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Purification and Properties of Endo-β-1,4-glucanase from Thermophilic *Clostridium thermocellum*

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고온성 *Clostridium thermocellum*이 생산하는 Endo-β-1, 4-glucanase의 정제 및 성질

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ABSTRACT: A new endo-β-1,4-glucanase was purified from the culture filtrate of thermophilic anaerobic *Clostridium thermocellum*. The purification procedure included two steps of ion exchange chromatography with DEAE-Sephadex A-50 and gel filtration chromatography with Sephadex G-75. Even though the 56 fold increase in CMCase specific activity was obtained, the actually recovered enzyme activity was relatively lower level of 0.7%. Judging from the two bands in SDS-polyacrylamide gel electrophoresis, the endo-β-1,4-glucanase consists of two subunits whose M.W. are 38,000 and 58,000, respectively. The optimum pH and temperature were determined to be 5.0 and 65°C, respectively. The enzyme was stable up to 70°C, but inactivated at 80°C. The kinetic parameters of the separated fraction were also determined. The purified enzyme did not show any significant hydrolytic activity against the highly ordered crystalline cellulose as well as filter paper.

KEY WORDS□*Clostridium thermocellum*, endo-β-1,4-glucanase, purification, enzyme properties.

A thermophilic anaerobic *Clostridium thermocellum* possesses not only the effective cellulolytic enzyme activity but also the fermentation capability. Therefore, it can directly convert cellulosic biomass to ethanol with the simultaneous accumulation of fermentable sugars (Cooney *et al.*, 1978; Ng *et al.*, 1977, 1981). Recently a great deal of attention has been given to the utilization of the organism to produce biofuel from biomass.

C. thermocellum is known to excrete a significant amount of extracellular cellulolytic enzyme, and their purification was attempted by several investigators. However, only a limited number of enzyme has been fractionated, and the mode of action of cellulase complex is barely understood. This is due to the fact that most of enzyme activity is lost during the fractionation procedures that are usually carried out in aerobic state in contrast to anaerobic growth condition.

The purification of *C. thermocellum* cellulase components has been also hindered by adherence of enzymes to cellulose and tendency of the proteins to form aggregates. The characterization of cellulase complex is also hindered by the difficulty of separation of the component especially exo-β-1,4-glucanase (Lamed *et al.*, 1983; Ng and Zeikus, 1981). So far two endo-β-1,4-glucanases have been purified from the culture filtrate of *C. thermocellum* by Petre *et al.* (1981) and by Ng and

Zeikus (1981). These enzymes act upon carboxymethyl cellulose (CMC) but not upon crystalline cellulose. In addition to these two components, a β-glucosidase was purified from the periplasmic space of *C. thermocellum* and its characteristics were determined by Ait *et al.* (1979).

Recently, multicomponent complex that was termed as cellulosome was separated and it was studied extensively (Lamed *et al.*, 1983, 1985; Bayer *et al.*, 1983; Johnson and Demain, 1984; Hon-nami *et al.*, 1986). A cellulosome is a high molecular weight, cellulose-binding and multicellulase-containing protein complex. Similar to the crude enzyme system, the purified cellulosome demonstrated a true cellulolytic activity on the basis of extensive solubilization of microcrystalline cellulose.

The concept of cellulosome reveals the overall scope of the cellulase complex of *C. thermocellum.* However, there are many questions to be answered about the cellulase complex itself. In order to understand the detailed reaction mechanism of cellulase complex, each component of cellulase complex has to be fractionated and their individual modes of action have to be investigated. In this paper, we attempted to fractionate various components of cellulase complex. As a consequence, a new endo-β-1,4-glucanase which is different from two endo-β-1,4-glucanases previously reported in literatures was purified, and its properties were determined.

MATERIALS AND METHODS

Organism

The strain used in this study was *Clostridium thermocellum* ATCC 27405.

Cultivation

C. thermocellum was grown anaerobically at 60°C for 5 days in 5*l* culture flask with CM3 medium (Weimer and Zeikus, 1977) that was slightly modified to contain 1g/*l* cysteine-HCl and 10g/*l* α-cellulose instead of the cysteine-Na_2S mixture and 9g/*l* α-cellulose. The initial pH of culture medium was adjusted to 7.5 and the medium was deoxygenated with N_2 gas and then sealed with a rubber stopper. The culture broth was centrifuged at 12,000g for 15min and the supernatant fluid was used as crude enzyme.

Determination of enzyme activity and protein

Endo-β-1,4-glucanase (CMCase or Cx) activity and filter paper (FPase) activity were measured by the methods described by Mandels *et al.* (1976). Soluble protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard or by measuring the absorbance at 280nm.

Concentration of crude enzyme

3*l* of crude enzyme was initially precipitated by the addition of cold acetone, which corresponds to 50% of saturation, and then keeping at -20°C for 3hrs. The residual acetone in the centrifuged precipitate was eliminated by keeping at room temperature. The precipitate was dissolved in 200 m*l* of 10mM phosphate buffer (pH 7.0), and then after centrifugation only the supernatant was applied on DEAE-Sephadex A-50 column.

First ion exchange chromatography

200 m*l* of the concentrated solution was applied on DEAE-Sephadex A-50 column (2.8× 20cm) which was equilibrated with 10mM phosphate buffer (pH 7.0), and then, the nonbinding fraction was washed out with the same buffer. The binding component was eluted with a linear NaCl gradient from 0 to 0.8M in the same buffer. The flow rate was 50m*l*/hr and the fraction volume was 4.5 m*l*. The fractions containing CMCase were collected and concentrated by the addition of cold acetone as described previously. The acetone eliminated residual pellets were redissolved in 50mM acetate-NaOH buffer (pH 5.0), and the solution was applied on Sephadex G-75 column.

Gel filtration chromatography

The further fractionation was carried out on Sephadex G-75 column (1.8×150cm) which was previously equilibrated with 50mM acetate-NaOH buffer (pH 5.0). 5m*l* of sample was applied to the column, and then it was eluted with same buffer. The fractions of 3.0m*l* were collected at a flow rate of 20m*l* per hour.

Second ion exchange chromatography

The fractions obtained from Sephadex G-75 column chromatography containing CMCase were dialyzed with dialysis bag (Sigma Co.) against 10mM phosphate buffer (pH 7.0) at 4°C overnight to obtain 45m*l* of dialyzed enzyme solution. It was applied on DEAE-Sephadex A-50 column (0.8×25cm) and then the column was eluted with pH gradient from 7.0 to 5.0 in 10mM citrate-phosphate buffer with flow rate of 20m*l*/hr and fraction volume of 2.0m*l*.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out by the method of Davis (1964) using 7% acrylamide gel with bromophenol blue as the tracking dye. A current of 3mA per gel was applied, and the protein was stained with Coomassie brilliant blue R-250. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed to estimate the molecular weight of the enzyme according to the method of Laemmli (1970) using 10% acrylamide gel.

RESULTS AND DISCUSSION

Fractionation of an endo-β-1,4-glucanase

Table 1 compares recovery efficiencies of the various methods used for concentration of culture filtrate, which include precipitation with acetone, salting out with ammonium sulfate, precipitation with ethanol or methanol, and direct evaporation at 60°C by rotary evaporator. In this experiment 60m*l* of culture filtrate was concentrated by adding various organic solvents and saturated ammonium sulfate to the level of 70% saturation, and then keeping at −20°C overnight. The precipitate was redissolved in 6m*l* of 50mM acetate-NaOH buffer (pH 5.0), and then the recovered CMCase activity was measured. The protein in the culture filtrate was readily precipitated in each case, however, the precipitate was not easily redissolved in acetate-NaOH buffer. The salting out of protein with ammonium sulfate was also unsuccessful even with up to 90% saturation. The most favorable recovery was achieved by the precipitation with acetone. The recovery yield of total enzyme activity in the case of evaporation at 60°C was similar to that of precipitation with acetone, however, the specific activity was lower than that recovered by precipitation with acetone. The enzyme produced from thermophilic bacteria is reported to be relatively stable against temperature and organic solvents (Sonnleiter and Fiechter, 1983). However, about 80% of CMCase activity of crude enzyme was lost during the acetone precipitation. In order to prevent the deactivaion of enzyme, the precipitation at the low level of saturation was attempted, but the protein was not precipitated any significant amount below 30% saturation.

The acetone precipitated fraction was further subjected to ion exchange chromatography on DEAE-Sephadex A-50 column eluted linearly with NaCl gradient. The major CMCase activity was found in the fractions eluted with 0.15-0.35M NaCl. The CMCase enriched fraction was collected and it was reconcentrated with acetone precipitation. The next step of separation was carried out through gel filtration chromatography on Sephadex G-75 column. The CMCase activity was

Table 1. *Concentration of crude enzyme solution from C. thermocellum*

Methods	Total activity (units)	Protein (mg)	Specific activity (units/mg)	Yield of activity (%)
Crude enzyme	10.66	48.0	0.22	100.0
Precipitation with methanol (70%)	0.35	0.95	0.37	3.3
Precipitation with ethanol (70%)	0.93	2.66	0.35	8.7
Precipitation with acetone (70%)	2.27	4.18	0.54	21.3
Salting out with ammonium sulfate (80%)	0.32	0.75	0.43	3.0
Evaporation at 60°C	2.52	9.12	0.28	23.6

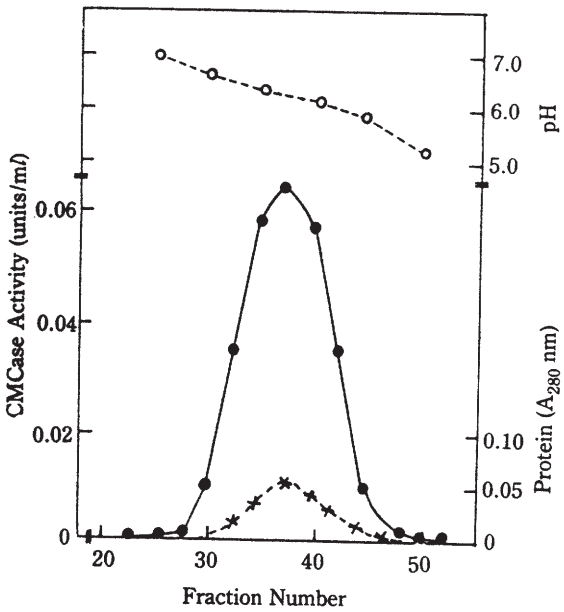

Fig. 1. *Ion exchange chromatography of F-I on DEAE-Sephadex A-50 column (0.8x25 cm).*
The column was eluted with pH gradient from 7.0 to 5.0 in 10 mM citrate-phosphate buffer. The flow rate was 20 ml/hr and the fraction volume was 2.0 ml.
Protein (×---×), CMCase activity (●—●), pH (○---○)

appeared mainly in the fraction number of 60-90 which assigned as F-I. The F-I fraction was dialyzed at 4°C overnight against 10mM phosphate buffer (pH 7.0), and then subjected to further purification by ion exchange chromatography on DEAE-Sephadex A-50 column eluted with pH gradient in 10mM citrate-phosphate buffer. The fractionation result is shown in Fig. 1. As can be observed in Fig. 1, single CMCase peak was identified, and the pH of the fraction with the highest CMCase activity was measured to be 6.3.

Table 2 summerizes the enzyme recovery efficiency of each purification steps used for fractionation of the CMCase. Even though CMCase specific activity was increased 56 fold after purification, the overall recovery yield was found to be very low. Only 0.7% of the total CMCase activity initially used was recovered.

The purity of the fractionated CMCase was analyzed by polyacrylamide gel electrophoresis as shown in Fig. 2. Only a single band was observed, which demonstrates that the separated fraction is pure component.

The purification of cellulase from *C. thermocellum* was known to be a very difficult task. This is due to the facts that the cellulase tends to form aggregates and that cellulases have low

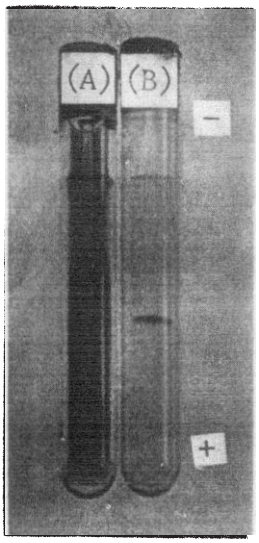

Fig. 2. *Polyacrylamide gel electrophoresis of the purified endo-β-1,4,-glucanase.*
(A); crude enzyme,
(B); purified endo-β-1,4-glucanase.

Table 2. *Purification of endo-β-1,4-glucanase from C. thermocellum*

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification fold	Yield of activity (%)
Crude enzyme	401.95	2400	0.17	—	100
Precipitation with acetone (50%)	81.61	235.8	0.35	2.1	20.3
First DEAE-Sephadex A-50	20.13	36.2	0.56	3.3	5.0
Sephadex G-75	8.28	1.9	4.25	25.6	2.1
Second DEAE-Sephadex A-50	2.85	0.3	9.50	55.9	0.7

mobility during procedure of gel filtration chromatography. As a consequence, so far only two endo-β-1,4-glucanases have been purified. One of them was purified by Ng and Zeikus(1981) through the procedures, centrifugation, ultrafiltration, ion exchange Sephadex chromatography and preparative gel electrophoresis. The other was purified by Petre *et al.* (1981) through the procedure of QAE-Sephadex A-50 chromatography in the presence of 6M urea.

Determination of molecular weight

The molecular weight of the purified CMCase was measured by SDS-PAGE, however, surprisingly two bands were observed in the SDS-PAGE diagram. The above observation demonstrates that the purified CMCase is composed of two subunits. The apparent molecular weight of the purified CMCase is 96,000 which is composed of one subunit of M.W. of 38,000 and the other subunit of M.W. of 58,000 as shown in Fig. 3.

Notice that the molecular weights of the CMCase purified by Petre *et al.* (1981) and by Ng and Zeikus (1981) were reported to be about 56,000 and between 83,000 and 94,000, respectively. The purified cellulase which is composed of two subunits has never been reported in the literature. Recently Wu and Demain (1985) have reported a large complex of enzyme so called cellulosome whose molecular weight is about 6.5×10^6. The complex has the ability to hydrolyze Avicel and CM cellulose associated with an enzyme complex, and it composes of several different polypeptide types, some of which has endo-β-1,4-glucanase activity. Also Lamed *et al.* (1983) reported that cellulolytic complex (cellulosome) isolated from the culture filtrate has a molecular weight of about 2×10^6 daltons and consists of at least 14 different types of polypeptide. Therefore, it can be postulated that the CMCase separated by us is one or more of the above constituents of the reported large cellulase complex. It seems that the purified CMCase is a new cellulase component that is different from previously reported ones.

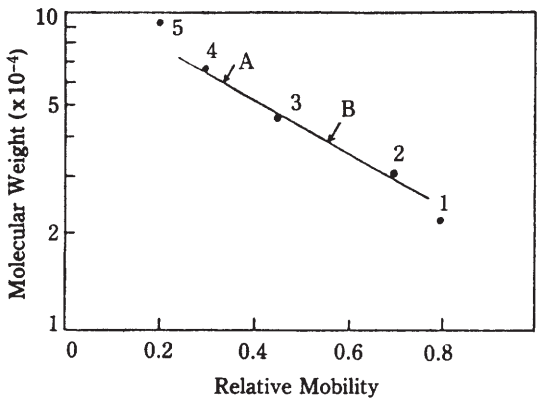

Fig. 3. *Molecular weight determination of endo-β-1,4-glucanase by SDS-polyacrylamide gel electrophoresis.*
Molecular weight markers: 1, soybean trypsin inhibitor (21,500); 2, carbonic anhydrase (31,000); 3, ovalbumin (45,000); 4, bovine serum albumin (66,200); 5, phosphorylase b (92,500). A,B: subunits of endo-β-1,4-glucanase.

Optima of pH and temperature

The optimum pH was estimated to be around 5.0 (see Fig. 4), in which 0.1M citrate-phosphate buffer (pH 3.5-6.5) and 0.1M phosphate buffer (pH 7.0-8.0) were used. Notice that the optimum pH of endo-β-1,4-glucanase purified by Petre *et al.* (1981) was 6.0, on the other hand, that purified by Ng and Zeikus (1981) was 5.2. Also Fig. 5 indicates the temperature effect with optimal temperature of 65°C.

Thermal and pH stability

The thermal stability was determined after

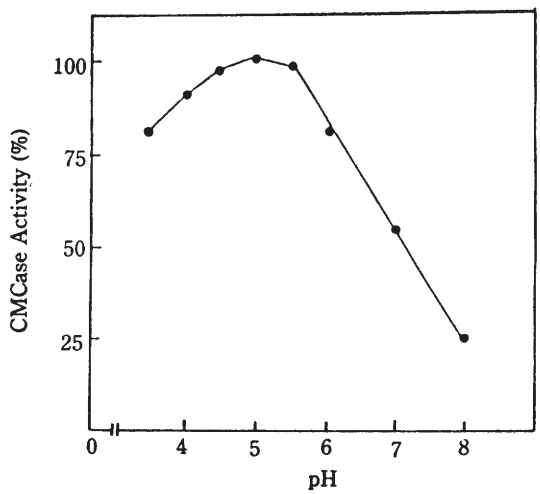

Fig. 4. *Effect of pH on the activity of endo-β-1,4-glucanase.*

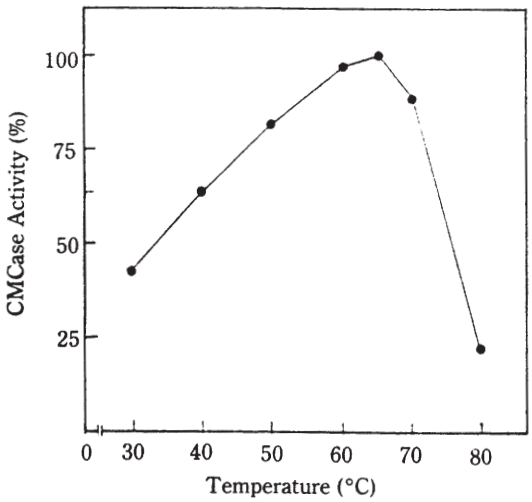

Fig. 5. *Effect of temperature on the activity of endo-β-1,4-glucanase.*

preincubation in pH 5.0 from 30°C to 80°C with 5°C intervals for 30 min. The enzyme was stable up to 70°C but inactivated severely at 80°C as shown in Fig. 6.

The pH stability of enzyme was measured by keeping at room temperature for 2hr at different pH. The enzyme was found to be stable at pH ranges of 3.5-6.0 as shown in Fig. 7.

Effects of metal ions and chemical agents

The effects of various metal ions and chemical agents on the separated CMCase activity are given in Table 3 and 4.

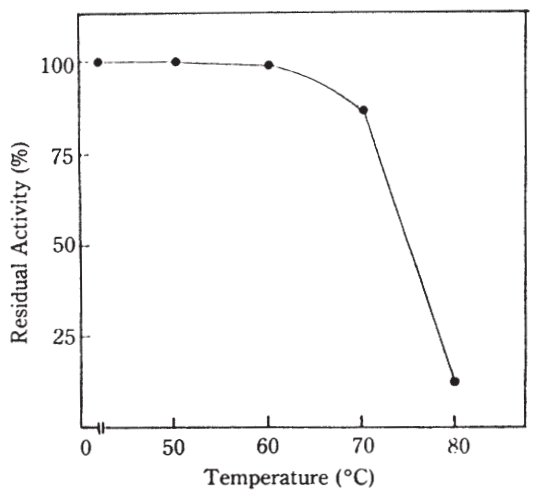

Fig. 6. *Thermal stability of endo-β-1,4-glucanase.*

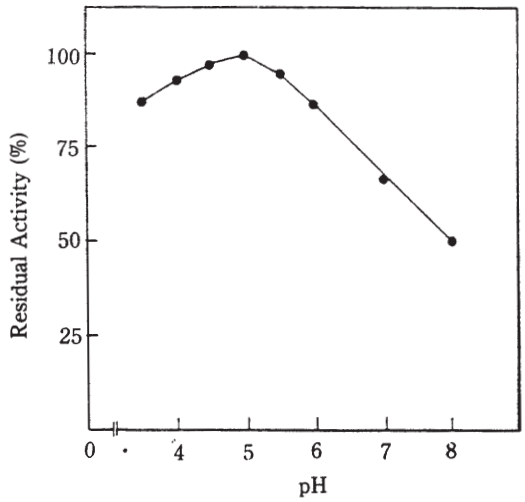

Fig. 7. *pH stability of endo-β-1,4-glucanase.*

The activity of enzyme was slightly stimulated with 1 mM Ni^{++} and Mg^{++}. But it was strongly inhibited by 1 mM *p*-CMB that forms mercaptides with sulfhydryl groups and by Pb^{++}, Hg^{++} and Fe^{++} at 1 mM concentration. It, however, was not affected by some reducing agents such as 1 mM cysteine-HCl and DTT. Therefore it can be postulated that the purified enzyme contains disulfide linkage of thiols and its CMCase activity is insensitive to oxidation inactivation or sulfhydryl reagents. This is thought to be the same result as the endo-β-1,4-glucanase component of cellulase complex was not susceptible to

Table 3. *Effect of metal ions on the activity of endo-β-1,4-glucanase*

Metal ion (1 mM)	Relative activity (%)
None	100.0
Zn^{++}	69.2
Mg^{++}	107.2
Ni^{++}	110.3
Ca^{++}	102.6
Cu^{++}	94.9
Mn^{++}	89.7
Fe^{++}	0
Pb^{++}	0
Hg^{++}	0
Na^{+}	92.3
K^{+}	71.8

Table 4. *Effect of chemicals on the activity of endo-β-1,4-glucanase*

Chemical agents	Concentration(mM)	Relative activity(%)
None	—	100.0
Urea	10	89.7
Sodium azide	10	59.0
β-Mercaptoethanol	10	97.4
Cysteine-HCl	10	102.6
EDTA	10	71.8
o-Phenanthroline	1	94.9
p-CMB	1	0
Sodium cyanide	1	60.1
DTT	1	102.6
Mono iodoacetate	1	87.2

oxidation inactivation and sulfhydryl reagents (Johnson *et al.*, 1982, 1984; Petre *et al.*, 1981). However, the endo-β-1,4-glucanase purified by Ng and Zeikus (1981) did not contain cysteine.

Effect of substrate concentration

The Km value for CMC calculated from Lineweaver-Burk plot was 6.56 mg/m*l*, on the other hand, Vmax was 0.22 units/m*l* as shown in Fig. 8.

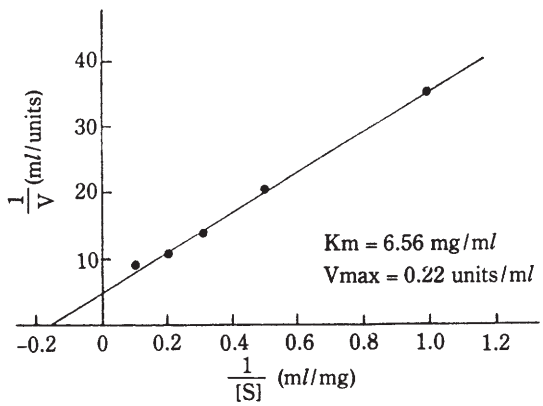

Fig. 8. *Lineweaver-Burk plot of endo-β-1,4-glucanase on CMC.*

Substrate specificity

The substrate specificity of CMCase for various cellulosic substrates was measured. The purified enzyme is found to be specific for CMC, on the meanwhile the hydrolyzing activity against the highly ordered crystalline cellulose such as Sigmacell, α-cellulose and filter paper was negligible.

적 요

고온 혐기성 *Clostridium thermocellum*의 배양액으로 부터 새로운 endo-β-1, 4-glucanase 를 ion exchange chromatography와 gel filtration chromatography를 통하여 정제하였다. 정제된 효소의 비활성은 56배 증가하였으나 수율은 0.7%로서 매우 낮았다. SDS-PAGE 결과, 정제된 효소는 분자량이 각각 38,000과 58,000으로 된 두개의 subunit로 구성되어 있었다. 이 효소의 반응 최적 pH는 5.0, 최적온도는 65℃였으며 70℃까지는 열에 안정하였으나 80℃에서 거의 실활되었다. 기타 여러가지 효소학적인 성질을 조사하였으며, 분리된 효소는 결정형 섬유소에 대한 효소활성을 나타내지 않았다.

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