Assessment of the Endo-1,4-β-Glucanase Components of Ruminococcus flavefaciens FD-1

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The extracellular endo-1,4-β-glucanase components of Ruminococcus flavefaciens FD-1 were analyzed by high-performance liquid chromatography (HPLC) by using DEAE ion-exchange, hydroxyapatite, and gel filtration chromatography and polyacrylamide gel electrophoresis (PAGE). Two endo-1,4-β-glucanase peaks were resolved by DEAE-HPLC and termed endoglucanases A and B. Carboxymethyl cellulose (CMC) zymograms were achieved by enzyme separation using nondenaturing PAGE followed by incubation of the gel on top of a CMC-agarose gel. This revealed no less than 13 and 5 endo-1,4-β-glucanase components present in endoglucanases A and B, respectively. Hydroxyapatite chromatography of endoglucanases A and B revealed one activity peak for each preparation, which contained 4 and 5 endo-1,4-β-glucanase components, respectively. Gel filtration chromatography of endoglucanase A following hydroxyapatite chromatography resolved the most active carboxymethylcellulase (CMCase) component from other endo-1,4-β-glucanase activities. Gel filtration of endoglucanase B following hydroxyapatite chromatography showed one CMCase activity peak. Protein stains of sodium dodecyl sulfate-PAGE and nondenaturing PAGE gels of endoglucanases A and B from hydroxyapatite and gel filtration chromatography revealed multiple protein components. When xylan was substituted for CMC in zymograms, identical separation patterns for CMCase and xylanase activities were observed for both endoglucanases A and B. These data suggest that both 1,β-4 linkage-hydrolyzing activities reside on the same polypeptide or protein complex. The highest endo-1,4-β-glucanase-specific activities were observed following DEAE-HPLC chromatography, with 16.2 and 7.5 μmol of glucose equivalents per min per mg of protein for endoglucanases A and B, respectively. CMCase activities decreased after the two subsequent purification steps, resulting in specific activities of 0.02 and 1.4 for endoglucanases A and B, respectively.

Extracellular cellulose hydrolysis typically results from the action of at least three classes of cellulase enzymes. According to some hypotheses, these enzymes act synergistically in the hydrolysis of the insoluble substrate. Endo-1,4-β-glucanase (EC 3.2.1.4; endoglucanase; carboxymethylcellulase) randomly hydrolyzes 1,4-β bonds along the interior of the cellulose chain, producing reducing and nonreducing ends while also creating shorter water-soluble polymers (up to seven glucose moieties) called cellulodextrins. Exo-1,4-β-glucanase (EC 3.2.1.91; exoglucanase) cleaves cellulose units from the nonreducing ends of the cellulose polymer. The third enzyme, β-D-glucoside glucohydrolase (EC 3.2.1.21; β-glucosidase), hydrolyzes cellulobiose, yielding two glucose molecules (36).

Even though in theory only three enzymes are required to hydrolyze cellulose, bacterial cellulase systems are typically composed of many β-glucanase enzymes. For example, the cellulase system of Clostridium thermocellum contains at least 15 polypeptides, many of which have glucanase activity (12, 18, 42). This multiplicity of enzyme activities appears to be the result of three major factors: the existence of multi-gene cellulase families (10–14, 43), glycosylation of cellulase gene products (3, 9, 15, 19–21, 22, 27, 44), and cleavage of cellulase gene products by host-produced proteolysis (6, 20, 23).

The cellulase system of Ruminococcus flavefaciens contains endoglucanase (29, 30) and exoglucanase (8) but differs from the scheme stated above in that no extracellular β-glucosidase activity has been reported. However, hydrolysis of cellulodextrins has been observed (31). This cellulodextrinase activity has been shown to be due to an enzyme(s) separate from those which act solely on insoluble cellulose. Cellulobiase and celalobiase are end products of cellulose degradation of R. flavefaciens (31). Pettipher and Latham (29) noted that the xylanase activity of R. flavefaciens copurified with carboxymethylcellulase (CMCase) activity but suggested that the active site(s) for each activity was distinct, possibly residing on the same polypeptide.

We addressed the complexity and nature of extracellular endo-1,4-β-glucanase components of R. flavefaciens FD-1 by using various column chromatography and polyacrylamide gel electrophoresis (PAGE) techniques. The results of the partial purification, characterization, and enumeration of these endo-1,4-β-glucanase components are presented here. (A preliminary account of these data has been presented [Abstr. Conf. Rumen Function, Chicago, Ill., 1987, p. 36] [Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, K-29, p. 211]. These data were also presented in part by K. C. Doerner [M.S. thesis, University of Illinois, Urbana, Ill., 1989].)

MATERIALS AND METHODS

Organism and culture conditions. R. flavefaciens FD-1 was obtained from the stock culture collection of the Department of Animal Sciences, University of Illinois, Urbana, Ill. Cells were maintained and grown at 39°C by using the defined medium of Gardner et al. (8) containing 0.12% (wt/vol) cellulose as the sole energy and carbon source.

Crude cellulase preparation. A 500-ml 5-day-old culture was used to inoculate 7 liters of defined medium in one carboy. After 8 days of growth, the culture supernatant was harvested by centrifugation (7,970 × g; 25 min; 1°C). During
centrifugation, CO₂ was used to overlay both the culture and the culture supernatant to prevent a pH change. The supernatant was concentrated by using a stirred ultrafiltration cell equipped with a 300,000-Mₚ cutoff membrane (YM30; Amicon Corp., Lexington, Mass.; 4°C; under 8 lb/in² of CO₂). Ultracentrifugation (105,000 × g; 1 h; 4°C) was used to further remove cells and any other insoluble material from the concentrated sample, and the resulting supernatant was used for further purification. This and all subsequent enzyme preparations were maintained aerobically at 4°C. These experiments were reproduced with five different enzyme preparations.

**Enzyme purification.** A preparative high-performance liquid chromatography (HPLC) anion-exchange column (Sphero gel TSK DEAE-5PW; 21.5 mm by 15 cm; Beckman Instruments, Inc., Fullerton, Calif.) was equilibrated with 0.05 M sodium phosphate (pH 8.0) at a flow rate of 6 ml/min. The crude cellulase preparation was then vacuum filtered through a 0.45-μm-pore-size membrane and applied to the column. Protein was eluted with a linear NaCl gradient of 0 to 0.5 M in the sodium phosphate buffer. Fractions were collected at 1-min intervals and immediately placed on ice. Further purification was achieved through the use of an HPLC hydroxylapatite column (Bio-Gel HPHT; 7.8 by 100 mm; Bio-Rad Laboratories, Richmond, Calif.) with the guard column recommended by the manufacturer (50 by 4.0 mm). This column was equilibrated with 0.01 M sodium phosphate (pH 7.0) −10⁻³ M CaCl₂ −0.02% (wt/vol) sodium azide at a flow rate of 0.5 ml/min. Pooled fractions from DEAE chromatography were diluted 1/4 with distilled water to decrease the ionic strength of the buffer, allowing protein to be retained on the resin. The sample was then pumped onto the column and eluted by increasing the ionic strength of the mobile phase to 0.35 M sodium phosphate. Fractions were collected and handled as described above.

Gel filtration chromatography was accomplished by HPLC by using either an analytical (Sphero gel TSK 4000-SW; 7.5 by 300 mm; flow rate, 0.5 ml/min; Beckman Instruments, Inc.) or preparative model of the same column (21.5 mm by 30 cm; flow rate, 4 ml/min). The recommended precolumn (TSK Pre-SW column; 21.5 mm by 7.5 cm) was used in conjunction with the preparative column. Protein was eluted isocratically after both columns were equilibrated with 0.02 M Pipes (piperazine-N,N',N''-bis(2-ethanesulfonic acid)), pH 6.8, containing 0.1 M NaCl. Gel filtration columns were calibrated before and after each chromatography run with a standard which included bovine thyroglobulin (M₀, 670,000), bovine gamma globulin (M₀, 158,000), chicken ovalbumin (M₀, 44,000), horse myoglobin (M₀, 17,000), and vitamin B₁₂ (M₀, 1,350) (Bio-Rad Laboratories).

Exo-1,4-β-glucanase A (Exo A) from *R. flavefaciens* FD-1 was partially purified by DEAE chromatography as previously described (8).

**Enzyme assays.** Endoglucanase assays were conducted with 1% (wt/vol) carboxymethyl cellulose (CMC) (low viscosity; Sigma Chemical Co., St. Louis, Mo.) in 0.02 M PIPES (pH 6.8) with 5% (vol/vol) of each mineral solution 1 and 2 (5) (PIPES 1 and 2) at 39°C. Xylanase assays were conducted with 0.5% (wt/vol) oat spelt xylan (Sigma Chemical Co.) in 0.02 M PIPES 1 and 2 (pH 6.8) at 39°C. An enzyme sample (0.01 to 0.05 ml) was added to the temperature-equilibrated assay mixture (0.2 ml, final volume) to start the reaction. Typical incubation time was 20 min but ranged between 10 min and 1 h. The reaction was stopped by adding 0.1 ml of 1% (wt/vol) NaOH with 1% (wt/vol) Na₂HPO₄ · 2H₂O (stop reagent) to the assay solutions, which were immediately placed on ice. Separate assay mixtures were incubated on ice with stop reagent added prior to the enzyme to correct for background reducing sugar. A modification of the potassium ferricyanide method (28) was used for colorimetric determination of the reducing sugar content. A sample of 0.15 ml of 0.116% (wt/vol) K₃[Fe(CN)]₆·1.0% (wt/vol) Na₂HPO₄ · 7H₂O·0.22% (wt/vol) NaOH was added to the assay tubes, which were placed in boiling water for approximately 3 min. Activity was determined by comparison to a D-glucose standard curve measured at A₄₂₀. Units are expressed in micromoles of glucose equivalents released per minute. All specific activities were determined to be linear with time and protein concentration.

Assays in which radiolabeled cellulose ([14C]cellulose, *Canna indica*, 3.4 Ci/mg; ICN Radiochemicals, Irvine, Calif.) was used were performed as described by Du Preez and Kistner (7), except that the buffer for the enzyme assay was PIPES 1 and 2.

**Electrophoresis.** Nondenaturing PAGE (ND-PAGE) was performed by using a discontinuous buffer system as described by Calza et al. (6). A 12% (wt/vol) polyacrylamide resolving gel in 0.06 M Tris hydrochloride, pH 8.0, was used with a 4% (wt/vol) polyacrylamide stacking gel in 0.086 M Tris hydrochloride (pH 7.0). A 1/10 volume of chilled 50% (wt/vol) sucrose and 0.2% (wt/vol) bromophenol blue solution was added to the sample before loading into the well. Sodium dodecyl sulfate-PAGE was performed as described by Laemmli (17), except that a 4% (wt/vol) polyacrylamide stacking gel and a 5% (wt/vol) polyacrylamide resolving gel were used. All PAGE was performed with slab gels (10.2 cm by 5 cm by 0.75 mm) (mini-PAGE) or larger slab gels (14.2 cm by 16 cm by 0.75 mm). Mini-PAGE gels were subjected to electrophoresis at 200 constant volts, otherwise PAGE was performed at 0.025 constant amperes. Protein bands were visualized by using the silver stain method of Merrill et al. (24).

**Zymograms.** Carboxymethylcellulose zymograms were carried out by using a ND-PAGE gel overlaid on a 0.7% (wt/vol) agarose gel containing 1.25% (wt/vol) CMC in PIPES 1 and 2 buffer. Xylan zymograms were carried out by using a ND-PAGE gel overlaid on a 0.7% (wt/vol) agarose gel containing 0.5% (wt/vol) polyacrylamide resolving gel and 5% (wt/vol) agarose gel. All PAGE and slab gels were placed on the substrate-containing gel, while not allowing air bubbles to form in the sandwich. The zymogram was incubated for 12 to 24 h in a humidified incubator at 39°C. The polyacrylamide gel was then removed, and the surface of the agarose-CMC or agarose-xylan gel was flooded with Congo red (1 mg/ml, filtered through a 0.65-μm-pore-size membrane) for 30 min, followed by 1 M NaCl treatment for 15 min to obtain clear activity bands against the red background (2, 4, 38).

**Protein concentration determination.** Protein concentration was determined by either the bicinchoninic acid method (37) or by using the equation described by Kalb and Bernlohr (16).

**RESULTS**

**Preparation of crude cellulase.** At 8 days of incubation, few whole cellulose disks were seen, while cellulose fragments remained. Harvesting the culture supernatant at this stage of growth yielded large amounts of soluble CMCase, evidenced by the observation that over 85% of the total endoglucanase activity present in the culture was in the culture supernatant. This supernatant, however, became turbid with residual...
cells and particulate material upon concentration. The particulate material in the cell culture supernatant clogged all ultrafiltration membranes tried. To minimize this problem, we used a high-molecular-weight-cutoff membrane \( (M_w, 300,000) \). This, however, still tended to clog, which in turn caused some leakage around the membrane; therefore, we operated the filtration unit at low pressures \( (8 \text{ lb/in}^2) \). The CMCase components which passed by the membrane yielded the same DEAE chromatogram as those which were retained by the membrane (Fig. 1). These data suggest that the sample retained by the membrane represents all of the extracellular CMCase components. The filtration step worked poorly as a molecular sieve but did concentrate the sample to a manageable volume \( (50 \text{ ml}) \). In an effort to develop alternative strategies for harvesting endoglucanase from the culture supernatant, however, we found that a 75% ammonium sulfate precipitation yielded less than 10% recoverable activity. We concluded that the ultrafiltration step was our best alternative. Before applying the ultrafiltration sample to HPLC columns, removal of the particulate material by ultracentrifugation was necessary. The supernatant from the ultracentrifugation was used for further experiments and represented 76% of the total CMCase activity present in the cell culture supernatant. The remaining activity was present in the filtered solution from the concentration step.

**HPLC of crude cellulase preparation.** DEAE chromatography of the supernatant from ultracentrifugation yielded two major endoglucanase peaks, designated endoglucanase A and endoglucanase B (Fig. 1). Endoglucanase A eluted at 0.22 M NaCl, and the second peak, endoglucanase B, eluted at 0.37 M NaCl. Exo A (8) was observed eluting between these two major endoglucanase activity peaks and coincided with a small CMCase peak, which could be due to synergistic activity between Exo A and the endoglucanases.

Both endoglucanase preparations showed sharp, coeluting, symmetrical activity and \( A_{280} \) peaks when fractionated by hydroxylapatite chromatography (Fig. 1). It is possible this coelution of the CMCase in each sample could have been due to a tendency of the proteins to aggregate. If the proteins were aggregating, it is probable that this is a specific interaction, as not all CMCase components eluted at this location. We did, however, explore alternative chromatography methods to determine whether separation could be improved. We determined that *R. flavefaciens* protein did not bind to an HPLC cation-exchange column equilibrated at \( pH \geq 4.6 \) and bound irreversibly to a phenyl-Sepharose column. These data indicated that neither resin would improve separation. Preparative isoelectric focusing (Rotofor cell apparatus; Bio-Rad) was also used, but carrier ampholytes interfered with the reducing sugar assay, and thus separation and detection were not possible since the enzyme preparations lost activity upon dialysis to remove the ampholytes. Betaine was included during anion-exchange chromatography to decrease protein-protein interactions but failed to alter protein elution patterns.

Endoglucanase A activity eluted in a broad peak with an estimated \( M_f \) of 140,000 when fractionated by using gel filtration chromatography (data not shown). Also, two broad shoulders with lower activity were observed following the 140,000-\( M_f \) peak. Mini-ND-PAGE of the 140,000-\( M_f \) sample stained for protein showed that no less than eight components were present. Since preparative gel filtration was used, only one chromatography experiment was performed; therefore, these data have not been duplicated.

Gel filtration chromatography of endoglucanase B revealed results similar to those for endoglucanase A. Not all of the CMCase components applied to the column were observed in the resulting fractions. The major activity observed corresponded to an \( M_f \) of 100,000 (data not shown). Again, mini-ND-PAGE containing a sample of this activity peak showed no less than eight protein components.

Zymograms of endoglucanases A and B following hydroxylapatite and gel filtration chromatography show that both endoglucanase A and endoglucanase B still contained multiple CMCase components (Fig. 2). Endoglucanase A following hydroxylapatite chromatography still contained no less than four CMCase components, while endoglucanase B following hydroxylapatite chromatography still contained no less than five CMCase components (Fig. 2). The major activity peak of endoglucanase A from gel filtration chromatography contained only one CMCase component (Fig. 2). The other CMCase components applied to the gel filtration column were not observed in the major activity peak. The fraction from endoglucanase B, with an \( M_f \) of 100,000,
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FIG. 2. CMC zymogram of hydroxylapatite and gel filtration samples of endoglucanase A and B. Lanes: 1, 10 \( \mu \)g of endoglucanase A following hydroxylapatite chromatography; 2, 24 \( \mu \)g of endoglucanase B following hydroxylapatite chromatography; 3, 43 \( \mu \)g of endoglucanase A following gel filtration chromatography; 4, 3 \( \mu \)g of \( M_r \) 100,000 from endoglucanase B gel filtration chromatography. A 1.5-mm-thick ND-PAGE gel was used for this experiment. Arrows denote the predominant activity bands.

contained one major CMCase component as well as other less active components (Fig. 2).

The CMCase polypeptides of endoglucanases A and B could only be partially purified. Maximum specific activities obtained were observed following DEAE chromatography, which were 16.2 U/mg of protein for endoglucanase A and 7.5 U/mg of protein for endoglucanase B. Specific activities decreased to 0.02 U/mg of protein and 1.4 U/mg of protein following gel filtration for endoglucanases A and B, respectively.

CMC zymograms of the catalytically active fractions from DEAE chromatography. We felt from the initial results that endoglucanase A and B complexes consisted of more CMCase components than revealed by mini-ND-PAGE and thus analyzed the individual fractions of DEAE chromatography with larger ND-PAGE gels (Fig. 3 and 4). The zymogram of the fractions which compose the endoglucanase A peak indicated that no less than 13 CMCase bands were present (Fig. 3). Samples from DEAE chromatography which eluted between endoglucanases A and B were also examined. These failed to produce any zones of hydrolysis on CMC zymograms (Fig. 4A, lanes 1 through 4). A CMC zymogram of the endoglucanase B region from DEAE chromatography is shown in Fig. 4. The endoglucanase B region contained no less than five CMCase bands, four of which appeared in the most active fraction. The polypeptide patterns of endoglucanase A or endoglucanase B clearly show many noncatalytic proteins present in these CMCase-positive fractions (Fig. 3B and 4B). The protein-stained gel containing the endoglucanase A region from DEAE chromatography shows poor banding of protein that eluted from minutes 29 to 31 (Fig. 3B, lanes 5 to 7). The reason for this is unknown and is probably an artifact of protein staining, as evidenced by the distinct bands detected by the activity gel.

However, it is interesting that these samples correspond to the most active fractions of the CMCase activity peak.

Polysaccharide hydrolyzing activities in endoglucanase A and endoglucanase B. Pettipher and Latham (29) have observed xylanase and CMCase coeluting from gel filtration columns and suggested that these activities of \( \textit{R. flavefaciens} \) are at different active sites but could reside on the same polypeptide or protein complex. We also observed a high xylanase activity in endoglucanases A and B following DEAE chromatography (Table 1). We therefore addressed the question of multifunctional enzymes by performing side-by-side CMC-xylan ND-PAGE zymograms of proteins from fractions with the highest CMCase activities from endoglucanase A and endoglucanase B following DEAE chromatography. Figure 5 shows that many, if not all, of the CMCase bands in endoglucanase A also hydrolyze xylan. The same relationship was observed for endoglucanase B (data not shown). These results support the hypothesis that both CMCase and xylanase active sites reside on the same polypeptide or protein complex, and thus these enzymes may be multifunctional.

Endoglucanases A and B from DEAE chromatography were also tested for hydrolysis of \( ^{14} \text{C} \)cellulose (Table 1).
Endoglucanase A was 1.55- and 1.8-fold higher in specific activity than endoglucanase B against CMC and [14C]cellulose, respectively. The ratio of CMCase to [14C]cellulose hydrolyzing activity was approximately the same for both enzyme preparations, indicating that the CMCase components were hydrolyzing the [14C]cellulose. No apparent synergism in the hydrolysis of CMC or [14C]cellulose was detected between endoglucanase A or B with partially purified Exo A (8). Furthermore, no apparent synergism in the hydrolysis of CMC or [14C]cellulose was detected between endoglucanases A and B either in the presence or absence of Exo A.

**DISCUSSION**

The endoglucanase components of *R. flavefaciens* 67 have previously been investigated (29). These workers reported that cell culture supernatant endo-1,4-β-glucanase existed in three forms, one of ≥3,000,000 daltons and 2 smaller fractions of ≥800,000 and 89,000 daltons, and each of these fractions also contained activity against xylan, filter paper cellulose, and Avicel. Although they suggested that different active sites were probably responsible for CMCase and xylan degradation, no further attempt was made to purify these enzymes and ascertain characteristics of the individual proteins responsible for the observed activities. Our work supports the observation of Pettipher and Latham (29) by showing that endo-1,4-β-glucanase is composed of a variety of components. We also present data suggesting that the high-molecular-weight enzyme complexes must be intact for maximal endo-1,4-β-glucanase activity.

CMCase zymogram experiments in which the fractions of endoglucanase A from DEAE-chromatography were used contained no less than 13 CMCase components, while endoglucanase B appeared less heterogeneous, with at least 5 CMCase components (Fig. 3 and 4). One explanation is that two endoglucanase complexes, each composed of different enzyme species, were resolved by DEAE chromatography. Likewise, it is also possible that some non-CMCase components had a high affinity for the endoglucanase complex and thereby eluted as an aggregate of proteins. The appearance of apparently noncatalytic polypeptides in the CMCase
complexes before and following hydroxyalpate and gel filtration chromatography supports the protein complex hypothesis. This hypothesis is consistent with the previous results of Pettipher and Latham with \textit{R. flavefaciens} 67 (29). Furthermore, it has been shown that endoglucanase components of many cellulolytic organisms exist in large aggregates and are composed of multiple endoglucanase species. \textit{C. thermocellum} is currently thought to have no less than 15 polypeptides with no less than six unique endoglucanase components and a noncatalytic cellulose-binding polypeptide present in a multienzyme complex called the cellulosome (12, 18, 42). Cellulose-grown \textit{Ruminococcus albus} cultures exhibit CMCase activity from a cell-bound complex of >1.5 \times 10^6 daltons (41), and multiple endoglucanase components in cell aggregates of \( M_r > 4 \times 10^6 \) daltons have also been reported in \textit{Bacteroides succinogenes} (33). Therefore, it is likely that the endoglucanases from \textit{R. flavefaciens} also exist in aggregates of catalytic and noncatalytic components. The natures and roles of these noncatalytic polypeptides are not known.

The heterogeneity of the CMCase components from \textit{R. flavefaciens} could be the result of post translational modification of cellulase gene products. Carbohydrate-containing cellulase has been observed in many cellulolytic species. The bacteria \textit{B. succinogenes} (15), \textit{Thermomonospora fusca} (6), and \textit{C. thermocellum} (9) as well as the fungi \textit{Trichoderma viride} (35) and \textit{Robellarda} sp. (40) possess glycosylated cellulases. Glycosylation is also present in \textit{Cellulomonas fimi} cellulase, which is thought to protect regions of nascent enzyme from proteolytic cleavage. This glycosylation does not affect enzyme kinetics or impart stability to changing pH or temperature (20). Alternatively, \textit{Saccharomyces cerevisiae} produces two cellulase enzymes from the same structural gene, having distinct kinetic characteristics due to differing degrees of glycosylation (26). \textit{R. flavefaciens} FD-1 has been shown to produce a variety of carbohydrate-containing proteins, but it remains unclear whether any cellulase is glycosylated (K. C. Doerner, M. S. thesis, University of Illinois, Urbana, 1989).

It should be noted that the heterogeneity of cellulase components in \textit{R. flavefaciens} is also most probably due to the existence of multigene families. Molecular cloning experiments of cellulases from \textit{R. flavefaciens} FD-1 have to date identified at least six different genes encoding CMCase (1; G. T. Howard and B. A. White, Anim. Biotech., in press; White et al., unpublished data; J. A. Thomson, personal communication).

The observation that many of the CMCase proteins also catalyzed the hydrolysis of xylan is interesting. This type of multifunctional cellulase has been reported in \textit{T. viride} (34, 39) and has been suggested for many other systems. This type of cellulase appears to be common to \textit{Ruminococcus}. It has been suggested that CMCases from \textit{R. albus} have activity towards xylan (25). Indeed, a CMCase cloned from \textit{R. albus} SY3 has both CMCase and xylanase activity in a single 56,000-dalton protein encoded from a 4.7-kilobase fragment (32). We have also cloned a CMCase from \textit{R. flavefaciens} FD-1 that appears to be a multifunctional protein with both CMCase and xylanase activity (Howard and White, Anim. Biotech., in press). This type of enzyme may play an important role in plant cell wall hydrolysis and warrants further investigation.

Our present research efforts are directed toward purifying some of these endoglucanase components. Furthermore, we are using molecular analysis of cellulase genes to address the heterogeneity of cellulase components and to determine whether both cellulase and xylanase activities are catalyzed by the same or different active sites.

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**LITERATURE CITED**


