Kor. J. Appl. Microbiol. Biotechnol.
Vol. 20, No. 5, 505-510 (1992)

Properties of a Novel *Clostridium thermocellum* Endo-β-1,4-glucanase Expressed in *Escherichia coli*

Jung, Kyung Hwa[1], Jin-Ho Lee[1], Yong-Tack Yi[1], Ha-Kun Kim[1,2] and Moo-Young Pack[1]*

[1]*Department of Biological Science & Engineering , Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea*
[2]*Department of Genetic Engineering, Pai Chai University, Taejon 302-735, Korea*

대장균에서 발현되는 *Clostridium thermocellum*의 섬유소 분해 효소의 특성

정경화[1] · 이진호[1] · 이용택[1] · 김하근[1,2] · 박무영[1]*

[1]한국과학기술원 생물공학과, [2]배재대학교 유전공학과

Abstract — An endo-β-1,4-glucanase gene of *Clostridium thermocellum* was cloned in *Escherichia coli* and was considered as a novel gene by comparison with the restriction patterns of the *C. thermocellum* cellulase genes so far reported. The endoglucanase from recombinant *E. coli* was purified by column chromatography after heat treatment. The purified enzyme was a monomer having molecular weight of 40,000. The enzyme hydrolyzed CMC to glucose and cello-oligosaccharides at maximum activities at pH 5.0 and 65℃. One of the endproducts, glucose, showed no inhibitory effect on the enzyme activity, while the other endproduct, cellobiose, inhibited slightly. The values of K_m and V_{max} of the enzyme for CMC were 0.39% (w/v) and 268 U/mg protein, respectively.

At least three kinds of enzymes, i.e., endo-β-1,4-glucanase, exo-β-1,4-glucanase, and β-glucosidase are needed to obtain glucose from cellulose in bacteria (1). *Clostridium thermocellum*, an anaerobic thermophilic bacterium, is known to produce a thermostable cellulase complex termed cellulosome, which contains at least 15 different polypeptides (2). Because of the inherent thermostability and resistance to the end product inhibition by glucose and cellobiose, the industrial potential of this microbial cellulase system has long been recognized. Since it is not easy to isolate the individual enzymes from its the cellulase complex of *C. thermocellum*, clonings of the individual genes in *E. coli* have been tried for the study of the cellulolytic mechanism. Genes encoding endoglucanases A, B, C, D, E and H have been sequenced (3-8) and corresponding enzymes are purified and characterized from recombinant *E. coli* strain (7, 9-12).

In this study we cloned an endoglucanase gene in *E. coli* from *C. thermocellum* and its restriction map was compared with those reported so far. And the enzyme produced by the *E. coli* transformant was purified and characterized.

Materials and Methods

Bacterial strains, plasmids and culture conditions

Clostridium thermocellum ATCC 27405 was used as an endoglucanase gene source, *Escherichia coli* JM83 as a cloning host, and pUC9 as a cloning vec-

Key words: *Clostridium thermocellum*, endo-β-1,4-glucanase, cloning, purification
*Corresponding author

Abbreviations: endo-β-1,4-glucanase, endoglucanase; carboxymethylcellulose, CMC; dinitrosalicylic acid, DNS; p-nitrophenyl-β-D-cellobioside, pNPC.

tor. *C. thermocellum* was grown anaerobically at 60℃ in CM3 medium (13) supplemented with cellobiose (9.72 g/liter). *E. coli* was grown in LB medium at 37℃. Ampicillin (50 μg/m*l*) was added to the medium when *E. coli* carrying plasmids was selected.

Isolation of the *Clostridium thermocellum* endoglucanase gene

Chromosomal DNA was isolated from *C. thermocellum* grown in CM3 medium anaerobically, and pUC9 DNA was prepared from *E. coli* JM83 by the alkaline lysis method (14). *C. thermocellum* chromosomal DNA and pUC9 DNA were digested with *Eco*RI. The vector DNA was treated with calf intestinal alkaline phosphatase. DNA ligation was carried out as described by Maniatis *et al.* (14) and the ligation mixture was transformed into *E. coli* JM83. LB medium containing CMC (0.5%, w/v) and ampicillin (50 μg/m*l*) was used for the selection of CM-Case-positive transformants by using the Congo Red dye method (15).

Preparation of the enzyme extracts

E. coli cells carrying recombinant plasmids were grown overnight at 37℃ in LB medium supplemented with ampicillin. Harvested cells (7 g wet weight) were suspended in 50 mM sodium citrate buffer, pH 5.0, and sonicated for 10 min with Branson sonifier (Model 350) at 40% output. Cell debris was removed by centrifugation at 16,000 g for 30 min.

Enzyme assay

Endoglucanase activity was determined by measuring the amount of reducing sugars released from CMC, filter paper and Avicel by the DNS method (8, 12, 16). The absorbance was measured at 550 nm. One unit of the enzyme activity is defined as the amount of enzyme that produces 1 μmole of reducing sugar per min. For viscometric assay, the reaction mixture containing 1% CMC (medium viscosity) was incubated for 60 min at 60℃ and the reaction was stopped by boiling for 5 min. The viscosity was determined at 20℃ by using the Haake viscometer (model UT 181). The amount of glucose was measured by using the PGO enzyme kit (Sigma Co.).

Endoglucanase purification

Streptomycin sulfate solution (final 1.5% (w/v)) was added to the crude extracts and the mixtures were centrifuged to remove the nucleic acids. The streptomycin sulfate treated extracts were heated at 60℃ for 20 min and the denatured proteins were removed by centrifugation at 16,000 g for 30 min. Then, the heat treated extracts were fractionated with ammonium sulfate (35~75% saturation) and dialyzed against 20 mM Tris-HCl, pH 8.0. The obtained enzyme solution was applied to a DEAE Sephadex A-50 column, which had been equilibrated with 20 mM Tris-HCl, pH 8.0. After washing with the same buffer, bound proteins were eluted by a linear NaCl gradient (0 to 0.4 M). The flow rate was 24 m*l*/hr and the fractions (8 m*l*/tube) were collected. The active fractions of DEAE Sephadex A-50 column chromatography were pooled and dialyzed against 10 mM sodium phosphate buffer, pH 7.0. The dialyzed enzyme solution was applied to hydroxyapatite column, which had been equilibrated with 10 mM sodium phosphate buffer, pH 7.0. After washing with the same buffer, the proteins were eluted with linear sodium phosphate gradient (10 to 200 mM). The flow rate was 12 m*l*/hr and the fractions were collected 4 m*l*, each. Protein concentrations were determined by the method of Lowry (17) with bovine serum albumin as the standard.

Determination of the molecular weight

The molecular weight of the purified enzyme was determined by 11% (w/v) SDS-polyacryamide gel electrophoresis according to Laemmli (18). The apparent molecular weight of the enzyme was also determined by using Sephadex G-100 gel filtration chromatography with aprotinin (M.W.: 6,500), cytochrome C (M.W.: 12,400), carbonic anhydrase (M.W.: 29,000), and bovine serum albumin (M.W.: 66,000) as molecular weight markers. The proteins were eluted with 50 mM sodium phosphate buffer, pH 7.0, containing 200 mM NaCl.

Results

Isolation of an endoglucanase gene

The chromosomal DNA digested with *Eco*RI was ligated to the EcoRI digested pUC9 and the ligation mixture was transformed into *E. coli* JM83. Among the transformants on the selective medium, one CMCase positive colony was identified by using Congo Red dye method (15) and a hybrid plasmid, designated pKH40, contaning 4 kb *Eco*RI chromosomal fragment was isolated from the positive colony. The restriction map of this recombinant plasmid is shown in Fig.1.

Enzyme purification and characterization

The purification procedure is summarized in Table 1. The heat susceptible *E. coli* proteins were removed by treating at 65℃ for 20 min . The ammonium sulfate fractionated proteins were resolved into four major protein components by DEAE Sephadex A-50 column chromatography (data not shown). Enzyme activity was detected in the first peak of the protein elution. Active fractions were collected, dialyzed, and applied to hydroxyapatite column. The final yield was 29% of original activity and the enzyme was purified to 40 fold in specific activity. The purified enzyme preparation displayed a single protein band on 11% (w/v) SDS-polyacryla-

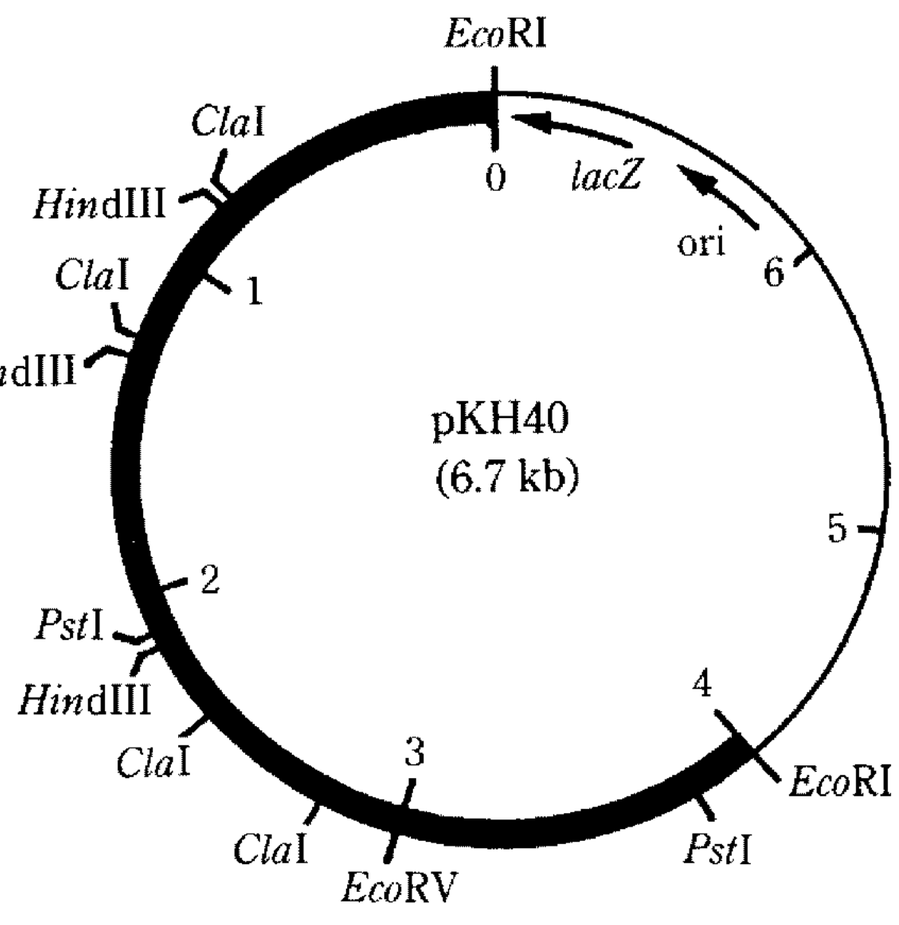

Fig. 1. Restriction map of recombinant plasmid, pKH 40.

The thick line indicates *Eco*RI fragment isolated from *C. thermocellum* and the thin line indicates a region of pUC9 DNA. The numbers denote DNA size in kilobases.

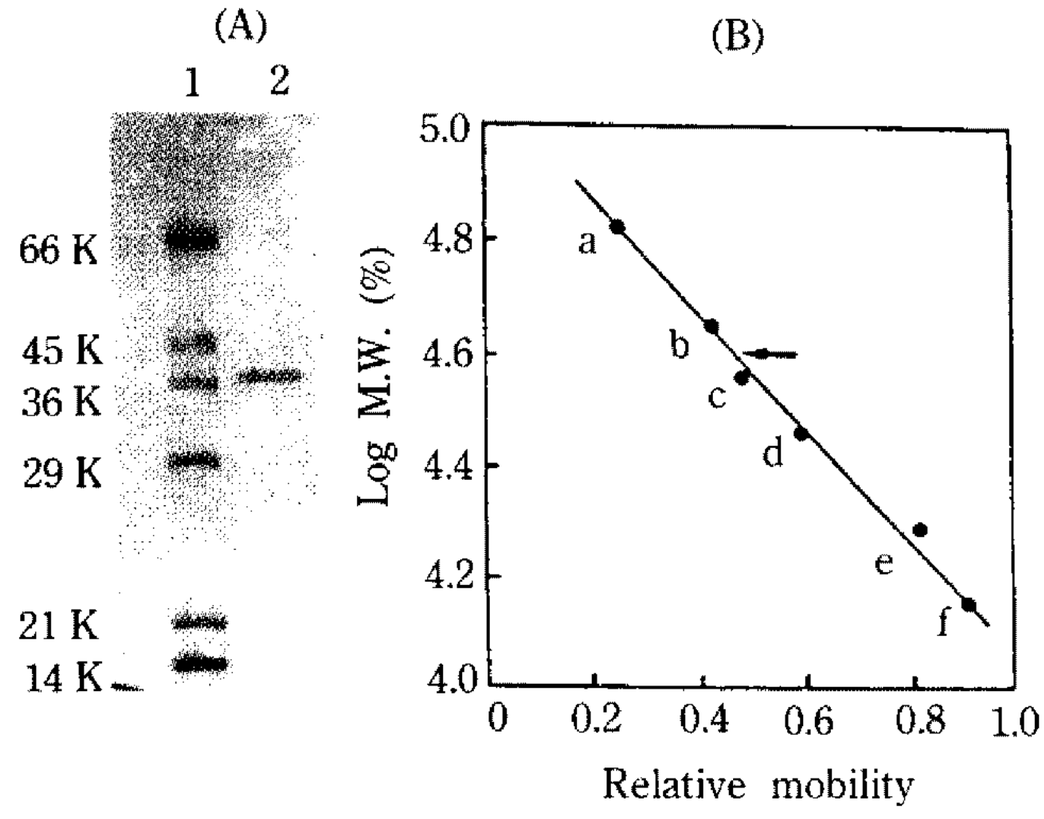

Fig. 2. Determination of molecular weight of the purified enzyme.

(A) 11% (w/v) SDS-polyacrylamide gel electrophoresis; lane 1, molecular weight standard markers; lane 2, purified enzyme. (B) the calibration curve; a, bovine serum albumin (M.W.: 66,000); b, egg albumin (M.W.: 45,000); c, glyceraldehyde 3-phosphate dehydrogenase (M.W.: 36,000); d, carbonic anhydrase (M.W.: 29,000); e, trypsin inhibitor (M.W.: 21,100); f, α-lactalbumin (M. W.: 14,200); ←, purified enzyme.

Table 1. Summary of purification of endoglucanase produced by *E. coli* JM83 (pKH40)

Procedure	Total activity (unit*)	Total proteins (mg)	Specific activity (unit/mg)	Fold	Yield (%)
Crude extracts	2401	405	5.9	1.0	100
Streptomycin sulfate fractionate	2168	283	7.7	1.3	90
Heat-treated extracts	2017	155	13.0	2.2	84
Ammonium sulfate fractionate	1110	34	33.0	6.0	46
DEAE Sephadex A-50	914	15	63.0	11.0	38
Hydroxyapatite	704	3	238.0	40.0	29

*μmoles of reducing sugars per min.

Table 2. Specific activities of the purified endoglucanase toward various substrates.
All reactions were incubated at 60℃ in 50 mM sodium citrate buffer, pH 5.0. Products measured with respective substrates were: reducing sugars (CMC, filter paper and Avicel); p-nitrophenol (pNPC); glucose (cellobiose).

Substrates	Specific activity[a]
CMC	238.0
pNPC	1.2
Filter paper	1.5
Avicel	1.0
Cellobiose	N.D.[b]

[a] specific activity is expressed as μmoles of reducing sugars liberated per min per mg protein. [b] Not detected.

mide gel (Fig. 2A).

The purified enzyme hydrolyzed CMC with maximum activities at pH 5.0 and 65℃. The proportions of the glucose to total reducing sugars produced from CMC and filter paper by the enzyme were 6% and 10%, respectively.

The specific activities of the endoglucanase were assayed on different substrates (Table 2). The enzyme rapidly hydrolyzed a soluble cellulose, CMC, but showed low activity on crystalline celluloses such as filter paper and Avicel, and the activity toward cellobiose was not detected. The enzymatic hydrolysis of CMC caused a rapid decrease in viscosity of the reaction mixture with an concommitant increase in reducing sugars (Fig. 3).

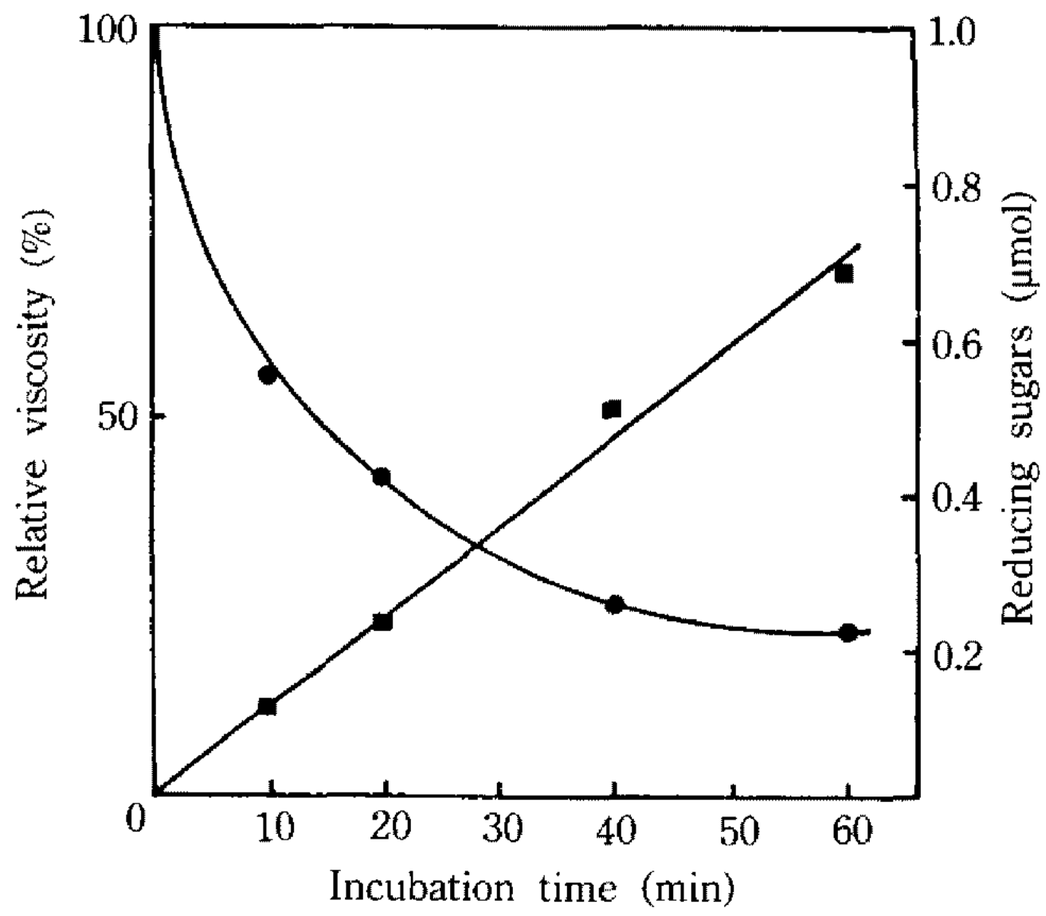

Fig. 3. Change of viscosity and release of reducing sugars during the CMC-hydrolysis by purified enzyme.
1% (w/v) CMC (medium viscosity) was hydrolyzed at 60℃ for 60 min by the purified enzyme and the viscosity was determined at 20℃ by Haake viscometer (model UT181). ●, change of viscosity; ■, release of reducing sugars.

Molecular weight

The molecular weight of the enzyme determined by 11% (w/v) SDS-polyacrylamide gel electrophoresis was about 40,000 (Fig. 2). The molecular weight estimated by electrophoresis was reconfirmed by gel filtration chromatography in the presence of 0.2 M NaCl (data not shown). These results suggested that the purified endoglucanase was a monomer having molecular weight of 40,000.

End product inhibition

The effects of glucose and cellobiose on the enzyme activity were investigated. As shown in Fig. 4,

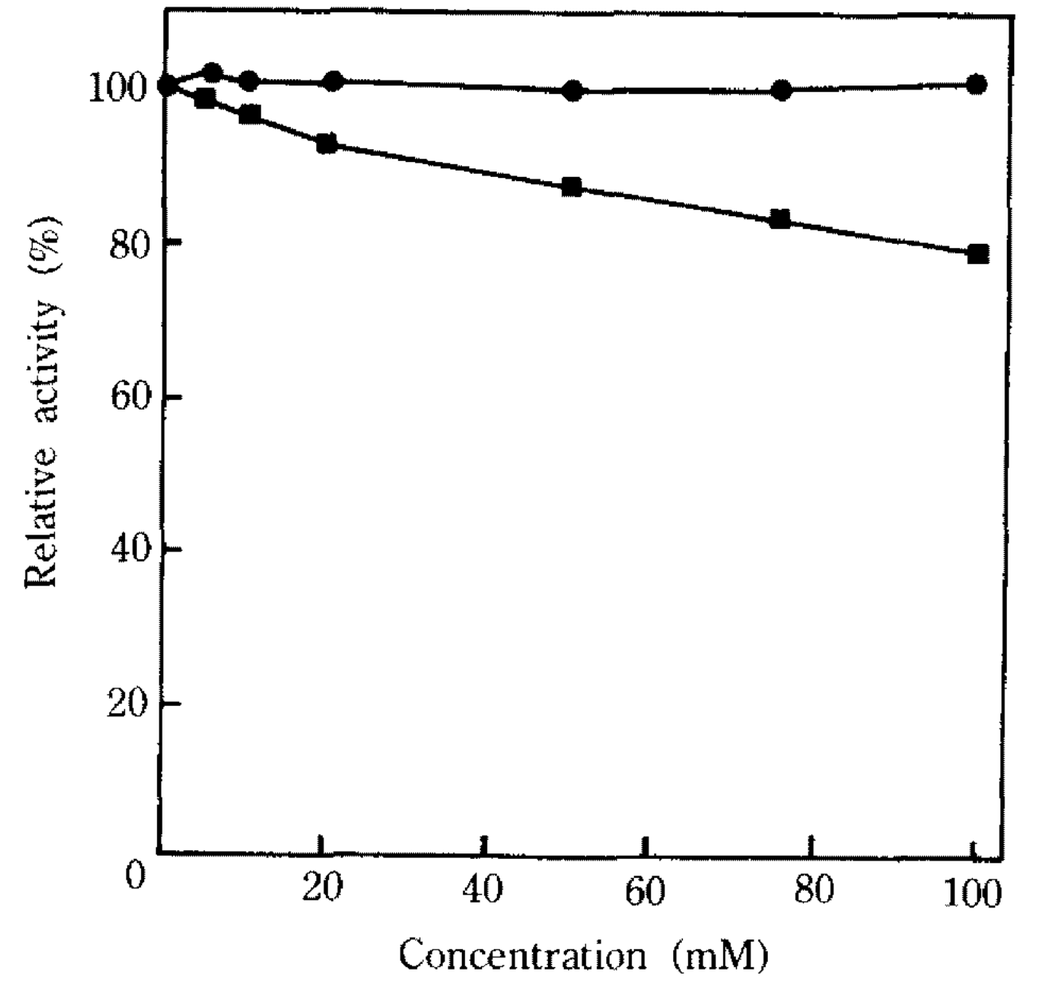

Fig. 4. Effects of glucose and cellobiose on the pNPC hydrolyzing activity.
All reactions were incubated at 60℃ and the activities were estimated by measuring the amount of p-nitrophenol released from pNPC. ●, effect of glucose; ■, effect of cellobiose.

the presence of glucose up to 100 mM showed no effect on pNPC hydrolysis by the endoglucanase, whereas some inhibition by cellobiose was observed.

Kinetic constants

The K_m and V_{max} values of the purified enzyme for CMC and pNPC were obtained from Lineweaver-Burk plots. The values of K_m and V_{max} of the enzyme for the CMC were 0.39% (w/v) and 268 U/mg protein, respectively. In the hydrolysis of the pNPC, K_m and V_{max} values were 0.75 mM and 1.32 U/mg protein.

Discussions

We have cloned an endoglucanase gene from *C. thermocellum* and compared the cloned gene with the *C. thermocellum* cellulase genes so far reported (19-21). This gene has some similarity with *celC* gene in respect to the size of chromosomal DNA insert and the molecular size of the gene product (4, 22). However, the restriction pattern of this gene is entirely differnt from that of the *celC* gene and from those of any other *C. thermocellum* cellulase genes reported so far. Therefore we believed that we might have cloned a new endoglucanase gene from *C. thermocellum*.

The endoglucanase encoded by the new gene cloned in *E. coli* was purified and characterized. The new endoglucanase hydrolyzed CMC, filter paper and Avicel, but not cellobiose. When this enzyme reacted with CMC, a rapid decrease in viscosity was observed, and this suggested that the purified cellulase was a typical endo-type enzyme. A significant amount of glucose was detected in the hydrolytic products. This glucose might have come from the direct split of cello-oligosaccharides by the enzyme instead of the hydrolysis of cellobiose. This enzyme showed optimum activity at pH 5.0 and at 65℃, which is common to other *C. thermocellum* cellulases. Glucose caused no endproduct inhibition, but cellobiose inhibited the enzyme activity a little.

요 약

고온성 혐기성 세균인 *Clostridium thermocellum*의 섬유소 분해 효소 유전자를 pUC9 플라스미드를 이용하여 대장균에 클로닝하였고, 지금까지 클로닝된 *C. thermocellum*의 섬유소 분해 유전자들과 제한효소 양상을 비교하여 새로운 유전자임을 알 수 있었다. 대장균에서 섬유소 분해 효소를 열처리와 column chromatography에 의해서 정제를 하였고, 분자량은 40,000이었다. 이 효소는 pH 5.0과 65℃에서 CMC에 대해서 최대 활성을 보였고 최종 산물인 포도당과 cellobiose에 의한 활성의 저해는 크게 나타나지 않았다. CMC에 대한 이 효소의 K_m과 V_{max}값은 각각 0.39% (w/v)와 268 U/mg protein이었다.

REFERENCES

1. Bisaria, V.S. and T.K. Ghose. 1981. Biodegradation of cellulosic materials: substrates, microorganisms, enzymes and products. *Enzyme Microb. Technol.* **3**: 90-104.
2. Lamed, R., E. Kenig and E.A. Bayer. 1983. The cellusome-discret cell surface organelle of *Clostridium thermocellum* which exhibits separate antigenic, cellulose-binding and various cellulolytic activities. *Biotechnol. Bioeng. Symp.* **13**: 163-181.
3. Béguin, P., P. Cornet and J.-P. Aubert. 1985. Sequence of a cellulase gene of the thermophilic bacterium *Clostridium thermocellum. J. Bacteriol.* **162**: 102-105.
4. Grépinet, O. and P. Béguin. 1986. Sequence of the cellulase gene of *Clostridium thermocellum* coding for endoglucanase B. *Nucleic Acids Res.* **14**: 1791-1799.
5. Schwarz, W.H., S. Schimming, K.P. Rücknagel, S. Burgschwaiger, C. Kreil and W.L. Staudenbauer. 1988. Nucleotide sequence of the *celC* gene encoding endoglucanase C of *Clostridiuim thermocellum. Gene* **63**: 23-30.
6. Jollif, G., P. Béguin and J.-P. Aubert. 1986. Nucleotide sequence of the celluase gene *celD* encoding endoglucanase D of *Clostridium thermocellum. Nucleic Acids Res.* **14**: 8605-8613.
7. Hall, J., G.P. Hazlewood, P.J. Barker and H.J. Gilbert. 1988. Conserved reiterated domains in *Clostridium thermocellum* endoglucanases are not essential for activity. *Gene* **69**: 29-38.
8. Yagüe, E.,P. Béguin and J.-P. Aubert. 1990. Nucleotide sequence and deletion analysis of the cellulase-encoding gene *celH* of *Clostridium thermocellum. Gene* **89**: 61-67.
9. Schwarz, W.H., F. Gr Bnitz and W.L. Staudenbauer. 1986. Properties of a *Clostridium thermocellum* endoglucanase produced in *Escherichia coli. Appl. Environ. Microbiol.* **51**: 1293-1299.
10. Béguin, P., P. Cornet,and J. Millet. 1983. Identification of the endoglucanase encoded by the *celB* gene of *Clostridium thermocellum. Biochimie.* **65**:

495-500.
11. Pètre, D., J. Millet, R. Longin, P. Béguin, H. Girard and J.-P. Aubert. 1986. Purification and properties of the endoglucanase C of *Clostridium thermocellum* produced in *Escherichia coli*. *Biochimie.* **68**: 687-695.
12. Joliff, G., P. Béguin, M. Juy, J. Millet, A. Ryter, R. Poljak and J.-P. Aubert. 1986. Isolation, crystallization and properties of a new cellulase of *Clostridium thermocellum* overproduced in *Escherichia coli*. *Bio/Technology* **4**: 896-900.
13. Weimer, P.J. and J.G. Zeikus. 1977. Fermentation of cellulose by *Clostridium thermocellum* in the absence and presence of *Methanobacterium thermoautotrophicum*. *Appl. Environ. Microbiol.* **33**: 289-297.
14. Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. *Molecular Cloning*: *A Laboratory Manual.* 2nd ed. Cold Spring Harbor, N.Y.
15. Park, S.H. and M.Y. Pack. 1986. Cloning and expression of a *Bacillus* cellulase gene in *Escherichia coli*. *Enzyme Microb. Technol.* **8**: 725-728.
16. Miller, G.L., R. Blum, W.E. Glennon and A.L. Burton. 1960. Measurement of carboxymethylcellulose activity. *Anal. Biochem.* **2**: 127-132.
17. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. The protein measurement with folin phenol method. *J. Biol. Chem.* **193**: 265-275.
18. Laemmli, U.K. 1970. Clevage of structural proteins during the asembly of the head of bacteriophage T4. *Nature* **227**: 680-685.
19. Millet, J., D. Pétré, P. Béguin, O. Raynaud and J.-P. Aubert. 1985. Cloning of ten distinct DNA fragments of *Clostridium thermocellum* coding for cellulases. *FEMS Microbiol. Lett.* **29**: 145-149.
20. Schwarz, W., K. Bronnenmeier and W.L. Staudenbauer. 1985. Molecular cloning of *Clostridium thermocellum* genes involved in β-glucan degradation in bacteriophage lamda. *Biotechnol. Lett.* 7: 859-864.
21. Romaniec, M.P.M., N.G. Clarke and G.P. Hazlewood. 1987. Molecular cloning of *Clostridium thermocellum* DNA and the expression of further novel endo-β-1,4-glucanase genes in *Eshechia coli*. *J. Gen. Microbiol.* **133**: 1297-1307.
22. Schwarz, W.H., S. Schimming and W.L. Staudenbauer. 1987. High-level expression of *Clostridium thermocellum* cellulase genes in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **27**: 50-56.
23. Gilkes, N.R., M.L. Langsford, D.G. Kilburn, R.C. Miller, Jr. and R.A.J. Warren. 1984. Mode of action and substrate specificities of cellulases from cloned bacterial genes. *J. Biol. Chem.* **16**: 10455-10459.

(Received July 27, 1992)