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Purification and Characterization of Two β-1,4-Endoglucanases from *Thermomonospora fusca*†

Roger E. Calza, Diana C. Irwin, and David B. Wilson*

Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853

Received April 2, 1985

ABSTRACT: Two β-1,4-endoglucanases were isolated in nearly pure form from the culture supernatant of *Thermomonospora fusca* strain YX. The purification procedures included gel chromatography, ion-exchange chromatography, hydroxylapatite chromatography, and preparative gel electrophoresis. These enzymes and their proteolytic breakdown products account for at least 90% of the total extracellular carboxymethylcellulase activity, although at least one other β-1,4-endoglucanase is present. Even though these enzymes are essential for the hydrolysis of filter paper by the extracellular fraction, they are not sufficient. The specific activity of the starting material on filter paper was 4 times that of the most active purified enzyme. Even though they are both endoglucanases, these two enzymes differ in their molecular weights, substrate specificities, and carbohydrate contents and are immunologically distinct proteins. The most active enzyme (E_1) has a specific activity greater than 700 IU/mg on carboxymethylcellulose (CMC) when assayed at 55 °C in 0.05 M potassium phosphate buffer, pH 6.5. Its molecular weight is 94 000 determined by both sodium dodecyl sulfate gel electrophoresis and glycerol gradient centrifugation, showing that it is a monomeric enzyme. It contains less than 1% carbohydrate and has an isoelectric point at pH 3.5. Its temperature optimum is 74 °C, and it has a broad pH optimum centered at pH 6. The products of cellulose hydrolysis catalyzed by E_1 are mainly cellobiose with a small amount of glucose. Its K_m for CMC is 360 μg/mL; it is stimulated by Ca^{2+} or Mg^{2+} and totally inhibited by Hg^{2+}. The other enzyme (E_2) has a specific activity of 77 IU/mg on CMC. Its molecular weight is 46 000, and it is also a monomeric enzyme. It is a glycoprotein containing 25% carbohydrate by weight and has an isoelectric point at pH 4.5. Its temperature optimum is 58 °C, and it has a broad pH optimum centered at pH 6. The products of cellulose hydrolysis catalyzed by E_2 are glucose, cellobiose, cellotriose, and higher oligomers. Its K_m for CMC is 120 μg/mL, and it is slightly inhibited by Ca^{2+} or Mg^{2+} and greatly inhibited by Hg^{2+}. Neither enzyme is inhibited appreciably by 10% glucose, but both are about 50% inhibited by 10% cellobiose.

The study of cellulases is of practical importance because of their potential role in the conversion of renewable biomass into chemicals or fuel. Cellulases are also interesting because cooperative interactions between two or more enzymes are required for the optimal degradation of crystalline cellulose. While considerable progress has been made in the characterization of the cellulases produced by the mesophilic fungi *Trichoderma* (Bissett, 1979; Farkas et al., 1982; Shoemaker & Brown, 1978; Weber et al., 1980) and *Sporotrichum pulverulentum* (Erickson & Pettersson, 1978a,b) and the anerobic bacterium *Chlostridum thermocellum* (Ng & Zeikus, 1981b, Petre et al., 1981), much less is known about cellulases produced by thermophilic aerobic bacteria.

The potential advantages of thermophilic enzymes in practical applications center around their great stability. In this work the organism *Thermomonospora fusca* was chosen because it is a thermophilic, aerobic, bacterium that produces a very active cellulase. There have been a number of studies of the crude cellulase produced by different *Thermomonospora* species (Moreira et al., 1981; Hägerdal et al., 1978a,b), but the detailed characterization of purified enzymes has not been reported. This paper describes the purification and characterization of the two most active *T. fusca* β-1,4-endoglucanases.

Materials and Methods

Organism. *Thermomonospora fusca* strain YX was isolated by W. D. Bellamy, Cornell University. This organism has been shown by M. P. Lechevalier to be a thermophilic, filamentous actinomycete. The bacterium contains type III cell wall, forms aerial mycelium, and reacts variably with the Gram stain.

Crude Enzyme Preparation. A 48-h, 200-mL shake culture of *T. fusca* YX was used to inoculate a 14-L fermentor (New Brunswick Scientific Corp., New Brunswick, NJ) containing

† This research was supported by Grant PCM-9319432 from the National Science Foundation and Grant DE-FG02-84 ER13233 from the Department of Energy.

0006-2960/85/0424-7797$01.50/0

9.8 L of medium. The media components are described by Hägerdal et al. (1978a,b). Solka floc (Brown Paper Products, Concord, NH) was added to 1% (w/v) as carbon source. The culture was grown with vigorous aeration at 56 °C for 30 h, and then phenylmethanesulfonyl fluoride (PMSF) was added to 0.1 mM and the culture cooled to ~10 °C. Culture solids were removed by continuous centrifugation through a refrigerated Sharples supercentrifuge rotor (Sharples-Stokes Division, Pennsalt Corp., Warminster, PA). All subsequent purification steps were done at 0–4 °C. Protein in the culture broth was precipitated by adding solid $(NH_4)_2SO_4$ to 80% saturation. The solution was held at 0–4 °C for 1 h, and then the precipitated proteins were collected by centrifugation at 9000*g* for 30 min. The resultant pellet was resuspended in approximately 150 mL of 20 mM potassium phosphate, pH 6.5, per 10 L of starting culture and clarified by centrifugation at 15000*g* for 10 min.

Enzyme Purification. The crude enzyme was initially fractionated on a 2.5 cm by 100 cm column of Ultrogel AcA34 (LKB-Producter AB, Bromma, Sweden). Fifty-milliliter portions of the dissolved $(NH_4)_2SO_4$ sample were run on this column, and the carboxymethylcellulase (CMCase) peak fractions were concentrated 10-fold by ultrafiltration on XM 50 or YM 10 membranes (Amicon Corp., Danvers, MA). Unless otherwise stated, column running buffers were 20 mM potassium phosphate, pH 6.5, and all buffers were degassed before use.

A column (2.5 cm by 50 cm) of DEAE-Sephadex A-50 (Sigma Chemical Co., St. Louis, MO) was used to prepare the E_2. A 50-mL sample (containing up to 200 mg of protein) of desalted AcA34 protein was adjusted to 0.1 M sodium acetate, pH 4.5, and placed on ice for 30 min. The precipitated proteins (containing little cellulase activity) were removed by centrifugation, and the sample was loaded onto the DEAE column, which had been equilibrated with the same buffer. The proteins were eluted from the column with a 1.5-L gradient of 0–700 mM NaCl (also containing 0.1 M sodium acetate, pH 4.5) at a flow rate of about 15 mL/h. Fractions of 5–10 mL were collected and assayed for CMCase activity, and the active samples were pooled, concentrated, and desalted by ultrafiltration.

A column (2.5 cm by 40 cm) of hydroxylapatite (Hyp C) was also used to separate E_1 from E_2. A 25-mL, 600-mg sample of desalted AcA34 protein was loaded onto a Hyp C column that had been equilibrated with 20 mM potassium phosphate, pH 6.5. The proteins were eluted from the column with a 2-L gradient of 20–200 mM potassium phosphate, pH 6.5, at a flow rate of about 10 mL/h. Fractions were collected and assayed for CMCase activity, the active samples were pooled and concentrated, and the buffer was adjusted as described above.

The various components present in each CMCase peak were separated by native polyacrylamide gel electrophoresis (PAGE). Preparative gel electrophoresis was performed with the Canalco Disc Prep 200 apparatus (Fort Lee, NJ) according to manufacturer's suggestions. A 4–6-cm, 7.5% separating gel with a 1–2-cm 2.4% stacking gel was used. The upper reservoir buffer contained 0.043 M tris(hydroxymethyl)-aminomethane (Tris)/0.052 M glycine, pH 8.7, while the lower (anode) reservoir buffer contained 0.1 M Tris and 0.06 M HCl, pH 8.1. From 5 to 50 mg of protein was loaded for a run, and the proteins were eluted by sweeping the elution chamber with 0.1 M Tris, pH 8.1, at a rate of about 1 mL/min. Fractions (2–3 mL) were collected, assayed for CMCase activity, pooled, and concentrated as before.

General Procedures. All chemicals were purchased from Fisher Chemical Corp. (Pittsburgh, PA) or Sigma Chemical Co. (St. Louis, MO) unless otherwise stated and were of analytical grade.

Cellulase activities were determined on either carboxylmethylcellulose (CMC) (Hercules type 4M6F-CMC), acid-swollen amorphous cellulose (SW) (Sigma type 100) prepared by the method of Ferchak et al. (1980), crystalline cellulose (MC) (Sigma type 100), or filter paper (Whatman No. 1) (Mandels & Weber, 1969). Xylanase activity was determined on larchwood or oat xylan (Sigma type 100). The assays were done at 56 °C, and the substrate concentrations (mg/mL) were 2.5 (CMC), 1.1 (SW), 1.25 (MC), 5.7 (filter paper), and 0.9 (xylan). Filter paper assays were set up to digest at least 15% of the substrate. Reducing sugar formation was determined by the dinitrosalicylic acid (DNS) method of Miller et al. (1960). Standardization was done with either glucose or xylose. One international unit of activity (IU) equals 1 μmol of reducing sugar produced/min. Cellulase activity was also measured viscometrically on a size 100 Ostwald-Fenske viscometer incubated in a 56 °C water bath. Enzyme (<100 μL) at the proper concentration was added to 10 mL of 0.3% (w/v) CMC in 20 mM potassium phosphate, pH 6.5. The relaxation time for the 0.3% CMC solution was 94 ± 2 s. The relaxation time of 0.3% glucose was 53 ± 1 s. It was assumed that a decrease of 50% in viscosity decreased the relaxation time by 20.5 s. $1/\tau$ is recorded as the inverse of the relaxation time in seconds. Cellobiase activity was measured by the method of Wood & McCrae (1982). Protein was determined by the dye binding assay of Bradford (1976) or by reading the OD_{280nm} on a Gilford 240 spectrophotometer. Salt concentration was determined with a conductivity meter. Total carbohydrate present in protein samples was determined by the following procedure. Fifty milligrams of anthrone (Eastman Organic Chemicals, Rochester, NY) was dissolved in 23.75 mL of concentrated H_2SO_4 and 1.25 mL of distilled H_2O. A 0.5-mL sample of protein solution was mixed with 1.0 mL of reagent and heated to 100 °C for 10 min and then cooled, and the absorbance at 620 nm was recorded. Standardization was done with known amounts of glucose. Enough protein (unknown) was used to detect at least 1% carbohydrate (by weight).

Thin-layer chromatography of soluble sugars was performed as described by Uchino & Nakama (1981). Reducing sugars were detected after chromatography as described by Touchstone & Dobbins (1978). The method allowed the detection of ~1 μg/cm^2 spot. Glucose and cellobiose standards were purchased from Sigma. Cellotriose and cellotetrose were generated from crystalline cellulose (Sigma cell 100) by the fuming HCl treatment. Xylose, xylobiose, and xylotriose were generated by cold phosphoric acid hydrolysis of xylan (Gorbacheva & Rodionova, 1977). Samples for chromatography were prepared by lyophilization or by binding soluble sugars to Norit decolorizing (acid-washed) charcoal and eluting with a small volume of 50% ethanol. Samples were dried, dissolved in distilled H_2O, spotted onto plates, and developed.

The electrofocusing gel electrophoresis system used was from Separation Sciences, Inc. (Attleboro, MA). Sodium dodecyl sulfate (SDS)–PAGE was performed as described by Weber & Osborn (1964). The proteins were located by staining with Coomassie Brilliant Blue R-250. Molecular weight standards (Bio-Rad, Richmond, CA) consisted to myosin H chain (M_r 200 000), phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), egg ovalbumin (M_r 43 000, α-chymotrypsinogen (M_r 25 700), β-lactoglobulin (M_r 18 400), and lysozyme (M_r

14 300). Analytical nondenaturing PAGE was run according to the procedure of Maurer (1971). Glycerol gradient (10–60% w/v) centrifugation was performed in a Beckman SW41 Ti rotor. The molecular weight standards used were ferritin apoprotein, BSA, and cytochrome *c*. Stained gels were scanned with a Quick scan densitometer (Helena Laboratory, Beaumont, TX). Protein N-terminal sequence determination was done at MIT (Boston, MA) on a Beckman sequenator (Model 390) using a single cleavage program (Edman) and standard methods. Phenylhydantoin derivatives were identified by high-pressure liquid chromatography (HPLC). The amino acid composition was determined on a (HPLC) reverse-phase column (Picotag) after 26 and 96 h of HCl acid hydrolysis and the formation of phenyl isothiocynate derivatives.

Antibodies were generated in large (>8-lb) female Flemish Giant/Chincilla rabbits. Antigens, 10–100 μg/injection, were injected subcutaneously. The first injection contained Freund's complete adjuvant (Sigma), and the follow-up injections (weekly over a period of 6–10 weeks) contained incomplete adjuvant. Serum antibodies were separated from whole cells by centrifugation and concentrated by $(NH_4)_2SO_4$ precipitation. Ouchterlony double-diffusion plates were run as described by Ouchterlony (1958, 1962). The plates were stained with Coomassie dye after nonprecipitated proteins were washed out. Immunoblots were performed as suggested by the manufacturer (Bio-Rad, Richmond, CA) with 0.05% Tween-20 in all solutions except during the color-development step. Antibody inhibition experiments were done by incubating a dilution series of antiserum with various amounts of antigen at 4 °C for 18 h (or 37 °C for several hours) and then assaying for CMCase activity at either 37 or 56 °C.

The purified enzymes indicated in Figure 7 were partially digested after precipitating 25-μg samples at 0 °C with 10% trichloroacetic acid (TCA), collecting the precipitate by centrifugation (10000*g* for 10 min), and redissolving in 0.1 M Tris-HCl, pH 7.5, and 0.1% SDS. Supernatant containing *Thermomonospora* YX protease (0.08 μg of total protein) was then added, and the mixture was incubated at 56 °C for up to 18 h. Samples were prepared for SDS–PAGE as described above.

Results

Purification. *T. fusca* can produce supernatant levels of CMCase as high as 30 units/mg within 24 h of growth on Solka floc as a carbon source. The culture supernatant also contains high levels of protease activity. To avoid possible degradation during purification, PMSF was added prior to harvesting. PMSF inhibited more than 90% of the detectable protease activity, and failure to add PMSF resulted in greatly altered chromatography patterns. The CMCase activity was isolated from the PMSF-treated culture supernatant by ammonium sulfate precipitation.

The next step was Ultragel AcA34 exclusion chromatography, which gave two well-defined peaks. The first peak, which contained >90% of the CMCase activity, was pooled and concentrated by amicon filtration. The protease activity remaining after PMSF treatment is located within the second AcA34 peak and so removed.

The third step could be either DEAE-Sephadex chromatography or hydroxylapatite chromatography. Enzyme E_2 passed through the DEAE matrix when run in the buffer system used (Figure 1). This activity peak contained two enzymes (A and D), which were similar with respect to both their substrate specificities and the products they produce from cellulose. These two activities were separated by preparative nondenaturing PAGE. SDS–PAGE and activity determinations were used to decide which fractions should be pooled. Unfortunately, the separation of the two components was not complete. E_2D prepared this way is contaminated with significant amounts of E_2A.

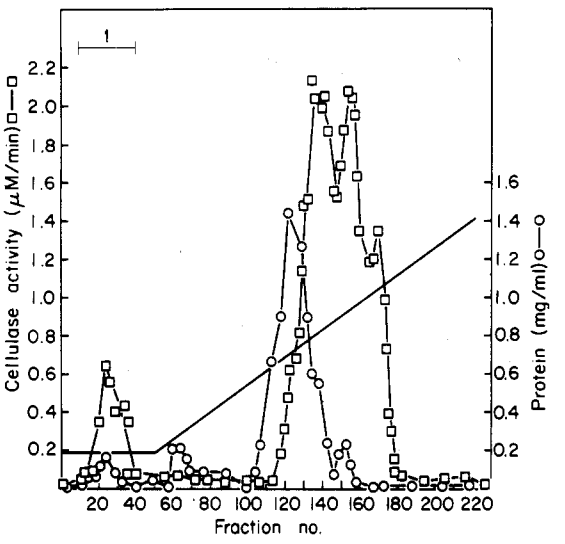

FIGURE 1: DEAE-Sephadex column chromatography of the AcA34 cellulase peak. The E_2 activities were further purified from pooled region 1. NaCl concentration (—) is 40–350 mM range.

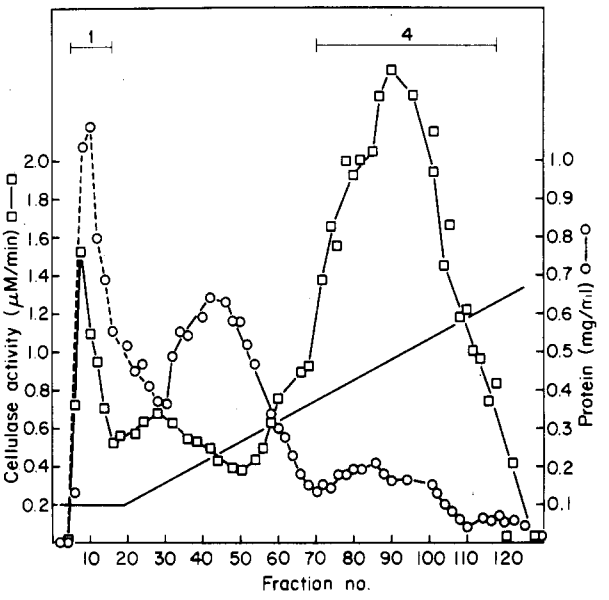

FIGURE 2: Hydroxylapatite column chromatography of the AcA34 cellulase peak. The E_1 activities were further purified from pooled region 4. E_2 was isolated from pooled region 1. Potassium phosphate concentration (—) is 20–170 mM range.

A much better purification of the *T. fusca* β-1,4-endoglucanases was obtained with hydroxylapatite chromatography for the third step. The results of a typical column are shown in Figure 2. Enzyme E_2D was obtained in high yield (4% of the initial CMCase activity) by chromatographing the first peak from the hydroxylapatite column on DEAE-Sephadex under the conditions described for the column shown in Figure 1. Peak 4 from the hydroxylapatite column contained an activity (E_1) that was very different from E_2. The CMCases present in this peak were separated by preparative gel electrophoresis, under the conditions described above. Three proteins with different molecular weights (E_1A, E_1B, E_1C) were separated, and these proteins all had similar substrate specificities and produced similar products from cellulose (data not shown).

Table I: Purification of Endoglucanases from *Thermomonospora fusca*

procedure	fraction	protein (mg)	CMC activity (units/min)	sp act. (units min^{-1} mg^{-1})	yield	purification (*x*-fold)
supernatant		1680	42000	25	1.00	1.0
$(NH_4)_2SO_4$		996	40480	41	0.96	1.6
AcA_{34}		255	28560	112	0.68	4.5
DEAE	peak 1	13.2	996	75	0.024	3.0
Hyp C	peak 1	157	5427	35	0.134	1.4
Hyp C	peak 4	58	20706	357	0.49	14.3
native gel (of DEAE peak 1)	E_2	3.8	293	77	0.007	3.2
native gel (of Hyp C peak 4)	E_1	8	6144	768	0.15	30.7
DEAE (of Hyp C peak 1)	E_2	33	1680	50	0.04	2.0

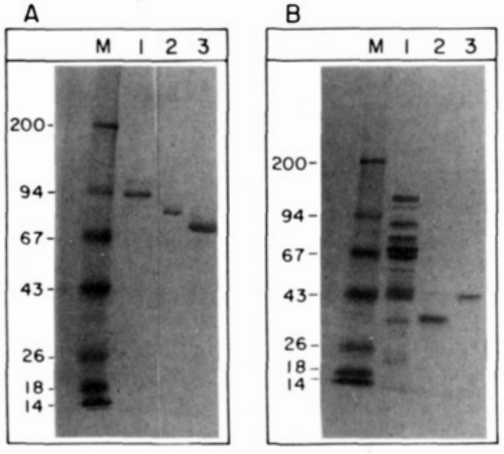

FIGURE 3: SDS–polyacrylamide gels of crude and purified cellulase proteins. Three to five micrograms of purified proteins and 25 μg of AcA34 were run on 7–14% gradient gels. (A) E_1C (lane 1), E_1B (Lane 2), and E_1A (lane 3). (B) AcA34 (lane 1), E_2A (lane 2), and E_2D (lane 3). Lane M represents molecular mass markers as indicated in kilodaltons.

Chromatography on immobilized concanavalin A, carboxylmethylcellulose, or phosphocellulose under various conditions did not give any further fractionation. Table I compares the various separation methods with respect to their yield and effectiveness in purifying the different endoglucanases. The yield of E_1-like components was 43% when E_1A, E_1-B, and E_1-C are included.

Purified proteins were stored at −80 °C. Concentrated solutions could be held at 0 °C for at least 1 week without any change in activity or molecular weight.

The purified CMCases were tested for purification by SDS–polyacrylamide gel electrophoresis. The results of these experiments are shown in Figure 3. The purified enzymes each contain a single major protein band and, except for E_1A, minor amounts of contaminating proteins. The major band contained more than 90% of the protein, as determined by densitometer scanning of the Coomassie Blue stained gels in every sample except E_1A.

Immunological Studies. Three methods, Ouchterlony double diffusion, immunoblotting on nitrocellulose, and inhibition of activity, were used to look for relationships between the various endoglucanases. In Table II the ability of different antisera to inhibit the CMCase activity of the different enzymes is shown. No preparation of preserum was found to inhibit or enhance any CMCase. For inhibition studies using antisera, a range of serum to enzyme concentration was tested, and the maximum inhibition is shown. Generally, 0.1 volume of antisera was added to the sample. The CMCase activity was determined by the DNS assay. It is clear that antiserum to E_1C completely inhibits E_1B and E_1A. The antiserum to E_2A also completely inhibits E_2D; however, it has no effect on any E_1 fraction. Antiserum to E_1C, conversely, does not affect the activity of E_2A or E_2D. The AcA34 antiserum, as expected, inhibits to varying degrees all of the enzymes.

Table II: Percent Inhibition of CMCase by Antisera

cellulase	antiserum	% inhibition	cellulase	antiserum	% inhibition
E_2A	normal	0	E_1B	normal	6
	E_2A	87		AcA34	24
	E_1C	2		E_1C	98
E_2D	normal	0		E_2A	0
	E_2A	99	E_1C	normal	0
	E_1C	0		AcA34	71
				E_1C	100
				E_2A	0

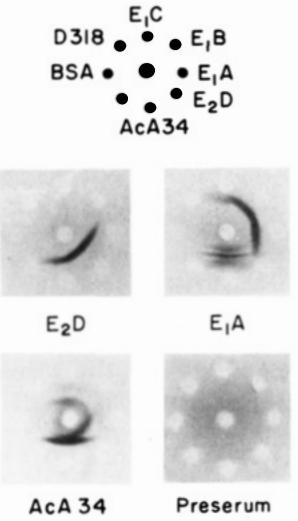

FIGURE 4: Ouchterlony double-diffusion plates of various cellulase proteins. Antisera (5 μL) as shown were added to center wells. The antigens (0.5 μg in 5 μL) were added around the outside in the pattern shown in the diagram.

The results of Ouchterlony double-diffusion experiments are shown in Figure 4. It is clear that preserum does not react with BSA or any purified CMCase. The E_1C antiserum forms lines of identity between E_1A, E_1B, and E_1C but does not react with E_2. Antiserum to E_2 does not react with any E_1 component but does react with E_2. The AcA34 fraction, as expected, contains all antigens. In fact, several additional precipitin lines are present, suggesting that other antigenically related proteins are in this fraction. The well, labeled D318, contains enzyme produced by the cloned *T. fusca* CMCase gene reported in Collmer et al. (1984).

In an effort to quantitate the degree of antigenic relatedness between the various cellulase proteins, we have performed immunoblots on nitrocellulose membranes. A spot's color intensity is correlated with its ability to bind antibody. It is clear from the results presented in Figure 5 that although antiserum to E_1C reacts to protein E_1C with the greatest intensity it also reacts strongly with E_1B and E_1A. All samples were adjusted to the same concentration before spotting.

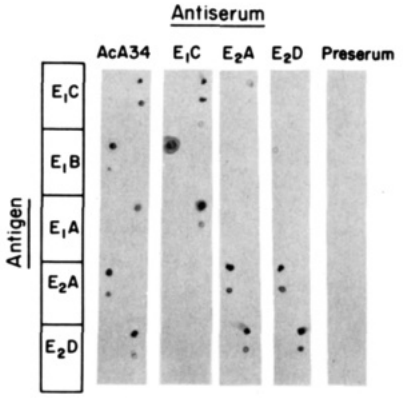

FIGURE 5: Nitrocellulose antibody/antigen immunoblotting of cellulase proteins. For each antigen tested, a series of 0.1, 0.01, and 0.001 μg were applied in 1-μL volumes with the 0.1-μg sample at the top of the zone and the 0.001-μg sample at the bottom of the zone.

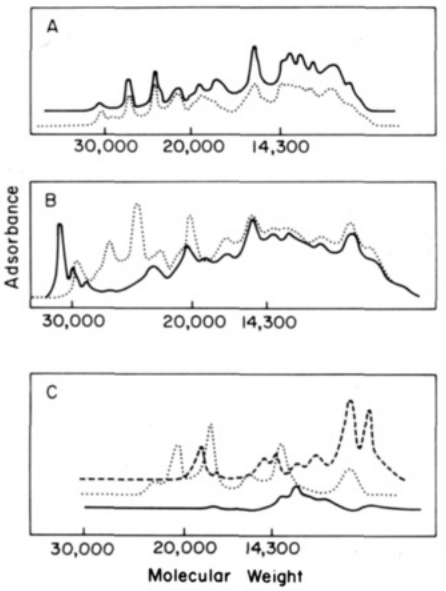

FIGURE 6: Densitometer scans of SDS–polyacrylamide gels of protease-treated proteins. (A) Gradient gel (10–20%) comparing a E_1A digest (—) with E_1B digest (···). (B) Straight gel (14%) comparing a E_1B digest (···) with a E_1C (—) digest. (C) Gradient gel (10–20%) comparing BSA (···), *Thermomonospora* xylanase (– –), and E_2A digests (—).

Antiserum to E_2A reacts about equally well with protein E_2A or E_2D. The most interesting finding here is that the antisera to E_2A and E_2D react to a measurable extent with E_1B and E_1C (although 100 times less strongly). We have additional evidence (data not shown) that antiserum to E_1C has a very low but significant amount of binding affinity for the xylanase proteins purified from *T. fusca* YX (unpublished results). The E_1C protein injected into rabbits certainly contained no detectable xylanase activity, and it was >95% homogeneous. This low-level affinity may reflect a common structure of all excreted proteins from this organism. No signal was evident with BSA on the membrane with any antiserum. Preserum was negative with all proteins tested.

Protease Studies. The relationships between E_1A, E_1B, and E_1C were also studied by comparing the products of partial proteolysis. Trypsin and chymotrypsin were unable to digest these cellulases. The protease in the culture supernatant did degrade acid-denatured samples of purified culture cellulases. As is evident in Figure 6, the protease cleavage patterns of the three enzymes are very similar. Densitometer tracing of the gels shows that many of the fragments are identical in molecular weight. There is considerable overlap in the pattern of peaks, particularly with those fragments having molecular weights less than 14000. Controls using completely unrelated proteins such as phosphorylase *b*, E_2A, and bovine serum albumin or a xylanase protein, also isolated from *T. fusca* YX, showed no similarity.

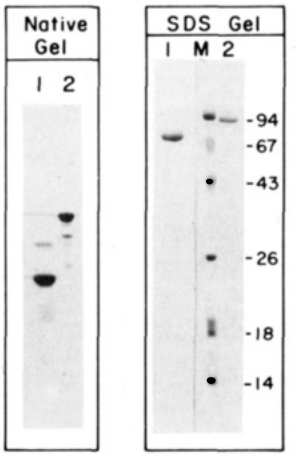

FIGURE 7: Effects of protease on the molecular weight of E_1. Samples of E_1 were held at 56 °C for 17 h with and without *Thermomonospora* culture supernatant containing protease (untreated with PMSF). The active protein fragment in each case was isolated from a native gel and subjected to SDS gel electrophoresis. Lane 1 of each gel represents the protease-digested sample, and lane 2 represents the untreated E_1 enzyme. Lane M represents molecular mass markers as indicated in kilodaltons.

The same protease preparation converted native E_1 protein to an active fragment of M_r of ~70000 (Figure 7). Even after extensive protease digestion as shown here (17 h), more than 81% of the activity is recovered from a M_r 70000 band on the native gel.

Physical and Catalytic Properties. The above studies indicate that at least two basically different types of endocellulases are present in the crude supernatant fraction. E_1C (E_1) and E_2D (E_2) were chosen as representative of the two types of enzymes. Both enzymes were studied in detail with respect to their pH and temperature optima, their effects of various metal ions on activity, their levels of glycosylation, and their rate constants, molecular weights, amino acid composition, and catalytic mechanisms.

The molecular weights of the purified components were determined from the SDS gel shown in Figure 3 and are 94000 for E_1C, 84000 for E_1B, 74000 for E_1A, 46000 for E_2D, and 37000 for E_2A. Using nondenaturing methods such as gel filtration to determine the molecular weight was unsuccessful. All proteins either bound to the matrix (at low salt) or ran with the void volume of the column (at high salt). However, the native molecular weights were determined by glycerol gradient centrifugation. The subunit molecular weights of E_1 and E_2 agree closely (within 10%) with their native, active molecular weights.

The amount of carbohydrate in E_1 and E_2 was determined by the anthrone assay, and E_1 contained less than 1% (by weight) sugar (glucose equivalents) while E_2A contained about 15% sugar. The higher molecular weight E_2 protein contained 25% carbohydrate. Catalase was used as a negative control, and ovalbumin was used as a positive control. The percentage by weight of carbohydrate in ovalbumin was 3.9%. This was in good agreement with the published literature value of 3.2% (Fletcher et al., 1965). The presence of carbohydrates was also tested by staining SDS–polyacrylamide gels run on the purified enzymes for carbohydrates by the PAS procedure of

Table III: Amino Acid Composition

amino acid	mol % E_1	mol % E_2	amino acid	mol % E_1	mol % E_2
Asp	10.8	11.9	Val	8.5	6.0
Glu	8.7	7.6	Leu	7.1	3.9
Ser	6.6	8.4	Ile	3.6	5.8
Thr	9.1	8.6	Phe	3.4	2.7
Gly	12.5	9.9	Tyr	3.6	2.4
Ala	9.5	13.8	Lys	2.5	1.7
Pro	7.5	8.5	His	1.8	2.4
Cys	1.0	1.1	Arg	2.7	3.4
Met	1.1	2.0			

Fairbanks et al. (1971). This procedure also showed the presence of carbohydrate in E_2 but not in E_1.

Using increasing amounts of CMC as substrate and measuring reducing sugar formation, we have calculated the K_m and V_{max} of E_1 at 56 °C to be 360 μg/mL and 25 μg/min, respectively. The K_m and V_{max} at 56 °C of E_2 were determined to be 120 μg/mL and 2.9 μg/min, respectively. The protein concentrations of E_2 and E_1 used for the assay were 1.2 and 0.75 μg/mL, respectively. Since the molecular weight of the CMC is an average value, calculations of accurate molar constants are not possible. The use of CMC as substrate for both enzymes allowed relative comparisons of rate constants to be made.

Each of the purified enzymes is quite stable to temperatures up to 60 °C. In fact, E_1 has an enzymatic maximum at 74 °C, and E_2 has a maximum at 58 °C. Experiments involving incubating either enzyme at 56 °C for 18 h showed that less than 20% of the activity was lost.

The pH optimums were determined in a potassium phosphate buffer system. Adjustments in pH were made by mixing KH_2PO_4 and K_2HPO_4 at the same molarity but in different ratios. Both enzymes have pH optimums at or near pH 6.5 with a broad maximum between pH 5 and pH 7. Enzyme E_1 has a ratio of the activity at pH 6 to that at pH 9 of about 5:1 whereas for enzyme E_2 the value of the ratio is 2:1.

By polyacrylamide electrofocusing, the approximate isoelectric point (p*I*) values of these proteins were determined. The value for E_1 is 3.6 and for E_2 is 4.5. These values are consistent with their behavior on ionic exchange columns.

The effects of heavy metal ions and other salts on the CMCase activity of E_1 and E_2 were determined. Certain salts interfered with the reducing sugar determinations, and so their inhibition was determined by viscometric methods. The most interesting results are that $HgCl_2$ (10 ppm) is completely inhibitory to both enzymes, $AgNO_3$ (100 ppm) is 50% inhibitory, and $CoCl_2$ (100 ppm) inhibits E_2 50% but not E_1. Finally, $CaCl_2$ is stimulatory to E_1 (2-fold at 2 or 10 mM) but not to E_2. Dithiothreitol (DTT) at concentrations up to 10 mM did not affect activity. Ethylenediaminetetraacetic acid (EDTA) alone did not inhibit enzymatic activity of either enzyme but EDTA plus Ca^{2+} did inhibit both enzymes 80%. KI, NaF, LiCl, ammonium molybdate, $CsCl_2$, $CuSO_4$, and $FeSO_4$ all at 200 ppm had no effect on either enzyme.

The large amount of purified E_1 allowed the determination of its N-terminal amino acid sequence. By using standard methods, it was determined that the sequence was X-1–Glu-2–Val-3–Asp-4–Glu-5–Phe(or Leu)-6–Cys(or Arg or His)-7–Asn-8–Gly-9–Asp-10. X means that this amino acid was destroyed by the procedure and could not be determined. The amino acid compositions of the two proteins are listed in Table III. The most interesting values are those for isoleucine, methionine, and leucine. E_1 contains nearly twice the mole percent of leucine of E_2 yet only about half the amounts of isoleucine and methionine.

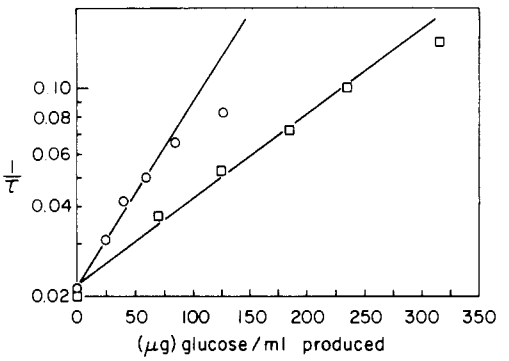

FIGURE 8: Relationship of CMC viscosity decrease to the reducing sugar produced by the cellulase enzymes: E_1 (□); E_2 (○).

In an effort to confirm that E_1 and E_2 are endocellulases, the rate of reducing sugar formation as a function of viscosity decrease of CMC was measured, and the products of cellulose hydrolysis were determined. The relative change in viscosity was plotted against the amount of reducing sugar produced as the amount of enzyme was varied. In one such experiment, Figure 8, it can be seen that both E_1 and E_2 reduce the viscosity of CMC. Comparison of the appearance of reducing sugar as a function of viscosity shows that the enzymes differ. E_1 generates reducing sugar significantly more rapidly (relative to viscosity decrease) than E_2. In fact at 50% reduction in relaxation time, E_1 had cleaved 1.6% of the β-1,4 bonds whereas E_2 had cleaved only 0.8% of the bonds. This shows a more exocellulase-like enzymatic function (mode of attack) for E_1. The fact that each enzyme reduces the viscosity of CMC faster than it release reducing sugar indicates that both are endocellulases.

It was expected, on the basis of other studies of cellulases, that an endocellulase would rapidly hydrolyze soluble cellulose (such as CMC) and that an exocellulase would prefer more crystalline (such as filter paper) types of substrates. In Table IV, it can be seen that E_1 is much more active on CMC than on crystalline cellulose. The ratio of its activities on CMC and acid-swollen (amorphorus) cellulose is approximately 9:1. E_2, however, shows approximately equal rates of hydrolysis of both CMC and acid-swollen cellulose and degrades microcrystalline cellulose faster than E_1. both E_1 and E_2 degrade filter paper at a much slower rate than does the crude AcA34 preparation. Both enzymes had extremely low cellobiase activity and no β-glucosidase activity.

The products produced by the two enzymes from cellulose are shown in Figure 9. The predominant product of E_1 is cellobiose while E_2 produces glucose, cellobiose, and higher

Table IV: Substrate Specificity of the Endoglucanases

sample	μmol min^{-1} mg^{-1} CMC	SW[a]	Solka floc	Sigma cell	filter paper	xylan	cellobiose
AcA34	162	19	[b]	0.26	0.036	13	[b]
E_2	77	44	0.107	0.160	0.004	0.0032	0.000 051 2
E_1	768	87	0.070	0.059	0.009	0.0024	0.000 004 4

[a] SW, phosphoric acid swollen cellulose. [b] Not determined in this experiment.

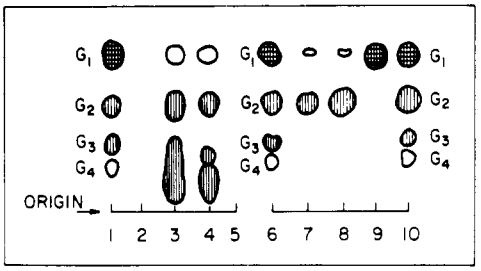

FIGURE 9: Thin-layer chromatography of cellulose hydrolysis products. Lanes 1, 6, and 10 contain the standards glucose (G_1), cellobiose (G_2), cellotriose (G_3), and cellotetrose (G_4). Lane 9 contains G1. Lane 2 contains CMC and SW incubated without enzyme. Lanes 3 and 4 show E_2 hydrolysis of CMC and SW, respectively, lanes 7 and 8 show E_1 hydrolysis of CMC and SW, respectively, and lane 5 is enzyme only.

Table V: End-Product Inhibition

	glucose		cellobiose	
	concn (mg/mL)	activity (%)[a]	concn (mg/mL)	activity (%)[a]
E_1	0.25	100	0.25	100
	2.5	100	2.5	98
	25	97	25	89
	100	93	100	71
	250	61	250	0
E_2	0.25	100	0.25	100
	2.5	100	2.5	97
	25	100	25	83
	100	104	100	49
	250	106	250	7

[a] As measured by specific change of viscosity of CMC.

oligomers of glucose. The higher oligomers can only be produced by an endoglucanase.

The activity of these enzymes in digesting oligodextrans was consistent with the suggestion that E_2 is a typical endoglucanase. When E_2 is incubated with cellotriose (G_3) and cellotetrose (G_4), very little of the G_3 was attacked and the main product of digestion was cellobiose. An endoglucanase is expected to prefer higher polymer number oligodextrans. E_1 hydrolyzed both oligodextrans, and both glucose and cellobiose were produced. The fact that E_1 can readily degrade oligodextrans probably explains why they do not accumulate during cellulose hydrolysis.

The relative reduction of viscosity of CMC in the presence of either glucose or cellobiose by both enzymes was measured to look for end-product inhibition. Cellobiose at 10% (w/v) or greater is significantly more inhibitory to E_2 than to E_1, whereas glucose has no effect on E_2 and little effect on E_1 (Table V).

If these enzymes truly possess very different modes of attack on cellulose, it was expected that synergism might occur when digesting insoluble cellulose. Experiments were performed to determine if reducing sugar generation from filter paper with both enzymes would occur at a higher rate than the sum of the rates of each enzyme by itself. There was no evidence of synergism between these two enzymes.

Discussion

Most of the cellulase activity present in the culture supernatant from *T. fusca* appears to require the two enzyme families we have isolated. This is indicated by the fact that antibodies to these enzymes inhibit more than 90% of the supernatant activity with either CMC or filter paper as substrate. However, other components are required for filter paper hydrolysis, since the specific activity of the starting material is 3 times higher than that of any purified enzyme.

The results of the immunological studies and the partial proteolysis experiments prove that E_1A and E_1B are proteolytic products of E_1C. E_2A probably is a proteolytic product of E_2D, but this has only been tested immunologically. It is quite surprising that almost one-third of the M_r 94 000 enzyme can be removed by protease digestion without changing its enzymatic activity. Erickson and Pettersson (1975a,b) have observed an increase in the endoglucanase activity produced by *Sporotrichum pulverulentum* as a result of proteolysis by endogenous proteases but do not know why this occurs.

E_2 does not increase in specific activity during purification even though it is separated from a very complex mixture of proteins. The crude mixture contains several cellulases with a wide range of rate constants. E_2 shows little substrate preference toward CMC so that it is responsible for only a fraction of the initial CMCase activity.

E_2 is clearly a β-1,4-endoglucanase since it produces large amounts of higher cellulose oligomers in addition to reducing the viscosity of CMC at a faster relative rate than it releases reducing sugars. This enzyme is a glycoprotein as are most cellulases that have been studied. To date, there have been only a few reports of glycosylated proteins in bacteria. The halophilic bacteria of the genus *Halobacterium* (Mescher & Stominger, 1976, 1978), *Cellulomonas* (Langsford et al., 1984), and *Clostridium* bacteria (Ng & Zeikus, 1981) contain glycoproteins.

E_1 produces cellobiose like an exocellulase, but it reduces the viscosity of CMC faster than it releases reducing sugars so it is also a β-1,4-endoglucanase. Furthermore, it attacks CMC much faster than crystalline cellulose, another characteristic of an endoglucanase. This enzyme is not a glycoprotein, yet it has the highest specific activity of the *T. fusca* CMCases, is very stable, and is excreted normally. Clearly, carbohydrate is not required for any of these properties.

Despite the fact that these are the two most active CMCases produced by *T. fusca*, they do not show synergism when they degrade crystalline cellulose or filter paper. All attempts at detecting synergism by mixing fractions of a DEAE column or a hydroxylapatite column have failed. It is unclear whether the *T. fusca* cellulase enzymes do not show typical synergism or as is most probable we have not yet identified all the cellulase components.

The cellulases of *Trichoderma* (Sprey & Lambert, 1983) and *Clostridium thermocellum* (Lamed et al., 1983) have been reported to be present in a complex, but *T. fusca* does not appear to produce a cellulase complex. The cellulose activity in the crude supernatant sediments at nearly the same rate as the purified enzymes and clearly more slowly than would a 1:1 complex of the E_1 and E_2. We have not been able to detect an exocellulase in the *T. fusca* culture supernatant, but as exocellulases often have low specific activities, it could have been missed. Neither the E_1 nor the E_2 antisera reacted with the cellulases produced by *Escherichia coli*, which contain the cellulase gene we had cloned previously (Collmer & Wilson, 1984) even though the AcA34 antisera did react with this activity. This provides additional evidence that we have not yet identified all of the cellulases produced by *T. fusca*.

The sensitivity of E_1 and E_2 to Hg^{2+} and Ag^{2+} resembles what was recorded for the cellulase enzymes of *Clostridia thermocellum* (Ng & Zeikus, 1981a). The Hg^{2+} sensitivity suggests sulfhydryl involvement during catalysis. Unlike the *Clostridium* enzyme (Johnson & Demain, 1984), however, DTT had no stimulatory effect on the cellulase enzymes of *T. fusca*. E_1 and E_2 differed in that E_1 was stimulated by 0.010

M Ca^{2+} and 0.005 M Mg^{2+} while E_2 was not. A very surprising result is that EDTA (0.01 M) alone did not inhibit either enzyme but EDTA and Ca^{2+} (each at 0.01 M) gave 83% inhibition of both enzymes.

E_1 has a recorded specific activity on CMC of approximately 750 μmol min^{-1} mg^{-1} at 56 °C. This is high compared to the *Trichoderma* (5 μmol min^{-1} mg^{-1} at 37 °C; Halliwell & Vincent, 1981) or *Clostridial* (65 μmol min^{-1} mg^{-1} at 60 °C; Ng & Zeikus, 1981b) enzymes, which are less than 10% of this value. The activity of E_1 can in fact be increased more than 3-fold over this value by incubating the enzyme at 74 °C in the presence of 4 mM $CaCl_2$. There are no similarities between our N-terminal sequence and the sequence reported for the *Trichoderma* cellulase enzyme (Hostomska, 1984) or for the *Clostridial* enzyme (Ng & Zeikus, 1981).

The usefulness these enzymes possess for industrial use are based on their tolerance to heat, their indifference to various ions, and their broad pH activity optimums. Certainly these parameters of E_1 and E_2 are promising.

Efforts are under way to isolate in pure form the other cellulase components from the culture supernatants of *Thermomonospora fusca* YX. These studies should help in determining mechanisms of cellulose degradation by this thermophilic bacteria.

ACKNOWLEDGMENTS

We are grateful to Joyce Broadhead for manuscript preparation.

Registry No. CMC, 9004-32-4; cellulase, 9012-54-8.

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