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A Role and Properties of C_1 Enriched Cellulase Fraction from Anaerobic *Clostridium thermocellum* in Cellulose Degradation

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섬유소 분해시 혐기성 *Clostridium thermocellum*이 생산하는 Cellulase의 C_1성분의 역할과 성질

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ABSTRACT: A C_1 enriched cellulase fraction was separated from culture filtrate of anaerobic *Clostridium thermocellum* by hydroxyapatite column chromatography. The separated fraction showed strong synergistic action with C_x component (endo-β-1,4-glucanase) in digestion of crystalline cellulose, similar to the other aerobic cellulolytic microorganisms. Unlike the C_x component the C_1 enriched fraction was rapidly inactivated by oxidation at the atmospheric condition. The enzyme activity was significantly enhanced by the addition of reducing agents, especially β-mercaptoethanol, which indicates that a C_1 component has a lot of sulfhydryl groups essential for the enzyme activity. The effect of metal ions on C_1 activity was also investigated. The C_1 fraction was found to be thermally stable compare to endo-β-1,4-glucanase. Optimal temperature and pH were found to be 60°C and 6.0, respectively.

KEY WORDS□*Clostridium thermocellum*, C_1 component, Cellulose degradation.

A thermophilic anaerobic *Clostridium thermocellum* has drawn a great deal of attention as a potential biofuel producing microorganism due to its ability of converting cellulosic biomass directly to ethanol with concomitant accumulation of sugar.

The fractionation of the constitute of cellulase complex is an essential step for understanding the cellulose degradation mechanism of above strain. Several research groups (Petre *et al.*, 1981; Ng and Zeikus, 1981; Kim *et al.*, 1987) attempted to fractionate the cellulase complex, as a result, three different kinds of endo-β-1,4-glucanase (C_x component) were purified and their properties were characterized. Also the presence of multicomponent enzyme complex (cellulosome) with molecular weight as large as 2.1×10^6 daltons was confirmed, its role in degradation of cellulosic biomass was discussed (Bayer *et al.*, 1985; Bayer and Lamed, 1986; Hon-nami *et al.*, 1986; Lamed *et al.*, 1983, 1985). This cellulosome may appear in being cell-bound to be associated with vesicular or membranous material and being cell-free forms (Bayer and Lamed, 1986; Hon-nami *et al.*, 1986). The presence of an affinity or binding factor required for the attachment of the cellulase complex to cellulose has been proposed for this organism (Ljungdahl *et al.*, 1983; Bayer *et al.*, 1983).

It is generally known that the degradation of crystalline cellulose is usually carried out by the synergistic action of at least three enzyme components: C_x, (endo-β-1,4-glucanase), C_1 (exo-β-1,4-cellobiohydrolase), and β-glucosidase (Lee

and Fan, 1980). Especially the role of C_1 component is critical for effective hydrolysis of crystalline portion of cellulose. However, the separation of C_1 component from *C. thermocellum* has not been succeeded. This may be due to the difficulty of separation caused by the inactivation during purification procedure. Therefore, the synergism among cellulase components of *C. thermocellum* could not be investigated. Johnson and Demain (1984), who studied the enzyme degradation mechanism using crude extracellular cellulase, suggested that C_1 component was oxidatively inactivated by air, on the other hand, C_x component was not susceptible to oxidative inactivation.

Recently, we separated a C_1 enriched cellulase fraction by hydroxyapatite column chromatography, and report a role and properties of C_1 enriched fraction of *C. thermocellum* in degradation of crystalline cellulose.

MATERIALS AND METHODS

Organism and cultivation

Clostridium thermocellum ATCC 27405 was cultivated anaerobically at 60°C for 5 days at CM3 medium (Weimer and Zeikus, 1977) containing 1% α-cellulose as a carbon source. 0.1% cystein-HCl was added to the medium as a reducing agent. The medium was deoxygenated with N_2 gas and sealed with a rubber stopper.

Determination of cellulase activities

Endo-β-1,4-glucanase (CMCase or C_x) activity; 0.5mL of enzyme solution was added to 0.5mL of 1% carboxymethyl cellulose (CMC) solution in 50mM acetate-NaOH buffer (pH 5.0). The mixture was incubated at 50°C for 30min, and then the amount of reducing sugar was determined by the method of DNS (Miller, 1959) with glucose as standard. The unit of enzyme activity was defined as 10μg glucose liberated at 50°C for 1hr.

Filter paper (FPase) activity: 0.5mL of enzyme solution was added to 1mL of 50mM acetate-NaOH buffer containing 50mg of Whatman No. 1 fiter paper (1 × 6cm strip). After incubation of the mixture at 50°C for 1hr, the amount of reducing sugar was determined. The unit of enzyme activity was defined the same with previous section.

Sigmacellase (C_1) activity; 1mL of enzyme solution was added to 1mL of 1% Sigmacell (Sigma Co.) suspension in 50mM acetate-NaOH buffer (pH 5.0). The mixture was incubated with continuous shaking at 50°C for 12hr. After centrifugation of the reactant at 3,000 × g for 30min, 1mL of the supernatant was taken. The amount of reducing sugar released from the reactant and the unit of enzyme activity were determined.

Protein determination

Soluble protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Separation of C_1 enriched fraction

The culture broth was centrifuged at 12,000 × g for 15min to eliminate the cell and solid materials. 0.3L of the supernatant was applied to hydroxyapatite column (2.8 × 19cm) that was previously equilibrated with 5mM phosphate buffer (pH 6.0). The nonbinding substance to the column was fractionated to obtain C_1 enriched fraction (F-A) with 50mL/hr of flow rate and 5.0mL of fraction volume. On the other hand, the binding substance was eluted with linear gradient from 0 to 1M phosphate buffer (pH 6.0) to obtain C_x enriched fraction (F-B) with the same flow rate and fraction volume.

RESULTS

Fractionation of cellulase complex

The cellulase components in the culture filtrate were separated into two fractions on the hydroxyapatite column chromatography; nonbinding fraction, and binding fraction but eluted with 1M phosphate buffer. Fig. 1 illustrates a typical elution profile of each cellulase component. The first eluted non-binding fraction (F-A fraction, fraction number 15-80) showed mainly filter paper activity (FPase), meanwhile, the relatively low activity on soluble carboxymethyl cellulose. On the other hand, the binding fraction (F-B fraction, fraction number 120-150) showed strong CMCase activity but low FPase activity.

C. thermocellum excretes a yellow substance in

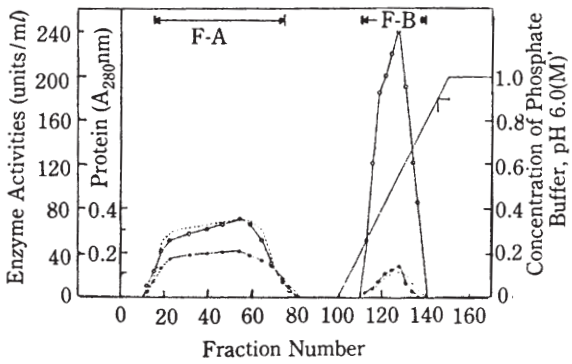

Fig. 1. *Fractionation of the cellulase complex of C. thermocellum on hydroxyapatite column (2.8x19 cm).*
The flow rate was 50ml/hr and the fraction volume was 5.0ml.
Protein(------), CMCase activity(○ — ○), FPase activity(● — ●).

culture broth during growth. The yellow substance which was known to be binding factor of endo-glucanase to cellulose-fiber and be involved in the cellulolytic process was presented in F-A but not in F-B.

Table 1 summerizes the distribution of soluble protein, CMCase activity, FPase activity on each separated fractions, and compares with those of crude enzyme. Around 78.1% FPase activity was recovered in F-A fraction, and the ratio of FPase activity over CMCase activity was 0.73 compared with those of F-B fraction of 0.11. Therefore, it can be assured that the separated F-A is C_1 component enriched fraction.

In other experiment, we attempted the separation of cellulase complex using Sigmacell column chromatography instead of hydroxyapatite column chromatography. The binding and nonbinding patterns of cellulase complex on Sigmacell column were found to be similar to those of hydroxyapatite column, even though the separation efficiency on cellulose column was much lower than those of hydroxyapatite column.

Synergistic action of C_1 enriched fraction with other cellulase components

The synergistic action of C_1 enriched fraction with other cellulase components from *Clostridium thermocellum* was also investigated. The other components include the binding fraction (F-B) and endo-β-1,4-glucanase that was previously purified in our laboratory (Kim *et al.*, 1987).

Table 2 shows synergistic action between C_1 enriched fraction and other components when each component was diluted to be equivalent to the 0.5mL of testing enzyme solution. The F-A and the F-B retained 37.5% and 16.5% of the FPase activity of the original culture filtrate, respectively. However, when the F-A and the F-B were recombined in their equivalent proportions, 94% of FPase activity of the original culture filtrate was recovered. On the other hand, when the F-A was recombined with the purified endo-β-1,4-glucanase in the same proportions, the FPase activity of the mixture was recovered up to 78%.

Table 2. *Synergism between C_1 enriched fraction (F-A) and other C_x components (F-B, Endo-β-1,4-glucanase) of cellulase complex*

Components	FPase activity(%)	Sigmacellase activity(%)
Crude enzyme	100.0	100.0
F-A	37.5	38.0
F-B	16.5	17.5
Endo-β-1,4-glucanase	0	0
F-A + F-B	94.0	90.7
F-A + Endo-β-1,4-glucanase	78.0	77.5

Table 1. *Distribution of enzyme activities in each fraction isolated from C. thermocellum on hydroxyapatite column chromatography*

Fractions	Total protein		Total CMCase activity		Total FPase activity		FPase activity
	(mg)	(%)	(units)	(%)	(units)	(%)	CMCase activity
Crude enzyme	235.3	100.0	60,000	100.0	16,000	100.0	0.27
F-A	134.0	56.9	17,000	28.3	12,500	78.1	0.73
F-B	13.6	5.8	22,800	38.0	2.520	15.7	0.11

The synergistic action on the digestion of Sigmacell, highly crystalline form of cellulose, was also determined. The magnitude of synergism was found to be similar to the result obtained from filter paper.

The synergistic action on FPase and Sigmacellase activities was significantly enhanced by Ca^{++}, Ni^{++}, β-mercaptoethanol and their combination as shown in Table 3. Especially, the activity of Sigmacellase was greatly increased up to about 9.5 times by the combination of Ca^{++} and β-mercaptoethanol.

Effect of reducing agents and chemicals on the activity of C_1 enriched fraction

The FPase activity of the C_1 enriched fraction is stimulated by reducing agents such as DTT, cystein-HCl and β-mercaptoethanol (Table 4). It was stimulated to 147% and 218% by 50mM DTT and cystein-HCl, respectively, but inhibited by above 50mM concentration of the reducing agents. The hightest stimulation up to 388% was obtained by 0.4M β-mercaptoethanol which can convert disulfides into thiols. But it was inactivated beyond the β-mercaptoethanol concentration of 0.4M. Meanwhile, it was inhibited by *p*-CMB, mono iodoacetate and sodium cyanide.

Effect of metal ions on the activity of C_1 enriched fraction

The FPase activity of C_1 enriched fraction was slightly stimulated by Ni^{++} and Ca^{++} but strongly inhibited by Cu^{++}, Hg^{++} and Fe^{++} (Table 5). It was stimulated up to 138% by 10mM Ca^{++} and 131% by 10mM Ni^{++}, respectively.

Table 3. *Effect of activators on the synergism between F-A and F-B*

Activators	FPase activity(%)	Sigmacellase activity(%)
None	100	100
Ca^{++}(10mM)	144	202
Ni^{++}(10mM)	136	252
β-Mercaptoethanol(400mM)	200	862
Ca^{++}(10mM)+Ni^{++}(10mM)	168	633
Ca^{++}(10mM)+β-Mercaptoethanol(400mM)	232	948

Optima of pH and temperature

As shown in Fig. 2 the optimum temperature and pH were estimated to be 60°C and 6.0, respectively. Optimum pH was different from puri-

Table 4. *Effect of reducing agents and chemicals on the FPase activity of C_1 enriched fraction*

Chemicals	Concentration (mM)	Relative activity(%)
None	—	100
DTT*	5	118
	10	135
	50	147
Cystein-HCl	5	135
	10	159
	50	218
β-Mercaptoethanol	5	106
	10	123
	50	147
	100	206
	400	388
p-CMB*	10	54
o-Phenanthroline	10	85
Mono iodoacetate	10	68
EDTA*	10	81
Urea	10	98
Sodium cyanide	10	12

* DTT; Dithiothreitol,
p-CMB; *p*-Chloromercuribenzoic acid,
EDTA; Ethylenediaminetetraacetic acid.

Table 5. *Effect of metal ions on the FPase activity of C_1 enriched fraction*

Metal ions (10mM)	Relative activity(%)
None	100
Ca^{++}	138
Ni^{++}	131
Mg^{++}	100
Pb^{++}	42
Zn^{++}	17
Cu^{++}	4
Hg^{++}	4
Fe^{++}	2
Mn^{++}	25
Na^{+}	65
K^{+}	60

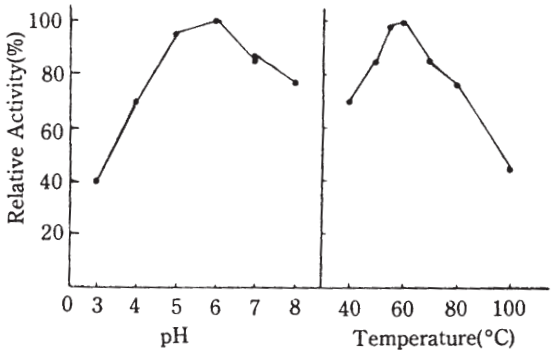

Fig. 2. *Effect of pH and temperature on the FPase activity of C_1 enriched fraction.*
Buffers used were 0.1M citrate-phosphate buffer (pH 3.0-7.0) and 0.1M phosphate buffer (pH 7.0-8.0).

fied endo-β-1,4-glucanases whose optimum pH were 5.2 and 5.0 (Ng and Zeikus, 1981; Kim *et al.*, 1987). The optimum temperature of C_1 component from *T. koningii* was measured to be 37°C (Halliwell and Griffin, 1973).

Thermal and pH stabilities

The C_1 enriched fraction showed remarkable thermal stability (Fig. 3). 60% of the initial FPase activity of the C_1 enriched fraction was remained after treatment for 20 minutes at boiling water. The C_x component was mostly inactivated at 80°C (Kim *et al.*, 1987). pH stability was determined by keeping at room temperature for 2 hours at each pH. The C_1 enriched fraction was shown to be relatively stable in wide pH ranges from 5.5 to 9.0.

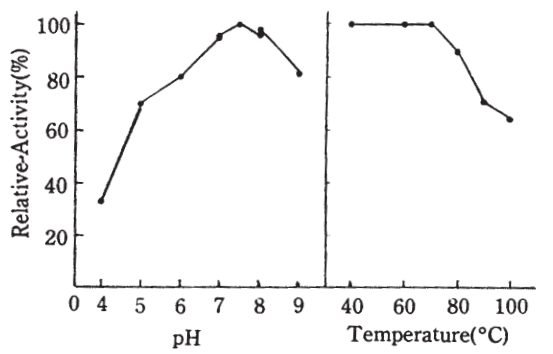

Fig. 3. *Effect of pH and temperature on FPase stability of C_1 enriched fraction.*
Buffers used for stability test were 0.1M citrate-phosphate buffer (pH 3.5-7.0), 0.1M phosphate buffer (pH 7.0-8.0) and 0.1M Tris-HCl buffer (pH 8.0-9.0).

DISCUSSION

Most of enzyme activity of original culture filtrate was recovered by combination of C_1 enriched fraction(F-A) with C_x fraction(F-B) and with endo-β-1,4-glucanase in their equivalent proportions. The synergism on cellulose degradation has been well documented in the case of aerobic cellulolytic strains, but not for the anaerobic *C. thermocellum*. The proportional original combination of C_1, C_x and β-glucosidase of *T. koningii* restored 96% of the activity of original culture filtrate, though each activity of C_1 and C_x+β-glucosidase was 4% and 3%, respectively (Wood, 1972). The cellulose degradation by anaerobic *C. thermocellum* is also carried out by synergistic action of cellulase components by similar mode of action with other aerobic microorganisms. In contrast to the extracellular presence of exo-glucanase and endo-glucanase, the β-glucosidase of *C. thermocellum* was known to be presented largely in periplasmic space (Ait *et al.*, 1979). In *C. thermocellum*, insoluble cellulose may be degraded extracellularly to oligosaccharide, cellotriose or cellobiose by the synergism of C_1 and C_x components, and then taken up by cells.

Unlike the C_x component, the separated C_1 enriched fraction from anaerobic *C. thermocellum* was easily inactivated in aerobic purification and storage conditions. The activity of C_1 enriched fraction was almost lost within two weeks at refrigerator. However, the enzyme activity was extensively stimulated by addition of reducing agents, especially in the presence of high concentration (0.4M) of β-mercaptoethanol. The synergistic action of C_1 fraction with C_x component was also strongly enhanced by reducing agents. This fact indicates that C_1 component contains a lot of sulfhydryl groups essential for the enzyme activity. The C_1 component is gradually inactivated by the formation of disulfide bonds from sulfhydryl groups under aerobic state or by the formation of acetic acid and lactic acid in culture broth during growth (pH in culture broth was lowered from 7.5

to around 5.0 with growing of the cells). However, C_1 activity may be capable of reactivation by the addition of β-mercaptoethanol that converts disulfide bonds to sulfhydryl groups. Meanwhile C_x component (endo-glucanase) of *C. thermocellum* was found to be independent of the addition of thiols or β-mercaptoethanol (Kim *et al.*, 1987). Johnson and Demain (1984) have reported that C_1 component in culture broth may have sulfhydryl groups and its activity is reactivated by the addition of 10mM DTT. The degradation of cellulose by mesophilic aerobic strains such as *Sporotichum pulverulentum, Polyporus adustus, Myrothecium verrucaria* and *Trichoderma viride* was known to be either enhanced or not influenced in oxygen atmosphere (Eriksson *et al.*, 1974). The degradation of insoluble cellulose by *C. thermocellum* can be carried out more actively in anaerobic condition.

C. thermocellum excretes a yellow colored substance during growth. Ljungdahl *et al.* (1983) suggested that the yellow substance helps the effective binding of endo-glucanase to cellulose fiber and involves in the cellulolytic process. The yellow substance was mostly recovered in nonbinding fraction(F-A), but not in binding fraction (F-B). Above observation reveals that the substance may not be participated in the binding of endo-glucanase (C_x) to cellulose fiber as suggested. It may be more closely related with C_1 component rather than C_x component, but need further investigation.

적 요

혐기성 *Clostrium thermocellum*의 배양액으로 부터 hydroxyapatite column chromatography를 통하여 cellulase complex중의 C_1 enriched fraction을 분리하였다. 다른 호기성 미생물과 마찬가지로 분리된 C_1 fraction과 다른 C_x 성분과의 synergism에 의해 불용성 섬유소의 분해가 현저히 촉진되었다. C_x 성분과는 달리 C_1 fraction은 공기중에서 산화에 의해 잘 실활되었으나 환원제, 특히 β-mercaptoethanol에 의해 효소 활성이 강하게 증가되는 것으로 보아 C_1 component는 다량의 sulfhydryl group을 가지고 있는 것으로 판단되었다. 이 fraction은 열에 매우 안정하였으며 최적 온도와 pH는 각각 60℃와 6.0 이었다.

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