Purification of Cellulase from *Trichoderma viride* and properties of Its Component Enzymes

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Major cellulase components, such as three endoglucanases (endoglucanases I, II, and III) and one exoglucanase (exoglucanase II), were isolated from a commercial cellulase (Meicelase TP 60) derived from the fungus *Trichoderma viride* by a series of chromatography procedures. These procedures were the gel filtration on Bio-Gel, the anion exchange on DEAE-Bio-Gel A, the cation exchange on SP-Sephadex C50, and the affinity chromatography on Avicel cellulose. The average molecular weights determined by SDS-polyacrylamide gel electrophoretic analysis were 51,000, 59,000, 41,000 and 62,000 Da for endoglucanases I, II and III and exoglucanase II, respectively. The extinction coefficients, $\varepsilon^{1\%}$ 280 nm, of these enzymes were 11.7, 3.3, 7.2 and 11.3, respectively. Among them, the endoglucanase II showed the very low value of the coefficient compared with the others. On the other hand, it was found that endoglucanase II and III were of more random hydrolytic mode on carboxymethylcellulose as compared with those of endoglucanase I and exoglucanase II. Especially, endoglucanase I showed less random action than that of exoglucanase II. In the hydrolysis of insoluble cellulose by the enzyme components, cellobiose was the major product, but glucose was the major product by endoglucanase III.

Introduction

The mechanism of cellulose degradation by cellulase is the difficult problem to be elucidated in some field of biochemistry. *Trichoderma* species as one of the most powerful fungi sources secreting cellulase have been considered with the enzymatic conversion of cellulosic materials to glucose.[1,2] The cellulase produced by cellulolytic funjgi consists of multiple form including three enzyme components, that is, 1,4-β-D-glucan glucanohydrolase (endoglucanase ; EC 3.2.1.4), 1,4-β-D-glucan cellobiohydrolase (exoglucanase ; EC 3.2.1.91), and β-D-glucoside glucohydrolase (β-glucosidase ; EC 3.2.1.21). These components from *Trichoderma viride* have been isolated by many investigators.[3-9] Generally, the hydrolysis mode of cellulose by multi-cellulases is as follows[10] : endoglucanases attack β-1,4-glycosidic bond at random positions in the cellulose chain, exoglucanases split off cellobiose or less commonly glucose from non-reduced chain ends, and β-glucosidase (EC 3.2.1.21) cleaves cellobiose to yield glucose. Most celullase complexes contain more than one endoglucanase and exoglucanase, and the isoenzymes may be modified forms derived from a single gene or may have separate genetic origins.[10-12] In many studies, at least, six endoglucanases, four exoglucanases, and three β-glucosidases were isolated from the cellulase complex. However, the comparative studies of the enzyme components are difficult to conduct, because enzyme characterization is carried out using different criteria and fungi species. Moreover, even the same species from different preparations secret distinct enzyme components from the complexes. The enzymatic hydrolysis of microcrystalline cellulose by the so-called 'synergistic action' which results from mutual interaction between both *endo-* and *exo*-type components[13] and two different *exo*-types[14,15] requires to be interpreted detailedly based on the physico-chemical properties of each component. For a study on cellulolytic action of cellulase, each component must be highly purified and the physico-chemical properties of each component must be examined. Even now, the hydrolysis mechanism by one exoglucanase and one endoglucanase have not been elucidated clear.

In this work, the isolation of a commercial cellulase derived from *Trichoderma viride* was fulfilled by column chromatography methods using various column materials. From this isolation experimental, major cellulase components of three endoglucanases (endoglucanases I, II, III) and one exoglucanase (exoglucanase II) were used to define their action patterns for hydrolysis of cellulose. Their properties were compared with each other.

Experimental

Analytical methods

Reducing sugar content was determined by the dinitrosalicylic acid (DNS) method[16] and total carbohydrate composition was estimated by the phenol-sulfuric acid method[17] using glucose as the standards. Protein concentration was also estimated by the Lowry method.[18]

Enzyme assay

Cellulase powder (Meicelase TP 60) of *Trichoderma viride* was a gift from Meiji Seika Kaisha Ltd., Japan. Avicel PH-101 (FMC Corporation, Philadelphia, PA, USA), Carboxymethylcellulose (CM-cellulose, medium viscosity ; Sigma Co., St. Louis, MO, USA), *p*-nitrophenyl β-D-glucopyranoside (PNPG ; Sigma Co., USA) and amorphous cellulose (H_3PO_4-treated cellulose) which was obtained by the precipitation of dissolved Avicel PH-101 by 85% H_3PO_4 with distilled water were used as cellulase substrates. All activities were described with International Units (IU) that one unit of activity is defined as the amount of enzyme required to liberate 1 μmol of product under experimental condition. Specific activities were measured by each assay method and described with protein concentration mg/mL per min.

Avicelase activity. This activity was determined by

placing 0.5 mL 1% Avicel suspension in 0.05 M sodium acetate buffer, pH 5.0 and 0.5 mL of enzyme solution in a test tube. The mixture was incubated at 30℃ for 20 h. After centrifugation, 0.5 mL of the supernatant was used to determine the reducing sugar by DNS method.[16]

CM-cellulase activity. This activity was measured using a mixture of 0.1 mL of 1% CM-cellulose solution in 0.05 M sodium acetate buffer, pH 5.0 and 0.1 mL of enzyme solution. After the mixture was incubated at 40℃ for 10 min and the reducing sugar was determined by DNS method. To determine viscometrically this activity, the mixture containing 10 mL of 0.5% CM-cellulose solution in 0.05 M sodium acetate buffer and 1 mL of enzyme solution was incubated in an Ubbelode viscometer at 25℃, and the specific viscosity (η_{sp}) of the mixture was measured with respect to reaction time.[7] Units of the activity are expressed in terms of the change in specific fluidity ($\Delta\Phi_{sp}$) per min. At the same condition, reducing sugar of the mixture was estimated by DNS method and compared with $\Delta\Phi_{sp}$.[3,19]

PNPGase activity. The assay mixture contained 1.0 mL of 0.5 mM PNPG in 0.05 M sodium acetate buffer, pH 5.0 and 0.1 mL of enzyme soultion. After incubation at 40℃ for 10 min. the mixture was poured into 9 mL of 1 M sodium carbonate solution. The concentration of *p*-nitrophenol liberated was measured at 400 nm.

Activity toward H_3PO_4-treated cellulose. A 1 mL suspension of 1% H_3PO_4-treated cellulose in 0.05 M sodium acetate buffer, pH 5.0 was mixed with 0.1 mL of enzyme solution. The mixture was incubated at 40℃ for 2 h. After centrifugation 0.5 mL of the supernatant was used to determine the reducing sugar by DNS method.[16]

Purification of cellulase components

Trichoderma viride cellulase solution was repeatedly chromatographied on various column at 4℃. Packed column materials were Bio-Gel P10 (100-200 mesh), Bio-Gel P100 (100-200 mesh), DEAE-Bio-Gel A (Bio-Rad Laboratories, Richmond, USA), SP-Sephadex C50 (Pharmacia Fine Chemicals, Uppsala, Sweden) and Avicel PH-101 cellulose.

SDS-gel electophoresis

An LKB 2001 Vertical Electrophoresis Unit was used for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was carried out on 10% polyacrylamide slab using the buffer system of Laemmli.[20] Samples were diluted 5 times with sample buffer consisting of 0.125 M Tri/HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.0025% bromophenol blue. Gel protein was stained with Coomassie brilliant blue.[21] Bovine serum albumin (66,000 Da), egg albumin (45,000 Da), glyceraldehyde-3-phosphate dehydrogenase (36,000 Da), carbonic anhydrase (29,000 Da), and trypsinogen (24,000 Da) were used as protein size markers.

HPLC analysis

Hydrolytic products released from cellulose were analyzed by high perfermance liquid chromatography (Waters Model 401 Unit, Waters Associates, Inc., Milford Mass. USA) with a differential refractometer. Soluble products were separated by using a Waters Carbohydrate Analysis Column, μ Bondarpak C18 Guard-Pak, and Cation Guard column with 75 : 25 (v/v) acetonitrile/water eluent system and 1.5 mL/min flow rate.

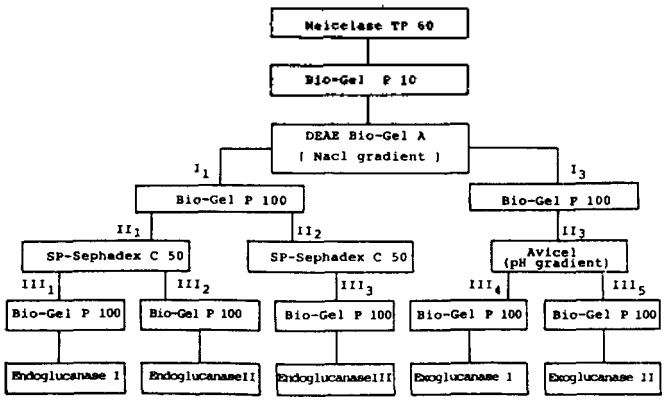

Figure 1. Flow sheet of the purification of endoglucanases and exoglucanases from a *Trichoderma viride* cellulase, Meicelase TP 60.

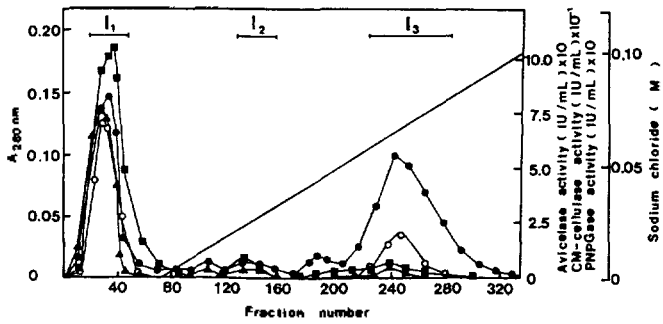

Figure 2. Fractionation of crude cellulase on DEAE Bio-Gel A column (30×200 mm). The flow rate was 25 mL/hr and the fraction volume was 5 mL. (●) Absorbance at 280 nm; (○) Avicelase; (■) CM-cellulase; (▲) PNPGase; (−) sodium chloride concentration.

Results

Purification

The chromatographic steps to the isolation of cellulase components are shown in Figure 1, collectively. 10 g of crude cellulase dissolved in 40 mL 0.01 M sodium acetate buffer, pH 5.0 was used as starting materials. After centrifugation for 20 min at 3000×g, the supernatant enzyme was desalted on a Bio-Gel P 10 column (30×900 mm) previously equilibrated with the same buffer. At this elution one peak only showed cellulase activities. The fraction was concentrated in an ultrafiltration stirred cell fitted by the disc membrane NMWC 10,000 (Cole-Parmer, Co., Chicago, IL, USA), and then applied to a DEAE Bio-Gel A column (30×200 mm) equilibrated with 0.01 M sodium acetate buffer, pH 5.0, and then eluted with sodium chloride gradient by using 0.1 M sodium chloride in the same buffer (Fig. 2). Three major peaks, I_1, I_2 and I_3, were obtained as shown in Figure 2.

Purification of endoglucanase I and II. The combined eluent of Peak I_1, containing various cellulase activities was concentrated by ultrafiltration and freeze-dried. This powder was dissolved in a small volume of buffer solution,

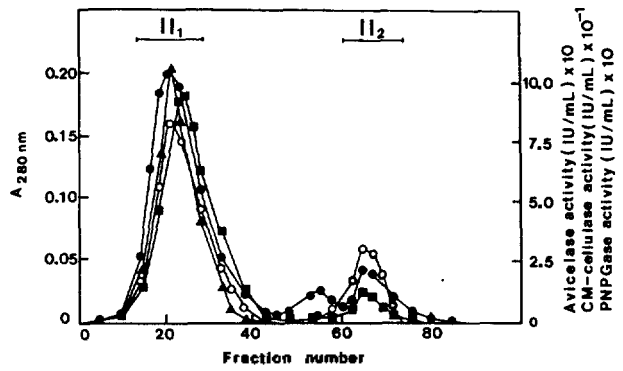

Figure 3. Fractionation of pool I_1 on Bio-Gel P 100 column (20×900 mm). The flow rate was 25 mL/hr and the fraction volume was 5 mL. (●) Absorbance at 280 nm; (○) Avicelase; (■) CM-cellulase; (▲) PNPGase.

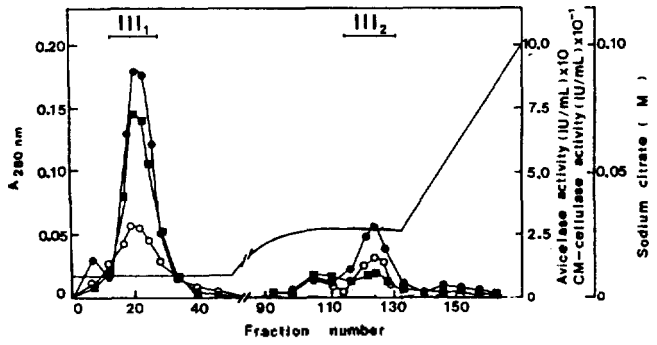

Figure 4. Fractionation of pool II_1 on SP-Sephadex C 50 column (25×300 mm). The flow rate was 6 mL/hr and the fraction volume was 5 mL. (●) Absorbance at 280 nm; (○) Avicelase; (■) CM-cellulase; (-) sodium citrate concentration.

applied to a Bio-Gel P 100 column (20×900 mm), and equilibrated in 0.02 M sodium citrate buffer, pH 3.5. As shown in Figure 3, two peaks (II_1 and II_2) showed cellulase activity. Peak II_1 and II_2 were dialyzed and concentrated by ultrafiltration, respectively. This concentrated II_1 was applied to a SP-sephdex C50 column (25×400 mm) equilibrated with 0.01 M sodium citrate buffer, pH 3.5. Stepwise elution was carried out with 0.025 M sodium citrate buffer and 0.1 M sodium citrate buffer, pH 3.5 (Fig. 4). Two peaks III_1 and III_2, which were associated with >70% of the protein absorbance of fraction eluted from the column, were isolated. Each peak was concentrated by ultrafiltration in 0.05 M sodium acetate buffer, pH 5.0, and applied to a Bio-Gel P 100 column (20×900 mm) equilibrated in the same buffer. After elution with the same buffer each single peak showed cellulase activity was obtained from the elution, respectively. They were dialyzed, concentrated in distilled water by ultrafiltration and then freeze-dried, and named endoglucanase I and endoglucanase II, respectively. They gave a single band on SDS-PAGE (Fig. 8), respectively. Endoglucanase I showed markedly high specific activities toward CM-cellulose and Avicel, while endoglucanase II showed very low specific activity toward Avicel (Table 1). From the HPLC analysis, glucose, cellobiose and cellotriose were detected in hydrolytic products of amorphous cellulose by these enzymes (Fig. 9). Thus

Table 1. Specific activities of endoglucanases I-III and exoglucanase II isolated from *Trichoderma viride* cellulase (Meielase TP 60)

Enzyme	Specific Activity[a] toward		
	CM-cellulose[b]	Avicel	H_3PO_4-treated cellulose[c]
endoglucanase I	17.0	19.4	24.0
endoglucanase II	2.5	0.6	1.2
endoglucanase III	1.2	2.3	1.6
exoglucanase II	1.0	1.7	2.6

[a]Activity at protein concentration mg/mL per min. For experimental details see the text. [b]Carboxymethylcellulose. [c]Amorphous cellulose regenerated with phosphoric acid.

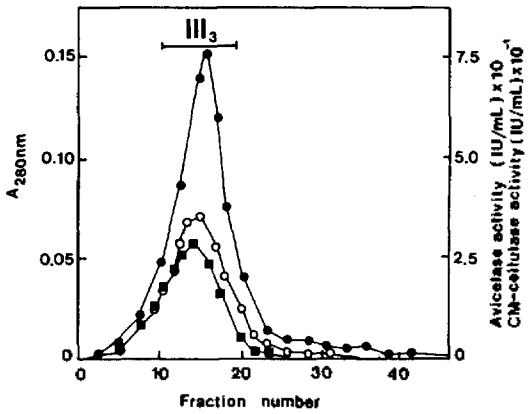

Figure 5. Fractionation of pool II_2 on SP-Sephadex C 50 column (20×200 mm). The flow rate was 20 mL/hr and the fraction volume was 10 mL. (●) Absorbance at 280 nm; (○) Avicelase; (■) CM-cellulase.

these enzymes were identified as endoglucanase I and II.

Purification of endoglucanase III. The peak II_2 with Figure 3 was also dialyzed and concentrated by ultrafiltration in 0.05 M sodium acetate buffer, pH 5.0. This concentrated II_2 was applied repeatedly to a SP-shepdex C50 column (25×200 mm) equilibrated in 0.02 M sodium citrate Buffer, pH 3.5 (Fig. 5). And the combined peak III_3 fraction containing cellulase activity was concentarated by ultrafiltration in 0.05 M sodium acetate buffer, pH 5.0, and then applied to a Bio-Gel P 100 column (20×900 mm). The major peak showed cellulase activity was obtained from the elution, this peak containing CM-cellulase activity and low Avicelase activity was dialyzed, concentrated rapidly in distilled water by ultrafiltration and then freeze-dried, and named endoglucanase III. From the HPLC analysis, glucose and cellobiose were detected in hydrolytic products of amorphous cellulose by this enzyme as typical endoglucanase (Fig. 9). This fraction gave a single band on SDS-PAGE (Fig. 8).

Purification of exoglucanase II. In Figure 2, the combined peak I_3 containing complex cellulase activity was desalted on a Bio-Gel P 10 column (30×900 mm), concentrated by ultrafiltration, and then eluted on a Bio-Gel P 100 column (20×900 mm) equilibrated in 0.05 M sodium citrate buffer, pH 4.0 (Fig. 6). After the elution, Peak II_3 was dialy-

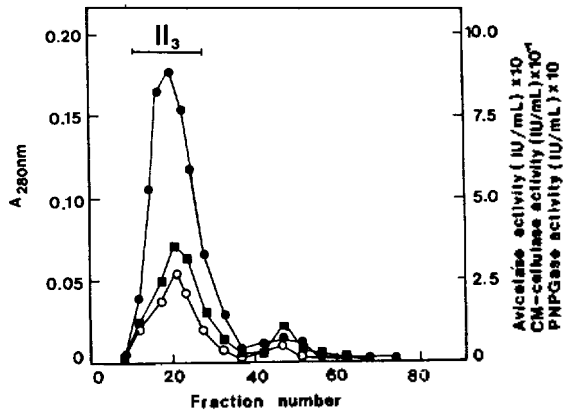

Figure 6. Fractionation of pool I_3 on Bio-Gel P 100 column (20×900 mm). The flow rate was 25 mL/hr and the fraction volume was 5 mL. (●) Absorbance at 280 nm; (○) Avicelase; (■) CM-cellulase.

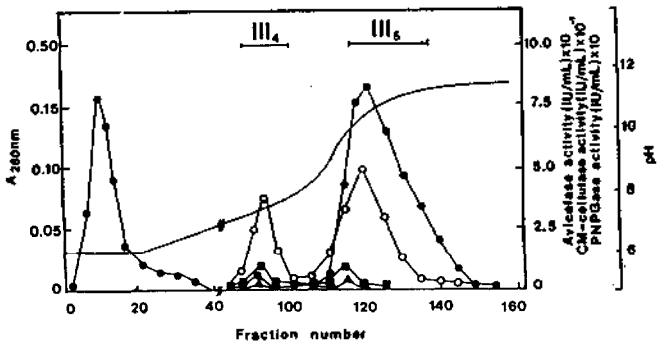

Figure 7. Fractionation of pool II_3 on Avicel column (22×100 mm). The flow rate was 50 mL/hr and the fraction volume was 5 mL. (●) Absorbance at 280 nm; (○) Avicelase; (■) CM-cellulase; (▲) PNPGase; (−) pH.

zed by ultrafiltration to 0.1 M potassium-sodium phosphate buffer, pH 6.0 and applied to the Avicel column (25×100 mm) and then eluted with a pH gradient in 0.1 M potassium-sodium phosphate from pH 6.0 to pH 11.8 as shown in Figure 7. The fraction tube containing 2.5 mL of 0.2 M potassium dihydrogen phosphate solution was used for neutralization to pH 7.0. Two peaks III_4 and III_5 containing high specific activity toward Avicel and very low activity toward CM-cellulose were isolated similarly as described by Beldman *et al.*[8] Main peak III_5 was dialyzed and concentrated by ultrafiltration, and then applied to a Bio-Gel P 100 column (20×900 mm) equilibrated in 0.05 M sodium acetate buffer, pH 5.0. The eluted main peak, exoglucanase II, gave a single band on SDS-PAGE (Fig. 8). Exoglucanase II as a major *exo*-type enzyme released cellobiose as the only product in the hydrolysis of amorphous cellulose, but glucose as negligible quantity (Fig. 9). This component acted synergistically with another endoglucanases purified in this work during the hydrolysis of celluose (unpublished data).

Properties of celulase components

Physico-chemical properties of the purified cellulase components, *i.e.*, endoglucanases (I, II and III) and exoglucanase II, were summerized in Table 2. Average molecular weights (M_r) of these enzymes were determined by SDS-PAGE (Fig.

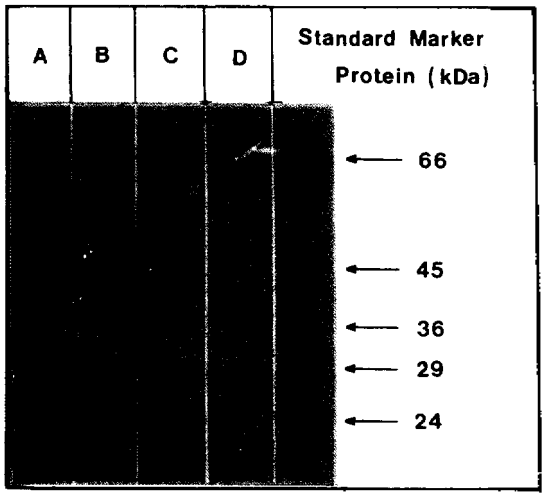

Figure 8. SDS/polyacrylamide gel electrophoresis of endoglucanases I-III and exoglucanase II purified from commercial cellulase derived from Meicelase TP 60. The gel had a polyacrylamide concentration of 10%. (A) endoglucanase I; (B) endoglucanase II; (C) endoglucanase III; (D) exoglucanase II. Proteins were stained with Coomassie Brilliant Blue. Standard proteins: bovine serum albumin (66 kDa), egg albumin (45 kDa), and glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa) and trypsinogen (24 kDa).

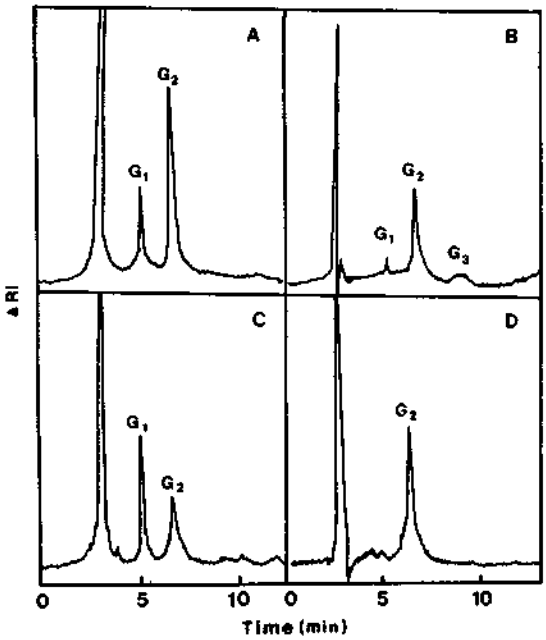

Figure 9. HPLC analysis of soluble products from endoglucanase I, II and III (A, B and C, respectively) and exoglucanase II (D) reaction with H_3PO_4-treated cellulose. G_1, glucose; G_2, cellobiose; G_3, cellotriose; RI, refractive index.

8). Their average molecular weights estimated for endoglucanases (I, II and III) and exoglucanase II were 51,000, 59,000, 41,000 and 62,000 Da, respectively.

Endoglucanases I, II and exoglucanase II were optimally active between pH 4.5-5.5 and decreased markedly above pH 6.0, but endoglucanase III showed a low pH optimum

Table 2. Properties of endoglucanases I-III and exoglucanase II isolated from *Trichoderma viride* cellulase (Meielase TP 60)

Enzyme	Optimum temp.(℃)	Optimum pH	Carbohyd-rate (%)	$\varepsilon^{1\%a}$ 280 nm	MW[b] (Da)
endoglucanase I	53-58	4.5	20.9	11.7	51,000
endoglucanase II	58	4.7-5.3	2.3	3.3	59,000
endoglucanase III	50-55	3.5-3.9	10.0	7.2	41,000
exoglucanase II	70	4.9-5.2	5.8	11.3	62,000

[a]Extinction coefficient. [b]Average molecular weight.

Table 3. Relationship between increase in fluidity (Φ_{sp}) and the release of reducing sugar for the hydrolysis of CM-cellulose

Enzyme	$\Delta\Phi_{sp}$/reducing sugar
endoglucanase I	0.28
endoglucanase II	0.90
endoglucanase III	2.55
exoglucanase II	0.63

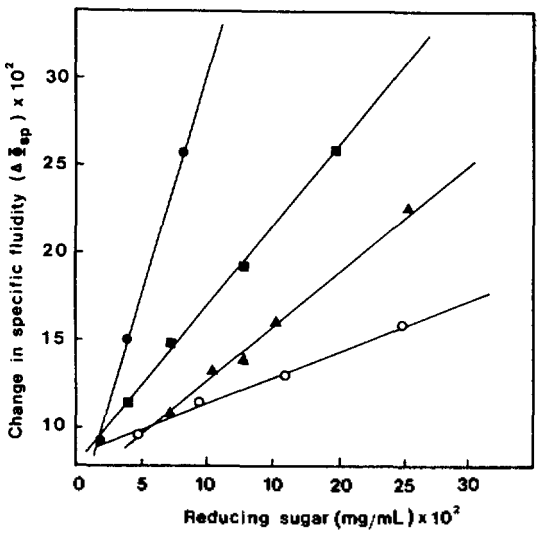

Figure 10. Relationship between change in fluidity and production of reducing sugar during the hydrolysis of CM-cellulose by cellulase components. (○) endoglucanase I; (■) endoglucanase II; (●) endoglucanase III; (▲) exoglucanase II.

range of 3.5-3.9. Optimum temperature of endoglucanases were 58-60℃, and *exo*-glucanase 70℃, but the stability of these enzymes was decreased sharply at higher than optimum temperatures. All the enzymes were identified as glycoproteins, and the carbohydrate contents determined by phenol-sulfuric acid method[17] were ranged from 2 to 21%. The extinction coefficients, $\varepsilon^{1\%}$ 280 nm, of these components were 11.7, 3.3, 7.2, and 11.3, for endoglucanases I, II and III, and exoglucanase II, respectively. Among them, endoglucanases II and III showed the low value of the coefficient compared with endoglucanase I and exoglucanase II (Table 2).

As shown in Table 3, the degree of randomness of the enzymes was tested depending on the decrease in viscosity after incubation of 1% CM-cellulose solution and each enzyme. At the same time, the amount of reducing sugar was measured by the DNS method. The change in specific fluidity and the released reducing sugar during the hydrolysis of CM-cellulose by each enzyme were drawn in Figure 10 as good linearity. In a larger positive slope, the enzyme has greater randomness in attacking substrate. A slope of exoglucanase II lies between those of endoglucanase I and endoglucanase II. Endoglucanase III gives the largest positive slope, resulting in a low CM-cellulase activity. Thus endoglucanase I and exoglucansse II were a less random type of enzyme. Endoglucanase I showed that of a least random type. As an *endo*-type enzyme, from the HPLC analysis for the released product by endoglucanase I, glucose, cellobiose and slightly cellotriose were identified as products. But, a distinct *endo*-action of both endoglucanase II and III resulted in a large positive slope.

Discussion

The commercial cellulases from *Trichoderma viride* or *Trichoderma reesei* showed different physico-chemical properties with respect to the sources of products.[8,9] First, in this experiment, three major enzyme components (endoglucanases I, II and III) showing high activity against CM-cellulose were classified as typical endoglucanase. However, the definition is not clear, since exoglucanase II isolated in this study showed appreciable activity toward CM-cellulose. Additional information for the classification was supplied by the HPLC analysis of hydrolysis products of amorphous cellulose. Three endoglucanases released various saccharides with cellobiose as a main product during the hydrolysis of amorphous cellulose, while exoglucanase II released cellobiose with a negligible quantity of glucose. When exoglucanase II combined with each endoglucanase, it showed a large synergistic effect on hydrolysis of cellulose (unpublished data). Therefore exoglucanase II had typical mode of *exo*-glucanase. All purified cellulase had not PNPGase activity. Each of the endoglucanases I, II and III appeared as a single band in SDS-PAGE. Schoemaker *et al.*[6,7] and Beldman *et al.*[8] isolated a low-molecular mass and a low carbohydrate content endoglucanase from a commercial cellulase from *Trichoderma viride*. We also obtained this component as endoglucanase III. In addition, it was reported that above mentioned low-molecular mass endoglucanses give a larger slopes as random-type by the measure of the degree of randomness of endoglucanase.[8]

Endoglucanase I is a less-random type of component, since it gave a very small slope in Figure 10. This characteristic component was described as endoglucanase III by Schoemaker *et al.*,[6,7] which was described as Endo V by Beldman *et al.*[8] However, this enzyme was markedly active against CM-cellulose and Avicel substrates in comparison to other components. Surprisingly, endoglucanase I yielded a very small slope which reflected the type of exoglucanase action. On the other hand, endoglucanase II isolated in this study is a more random type of enzyme, and this component is similar to Endo II and endoglucanase II described by Beldman *et al.*[8] and Schoemaker *et al.*,[6,7] respectively, with respect to optimum temperature, degree of randomness in hydrolysis of CM-cellulose and low activity of Avicel. Similar endogluca-

nase was purified as cellulase III by Okada.[3] Nevertheless, its extinction coefficient at 280 nm is much less than the value of endoglucanase II isolated by Schoemaker *et al.*[7] This coefficient for cellulase component was rarely to be reported. Moreover, the molecular weight of this enzyme is larger than the molecular weight corresponding to this enzyme.

Beldman *et al.*[8] purified three exoglucanases, and two of them as Exo I and II were isolated by the affinity chromatography using crystalline cellulose with pH gradient, which resulted in a high affinity to Avicel. Gum *et al.*[5] purified three exoglucanases from Meicelase P from *Trichoderma* species and suggested that their carbohydrate content is the principal factor which differentiates the cellobiohydrolase enzymes. However, it was reported that no distinct differences in the elution process were found. Exoglucanase II isolated in this study as a major *exo*-type enzyme gives a low activity toward CM-cellulose and Avicel as like as Exo III isolated by Beldman *et al.*[8] and CBH III by Gum *et al.*[5] These enzymes have produced only cellobiose from cellulose. Similar exoglucanase was isolated by Shikata *et al.*[4] from *T. viride*. All exoglucanase III-type enzymes reported showed a high affinity to crystalline cellulose with a very low activity to crystalline cellulose.

From the results presented here, these similarities between the enzyme components classified by several investigators indicate partially that the multiplicity of endoglucanases are determined genetically. Nevertheless, endoglucanases I and II showed a less similarity in hydrolytic and spectroscopic properties. Further work considering these differences is required to understand the hydrolytic characteristics of these component enzymes on insoluble cellulose.

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Theoretical Investigation on the Effects of Additive Oxygen in HF Chemical Laser Performance

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The effect of oxygen for the HF chemical laser performance has been theoretically investigated. Due to the inhibition mechanism of O_2 in H_2/F_2 chain reaction, the rate for the formation of HF is reduced by the addition of O_2. As the concentration of O_2 in the reaction mixture increases, the pulse power and temperature of the system becomes lower, while total output energy does not change significantly. But addition of O_2 makes the system easy to be controlled and the composition of H_2+F_2 can be high at constant total pressure. With this system, it is possible to obtain higher output energy than oxygen free environment.

Introduction

During last two dacades a great interest has been made for the utilization of hydrogen-fluorine reaction in HF chemical laser system.[1,2] The reaction of hydrogen with fluorine releases large exothermic energy and makes it possible to