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Purification and Characterization of Wall-Localized Cellulase from Maize Coleoptiles

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Wall-localized cellulase was partially purified from freeze-dried maize coleoptiles by a combination of DEAE-Sepharose, Superdex-200 gel filtration and Hydroxyapatite column chromatography. Activity was measured by both reducing sugar assay and dot assay on agarose gel containing carboxymethylcellulose(CMC). In situ activity staining on a nondenaturing gel overlaid on agarose gel containing CMC turned out to be a quite reliable method to detect cellulase activity. The molecular mass of partially-purified cellulase was determined to be about 53 kD based on SDS-PAGE, and the N-terminal amino acid sequence of this cellulase was NH_2-AGAKGANXLGGLXRA. The enzyme hydrolyzed CMC with an optimal pH of 4.5 and optimal temperature of 40°C. It also catalyzed carboxymethylcellulose with a K_m of 2.02 mg/mL and a V_{max} of 160 μg/h/mL. The β-1,4-glucosyl linkages of CMC, fibrous cellulose and lichenan were cleaved specifically by this enzyme. Reducing reagents such as cysteine-HCl, dithiothreitol and glutathione strongly enhanced the activity, suggesting that SH-groups of the enzyme were protected from oxidation. N-ethylmaleimide which is a sulfhydryl-reacting reagent did not seem to inhibit the activity, indicating that cysteine residues were not located near the active site of the enzyme. These results will be valuable in understanding the structure of wall-localized cellulase in maize coleoptiles and in predicting its possible function in the cell wall.

Key words: cell wall, cellulase, maize coleoptile, carboxymethylcellulose

One of the most significant differences between plant and animal cells is that plant cells are surrounded by walls in nearly all stages of development. Cell walls are dynamic parts of cells that can grow and change their shape and composition. The plant cell wall is thought to provide rigidity and protection to the plant cell without preventing diffusion of water and ions from the environment to the plasma membrane (Taiz and Zeiger, 1991).

The concept is now well established that the primary cell wall is a metabolically-active compartment of the cell (Hoson, 1993). Evidence partially supporting this concept is the occurrence of cell wall enzymes. The substrate specificities of these enzymes indicate that they can modify the structural components of the wall (Fry, 1995).

Cell wall hydrolases play an important role in many developmental processes in plants. Cellulases (endo-1,4-β-D-glucanases) constitute one class of such cell wall hydrolases. Many fungal and bacterial species survive in nature by producing a mixture of hydrolytic enzymes that act in synergy to degrade plant polysaccharides-especially cellulose into nutrient. Thus, cellulases and other β-glucanases are widely reported in bacteria and fungi where they are usually assigned a role in nutrition or in infective processes (Allen et al., 1984).

Unlike microbial endo-1,4-β-glucanases, whose function is to attack exogenous substrates, plant endo-1,4-β-glucanases are presumed to act in vivo by selectively disassembling portions of their own cell wall during development (Brummell et al., 1994). In vascular plants, it has been proposed that plant endo-1,4-β-glucanases control various aspects of plant development, such as leaf abscission, fruit ripening, loosening of the cell wall, vascular differentiation and symbiosis (Nakamura et al., 1995). The endo-1,4-β-glucanases may act by hydrolyzing wall materials during these processes (Ohmiya et al., 1995). Not all cellulases have the same pI, suggesting that there are different cellulases with distinct cellular functions (Kemmerer and Tucker, 1994).

Extracellular and wall-bound endo-1,4-β-glucanase have been reported in suspension-cultured poplar cells and Ginseng seed (Yu et al., 1994; Nakamura et al., 1995; Ohmiya et al., 1995). There were several differences between the two forms in spite of similarities in their isoelectric points and reaction kinetics. The wall-bound form did not bind to anion-exchange resins at pH 7.0 but bound to cation-exchange resins, whereas the extracellular form bound to anion-

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exchange resins at this pH. However, the N-termini of both enzymes were 90% homologous to each other.

Some possibilities exist for the involvement of endo-1,4-β-glucanase in the synthesis of cellulose (Nakamura et al., 1995). Since an endo-1,4-β-glucanase has been found to be encoded 1 kb upstream of the cellulose synthase operon (*bcs* operon) in *Acetobacter xylinum*, it is possible that cellulase and cellulose synthase function in concert (Standal et al., 1994).

In this study, partial purification and characterization of cellulase from maize coleoptiles are reported. For characterization, pH-dependence, temperature-dependence, substrate specificity and reaction kinetics of partially-purified cellulase were examined. These results should be very informative in understanding the structure of wall-localized cellulase in maize and in determining its possible function in the cell wall.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Maize (*Zea mays* L.) seeds were soaked in running water for 6-7 h. The seeds were sown on moist vermiculite and grown in the dark for 5-7 days at 22-26°C. Coleoptiles were cut with a razor blade and sectioned into 1-2 cm segments. The materials were put into a bottle containing liquid nitrogen soon after sectioning. The frozen coleoptiles were freeze-dried for 5 days. Freeze-dried coleoptile tissues were stored at −20°C until used for the extraction of wall proteins.

Extraction of Wall Proteins

All procedures were carried out at 4°C. The wash buffer was composed of 10 mM HEPES and 7.5 mM Tris-HCl (pH7.8). 4 μg/mL aprotinin / 2.1 mM $Na_2S_2O_5$ were added to the initial wash buffer. The Triton X-100 wash buffer contained 1% (v/v) Triton X-100 in the wash buffer. To make the extraction buffer, 100 mM $CaCl_2$, 4 μg/mL aprotinin and 2.1 mM $Na_2S_2O_5$ were added to 50 mM HEPES / 37.5 mM Tris-HCl (pH7.8). 10 g of freeze-dried tissue was homogenized in 133 mL of initial wash buffer using a homogenizer (X620, CAT, Germany). The homogenized tissue was filtered through two layers of miracloth in a Buchner funnel. Wash buffer was added and the tissue was filtered additionally. The slurry was put into 70 mL of Triton X-100 wash buffer, stirred for 5 min, then filtered and washed with wash buffer through two layers of miracloth. The washed slurry was mixed with 25 mL of extraction buffer in a 250 mL centrifuge tube for 1 min and then centrifuged at 12,000g at 4°C for 15 min. After centrifugation, the supernatant was saved and this step was repeated once with an additional 25 mL of extraction buffer. Both supernatants of crude extract were pooled and their volume was usually 60-70 mL in each preparation.

Purification of Cellulase from Cell Wall Extract

DEAE-Sepharose, Superdex-200 and HA (Hydroxyapatite) chromatography columns were used for purification. All column separations were conducted at 4°C.

1) DEAE-Sepharose Column

The crude extract of cell wall materials was dialyzed against 50 mM Tris-HCl (pH 7.8) for about 10 h. The dialyzed crude extract was applied to a DEAE-Sepharose column (1.2 × 7 cm) equilibrated with 50 mM Tris (pH 7.8) using the Bio-Rad Econo System. After loading a sample, the column was washed with 60 mL of equilibration buffer and then eluted with 80 mL of a NaCl gradient from 0 to 0.5 M. Individual 4 mL fractions were collected at a flow rate of 1 mL/min. Each fraction was assayed for protein concentration and enzyme activity. Protein concentration was estimated either by reading absorbance at 280 nm on a spectrophotometer (Spectronic 1001, Milton Roy Co.) or by the microassay procedure using a Bio-Rad protein assay kit (Bradford, 1976). For the latter method, lysozyme was used as a standard.

2) Superdex-200 Column

The active fractions having cellulase activity from the DEAE-Sepharose column were pooled and concentrated using Centriprep-10. The concentrated sample was applied to a Superdex-200 column (1.6 × 60 cm) equilibrated with 100 mM Tris-HCl (pH 7.5) / 100 mM NaCl. The column was run with equilibration buffer at a flow rate of 1 mL/min and the volume of each fraction was 5 mL. The FPLC system (Pharmacia) was used for this chromatography step.

3) HA Column

The active fractions with cellulase activity from the Superdex-200 column were pooled and applied to an HA column (1.2 × 8 cm) equilibrated with 10 mM potassium phosphate (pH 6.8) / 5 mM $MgCl_2$ using the Bio-Rad Econo system. The column was washed with 20 mL of equilibration buffer followed

by 40 mL of 50 mM potassium phosphate. The column was then eluted with a gradient of potassium phosphate from 50 to 400 mM. Individual 4 mL fractions were collected at a flow rate of 1 mL/min.

Assay of Cellulase Activity

1) Assay by Somogyi-Nelson Method

The cellulase activities of crude extract and column fractions were assayed for reducing sugars by the Somogyi-Nelson method (Somogyi, 1952). The substrate consisted of 0.5% CMC (carboxymethylcellulose) in 10 mM citric acid (pH 4.5). A mixture of 0.1 mL of enzyme solution and 0.4 mL of substrate solution was incubated at 37°C for 1 h with shaking. After treatment with Somogyi I, II, and Nelson reagent, the absorbance was read at 650 nm. One unit of activity was defined as the amount of enzyme that could liberate 1 μM glucose from the substrate in 1 h.

2) Detection of Cellulase Activity Using Agarose Gel

A gel of 0.75 mm thickness was cast between two glass plates with 2% agarose containing 0.1% CMC, 50 mM disodium hydrogen phosphate, and 12.5 mM citric acid (pH 6.3) (Beguin, 1983). Each 3 μL of various column fractions were dotted on the gel, and the gel was incubated at 37°C for 2 h on a glass plate. The gel was soaked in 0.1% Congo red, shaken gently for 30 min, then washed with 1 M NaCl until excess stain was removed from active dots. The NaCl-washed gel was stored in 5% acetic acid.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was performed using minigels following the method of Laemmli (1970). For vertical minigels, 10 or 12% separating gel and 4% stacking gel were used.

N-Terminal Amino Acid Sequences of Purified Cellulase

N-terminal amino acid sequences of the purified cellulase was determined by direct sequencing with an automatic sequencer (Applied Biosystems 476A, Foster City, USA), as described in Jung et al. (1995).

Characterization of Purified Cellulase

In order to characterize partially-purified cellulase, temperature-dependence, pH-dependence, substrate specificity, effects of reducing reagents and sulfhydryl reagents, and reaction kinetics experiments were conducted. Enzyme activities at temperatures from 20 to 50°C were compared. Enzyme activities at different pH of reaction mixtures were also examined. CMC, α-cellulose, fibrous cellulose, xylan, laminarin and lichenan were used for the substrate specificity tests. The concentration of each substrate solution for activity assays was 0.5% (w/v) in 0.01 M citric acid (pH 4.5). The reducing reagents tested were cysteine-HCl, dithiothreitol and glutathione. N-ethylmaleimide was tested as a sulfhydryl-reacting reagent. Each reagent was added into substrate mixtures at a 1 mM concentration.

RESULTS

Purification of Cellulase

Cell wall proteins were extracted from maize coleoptiles. Most soluble proteins were recovered by homogenizing coleoptiles with initial wash buffer, and membrane-bound proteins recovered using Triton X-100 wash buffer. The pellet, considered to be cell wall material, was washed with wash buffer and used for the extraction of cell wall proteins by extraction buffer containing 100 mM $CaCl_2$. Crude extract from 10 g of coleoptiles contained about 150 mg of total proteins, but the amount of total protein was slightly reduced by dialysis against 50 mM Tris-HCl (pH 7.8).

The dialyzed crude extract was applied to a DEAE-Sepharose column, a weak anionic exchanger, and the elution profile is shown in Figure 1A. Cellulase activities were found in both unbound and bound fractions, but the major activity was found in bound fractions. The major cellulase activity was eluted with 0.15 to 0.25 M NaCl. Fractions #23 to #26 exhibited especially strong activity. The activity profile measured by Somogyi-Nelson's method was consistent with the dotting results on agarose gel containing CMC (Fig. 2).

Fraction #24 was concentrated and loaded onto a 7.5% nondenaturing polyacrylamide gel. After electrophoresis, the gel was overlaid on a 2% agarose gel containing 0.1% CMC as substrate. After transfer and CMC hydrolysis at 37°C for 2 h, cellulase in the nondenaturing gel degraded CMC in the agarose gel, resulting in a transparent band (Fig. 3). Fractions #23 to #26 were pooled and applied to a Superdex-200 size-exclusion chromatography column. The elution profile of the Superdex-200 column is shown in Figure 1B. Activity was found in fractions #16 through

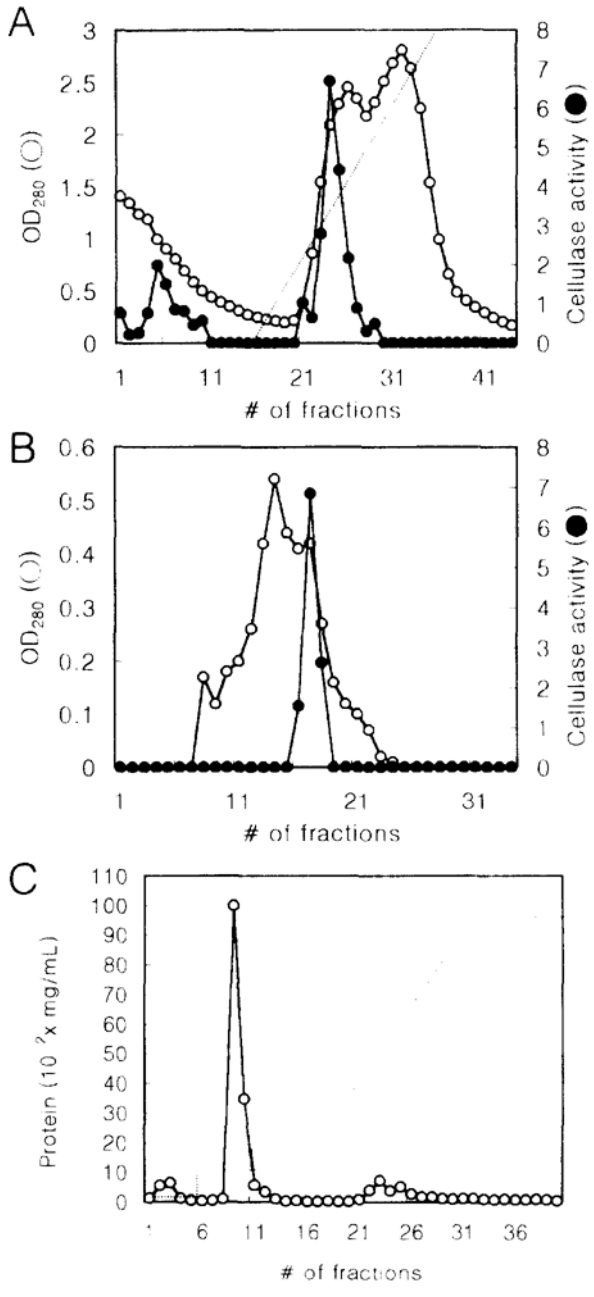

Figure 1. Elution profiles of DEAE-Sepharose (A), superdex-200 gel filtration (B), and HA (C) column chromatography. Crude extract from cell wall material was dialyzed, applied to a DEAE-Sepharose column (A) and eluted with a linear NaCl gradient from 0 to 0.5 M. Active fractions from the DEAE-Sepharose column were concentrated and applied to a superdex-200 column (B). Active fractions from the superdex-200 column were pooled and applied to an HA column (C), and developed with 50 mM potassium phosphate, followed by a linear gradient of 50 to 400 mM of potassium phosphate. Symbols in A and B: , OD_{280}; ●, cellulase activity; - - - -, concentration of NaCl. Symbols in C: , protein concentration ($\times 10^{-2}$ mg/mL); - - - -, concentration of potassium phosphate. ❋ Cellulase activity : $\Delta A_{\eta,d}$/ml·h.

Figure 2. Cellulase activity of DEAE-Sepharose fractions on agarose gel containing carboxymethylcellulose. Each 3 μL of DEAE-Sepharose fractions #21 to #45 was dotted on 2% agarose gel containing 0.1% CMC. The dotted gel was incubated at 37°C for 2 h and stained with 0.1% Congo red for 30 min, followed by destaining in 1 M NaCl. Fractions #23 to #26 (marked) showed transparent spots on the gel, indicating that cellulases in those fractions degraded CMC.

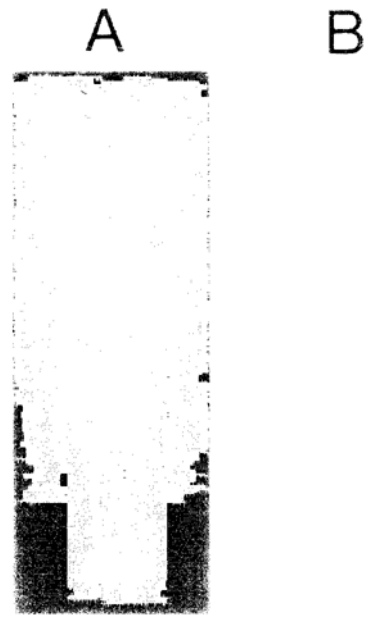

Figure 3. Detection of cellulase activity of DEAE-Sepharose active fraction using nondenaturing gel and agarose replica. Fraction #24 was concentrated and loaded on 7.5% nondenaturing polyacrylamide gel. After electrophoresis, it was overlaid on a 2% agarose gel containing 0.1% CMC at 37°C for 2 h. After transfer and CMC hydrolysis, the nondenaturing gel was stained with Coomassie Brilliant Blue (A) and the agarose replica was stained with Congo red (B).

#18, with fraction #17 containing the highest activity. The result was consistent with the bright spots on agarose gel shown in Figure 4. In Figure 5, the cellulase activity in fraction #17 was shown by activity staining on agarose replica containing CMC.

Fractions #17 and #18 of the Superdex-200 column were pooled, concentrated and loaded onto an

Figure 4. Cellulase activity of Superdex-200 fractions on agarose gel containing carboxymethylcellulose. Each 3 μL fraction from #1 to #25 was dotted on 2% agarose gel contaning 0.1% CMC. The dotted gel was treated similarly as described in Figure 2. Fractions #17 and #18 (marked) showed transparent spots on the gel.

Figure 5. Detection of cellulase activity of Superdex-200 active fraction using nondenaturing gel and agarose replica. Fraction #18 was concentrated and loaded on a 7.5% nondenaturing polyacrylamide gel. After electrophoresis, it was identically treated as described in Figure 3. The gel was stained with Coomassie Brilliant Blue (A) and the agarose replica was stained with Congo red (B).

HA column. Figure 1C shows the elution profile from HA chromatography. Activities were so weak that the Somogyi-Nelson assay method was not useable. Moreover, potassium phosphate seemed to inhibit the activity. Activity was confirmed by dotting samples on agarose gel containing CMC (data not shown).

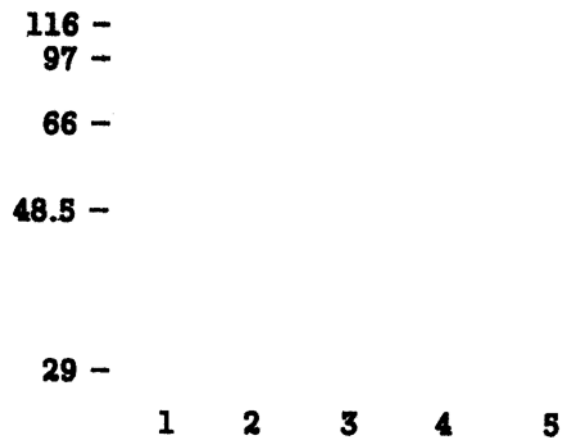

Figure 6. SDS-polyacrylamide gel electrophoresis of samples at various stage of purification. Active fractions of each column were separated by a SDS-polyacrylamide gel (4% stacking / 10% separating) and proteins were visualized by Coomassie Brilliant Blue staining. The standard molecular masses (kD) are indicated in lane 1. Lane 2, Crude extract; lane 3, DEAE active fraction; lane 4, Superdex-200 active fraction; and lane 5, HA active fraction.

Activity of the eluted samples was greatest in fraction #24, although it showed reduced activity compared to that from either DEAE-Sepharose or Superdex-200 purifications (data not shown).

In Figure 6 is shown the protein pattern of active fractions from each chromatography step on 10% SDS-polyacrylamide gel. The number of protein bands was reduced gradually as purification proceeded. In the active fraction from the HA column, one protein band was apparent whose molecular mass was estimated to be 53 kD.

The purification steps for cellulase are presented in Table 1. The amount of total protein was reduced from 258.99 mg of crude extract to 0.3 mg of active fractions recovered from the HA column. The specific activity of partially-purified cellulase was 1.2 Unit/mg. Enzymatic activity was purified only 10-fold relative to the activity of the crude extract, partly because the activity dropped significantly after HA chromatography. The N-terminal amino acid sequence of this cellulase was found to be NH_2-AGAKGANXLGGLXRA.

Characterization of Purified Cellulase

Partially-purified cellulase from Superdex-200 chromatography was used for the characterization study, because the activity of highly-purified cellulase from

Table 1. Purification of wall-localized cellulase from maize coleoptiles.

Purification step	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)	Purification fold	Recovery (%)
Crude extract	258.99	29.90	0.12	1.0	100
DEAE	54.92	8.87	0.16	1.3	30
Superdex-200	5.45	4.73	0.87	7.3	16
HA	0.30	0.36	1.20	10.0	1

*Unit : amount of enzyme liberating 1 μM glucose from CMC in 1 h.

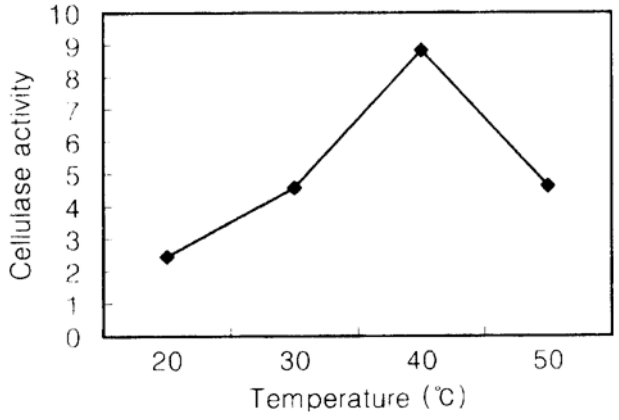

Figure 7. The effect of temperature on cellulase activities. Fractions #17 and #18 of the Superdex-200 column were pooled and concentrated. The concentrated sample was assayed for reducing sugar by Somogyi-Nelson's method. Incubation time of the mixture of enzyme and substrate solution was 1 h. ※ Cellulase activity : ΔA_{650}/mg·h.

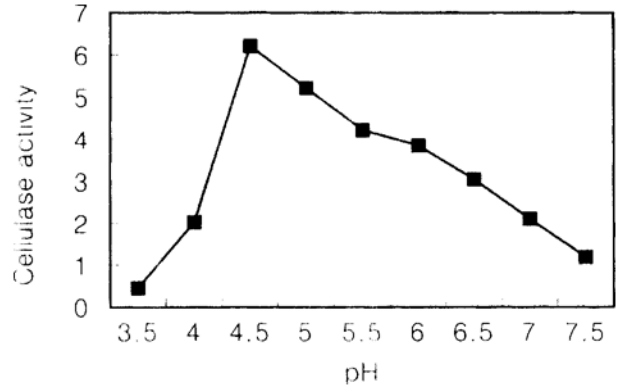

Figure 8. The effect of pH on cellulase activities. Samples were prepared as described in Figure 7, and assayed for reducing sugar by Somogyi-Nelson's method. Incubation time of the mixture of enzyme and substrate solution was 1 h. Buffers for the assay were as follows: 0.1 M citric acid (pH 3.5, 4, 4.5, 5); 0.1 M MES (pH 5.5, 6, 6.5); 0.1 M Tris-Cl (pH 7, 7.5). ※ Cellulase activity : ΔA_{650}/mg·h.

the HA column was too low to be used. The optimal temperature for activity in the partially-purified cellulase was assumed to be 40°C (Fig. 7). The activity declined at temperatures above 40°C. The activity of unbound active fractions from DEAE-Sepharose column was also examined using similar assay conditions. Activity of the unbound active fraction from DEAE-Sepharose was not seriously affected by temperature (data not shown). The pH effect on the activity of the Superdex-200 active fraction is shown in Figure 8. The optimal pH of this partially-purified cellulase was pH 4.5; activity declined rapidly below this pH.

Table 2 summarizes the activity of partially-purified cellulase with a variety of substrates having 1,4-β-glucosyl linkages in their structure. Under the standard conditions, the enzyme did not hydrolyze cellobiose at all. The enzyme specifically cleaved the 1,3- or 1,4-β-glucosyl linkages of carboxymethylcellulose, lichenan, fibrous cellulose, laminarin, α-cellulose and xylan, but the latter two substrates were hydrolyzed very slowly. It was shown that this partially-purified cellulase had even higher affinity for fibrous cellulose

Table 2. Substrate specificity of cellulase.

Substrate	Relative activity (%)
Carboxymethylcellulose	100
Fibrous cellulose	193.68
α-Cellulose	26.48
Lichenan	122.13
Laminarin	67.98
Xylan	10.28
Cellobiose	0

Concentration of each substrate solution was 0.5% (w/v) in 0.01 M citric acid (pH 4.5). The activity against carboxymethylcellulose was taken as 100%.

Table 3. Effects of reducing and sulfhydryl-reacting reagents on cellulase activity.

Compound (1mM)	Relative activity (%)
None	100
Cysteine-HCl	655.0
Dithiothreitol	960.7
Glutathione	396.4
N-Ethylmaleimide	106.4

Activity against 0.5% carboxymethylcellulose as substrate in the presence of 1 mM of each reagent was measured. The activity with no reagent was taken as 100% for comparison.

rather than carboxymethylcellulose.

The effects of reducing and sulfhydryl-reacting reagents on the partially-purified enzyme were examined (Table 3). The activity of the enzyme was strongly enhanced by cysteine-HCl, dithiothreitol and glutathione. N-Ethylmaleimide as a sulfhydryl-reacting reagent did not seem to change the activity. The K_m and V_{max} values of partially-purified cellulase were determined at pH 4.5 and 37°C with CMC in 0.01 M citric acid. Its K_m and V_{max} were estimated to be 6.1 mg/mL and 81 μg/h/mL respectively.

DISCUSSION

Cellulase was partially purified from crude extract of cell wall proteins from maize coleoptiles and its biochemical characterization was performed in this study. Salt was generally used for the extraction of cell wall proteins. Likewise, 1 M NaCl was used for the extraction of cell wall proteins from stems of pea seedlings (Byrne et al., 1975) and 0.1-0.5 M NaCl was added for extraction and fractionation of wall-bound proteins from suspension-cultured poplar cells (Ohmiya et al., 1995). Others have used 3 M LiCl to extract cell wall proteins from *Z. mays* seedlings (Hatfield and Nevins, 1987). We used 0.1 M $CaCl_2$ for the extraction of cell wall proteins. This concentration of $CaCl_2$ was also used for extraction of cell wall proteins from maize seedlings (Nematollahi, 1996).

The viscometric method (Awad and Lewis, 1980) and reducing sugar method (Somogyi, 1952) have been generally used for cellulase activity assays. We used the reducing sugar method because it is generally more rapid and sensitive than the viscometric one. However, the reducing sugar method is adversely affected by several substances such as ammonium sulfate and cellobiose.

For the purification of cellulase from crude extracts, a variety of columns were tried. Cellulose affinity columns have been used effectively for purification of cellulase from tobacco callus (Truelsen and Wyndaele, 1991), kidney bean abscission zone (Koehler et al., 1981) and ripening avocado fruit (Bennett and Christoffersen, 1986). We tried to use a cellulose affinity column but the detection of cellulase activity was too difficult, with the cellobiose used for eluting cellulase from the column deterring the reducing sugar assay.

Hydrophobic interaction columns are very effective in isolating particular proteins. A Phenyl-Sepharose column was also tested in this project. However, ammonium sulfate salt used for the equilibration and elution proved to inhibit the reducing sugar assay and detection of activity on agarose gels containing CMC. Therefore, DEAE-Sepharose, Superdex-200 and HA columns were used for the purification, but only a 10-fold increase of specific activity was obtained as a result (Table 1).

The optimum temperature for the cellulase activity of the two different fractions, the DEAE unbound and the Superdex-200 active fractions, was examined in the temperature range of 20 to 50°C The optimum temperature of partially-purified cellulase was determined to be 40°C (Fig. 7), whereas activity of the DEAE unbound fraction was maximum in the range of 30 to 40°C (data not shown).

The pH-dependence of the enzyme was studied from pH 3.5 to 7.5. The optimum pH of the DEAE unbound active fraction was 5.0 (data not shown), whereas the partially-purified cellulase showed an optimum pH at 4.5 (Fig. 8). The various cellulases examined so far have optimum pH as follows: pH 5.5-6.0 for pea epicotyl (Byrne et al., 1975), pH 5.5-6.5 for tobacco callus (Truelsen and Wyndaele, 1991), pH 6.0 for cultured poplar cell (Nakamura and Hayashi, 1993), pH 5.7-6.2 for bean cotyledon (Lew and Lewis, 1974) and pH 6.0-8.5 for bean abscission zones (Durbin and Lewis, 1988).

Substrate-specificity of the partially-purified cellulase with several substrates containing 1,4-β-glucosyl or 1,3-β-glucosyl linkages was also examined (Table 2). CMC, fibrous cellulose and lichenan proved to be effective substrates. CMC and fibrous cellulose have 1,4-β-glucan in their structures and lichenan has a mixed 1,3/1,4-β-glucan. It is interesting that this cellulase had even higher affinity for fibrous cellulose than CMC. Previously, no hydrolytic activity of purified cellulase from pea epicotyls was detected against 1,4-β-xylan or 1,3-β-glucan laminarin (Wong et al., 1977; Hatfield and Nevins, 1986). But our partially-purified cellulase from maize hydrolyzed laminarin and xylan slightly. Further experiments will be needed to see whether this is due to any contaminant enzyme in the preparation. In substrate specificity experiments, reducing sugar assays were found to give more accurate activity values than viscometric assays (Truelsen and Wyndaele, 1991). This might reflect the different chain lengths of the substrates. The substrate specificities of purified cellulase from various plant species have been determined in only a few studies. Although the available data are limited, purified enzymes in general show higher activity in vitro toward CMC and mixed-linkage 1,3/1,4-β-glucan but

exhibited differing affinities for xyloglucan, a potential in vivo substrate (Brummell et al., 1994).

Reducing reagents such as cysteine-HCl, dithiothreitol and glutathione strongly enhanced the activity (Table 3). According to Ohmiya et al. (1995), the SH-groups of the enzyme seem to be protected by such reducing reagents from oxidation. In the case of the sulfhydryl-reacting reagent, N-ethylmaleimide did not inhibit activity (Table 3). Sulfhydryl-reacting reagents are known to form disulfide bonds in the enzyme at irregular positions. If cysteine residues were located at the active site of the enzyme, activity should have been reduced by the sulfhydryl-reacting reagent. Therefore, it is assumed that cysteine residues are not located near the active sites of cellulase.

The partially-purified cellulase catalyzed the endo-hydrolysis of CMC with an apparent K_m of 6.1 mg/mL and a V_{max} of 81 g/h/mL. Compared with the K_m of 1.2 mg/mL of wall-bound endo-1,4-β-glucanase from suspension-cultured poplar cells (Ohmiya et al., 1995) and 2.5 mg/mL of pea endo-1,4-β-glucanase (Maclachlan, 1988), the K_m value of maize cell wall cellulase is higher, indicating a possible lower affinity for CMC. The V_{max} of other cellulases were 93 μmol Glc/mol enzyme/min from pea stem buffer-soluble endo-1,4-β-glucanase, 201 μmol Glc/μmol enzyme/min from pea stem buffer-insoluble endo-1,4-β-glucanase (Wong et al., 1977) and 5.0 μg Glc/h/mL from tobacco (Truelsen and Wyndaele, 1991). Very few plant endo-1,4-β-glucanases have been purified sufficiently and in adequate quantities to allow accurate determinations of K_m and V_{max} versus different substrates (Brummell et al., 1994).

The molecular mass of partially-purified cellulase in this study was estimated to be 53 kD by SDS-PAGE (Fig. 6). With the exception of the 20 kD cellulase of pea epicotyls, plant cellulases that have been purified to homogeneity possess apparent molecular weights of 50 to 70 kD (Brummell et al., 1994).

It has been thought that the cellulases located at plant cell walls are related to cell growth. Cellulase seems to play a key role in cell wall loosening. In auxin-treated plants, wall-bound cellulases degrade xyloglucan tightly bound to the cellulose microfibrils (McDougall and Fry, 1990). This action of cellulase may account for the wall-loosening and hence growth-promoting effect of auxin (Fry, 1989).

Further characterization of this purified cellulase is in progress. Although we have obtained some partial N-terminal amino acid sequences of this purified cellulase, they did not show higher homology to any known cellulase sequences. In higher plants, the N-terminal sequences of poplar (wall-bound, extracellular), avocado, bean, pea (wall-bound) and tomato were previously reported and compared (Brummell et al., 1994). It will be very useful to raise antibodies against our partially-purified cellulase in the near future. The function and exact location of this cellulase in maize coleoptiles could be determined precisely when either polyclonal or monoclonal antibodies against this enzyme are available.

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LITERATURE CITED

Allen RD, Lessler CL, Galewsky S, Newmann AJ (1984) The role of cellulase in hyalocyst pore formation in the moss *Syrrhopodon texanus*. Annals of Botany 53: 431-438

Awad M, Lewis LN (1980) Avocado cellulase: extraction and purification. Food Sci 45: 1625-1628

Beguin P (1983) Detection of cellulase activity in polyacrylamide gels using Congo red-stained agar replicas. Anal Biochem 131: 333-336

Bennett AB, Christoffersen RE (1986) Synthesis and processing of cellulase from ripening avocado fruit. Plant Physiol 81: 830-835

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Brummell DA, Lashbrook CC, Bennett AB (1994) Plant endo-1,4-β-D-glucanases: structure, properties, and physiological function. *In* ME Himmel, JO Baker, RP Overend, eds, Enzymatic conversion of biomass for fuels production, ACS Symposium Series 566, pp 100-129

Byrne H, Christou NV, Verma DPS, Maclachlan GA (1975) Purification and characterization of two cellulases from auxin-treated pea epicotyls. J Biol Chem 250: 1012-1018

Durbin ML, Lewis LN (1988) Cellulase in *Phaseolus vulgaris*. Methods in Enzymol 160: 342-351

Fry SC (1989) Cellulases, hemicelluloses and auxin-stimulated growth: a possible relationship. Physiol Plant 75: 532-536

Fry SC (1995) Polysaccharide-modifying enzymes in the plant cell wall. Annu Rev Plant Physiol Plant Mol Biol 46: 497-520

Hatfield RD, Nevins DJ (1986) Characterization of the hydrolytic activity of avocado cellulase. Plant Cell Physiol 27: 541-552

Hatfield RD, Nevins DJ (1987) Hydrolytic activity and substrate specificity of an endoglucanase from *Zea mays* seedling cell walls. Plant Physiol 83: 203-207

Hoson T (1993) Regulation of polysaccharide breakdown during auxin-induced cell wall loosening. J Plant Res 106: 369-381

Jung HI, Kim SI, Ha KS, Joe CO, Kang KW (1995) Isolation and characterization of guamerin, a new human leukocyte elastase inhibitor from *Hirudo nipponia*. J Biol Chem 270: 13879-13884

Kemmerer EC, Tucker ML (1994) Comparative study of cellulases associated with adventitious root inhibition, apical buds, and leaf, flower, and pod abscission zones in soybean. Plant Physiol 104: 557-562

Koehler DE, Lewis LN, Shannon LM, Durbin ML (1981) Purification of a cellulase from kidney bean abscission zones. Phytochemistry 20: 409-412

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277: 680-685

Lew FT, Lewis LN (1974) Purification and properties of cellulase from *Phaseolus vulgaris*. Phytochemistry 13: 1359-1366

Maclachlan G (1988) β-Glucanases from *Pisum sativum*. Methods in Enzymol 160: 382-391

McDougall GJ, Fry SC (1990) Xyloglucan oligosaccharides promote growth and activate cellulase: evidence for a role of cellulase in cell expansion. Plant Physiol 93: 1042-1048

Nakamura S, Mori H, Sakai F, Hayashi T (1995) Cloning and sequencing of a cDNA for poplar endo-1,4-β-glucanase. Plant Cell Physiol 36: 1229-1235

Nakamura S, Hayashi T (1993) Purification and properties of an extracellular endo-1,4-β-glucanase from suspension-cultured poplar cells. Plant Cell Physiol 34: 1009-1013

Nematollahi WPS (1996) Studies on a β-glucosidase from the cell walls of maize (*Zea mays* L.). Ph. D. dissertation. Univ of Texas at Austin, TX, USA

Ohmiya Y, Takeda T, Nakamura S, Sakai F, Hayashi T (1995) Purification and properties of a wall-bound endo-1,4-β-glucanase from suspension-cultured poplar cell. Plant Cell Physiol 36: 607-614

Somogyi N (1952) Notes on sugar determination. J Biol Chem 195: 19-23

Standal R, Iversen TG, Coucheron DH, Fjærvik E, Blantny JM, Valla S (1994) A new gene required for cellulose production and a gene encoding cellulolytic activity in *Acetobacter xylinum* are colocalized with the *bcs* operon. J Bacteriol 176: 665-672

Taiz L, Zeiger E (1991) Plant Physiology, Benjamin/Cummings Publishing Co, Redwood City, CA, pp 22-25

Truelsen TA, Wyndaele R (1991) Cellulase in tobacco callus: regulation and purification. J Plant Physiol 139: 129-134

Wong YS, Fincher GB, Maclachlan G (1977) Kinetic properties and substrate specificities of two cellulases from auxin-treated pea epicotyls. J Biol Chem 252: 1402-1407

Yu SC, Kim ES, Kim WK (1994) Immunocytochemical localization of cellulase on endosperm of ginseng seed. J Plant Biol 37: 435-439