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Purification and Some Properties of a Cellulase Active on Crystalline Cellulose from *Cellulomonas uda*

KATSUMI NAKAMURA and KUMPEI KITAMURA

Applied Bioscience Laboratory, Kirin Brewery Co., Ltd., Takasaki, Gunma 370–12, Japan

A cellulase was purified by ammonium sulfate precipitation, DEAE-Sepharose chromatography and Toyopearl HW-55F gel filtration from the culture filtrate of *Cellulomonas uda* CB4. The purified enzyme appeared homogeneous on disc-electrophoresis. Its isoelectric point was 4.4 and its molecular weight was estimated to be 66,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme rapidly hydrolyzed crystalline cellulose and produced cellobiose, but showed little activity on CM-cellulose or filter paper. It hydrolyzed cellotetraose to cellobiose but did not hydrolyze cellotriose.

Previously,[1] we reported the isolation of *Cellulomonas uda* CB4, which produced an extracellular cellulase with strong hydrolytic activity on crystalline cellulose (Avicel) and described the enzymatic properties of the culture filtrate of strain CB4. The rate limiting step of hydrolysis of native cellulose was considered to be breakdown of the crystalline region.[2] Fungal cellulases which hydrolyze crystalline cellulose have been studied extensively and are considered to be β-1,4-glucan cellobiohydrolase[3,4] or a "less random" cellulase.[5,6] This paper describes the purification and some properties of a cellulase active on crystalline cellulose from *Cellulomonas uda* CB4.

Materials and Methods

Production of cellulolytic enzymes *Cellulomonas uda* CB4 was cultured at 30°C for 48 h with shaking in the medium described previously.[1]

Assay of enzyme activity The hydrolysis of crystalline cellulose (Avicel, E. Merck AG.) and CM-cellulose (Sigma Co.) was measured as described previously.[1] Activity for hydrolysis of crystalline cellulose was assayed by measuring the liberation of reducing sugar by the Somogyi-Nelson method.[7]

Determination of protein concentration Protein concentration was determined by the method of Lowry *et al.*,[8] with bovine serum albumin as a standard, or estimated from absorbance at 280 nm. The value of absorbance at 280 nm for 0.1% cellulase (final step in Table 1) was 0.989.

Electrophoresis Disc electrophoresis was carried out by the method of Ornstein[9] and Davis.[10] The molecular weight was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel disc electrophoresis by the method of Weber and Osborn.[11] Marker proteins were obtained from Sigma Co. The isoelectric point of the enzyme was determined by electrofocusing on an Ampholine polyacrylamide gel plate (LKB. Co.).

Preparation of cellooligosaccharides Cellotriose and cellotetraose were prepared from cellulose powder (Whatman Co., CF11) by the method of Sasaki *et al.*[12]

Thin layer chromatography Samples were spotted on a Silica Gel G plate and developed for 3 h with *n*-butanol-ethanol-water (2 : 2 : 1, v/v). Sugars on the plate were detected by spraying the plate with 50% sulfuric acid and heating it at 110°C.

Results and Discussion

Purification of cellulase All operations were performed at 4°C. The culture broth (6 *l*) was fractionated by 10 to 50% saturation with ammonium sulfate and the resulting precipitate was dissolved in a small volume of 0.05 M Tris buffer, pH 7.2, and applied to a Sephadex G-25 column (3×90 cm) equilibrated with the same buffer. Protein fractions of larger molecular size were collected and precipitated with ammonium sulfate (50% saturation). The precipitate obtained was dissolved in a small volume of the buffer, desalted by gel filtration on Sephadex G-25, and applied to a DEAE-Sepharose column (2.6×34 cm) equilibrated

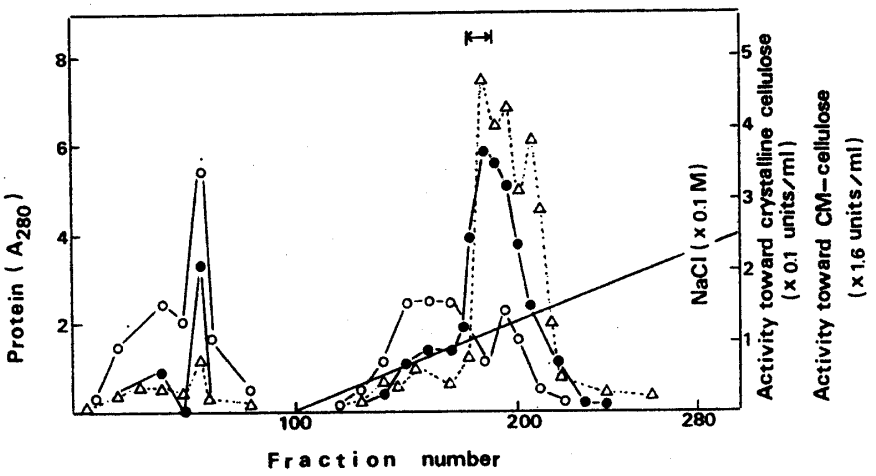

Fig. 1. Elution pattern of cellulolytic enzymes on the first DEAE-Sepharose column (3×90 cm) chromatography.
Fractions of 9.5 ml were collected.
△, Protein (absorbance at 280 nm); ●, Activity toward crystalline cellulose; ○, Activity toward CM-cellulose.

with the Tris buffer. The column was washed with the same buffer and enzyme protein was eluted with a linear gradient of 0 to 0.3 M NaCl in the buffer, as shown in Fig. 1. Fraction nos. 178–189, which showed high activity on crystalline cellulose, were collected and concentrated by the above procedures. The pooled fractions were re-chromatographed on a DEAE-Sepharose column with a linear gradient of 0 to 0.2 M NaCl. Fractions were treated as above, and applied on a Toyopearl HW-55F column (3×63 cm) equilibrated with the Tris buffer containing 0.2 M NaCl. As shown in Fig. 2, two protein peaks were eluted. Fraction nos. 60–65, which showed high activity on crystalline cellulose, were treated as above and rechromatographed on a Toyopearl HW-55F column under the same conditions. The cellulase was eluted as a single peak. The purification steps are summarized in Table 1. The recovery of the activity on crystalline cellulose was 0.4%. From the electrophoretic pattern shown in Fig. 3, the enzyme appeared homogenous.

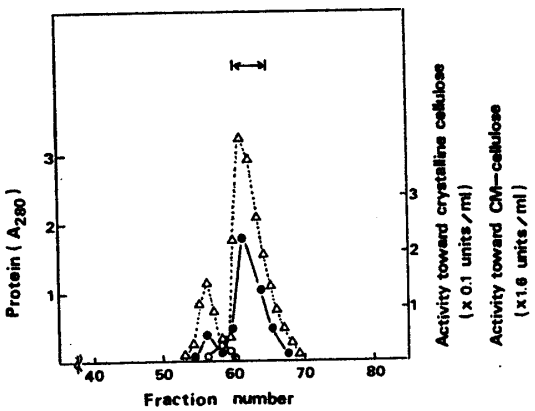

Fig. 2. Elution pattern of cellulolytic enzymes on a Toyopearl HW-55F column (3×63 cm).
Fractions of 4.5 ml were collected.
△, Protein (absorbance at 280 nm); ●, Activity toward crystalline cellulose; ○, Activity toward CM-cellulose.

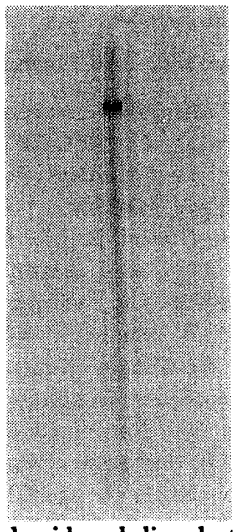

Fig. 3. Polyacrylamide gel disc electrophoresis of the purified cellulase. Electrophoresis was carried out at pH 8.9 at 2 mA/tube for 180 min.

Table 1. Summary of purification.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Culture filtrate	31,100	1,410	0.045	100
Ammonium sulfate	5,560	428	0.077	30.4
DEAE-Sepharose (2nd)	203	20	0.099	1.4
Toyopearl HW-55F (2nd)	56	6	0.11	0.4

Cellulase activity was measured with Avicel as a substrate.

Properties of the cellulase As shown in Fig. 4, the molecular weight of the purified enzyme was estimated to be 66,000. The isoelectric point of the enzyme was estimated to be 4.4. The optimum pH for the hydrolysis of Avicel was pH 5.5 to 6.5. The enzyme was stable between pH 5.5 and 8.0. The optimum temperature of the enzyme was 45–50°C. The activity of the enzyme was completely lost on incubation at 60°C for 10 min in 0.2 M acetate buffer, pH 5.5, without substrate. The enzyme was activated about 2 times by addition of 1 mM Co^{2+} or Mn^{2+}.

Hydrolysis of cellulose substrates The substrate specificity of the purified enzyme was studied. As shown in Table 2, Avicel was the best substrate and the rates of the hydrolysis of cotton, CM-cellulose and filter paper were 16, 13 and 1% of that of Avicel, respectively. The apparent K_m value for Avicel was about 2.9 mg/ml. As shown in Fig. 5, the main product from Avicel was cellobiose, with a trace of cellotriose. Figure 6 shows that cellotetraose was readily hydrolyzed to cellobiose, but cellotriose was not hydrolyzed. These results suggested that the cellulase from *C. uda* CB4 was a β-1,4-glucan cellobiohydrolase. Although β-1,4-glucan cellobiohydrolase from *T. viride* isolated by Wood *et al.*[3] had little ability to hydrolyze highly ordered forms of cellulose, our purified enzyme had high activity on Avicel. The cellulase with "highly less random" specificity, purified from *Trichoderma* by Nishizawa *et al.*[5] and Toyama *et al.*,[6] hydrolyzed cellotriose readily to cellobiose and glucose. Our enzyme from *Cellulomonas uda* CB4 did not hydrolyze cellotriose, but had high activity on Avicel. These differences in substrate specificity must

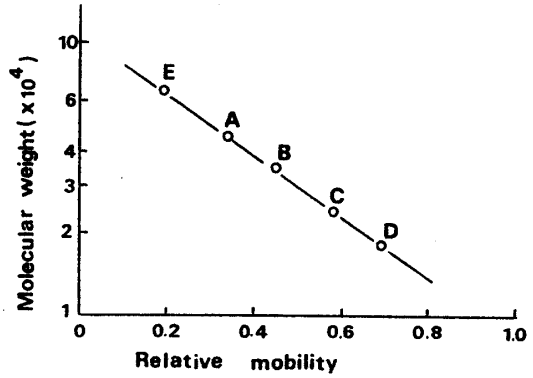

Fig. 4. Estimation of molecular weight of the purified cellulase by SDS-gel electrophoresis.
A, egg albumin (45,000); B, pepsin (34,700); C, trypsinogen (24,000); D, β-lactoglobulin (18,400); E, purified cellulase and bovine albumin (66,000).

Table 2. Substrate specificity of purified cellulase.

Substrates	Activity (units/ml)
Avicel	0.0740
CM-cellulose	0.0095
Cotton	0.0117
Filter paper	0.0009

A reaction mixture consisting of 2.0ml of 1% substrate solution, pH 5.5, and 0.1ml of purified cellulase (61 μg) was incubated at 45°C for 60 min. Reducing sugar was measured as glucose by the Somogyi-Nelson method. One unit of activity is defined as the amount liberating 1 μmol of glucose per min.

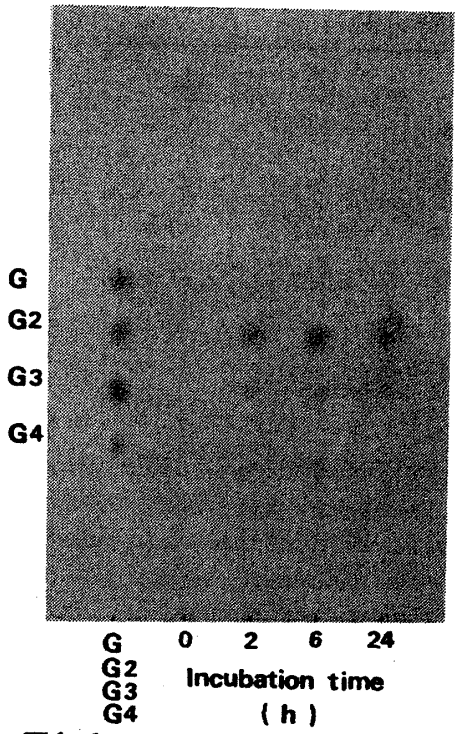

Fig. 5. Thin layer chromatogram of products from Avicel with the purified cellulase.
G, glucose; G2, cellobiose; G3, cellotriose; G4, cellotetraose. Avicel (20 mg) was incubated at 45°C with 61 μg of purified cellulase and 0.05 M acetate buffer (pH 5.5) for 2, 6 and 24 h (total volume, 2 ml). The reaction was stopped by heating the mixture for 5 min. Then the mixture was centrifuged and the supernatant deionized with Amberlite CG 120 (H) and Dowex 1×4 (OH), and aliquots of the reaction products were spotted on a Silica Gel G plate. About 4.0% of Avicel was solubilized after 24 h incubation.

be due to the differences in origin of the enzyme, *i.e.*, bacteria and fungi.

Acknowledgements

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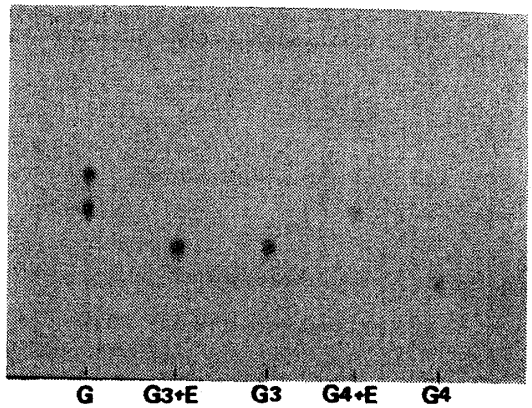

Fig. 6. Thin layer chromatogram of products from cellotriose and cellotetraose with the purified cellulase.
G, glucose; G2, cellobiose; G3, cellotriose; G4, cellotetraose. Purified cellulase (61 μg) was incubated with 350 μg of cellotriose of 150 μg of cellotetraose in 0.2 M acetate buffer (pH 5.5) at 45°C for 2 h (total volume, 0.5 ml). The mixture was deionized with Amberlite CG 120 (H) and Dowex 1×4 (OH), and aliquots of the reaction products were spotted on a Silica Gel G plate.

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