposed that the endoglucanase plays an important role in the depolymerization of β-D-glucan leading to the release of glucose during autolysis of isolated Zea cell walls. Additionally Luttenegger and Nevins demonstrated a close correlation between β-D-glucan content in cell walls and the growth pattern of Zea coleoptiles (16). The role of the endoglucanase in growth may be one of controlled depolymerization of the β-D-glucan molecule. Perhaps the incorporation of β-D-glucan into cell walls during elongation allows for a more flexible matrix. The regulation of the endoglucanase may play a critical role in the shift from rapid elongation to a reduced rate of elongation.

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Table I. Analysis of *Zea* endoglucanase recognition sites in β-D-glucans

The substrate specificity of the endoglucanase was determined by measuring the hydrolytic activity against β-D-glucans with various linkage arrangements. Hydrolytic activity was assessed by measuring the increase in reducing equivalents.

Substrate	Reducing sugar equivalents generated (μg/mg)[a]
A. Intramolecular (1→3) and (1→4) linkages	
Lichenan, *Cetraria islandica*	59.0
β-D-glucan, *Avena* endosperm	33.8
β-D-glucan, *Hordeum* endosperm	36.7
β-D-glucan, *Zea* cell wall (soluble)	21.5
Pneumococcal polysaccharide, reduced (RS-III)	0.0
B. (1→4) linkages	
Xyloglucan, soybean hypocotyl	10.5
Carboxymethylcellulose	22.1
Hydroxyethylcellulose	0.0
Hydroxypropylcellulose	0.0
Avicel (swollen cellulose)	0.0
C. (1→3) linkages	
Laminarin, *Laminara digitata*	0.0
Laminarin, *Eisenia bicyclis*, also (1→6)	0.0
Pachyman	0.0

[a] μg/mg= μg of reducing equivalents produced/ mg of substrate.

Table II. Analysis of hydrolytic activity of Zea endoglucanase against β (1→3) and β (1→4) oligosaccharides (0.5 mg/ml)

Substrate	D.P.	Rate of Reducing Sugar Equivalents Increase µg/h/µg[a]
β (1→3)		
	3	0.0
	4	0.0
	5	0.0
	6	0.0
β (1→4)		
	3	0.0
	4	0.50
	5	2.86
	6	2.90

[a] µg of reducing equivalents produced per h per µg of endoglucanase used in each treatment.

Table III. Glucosyl linkage hydrolyzed in β-D-Glucan Oligosaccharides

Methylation analysis of the hydrolytic activity of Zea endoglucanase against unique oligosaccharides released from Zea cell walls by Bacillus subtilis endoglucanase.

	Amount[a] %							
D.P.	4	4	5	5	6	6	7	7
linkage	control[b]	endo[c]	control	endo	control	endo	control	endo
T-Glc	23.0	22.2	17.8	22.4	12.9	17.8	16.5	21.2
3-Glc	14.2	14.3	9.6	14.0	12.5	13.4	14.0	17.7
4-Glc	62.8	63.5	72.6	63.6	74.6	68.7	69.4	61.0

[a] T-Glc (non-reducing terminal glucose residue) + 4-Glc (4-linked glucose residue) + 3-Glc (3-linked glucose residue) = 100%.

[b] refers to oligosaccharides that were not treated with endoglucanase.

[c] refers to oligosaccharides treated with the endoglucanase.

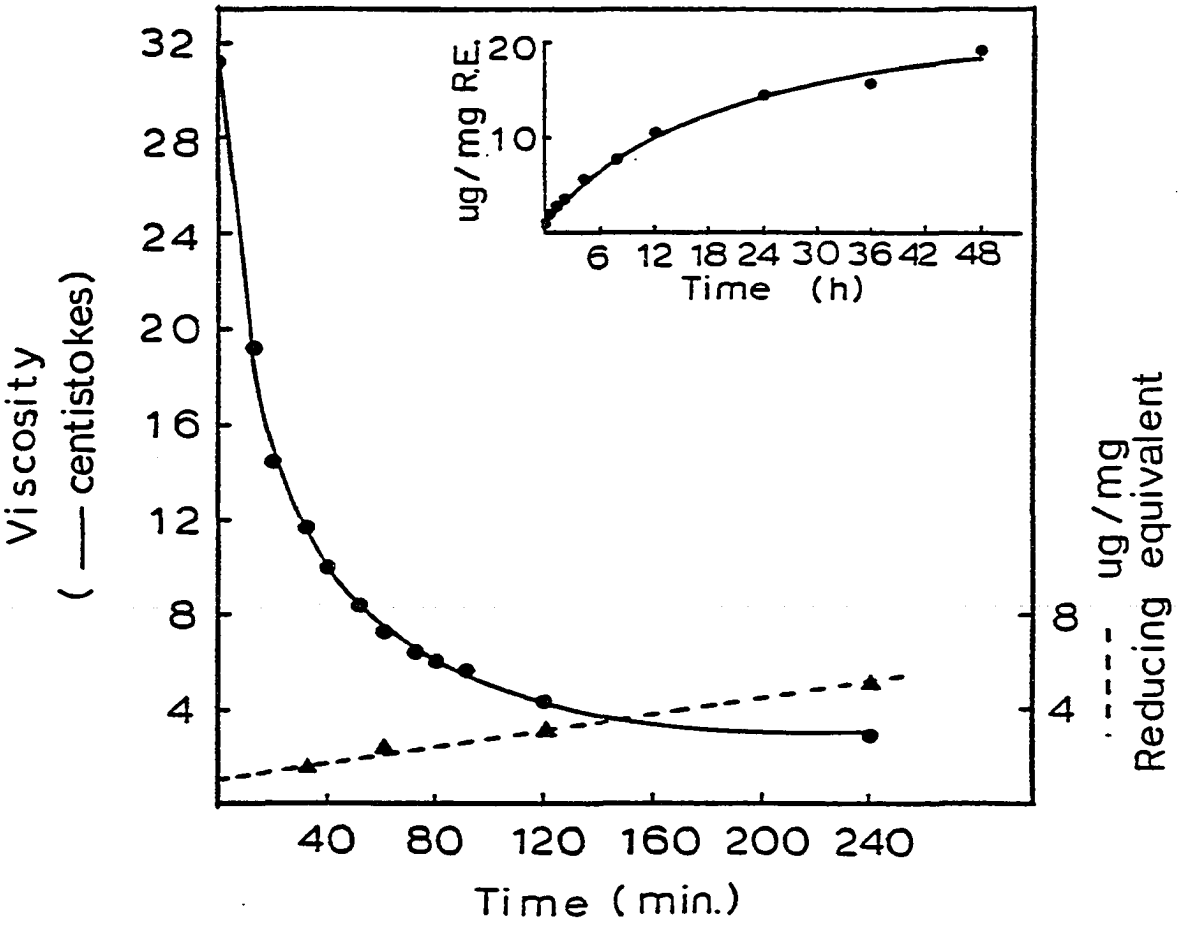
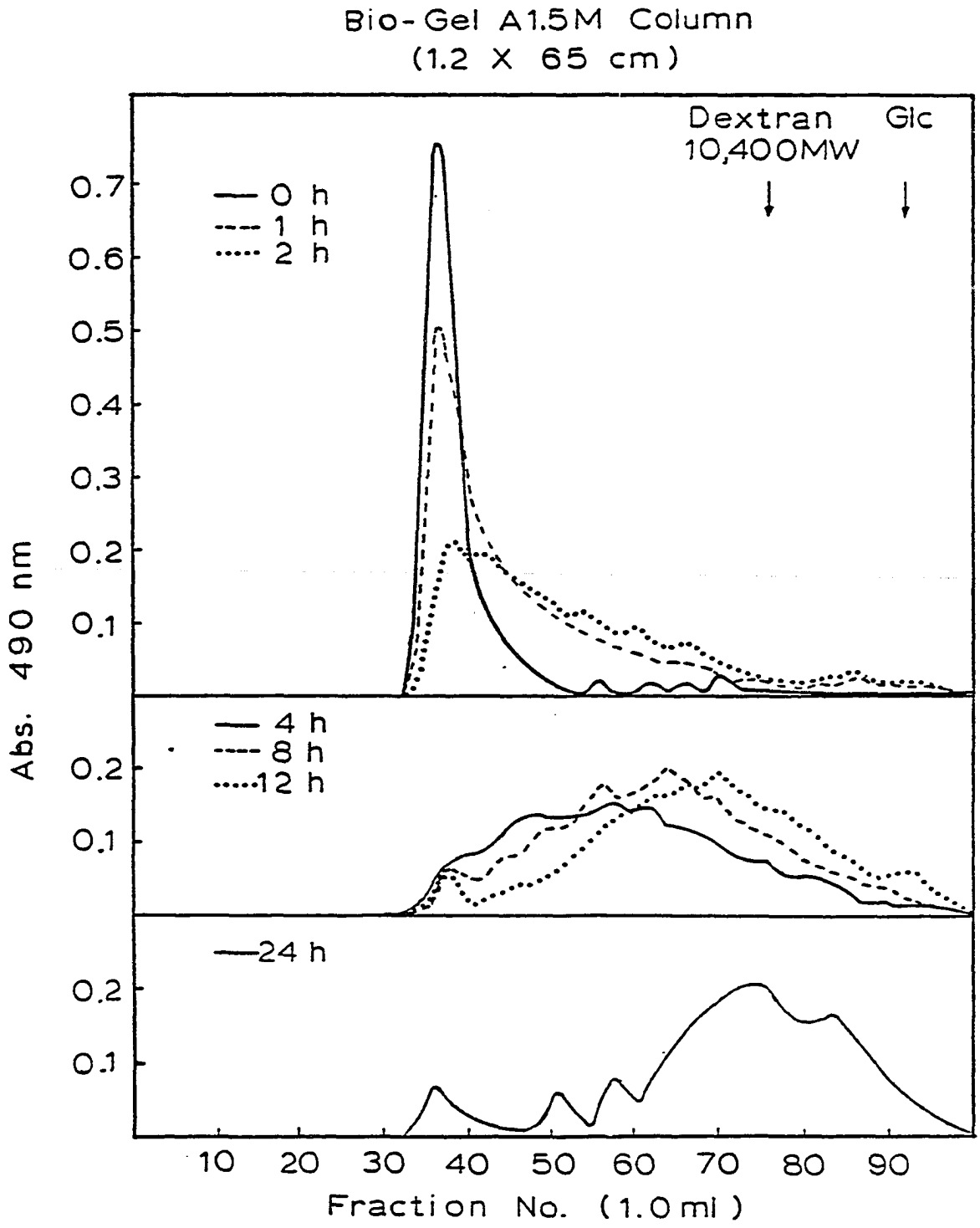


Fig. 1 Hydrolytic action pattern of Zea endoglucanase on Avena (1→3),(1→4)-β-D-glucan. (●——●) viscosity, (▲----▲) reducing equivalents Inset represents the increase in reducing equivalents over extended incubations time

Fig. 2 Changing Bio-Gel A-1.5M profile of hydrolytic products released from *Avena* (1→3),(1→4)-β-D-glucan by *Zea* endoglucanase as a function of increased incubation time


Bio-Gel A1.5M Column
(1.2 X 65 cm)
Dextran 10,400MW
Glc
0 h
1 h
2 h
4 h
8 h
12 h
24 h
Abs. 490 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
80
90
Fraction No. (1.0 ml)

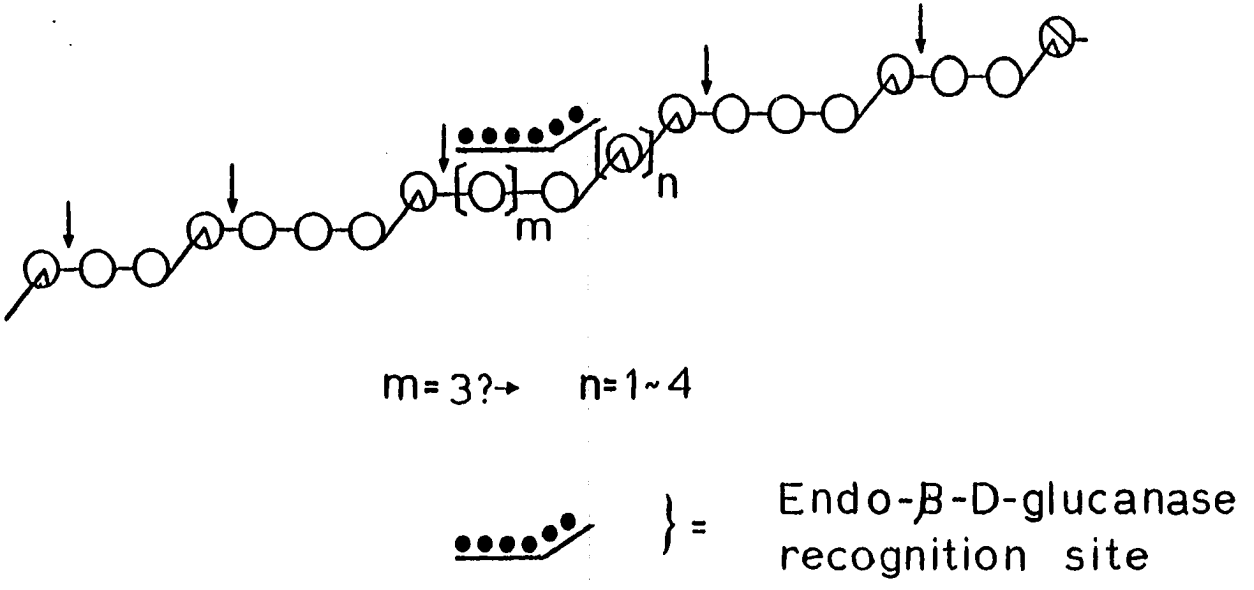


Fig. 3 Proposed Zea endoglucanase recognition site within the Zea (1→3),(1→4)-β-D-glucan molecule. Arrows represent the hydrolytic sites of Bacillus subtilis (1→3),(1→4)-β-D-glucan 4-glucanohydrolase

GENERAL SUMMARY

The primary cell walls of plants must be rigid yet amendable to permit organized cell expansion. Cell wall modifications must be under stringent regulation to insure organized wall expansion. Although specific cell wall hydrolases most likely mediate wall loosening events, the mechanism of auxin regulation of cell expansion is not known. The major objective of the work described here was to characterize specific proteins asssociated with the cell walls of Zea seedlings. Information gained is useful in elucidating the metabolic role of such proteins during auxin induced growth.

Characterization of the protein fraction responsible for eliciting anti-growth antibodies indicated that this fraction did not contain cell wall hydrolase activity. The lack of hydrolytic activity does not decrease the significance or the potential usefulness of these antibodies. It is possible that the active antibodies are elicited by a closely related family of proteins which have a structural role. Such proteins may be lectin-like in nature and form non-covalent linkages that can be dissociated during growth. If these proteins are lectins, there should be specific polysaccharides or glycoproteins within the wall matrix with appropriate binding sites.

Antibodies could have been elicited against specific polysaccharides dissociated from the cell wall. The two most probable polysaccharides associated with this cell wall fraction

are arabinogalactan and glucuronoarabinoxylan. Interestingly, arabinogalactan is found associated with rapidly elongating tissues of some grasses but absent from mature non-growing tissues (38). This would suggest a possible role in rapid cell wall extension. Binding of antibodies to this polysaccharide may disrupt transitional changes limiting matrix flexibility. Whether the same temporal pattern exists in Zea seedlings is not known.

Glucuronoarabinoxylan has been proposed to interact with β-D-glucans preventing intermolecular binding resulting in greater wall extensibility (35). Perhaps antibodies binding to glucuronoarabinoxylan could prevent this molecular interaction, reducing wall extension. It has been shown that the glucuronoarabinoxylan found in Zea seedling cell walls is feruloylated (14). Binding of the antibody to these polysaccharides may mimic diferulic acid cross-links. The formation of diferulic acid cross-links has been proposed to play a role in growth regulation by tightening the wall matrix thereby limiting growth (7). These antibodies provide potential probes to investigate the metabolic sequence leading to cell wall extension.

The direct involvement of the Zea endoglucanase in growth has not been substantiated. The original observation suggesting a relationship between the endoglucanase and the anti-growth antibodies has been disproven. Characterization of the endoglucanase indicated a high specificity for mixed linked β-D-glucans. For Zea β-D-glucan, the recognition-hydrolytic site

appears to be regions containing blocks of contiguous (1→4) linkages with more than one contiguous (1→3) linkage. Such regions have been proposed to impart a folded anti-parallel conformation to the β-D-glucan molecule (13). Whether such a conformation actually exists in the wall matrix is not known. Hydrolytic activity by the endoglucanase at these unique regions of the β-D-glucan molecule may result in a relaxation of the matrix leading to cell wall extension. This hypothesis would suggest a major structural role for the β-D-glucan molecule within the wall matrix. Such a role for the β-D-glucan has not been identified to date.

Although enzymes have not been identified the evidence suggests that specific hydrolases within the cell wall matrix are responsible for the cleavage of load bearing bonds (3,35). The process of hydrolysis is most likely balanced with the synthesis of new matrix material which itself may be hydrolyzed at a later time. It is quite possible therefore that a cell that maintains extensibility is one that is able to maintain a proper balance of wall degradation with wall synthesis. This balance of degradation/synthesis need not involve all the polysaccharides that make up a given wall matrix. Some wall components such as cellulose may show a continued synthesis with little or no degradation. Other components such as β-D-glucan may have rapid turnover rates at specific stages of cell growth. This may lead to cell walls with variable amounts of the polysaccharide

depending upon whether the cell walls are undergoing rapid extension or are quiescent. Enzymes such as the endoglucanase may play critical roles in maintaining a balance between synthesis and degradation. The developmental pattern and/or regulation of this enzyme may in turn regulate the shift from rapid elongation to cells that are no longer undergoing extension.

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