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Comparison of the Enzymatic Activities of Endo-1,4-β-Glucanases Derived from *Clostridium josui*

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Abstract

Endoglucanase EG-1C, -2C and -3 (EC 3.2.1.4) derived from *C. josui* were highly active in degrading of carboxymethyl cellulose (CMC), but only slightly active on Avicel. All enzymes could not hydrolyze xylan and *p*-Nitrophenyl-β-D-glucoside. *p*-Nitrophenyl-β-D-cellobioside was degraded only by EG-1C. By CMC and cellooligosaccharide degrading analysis, the specific activity of EG-2C against CMC was 5 times higher than the others and its action mode was most endowisely. In degrading cello-oligosaccharides, these enzymes could degrade cellotetraose and cellopentaose, but not cellobiose and cellotriose. The specific activities increased as degree of polymerization increases from four to five.

Key Words : endo-1, 4-β-glucanase • *Clostridium josui* • cellooligosaccharide

Introduction

Clostridium josui, a mesothermophilic anaerobe, having cellulose degrading activity, produces ethanol and other valuable compounds, including highly active cellulolytic enzymes capable of degrading tough (crystalline) cellulosic materials such as Avicel, rice straw, and water hyacinths (21). Its cellulolytic enzyme system is assumed to resemble the cellulosome of *C. thermocellum* (1, 12), which consists of many cellulases and cellulosome-integrating-protein CipA (4, 5, 9), since the nucleotide sequence of *C. josui cipA* gene has been determined (11).

In the previous studies, isolation of individual cellulase has been attempted to clarify *C. josui* cellulolytic system. One endoglucanase (EG-3) was purified from *C. josui* culture broth in the presence of 6 M urea (8) and the other two endoglucanases were cloned into *E. coli* (15, 6) and translation products (EG-1C and EG-2C) were purified from transformants (6, 7) and their properties were partially characterized. According to the classification of glycosidases (10), EG-1 (7) and EG-2 (6) belong to family 5 and 8, respectively, although EG-3 is still unknown to be classified into which family, it was

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supposed to belong to family 5 by its N-terminal amino acid sequence similarity with *C. cellulolyticum* CelCCA (3) which has been classified into family 5.

The optimum temperature of these enzymes against CMC were between 60-70℃, and these values were remarkably higher than that of cultivation of *C. josui*. The optimum pH of EG-1C (7) and EG-3 (8) were estimated at 7.2 and 6.8, respectively, and EG-2C showed pH optimum at 5.5 (6). In addition, cellooligosaccharide degrading patterns by EG-1C and EG-3 were different from that of EG-2C. These specificities might be necessary for the host organism to survive in various environments.

This report describes comparison and characterization of *C. josui* endoglucanases in kinetic pattern and degrading pattern of substrates such as CMC and cellooligosaccharides.

Materials and Methods

Enzyme preparations

Endoglucanases, EG-1C (7), -2C (6), and -3 (8) have been purified from *E. coli* JM103 (pUCJ1), *E. coli* JM103 (pUCJ2) and *C. josui* culture supernatant, respectively. Physical and chemical features of each enzyme are summarized in Table 1.

Substrates

CMC (DS = 0.6 ; molecular weights, 180,000 and 30,000 ; Cellogen WS-C) was purchased from Daiichi Seiyaku Kogyo (Kyoto, Japan). Cellooligosaccharides (cellobiose to cellohexaose) were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). The microcrystalline cellulose used was Avicel (E. Merck AG, Darmstadt, Germany). Ball-milled cellulose (BMC) was prepared as a 3% water suspension of pure cellulose (KC flock W-300; Sanyo Kokusaku Pulp Co., Tokyo, Japan) by ball-milling for 3 days. *p*-Nitrophenyl-β-D-glucoside (PNPG), *p*-Nitrophenyl-β-D-cellobioside (PNPC) and xylan were purchased from Sigma Chemicals Co. (Tokyo, Japan).

Enzyme assays

Viscometric assay was determined by evaluating the viscosity changes of a reaction mixture, containing CMC at 37℃ and was monitored for 5 min by using a cone plate-type viscometer (Tokyo Keiki Co., Tokyo, Japan). The reaction mixture contained 1% CMC solution in 50 mM sodium phosphate

Table 1. Summary of physical and chemical properties of endoglucanases derived from *C. josui*

Properties	EG-1C (*E. coli* JM103/pUCJ1)	EG-2C (*E. coli* JM103/pUCJ2)	EG-3 from *C. josui*
Molecular weight[a] (kDa)	39[d]	42	45
Optimum temperature (℃)	65-70	60	60
Thermal stability[b] (℃)	55	55	55
Optimum pH	7.5	5.5	6.8
Specific activity[c] (U/mg)	2.8	11.0	2.0
Cellulase family	5	8	5(?)

[a] Estimated by SDS-PAGE.
[b] Maximum temperature of 80% recovery after incubation for 1 h at given temperature.
[c] Enzymatic activity against CMC as described previously.
[d] Purified catalytic domain.

buffer (5 ml; pH 6.8) and enzyme solution (1ml). One unit of enzymatic activity was defined previously (14) as the amount of enzyme required to reduce the viscosity of CMC by 1 cP in 1min. The release of reducing sugar was compared graphically with the viscosity change in order to compare the modes of attack of endoglucanases on CMC. Enzyme was incubated at 37℃ with CMC and reducing sugar release and viscosity were measured at various intervals. The reducing sugar formed was determined as glucose by the Somogyi method (20). BMC, Avicel, and xylan-hydrolyzing activities were assayed and compared by determining the amount of reducing sugars released during incubation of the enzyme with 10 mg/ml BMC, 10 mg/ml Avicel, and 10 mg/ml xylan, reaction mixtures were incubated for 1 h at 37℃ in 50 mM sodium phosphate buffer (pH 6.8). Each reaction mixture was incubated at 100℃ for 10 min to stop the enzymatic reaction. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar (expressed as glucose) per min. Assays for PNPGase and PNPCase activities were carried out with 1.0 mM-substrate and a enzyme solution in 2 ml of 50 mM sodium phosphate buffer (pH 6.8). Similarly, reactions were stopped by incubating in the boiling water and p-nitrophenol liberated was quantified at 405 nm.

The kinetic constants, Km and Vmax, for cellotetraose and cellopentaose hydrolysis by endoglucanases were attempted using HPLC. The decrease of substrate concentrations were measured after incubation with enzymes at 37℃, pH 6.5 for a period of time during which less than 20% of substrate was degraded.

Results

The mode of action on CMC

The reduction in viscosity versus the liberation of reducing sugars of each enzyme on CMC used as the substrate are presented in Fig. 1. Remarkable differences were observed in the pattern of reducing sugar liberations. Hydrolyzing of CMC with EG-1C (Fig. 1, ○, ●) exhibited that the reducing sugar increased in propotion to the time of incubation. On EG-2C (Fig. 1,□, ■) and EG-3 (Fig. 1, △, ▲), the release of reducing sugar rose rapidly in the early stage and then more slowly as time progressed. This may have been due either to end product inhibition or to reduction of affinity of the enzymes for CMC with the decrease of the degree of polymerization. When plots of fluidity (inverse of viscosity) versus liberation of reducing sugars were constructed (Fig. 2), the slope reflects the type of enzymatic reaction on CMC; a predominantly endo-type reaction results in a large slope. The slopes of EG-1C and EG-3 were closely identical, but EG-2C exhibited large positive slope which reflects the ability of an enzyme to cleave more internal glucosidic bond in substrate molecule.

Substrate specificity of each enzyme

The substrate specificity of each endoglucanase is shown in Table 2. CMC and BMC were hydrolyzed by all of enzymes. The specific activity against CMC of EG-2C was about 5 times higher than the others. EG-2C and EG-3 revealed high specificity for large molecule CMC (M.W. = 180,000) in compared with small molecule CMC (M.W. = 30,000). Avicel was slightly degraded by EG-2C and EG-3. All enzymes could not hydrolyze xylan and PNPG. PNPC was cleaved only by EG-1C.

The resulting Lineweaver-Burk plots for each enzyme permitted calculation of Km and Vmax values (Table 3). In each enzyme, all Vmax increased with the larger substrate, and Km values of EG-2C and EG-3 decreased but that of EG-1C did not decrease. The data of Vmax show that each enzyme has high

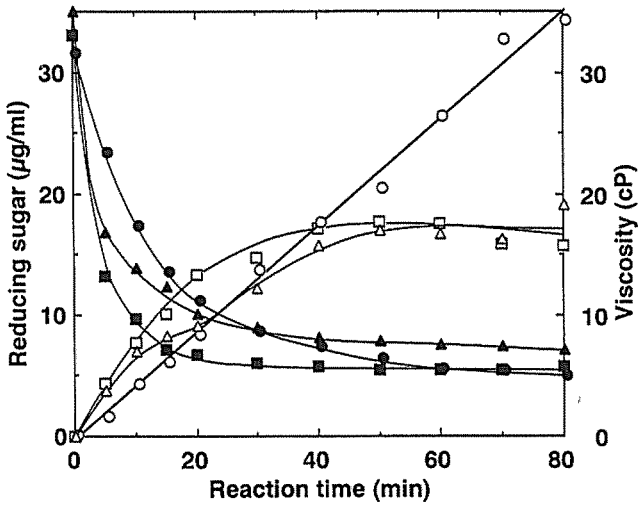

Fig. 1. Viscometric assays of EG-1C (○, ●), EG-2C (□, ■), and EG-3 (△, ▲) with 1% CMC used as the substrate. Same amount of enzymes liberating reducing sugar after 40 min of incubation were added. Symbols: ○, □, and △ show the liberated reducing sugar of EG-1C, EG-2C, and EG-3, respectively. ●, ■, and ▲ show the viscosity of EG-1C, EG-2C, and EG-3, respectively.

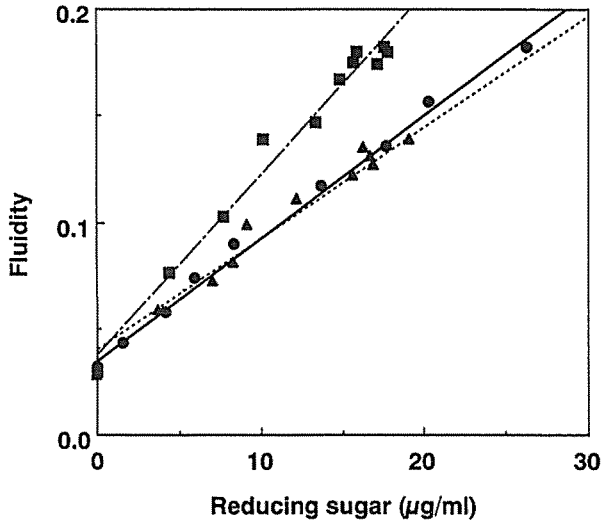

Fig. 2. Plot of fluidity versus reducing sugar for EG-1C (●), EG-2C (■) and EG-3 (▲). Data were derived from Fig. 1, which fluidity defined as the inverse of specific viscosity.

affinity with cellopentaose. EG-1C and EG-2C degraded cellopentaose with almost same velocity but EG-3 was half of their velocities. Km value of EG-3 against cellopentaose were relating higher than those of EG-1C and EG-2C.

Table 2. Substrate specificity of purified endoglucanases derived from *C. josui*.

Substrate	Concentration	Specific activity (μ mol/min per mg of protein) of:		
		EG-1C	EG-2C	EG-3
CMC[a]	10 mg/ml	7.6	44.3	8.5
CMC[b]	10 mg/ml	7.5	50.8	9.2
BMC	10 mg/ml	0.4	0.5	0.2
Avicel	10 mg/ml	ND	0.5	0.07
Xylan	10 mg/ml	ND	ND	ND
PNPG	1.0 mM	ND	ND	ND
PNPC	1.0 mM	4.8	ND	ND

[a] Molecular weight = 30,000. [b] Molecular weight = 180,000. BMC, Ball-milled cellulose; PNPG, *p-Nitrophenyl-β*-D-glucoside; PNPC, *p-Nitrophenyl-β*-D-cellobioside; ND, not detectable.

Discussion

All enzymes revealed higher affinity for amorphous cellulose than crystalline cellulose. In many cases, graphical comparison of viscosity decrease and reducing sugar production during enzyme reaction on CMC provides the mode of enzyme attack. Nishizawa *et al.* (13) and Okada (16, 17) differentiated the endo-1,4-β-D-glucanases of *Irpex lacteus* and *Trichoderma viride*, respectively. Large positive slops reflect the ability of an enzyme to cleave internal bonds in each number of cellulose molecules resulting in a large decrease of viscosity. The slopes obtained from exocellulases result in more close to horizontal line.

EG-1C and EG-3 revealed similar slope, suggesting that their mode of attack on CMC are similar. EG-1C produced reducing sugar proportionally and viscosity was decreasing through the enzyme reaction. However, enzymatic reaction of EG-3 was satisfied at 40 min after the start of reaction, and then viscosity change and reducing sugar production did not change. This may be due to inhibition by the products released in enzymatic reaction or its to low affinity for low molecular size substrate. This tendancy was observed in cellooligomer degradation shown in Table 3. The values of Vmax and Km of EG-3 on G4 indicated lower affinity and lower reaction velocity than those of G5.

EG-2C revealed the largest positive slope value among them. This enzyme could break down CMC rapidly but reducing sugar release were lower than EG-1C after 80min-incubation. The reducing sugar

Table 3. Kinetic constants of endoglucanases with cellooligosaccharides.

Substrate	EG-1C	EG-2C	EG-3
Cellotetraose			
Km(mM)	2.3	9.1	15.4
Vmax ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	7.8	1.1	1.4
Cellopentaose			
Km(mM)	5.9	1.7	6.7
Vmax($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	14.1	16.1	8.1

release of EG-2C was similar to that of EG-3 but EG-2C revealed lower viscosity, it suggested that EG-2C cleaved more internal glucosidic bonds in CMC molecule. In the time courses of enzymatic reaction shown in Fig. 1, three enzymes degraded CMC and their viscosities were almost same but reducing sugar quantity of EG-1C was twice of the others, after 80min-incubation. These results suggested that EG-1C reaction mixture contained smaller hydrolysis products of CMC than the others and its production increased in proportion to the reaction time.

All enzymes recognized G4 as a minimum substrate, however, PNPC which is regarded as model substrate of G3 was degraded by EG-1C. This property of EG-1C was similar to CelD from *C. thermocellum* (2). Many endoglucanases were purified and characterized about cellooligomer and synthetic substrate degradations, some endoglucanases are able to recognize the difference between PNPC and G3, for example E-I (endoglucanase-I) from *Bacillus* sp. KSM-522 (18) was able to degrade G3 or more than G3 of cellooligomers but not act on PNPC. EG-2C of *C. josui* and CelA revealed 62% identity with ***C. thermocellum*** in the catalytic domain but cellooligosaccharides degradation patterns were different, since EG-2C acts on G4 but CelA does not act on it (19). Therefore, some substitution, insertion, or missing of amino acids in the enzyme might affected their enzyme properties especially in the degradation of cellooligosaccharides.

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Clostridium josui 由来のエンド-1，4-β-グルカナーゼ群の酵素作用の特性比較

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要　約

C. josui 由来のエンドグルカナーゼ EG-1 C，-2C, および-3(EC3. 2. 1. 4)は共にカルボキシメチルセルロース(CMC)を強く分解するが，アビセルにはわずかしか作用せず，またキシランや p-ニトロフェニール-β-D-グルコシドを分解しなかった。しかし p-ニトロフェニール-β-D-セロビオシドは EG-1C によってのみ分解された。CMC およびセロオリゴ糖に対する作用を調べた結果，EG-1C の CMC に対する比活性は，他の酵素に比べて 5 倍も高く，エンド型の作用特性が最も強かった。セロオリゴ糖の分解に関しては，これらの酵素は，セロテトラオース，ならびにセロペンタオースを分解するが，セロビオースやセロトリオースは分解しなかった。これら酵素の比活性は，セロオリゴ糖の重合度が 4 から 5 に増加するにつれて増加した。