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Purification and Properties of Two *Endo*-cellulases from *Acremonium cellulolyticus*

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Two distinct *endo*-cellulase components derived from *Acremonium* cellulase, a commercial cellulase preparation from *Acremonium cellulolyticus*, were extensively purified by consecutive column chromatography and designated as cellulase III-A and cellulase III-B. Cellulases III-A and III-B were each homogeneous on both Native- and SDS-PAGE, and were completely free from β-glucosidase. The molecular mass (SDS-PAGE) and p*I* values of cellulases III-A and III-B were 58 kDa and 4.6, and 49 kDa and 4.2, respectively. Both enzymes contained 14–16% carbohydrates (as glucose). The N-terminal amino acid sequences from the 2nd up to the 20th residue of both enzymes were determined by Edman degradation. Some enzymatic properties of the purified cellulases were investigated. The optimum pH and temperature for cellulases III-A and III-B were pH 5.5 and 55°C, and pH 5.5 and 65°C, respectively. Cellulases III-A and III-B were completely stable over the range of pH 4.2–8.0 at 4°C for 24 h and at temperatures below 55°C, and pH 3.3–7.8 and below 60°C, respectively. Cellulases III-A and III-B retained 25 and 88% of the original CMC-saccharification activities, respectively, after heating at 70°C for 10 min. The hydrolysis of CMC by cellulase III-B was more *endo*-lytic than that by cellulase III-A. Both enzymes split various soluble and insoluble substrates to produce predominant cellobiose and a small amount of glucose as the final hydrolysis products.

Cellulose is the major constituent of the cell-wall material of all higher plants. It is thus by far the most abundant biomass resource on earth. In most cellulolytic microorganisms, it is well-known that several cellulase [1,4-(1,3;1,4)-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4] components with different substrate specificities are secreted into the culture medium. They together constitute a "cellulase system," and insoluble cellulosic materials are converted to soluble sugars by their synergistic actions.[1–4]

A central goal of cellulase research has been to determine the role of each enzyme and to establish the number of components necessary and sufficient for the effective conversion of native cellulose to glucose. Therefore, the strict purification and characterization of each cellulase component is absolutely necessary. In this respect, Okada *et al.* have intensively studied the cellulase system of a filamentous fungus, *Trichoderma viride*, and reported a series of their works.[5–13]

In a previous paper,[14] the authors reported that

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Abbreviations: QAE, quaternary amino ethyl; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; CMC, sodium carboxymethylcellulose; PNPG, *p*-nitrophenyl β-D-glucoside; DS, degree of substitution; G_1, D-glucose; G_2–G_6, cellooligosaccharides from cellobiose to cellohexaose; NBS, *N*-bromosuccinimide; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography.

Acremonium cellulase, a commercial cellulase preparation from *Acremonium cellulolyticus*, could be resolved into three cellulase peak fractions (Peaks I, III and IV) upon chromatography on a QAE-Toyopearl 550 C column. A highly purified cellulase IV was obtained from the Peak IV cellulase fraction, and was characterized physicochemically and enzymologically.

In a further investigation of the cellulase system of *A. cellulolyticus*, the Peak III cellulase fraction was extensively examined and two distinct cellulase components with different properties were obtained by careful fractionation procedures. The results are described in this paper.

MATERIALS AND METHODS

Starting enzyme material. The commercial cellulase preparation from the culture filtrate of *Acremonium cellulolyticus* (*Acremonium* cellulase, lot No. AUS-0301, Meiji Seika Kaisha, Ltd., Japan) was used as the starting material for the purification of cellulases.

Substrates. (i) *p*-Nitrophenyl β-D-glucoside (PNPG): A commercial product of Tokyo Kasei Kogyo Co., Ltd., Japan. (ii) Cellooligosaccharides: Cellobiose to cellohexaose were commercial products of Seikagaku Kogyo Co., Ltd., Japan. (iii) Sodium carboxymethylcellulose (CMC): Cellogen WS-C ($\overline{DS}$=0.62–0.68) was kindly supplied from Daiichi Industrial Pharmaceutical Co., Ltd., Japan. (iv) Avicel: A commercial product of microcrystalline cellulose powder, manufactured by Asahi Kasei Co., Ltd., Japan. (v) Filter paper: Whatman No.1 filter paper, produced by Whatman International, Ltd., England. (vi) Absorbent cotton: A commercial product of Japanese Pharmacopoeial Standard, manufactured by Warabieizai Co., Ltd., Japan.

Materials. QAE-Toyopearl 550C and Butyl-Toyopearl 650M were the products of Tosoh, Japan, and Mono Q and Alkyl Superose were from Amersham Pharmacia Biotech, Sweden. Polyacrylamide slab gel (PAGEL, NPU-12.5L), Ampholine PAGplate (pH 3.5–9.5), Coomassie Brilliant Blue R-250, SDS-PAGE molecular mass standards (low range) and IEF Calibration Broad p*I* Kit (pH 3–10) were the same products as used in a previous study.[14] Silver Stain II Kit Wako was a product of Wako Pure Chemical Industries, Japan. Pyroglutamate aminopeptidase from *Pyrococcus furiosus* was a product of Takara Shuzo Co., Ltd., Japan. All other reagents were of analytical grade.

Enzyme assays. CMC- and Avicel-saccharification activities were measured as described previously[14] by the colorimetric method of Somogyi[15] and Nelson.[16] One unit (U) of each saccharification activity was defined as the amount of enzyme liberating reducing sugars equivalent to 1.0 μmol of D-glucose from the substrate per minute under the assay conditions.

β-Glucosidase [EC 3.2.1.21] activity was measured as described previously.[14] One unit (U) of the enzyme activity was defined as the amount of enzyme that produces 1.0 μmol of *p*-nitrophenol under the assay conditions.

Protein assays. The protein concentrations of column effluents at each purification step were determined from the absorbance at 280 nm. When necessary, protein concentration was also estimated by the colorimetric method of Lowry *et al.*[17] using crystalline bovine serum albumin (Miles Laboratories, Inc.) as a standard.

Determination of carbohydrates. Total carbohydrate contents in both purified enzymes were determined by the phenol-sulfuric acid method[18] using D-glucose as a standard.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was done in 12.5% polyacrylamide with Tris-glycine buffer (pH 6.8) according to the method of Davis.[19] SDS-PAGE was done in 12.5% polyacrylamide containing 0.1% SDS by the method of Laemmli.[20] In both cases, the gels were stained with a silver staining kit (Silver Stain II Kit, Wako). The p*I* values of the purified enzymes were estimated by the method of Vesterberg[21] using Ampholine PAGplate (pH 3.5–9.5) and a LKB Multiphor II Electrophoresis Unit (Model 2117). Standard proteins with p*I* values ranging from 3 to 10 (Pharmacia Biotech) were run simultaneously. Gels were stained with 0.25% Coomassie Brilliant Blue R-250 for 10 min and destained with 8.0% (v/v) acetic acid until the

background became clear.

Analysis of N-terminal amino acid sequence. The N-terminal amino acid sequence analysis of the purified enzymes were performed by N-terminal Edman degradation employing a liquid-phase protein sequencer, PE Model 492 (Perkin-Elmer Co., USA).

Viscometry. The changes in viscosity during the enzymatic hydrolysis of CMC were measured at intervals with a Cannon-Fenske viscometer (c = 0.0369). The enzyme activity was expressed in terms of specific fluidity, $\phi_{sp.}$ (*i.e.*, $1/\eta_{sp.}$).

High-pressure liquid chromatography (HPLC). Sugar composition in the reaction mixture was quantitatively determined using HPLC on a Shimadzu LC-10 system as described previously.[14]

RESULTS

Purification of the enzymes.

Unless otherwise stated, all purification procedures for the enzymes were carried out in a cold room (4°C). The operations by FPLC were done at room temperature (25°C).

A commercial cellulase powder (5 g) was fractionated by QAE-Toyopearl 550C column chromatography, according to the procedures reported in a preceding paper,[14] into three cellulase fractions, named Peaks I, III and IV. Of these three cellulase fractions, Peak III was subjected to chromatography on a Butyl-Toyopearl 650M column for further purification.

(*1*) ***Butyl-Toyopearl 650M column chromatography.*** A concentrate of Peak III by ultrafiltration with a Diaflo PM10 membrane was dialyzed overnight in a Sartorius collodion bag against 20 mM acetate buffer (pH 4.0) containing 0.7 M $(NH_4)_2SO_4$. The dialyzed sample was put on a Butyl-Toyopearl 650M column (2.0×40.5 cm) preequilibrated with the same buffer. Stepwise elution was done with the same buffer containing 0.7, 0.5, 0.4, 0 M $(NH_4)_2SO_4$, and distilled water, respectively, at a flow rate of 0.6 mL/min. Each 5 mL fraction was collected and assayed for cellulase activity. As shown in Fig. 1, at least four cellulase peaks were eluted by this hydrophobic chromatography. The main active fraction (fraction Nos. 265–355 in Fig. 1) was pooled, concentrated and dialyzed overnight against 20 mM acetate buffer (pH 4.5).

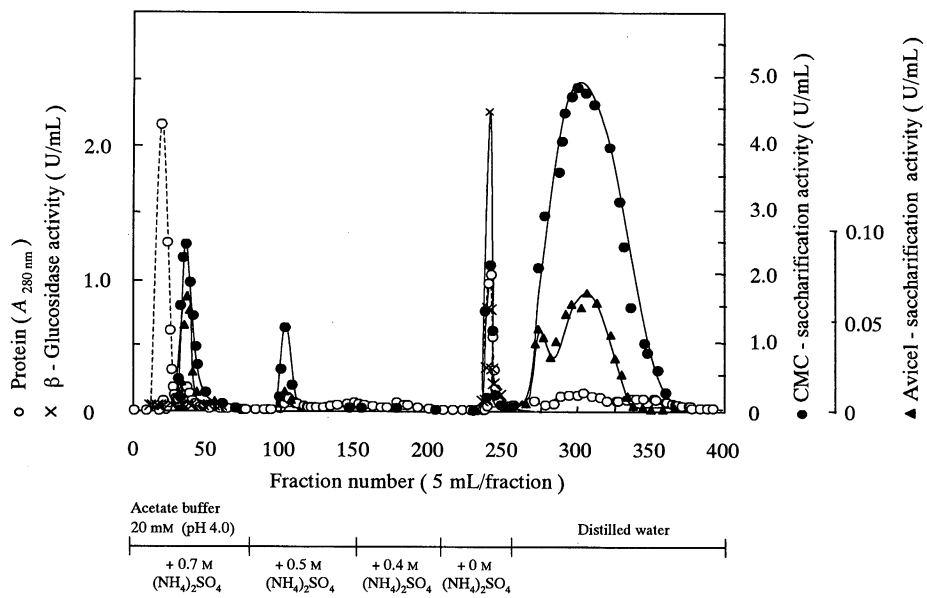

Fig. 1. Elution profiles of Peak III cellulase fraction after QAE-Toyopearl 550C column on Butyl-Toyopearl 650M column chromatography.

○, Protein ($A_{280\ nm}$); ●, CMC-saccharification activity (U/mL); ▲, Avicel-saccharification activity (U/mL); ×, β-glucosidase activity (U/mL).

(2) ***Mono Q column chromatography.*** The above sample was further applied to a Mono Q HR 5/5 FPLC column (0.5×5 cm) pre-equilibrated with 20 mM acetate buffer (pH 4.5) before the sample loading. The column was then eluted by a linear gradient of 0 to 100 mM NaCl in 20 mM acetate buffer (pH 4.5) at a flow rate of 0.5 mL/min. As shown in Fig. 2, two protein fractions (A and B) were obtained and both fractions possessed potent cellulase activity. The eluates forming Peak B (fraction Nos. 23–25 in Fig. 2) were then combined. This fraction, named cellulase III-B, gave a single protein band on both Native- and SDS-PAGE (Fig. 4).

(3) ***Alkyl Superose column chromatography.*** The other cellulase fractions in Peak A (fraction Nos. 17–19 in Fig. 2) were pooled and concentrated by Diaflo ultrafiltration. The concentrate was dialyzed overnight against 20 mM acetate buffer (pH 4.5) containing 1.5 M $(NH_4)_2SO_4$. The dialyzed sample was applied to an Alkyl Superose HR 5/5 FPLC column (0.5×5 cm) pre-equili-

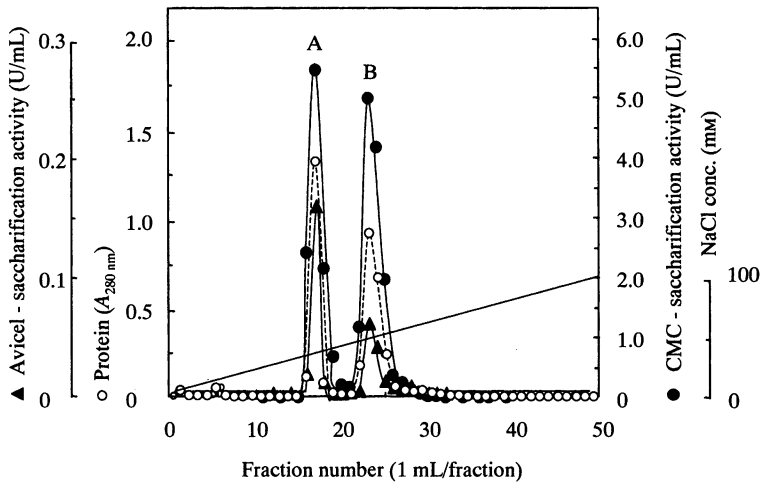

Fig. 2. FPLC elution profiles of cellulase fraction after Butyl-Toyopearl 650M column on Mono Q HR 5/5 column chromatography.
○, Protein ($A_{280\ nm}$); ●, CMC-saccharification activity (U/mL); ▲, Avicel-saccharification activity (U/mL).

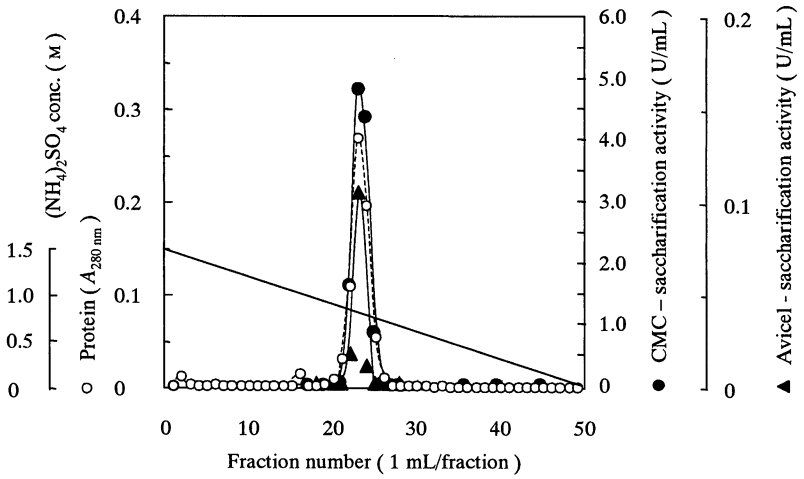

Fig. 3. FPLC elution profiles of Peak A cellulase fraction after Mono Q HR 5/5 column on Alkyl Superose HR 5/5 column chromatography.
○, Protein ($A_{280\ nm}$); ●, CMC-saccharification activity (U/mL); ▲, Avicel-saccharification activity (U/mL).

Table 1. Summary of the purification of cellulases III-A and III-B from *Acremonium cellulolyticus*.

Purification step	Protein[a] (mg)	Activity			Purification (fold)
		Unit	Yield (%)	Specific activity[b] (U/mL)	
1. Crude cellulase extract	3750.0	29325.0	100.0	7.8	1.0
2. QAE-Toyopearl 550C	1187.0	13828.6	47.0	11.7	1.5
3. Butyl-Toyopearl 650M	354.0	7257.0	24.7	20.5	2.6
4. Mono Q Peak A	119.0	2784.6	9.5	23.4	3.0
4. Mono Q Peak B (cellulase III-B)	80.6	2369.6	8.1	29.4	3.8
5. Alkyl-Superose (cellulase III-A)	78.6	1988.6	6.8	25.3	3.2

[a] Measured by the method of Lowry *et al.*[17] [b] CMC was used as a substrate.

brated with the same buffer. The column was then eluted by a linear gradient of 1.5 to 0 M $(NH_4)_2SO_4$ in the same buffer at a flow rate of 0.5 mL/min. A symmetrical protein peak containing only cellulase activity was obtained (Fig. 3). The active fractions (fraction Nos. 23–25) were combined as the purified enzyme. The purified enzyme was designated as cellulase III-A, and gave a single protein band on both Native- and SDS-PAGE.

Table 1 summarizes the specific activity and yield of cellulases III-A and III-B during the purification procedure. Cellulases III-A and III-B had specific CMC-saccharification activities of about 25 and 29 U/mg of protein in the final yields of about 7 and 8%, respectively, and were used in the subsequent characterization.

Physicochemical properties of the purified enzymes.

The molecular masses of cellulases III-A and III-B were estimated to be about 58 and 49 kDa by comparison of their relative mobility on SDS-PAGE with those of standard proteins, respectively (Fig. 4). The isoelectric points of cellulases III-A and III-B determined by isoelectric focusing were 4.6 and 4.2, respectively. Cellulases III-A and III-B contained approximately 14 and 16% carbohydrate (as glucose), respectively. The data seem to show that both purified cellulases are glycoproteins.

Concerning the N-terminal amino acid sequence of each purified enzyme, the first amino acid by Edman degradation was not detected, indicating the presence of a modified amino acid residue at the N-terminus. Polyglutamic acid was strongly

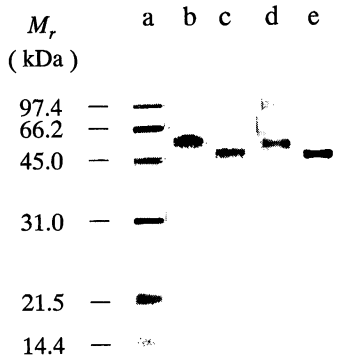

Fig. 4. Native- and SDS-PAGE of the two purified enzymes.
Lane a, standard protein mixture for SDS-PAGE containing phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), soybean trypsin inhibitor (21.5), and lysozyme (14.4); lanes b and c, cellulases III-A and III-B on SDS-PAGE, respectively; lanes d and e, cellulases III-A and III-B on Native-PAGE, respectively.

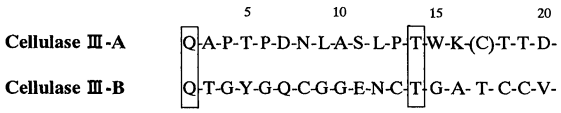

Fig. 5. N-terminal amino acid sequences of cellulases III-A and III-B.

suggested as the N-terminus amino acid in both purified cellulases judging from the result of polyglutamate aminopeptidase treatment. The N-terminal amino acid sequences from the 2nd up to the 20th residue of cellulases III-A and III-B are shown in Fig. 5.

Effects of pH on the activities of the enzymes.

The effects of pH on the activities of the puri-

fied enzymes were studied under the standard assay conditions against 0.25% CMC at 30°C for 10 min using 50 mM McIlvaine buffers at pH 3.0–8.0. Optimal activities of both enzymes were found to be at pH 5.5.

Stabilities of the enzymes toward pH and temperature.

Two series of enzyme solutions were prepared, each solution containing an amount (0.01 mL) of purified enzyme (0.1 U each) individually adjusted to a pH value from 2.5 to 11.0 by adding 0.04 mL of 50 mM McIlvaine buffer or Britton-Robinson buffer. After one set of solutions of each enzyme had been kept at 4°C for 24 h and the other at 45°C for 2 h, both series of buffered enzyme solutions were diluted 25-fold with 50 mM McIlvaine buffer (pH 5.5). Each 0.25 mL of enzyme sample was then examined for remaining CMC-saccharification activity by the standard assay at 30°C for 10 min. Cellulases III-A and III-B were completely stable over the range of pH 4.2–8.0 and pH 3.3–7.8 at 4°C, respectively. On the other hand, cellulases III-A and III-B were stable over the range of 4.2–6.5 and 4.6–7.8 at 45°C, respectively.

Cellulase solutions (0.1 and 0.15 U of cellulases III-A and III-B, respectively) in 0.05 mL of 50 mM McIlvaine buffer (pH 5.5) were heated at different temperatures for 10 min, and then cooled immediately in an ice-bath. They were diluted 50-fold with the same buffer. The remaining CMC-saccharification activity was then measured with 0.25 mL of each enzyme solution by the standard assay at 30°C for 10 min. Cellulases III-A and III-B were completely stable at temperatures below 55 and 60°C, respectively. Cellulases III-A and III-B retained 25 and 88% of their original activities on heating at 70°C for 10 min, respectively. Cellulase III-A was completely inactivated by heating at 80°C for 10 min, whereas cellulase III-B retained *ca.* 10% of its original activity even on heating at 100°C for 10 min.

Effects of temperature on the activities of the enzymes.

Cellulase solutions (0.01 and 0.015 U of cellulases III-A and III-B, respectively) in 0.75 mL of 50 mM McIlvaine buffer (pH 5.5) were incubated with 0.25 mL of 1% CMC solution at different reaction temperatures for 10 min. The cellulase activities per 1.0 mL of reaction mixture were assayed. The optimum temperatures for the activities of cellulases III-A and III-B were 55 and 65°C, respectively.

Effects of metal ions and inhibitors on the activities of the enzymes.

Each purified enzyme (0.96 U) was incubated with an equal volume (0.05 mL) of each metal ion or inhibitor solution (10 mM, pH 6.0) at 30°C for 30 min. Then the mixtures were diluted 10-fold with 50 mM McIlvaine buffer (pH 5.5), and the remaining enzyme activity per milliliter of each reaction mixture containing 0.24 U of enzyme was examined by the standard assay at 30°C for 10 min. Inactivations of cellulases III-A and III-B were found to be partial with 5 mM Mn^{2+}, Fe^{3+}, Hg^{2+} and $KMnO_4$, being about 13, 33; 52, 48; 92, 74 and 98, 90% inhibition, respectively. EDTA, NBS and other metal ions tested had no inhibitory effect on the activities of the enzymes.

Mode of action of the enzymes toward CMC.

The mode of action of the enzymes was analyzed using viscometry as described previously.[14] The reaction mixture was composed of 6 mL of 50 mM acetate buffer (pH 5.5) containing 0.5% CMC and 6 mL of the enzyme solution (0.04 U each for cellulases III-A and III-B, respectively). The results are shown in Fig. 6. Plots obtained for cellulase IV[14] are also shown in this figure: straight lines with distinctly different slopes were obtained for the three cellulases. The three lines were not parallel with the abscissa even at the early stages of hydrolysis. This indicates that the hydrolysis of CMC by these three cellulases proceeds according to an *endo*-lytic mechanism. The differences in slopes reflect differences in *endo*-lytic action, so that the hydrolysis of CMC by cellulase IV is by far the most *endo*-lytic among these three cellulases.

Action of the enzymes on cellooligosaccharides.

Each reaction mixture was comprised of 4 mM

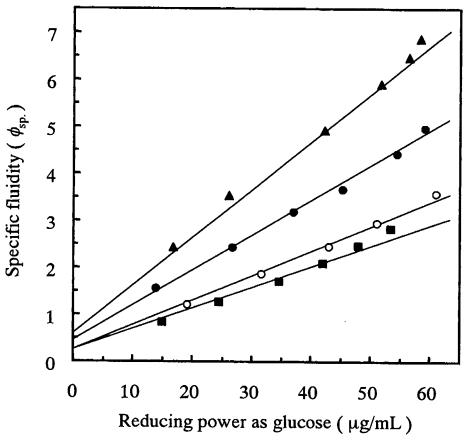

Fig. 6. Relationship between increases in fluidity and reducing power during the hydrolysis of CMC by cellulases III-A and III-B.

For comparison, the data obtained for a crude enzyme preparation and cellulase IV[14] are also shown. ■, Cellulase III-A; ●, cellulase III-B; ▲, cellulase IV; ○, crude enzyme preparation.

of each cellooligosaccharide (G_2–G_6) and cellulase solutions (0.01–0.05 U of each cellulase) in 0.4 mL of 10 mM acetate buffer (pH 5.5). The reaction mixtures were incubated at 30°C for an appropriate period. The hydrolysis products from cellooligosaccharides by cellulases III-A and III-B were analyzed by HPLC. Tables 2 and 3 show the degree of hydrolysis and product distribution due to the actions of two purified cellulases at the initial and late stages of incubation. Both cellulases III-A and III-B hydrolyzed G_6, G_5 and G_4 to give G_2 as the major end product. Cellulase III-A seemed to prefer cellooligosaccharides (G_3–G_6) as substrates rather than cellulase III-B. However, G_3 was fairly hard to attack by both enzymes compared to G_4–G_6. Both enzymes did not act entirely on G_2.

Action of the enzymes on insoluble substrates. Avicel, filter paper and absorbent cotton were

Table 2. Degree of hydrolysis and product distribution for cellooligosaccharides due to the action of cellulase III-A at the initial and late stages of incubation.

Substrate (4 mM)	Degree of hydrolysis (%)[a]	Time (min)	Product distribution (mM)					
			G_6	G_5	G_4	G_3	G_2	G_1
Cellohexaose	72.9	2	1.08	0.39	1.33	1.30	2.69	0.64
	100.0	240	—	0.13	0.22	0.47	7.71	4.64
Cellopentaose	53.4	2	—	1.86	0.47	1.63	1.66	0.39
	100.0	240	—	—	0.07	0.19	6.54	5.14
Cellotetraose	28.5	2	—	—	2.86	0.51	0.22	0.22
	98.8	240	—	—	0.05	0.13	6.29	2.32
Cellotriose	10.0	2	—	—	—	3.60	0.46	0.49
	94.7	240	—	—	—	0.13	3.51	3.36

[a]Calculated from the amount of residual substrate.

Table 3. Degree of hydrolysis and product distribution for cellooligosaccharides due to the action of cellulase III-B at the initial and late stages of incubation.

Substrate (4 mM)	Degree of hydrolysis (%)[a]	Time (min)	Product distribution (mM)					
			G_6	G_5	G_4	G_3	G_2	G_1
Cellohexaose	49.9	2	2.01	0.40	1.01	0.98	1.38	<0.01
	100.0	240	—	—	1.44	2.24	5.40	0.15
Cellopentaose	13.3	2	—	3.47	0.04	0.33	0.72	<0.01
	76.0	240	—	0.96	0.77	1.40	3.68	0.23
Cellotetraose	6.25	2	—	—	3.75	0.10	2.07	0.09
	95.4	240	—	—	0.18	0.94	5.50	1.02
Cellotriose	4.0	2	—	—	—	3.84	0.14	0.15
	61.9	240	—	—	—	1.53	2.25	0.72

[a]Calculated from the amount of residual substrate.

used as insoluble substrates. The reaction mixture contained 40 mg of each substrate and 2.0 mL of each purified enzyme (0.54 and 0.59 U of cellulases III-A and III-B, respectively) in 50 mM acetate buffer (pH 5.5). Other assay conditions were identical as described previously.[14] Reducing sugars and glucose formed in the reaction mixture were colorimetrically measured by the method of Somogyi[15]-Nelson[16] and of glucose oxidase,[22,23] respectively.

As shown in Fig. 7A, it is clear that cellulase III-A produces a high level of reducing sugars and a small amount of glucose from Avicel and filter paper. On the other hand, absorbent cotton was fairly hard to attack by this enzyme in a short-time incubation. HPLC analysis indicated that most reducing sugars produced were G_2. The amounts of reducing sugars and glucose produced by cellulase III-A were approximately 6 times higher than those by cellulase III-B (Fig. 7A and B).

DISCUSSION

Two highly purified cellulase components were separated from *Acremonium* cellulase, a crude cellulase preparation of *A. cellulolyticus*, by consecutive column chromatographies of QAE-Toyopearl 550C, Butyl-Toyopearl 650M, Mono Q and Alkyl Superose, until persistently contaminating β-glucosidase activity was completely removed. Both purified cellulases showed a single protein band on Native- and SDS-PAGE. They were designated as cellulase III-A and cellulase III-B.

As shown in Table 1, the specific activity of cellulases III-A and III-B increased 3.8- and 3.2-fold, respectively, compared with that in the original crude cellulase extract. Cellulase IV[14] showed a much higher saccharification activity toward CMC than both cellulases III-A and III-B. This strongly suggests that the crude cellulase extract is composed of several kinds of cellulase components with different substrate specificities, at least so far as CMC-saccharification activity is concerned.

The molecular masses of both cellulases III-A and III-B showed much larger values compared to that of cellulase IV (38 kDa) reported in a previous paper.[14] As is the case of cellulase IV[14] ($pI \leqq 3.4$), both purified cellulases were acidic proteins judging from their p*I* values. Cellulases III-A and III-B contained a considerable amount of carbohydrates (14–16% as glucose). This suggests that these two cellulases seem to be glycoproteins, as most extracellular enzymes from fungi are. As in the case of cellulase IV,[14] the N-terminal amino acids of both purified cellulases were also modified. According to the data of amino acid se-

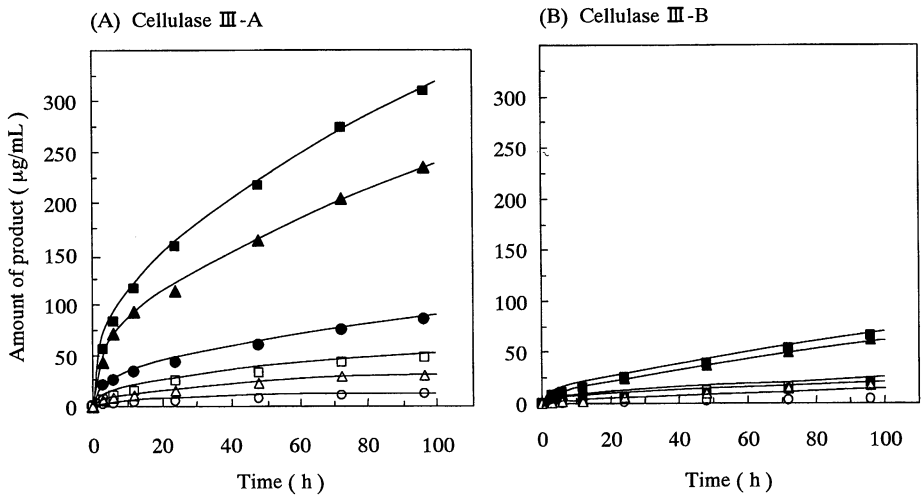

Fig. 7. Courses of reducing sugar and glucose formation due to the actions of cellulases III-A and III-B on insoluble substrates.

●, Reducing sugar from absorbent cotton; ○, glucose from absorbent cotton; ■, reducing sugar from filter paper; □, glucose from filter paper; ▲, reducing sugar from Avicel; △, glucose from Avicel.

quences by BLASTP, cellulase III-B showed that the enzyme has a high level of homology (*ca.* 70%) with *Penicillium jcenthinellum* endoglucanase II[24] from the N-terminus up to the 20th residue. On the other hand, cellulases III-A and III-B conserve identical amino acids at the positions of only the 2nd and 14th residue from each N-terminus sequenced up to the 20th residue.

The two purified cellulases were similar in regard to optimal pH and pH stability, but there were clear differences in the optimum reaction temperature (55 and 65°C for cellulases III-A and III-B, respectively). The activities of both cellulases were partially inactivated by 5 mM Mn^{2+}, Fe^{3+}, Hg^{2+} and $KMnO_4$ to various inhibition extents. On the other hand, EDTA and NBS showed no effect on the activities of both cellulases. This suggests that metals and sulfhydryl groups are not essential for the cellulolytic actions of the enzymes, as is the case with cellulase IV[14] and also highly purified cellulases from *T. viride*.[8,10]

The degree of *endo*-lytic action in the hydrolysis of CMC by three purified cellulases from *A. cellulolyticus* is shown in Fig. 6. There are significant differences in the slopes ($\phi_{sp.}$/R.P.) of the lines obtained for the three cellulases. This indicates that the mode of degradation by cellulase III-B is more *endo*-lytic than that by cellulase III-A, and cellulase IV[14] is by far the most *endo*-lytic among these three cellulases.

To investigate substrate specificity, saccharification activities of cellulases III-A and III-B were examined as a measure of the cellulolytic capacity using cellulosic substrates. Both enzymes were incapable of attacking either cellobiose or PNPG, however hydrolyzed various other substrates such as cellooligosaccharides (G_3–G_6), CMC, Avicel, filter paper, and absorbent cotton to various extents. As in the case of purified cellulases from *T. viride*,[9,10] the hydrolytic activities toward cellooligosaccharides by cellulases III-A and III-B increased with an increase in the chain length (degree of polymerization) of substrates. Cellulases III-A and III-B seemed to prefer G_3 rather than cellulase IV.[14] Both purified cellulases split various soluble and insoluble substrates to produce predominant cellobiose and a small amount of glucose as the final hydrolysis products, indicating the enzymes possess broad substrate specificities.

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糸状菌 *Acremonium cellulolyticus* 起源の2種エンドセルラーゼの精製と基本性質

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糸状菌 *Acremonium cellulolyticus* の市販酵素製剤から，エンドセルラーゼの二成分を各種カラムクロマトグラフィーを組み合わせ，高純度に精製し，それぞれをセルラーゼ III-A および III-B と呼称した．精製酵素は，いずれも電気泳動的に単一なタンパク質であり，両者は β-グルコシダーゼ活性を全く示さない標品であった．精製セルラーゼ III-A および III-B の分子量（SDS-PAGE 法）および等電点は，それぞれ 58 kDa，4.6 および 49 kDa，4.2 であった．両酵素はいずれも 14–16 %の糖含量（グルコース換算）を示した．また，これら 2 種の精製酵素の N 末端側第 2–第 20 番目までのアミノ酸配列をエドマン分解法により決定した．

セルラーゼ III-A および III-B の反応至適 pH および温度は，それぞれ 5.5，55°C および 5.5，65°C であり，セルラーゼ III-A および III-B の pH および温度に対する安定領域はそれぞれ pH 4.2–8.0，55°C 以下および pH3.3–7.8，60°C 以下であった．また，セルラーゼ III-A および III-B は，70°C，10 分間の加熱処理後，それぞれ 25 および 88% の残存 CMC 糖化活性を示した．セルラーゼ III-B による CMC の加水分解は，セルラーゼ III-A に比し，よりエンド水解度の高いものであった．両精製酵素を種々のセルロース性基質に作用させると，いずれの基質からも多量のセロビオースおよび少量のグルコースが最終生成糖として得られた．