The Journal of Natural Science,
Pai Chai University, Korea
Vol.8 No2: 57~61, 1996

Characteristics of *Cellulomonas fimi* β-glucosidase expressed in *Escherichia coli*

Ha-Kun Kim
Department of Genetic Engineering, Pai Chai University

대장균에서 발현되는 *Cellulomonas fimi* β-glucosidase의 효소학적 특성

김 하 근
배재대학교 유전공학과

The β-glucosidase enzyme was purified from *E. coli* carrying *Cellulomonas fimi* β-glucosidase gene. SDS-PAGE and analytical gel filtration revealed that molecular weight of this enzyme was 56,000 dalton and consisted of a single polypeptide.
Inhibition caused by heavy metals and activation by dithiothreitol suggest the existence of essential thiol group in the enzyme. The enzyme was not active on maltose (glucose α-1,4-glucose) which has a α-linkage, whereas it was active on lactose (glucose β-1,4-glucose), PNPG (p-nitrophenyl β-D-glucopyranoside) and PNPC (p-nitrophenyl β-D-cellobioside), although its reaction rates were different.

*Cellulomonas fimi*에서 유래한 β-glucosidase 유전자를 갖고 있는 대장균으로부터 β-glucosidase 효소를 정제하였다. 전기 영동과 크로마토그라피 실험을 수행함으로써 정제된 효소의 분자량은 56,000 달톤이며 단일 폴리펩티드로 구성되어 있음을 알 수 있었다. 정제된 β-glucosidase 효소는 당이 β-결합을 하고 있는 cellobiose, PNPG, PNPC 등의 기질에 대하여 작용하여 분해시킬 수 있었으나, α-결합을 갖고 있는 maltose는 분해할 수 없었으므로, β-결합에 대한 기질 특이성을 갖고 있음을 알았다. 철, 수은, 구리 등의 중금속 이온들에 의해 효소 활성이 저해되었고 DTT에 의해 효소의 활성이 활성화됨을 보임으로써 β-glucosidase 효소의 활성화 부위는 -SH 기가 중요하게 작용하고 있음을 시사하였다.

Key words : *Cellulomonas fimi*, β-glucosidase, cellobiose, PNPG

Introduction

At least three kinds of cellulases, endo-β-1, 4-glucanase, exo-β-1,4-glucanase, and β-glucosidase are equally needed to obtain glucose from cellulose in bacteria. By the action of the first two enzymes, cellulose is degraded mainly to cellobiose with some cellodextrins. Three different pathways for the cellobiose degradation are known. They are : hydrolysis (cellobiose + H_2O → 2 glucose), inorganic phosphate-dependent phospholysis (cellobiose + Pi → glucose + glucose-1-phosphate)[1, 2], and ATP-dependent phospholysis (cellobiose + ATP + H_2O → glucose + glucose-6-phosphate + ADP).[3, 4] β-Glucosidase hydrolyses cellobiose directly to two molecules of glucose. Recently, gene coding for β-glucosidase was cloned from *Cellulomonas fimi*.[5]

In this study *Cellulomonas fimi* β-glucosi-

dase enzyme was purified from *E. coli* and several enzymatic characteristics were investigated.

Materials and Methods

1. Preparation of enzyme extracts

E. coli cells carrying pCF18 plasmid were grown overnight at 37℃ in LB medium containing ampicillin (50 ug/ml). Harvested cells were suspended in 50 mM sodium phosphate buffer, pH 7.0, and sonicated for 2 min with Branson sonifier (VWR Model 350) at 40 % output. After removing the cell debris by centrifugation, the supernatants were used to determine the β-glucosidase activities.

2. Assay of β-glucosidase activity

The β-glucosidase enzyme activity was measured using cellobiose and p-nitrophenyl β-D-glucopyranoside (PNPG) as substrates. When PNPG was used, the reaction mixtures contained 1 ml of 1 mM PNPG in 50 mM sodium phosphate buffer (pH 7.0) and 0.1 ml of the cell extract. The hydrolysis was stopped by adding 2 ml of 1 M Na_2CO_3. Optical density of liberated p-nitrophenol was measured at 400 nm. To measure glucose released from cellobiose, the same reaction mixture as above except for 40 mM cellobiose as substrate was used. The cellobiose hydrolysis was stopped by steaming the reaction mixtures for 5 min and the amount of glucose released from cellobiose was measured using Sigma glucose detection kit (No. 510-A). A unit of the enzyme is defined as that causing the release of 1 nmole of p-nitrophenol or glucose per min. Proteins were determined by the method of Lowry with a bovine serum albumin as standard.[6]

3. Molecular weight determination of the purified enzyme

Molecular weight of the purified β-glucosidase was determined by electrophoresis in 10 % polyacrylamide gel containing 0.1 % SDS according to Laemmli.[7] Gels were stained with 0.25 % Coomassie brilliant blue in the solution A of methanol, acetic acid, and H_2O (400 : 70 : 530) mixture and destained by soaking in the same solution A. The apparent molecular weight of the enzyme was determined using Sephadex G-150 gel filtration chromatography with aprotinin (MW 6,500), cytochrome C (MW 12,400), carbonic anhydrase (MW 29,000), and bovine serum albumin (MW 66,000) as molecular weight standards. The column was eluted with 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl.

Results and Discussion

1. Purification of *Cellulomonas fimi* β-glucosidase

In order to purify the β-glucosidase enzyme, *E. coli* harboring pCF18 plasmid was grown in LB medium. The late log phase cells were harvested by centrifugation for 20 min at 5,000 rpm. The washed cells were suspended in 50 mM sodium phosphate buffer (pH 7.0). The suspended cells were sonicated using Sonifier Cell Disrupter equipped with a intermediate tip. Temperature was maintained below 4℃ with ice water bath during the disruption steps. The extracts were clarified by centrifugation at 15,000 rpm in SS34 rotor for 60 min and poured on to a DEAE Sephadex A-50 column which was previously equilibrated against the 50 mM sodium phosphate buffer (pH 7.0). The column was eluted with a linear NaCl gradient from 0 mM to 600 mM. The β-glucosidase was emerged at around 300 mM NaCl. The active fractions were pooled, concentrated using ultrafiltration (Amicon, PM 10), dialyzed and applied to a Sephadex G-100 column equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The active fractions were dialyzed against 10 mM AMP (2-amino-

2-methyl-1,3-propandiol) buffer (pH 9.0), and applied to QAE-Sephadex A-50 column equilibrated with the same buffer. As results of purification steps, the final preparation of this enzyme was enriched 38-fold in specific activity with yield of 24 % (Table 1).

Table 1. Purification of the β-glucosidase from *E. coli* carrying pCF18 plasmid.

purification steps	total activity (U)	protein (mg)	specific activity (U/mg)	Yield (%)
crude extract	285,000	1,400	203	100
DEAE Sephadex A-50	200,000	114	1,754	70
Sephadex G-100	150,000	47	3,190	52
QAE Sephadex A-50	68,750	10	6,875	24

2. Purity of purified enzyme and determination of molecular weight

Relative electrophoretic mobilities of purified β-glucosidase and of the reference proteins on SDS-polyacrylamide gel electrophoresis were plotted versus their molecular weight. The mobility of the purified β-glucosidase correspond to a molecular weight of 56,000 (Fig. 1). The molecular weight of intact enzyme determined by Sephadex G-150 was 57,000 (data not shown). These results indicate that the β-glucosidase enzyme is monomeric.

3. Substrate specificity

Using the purified β-glucosidase enzyme, several β-glucosides were tested as substrates for the enzyme (Table 2). The enzyme was not active on maltose which has α-linkage but readily hydrolyzed lactose which has β-linkage. The enzyme was active on cellobiose, p-nitrophenyl β-D-glucopyranoside, but the activity on p-nitrophenyl β-D-cellobioside was very low.

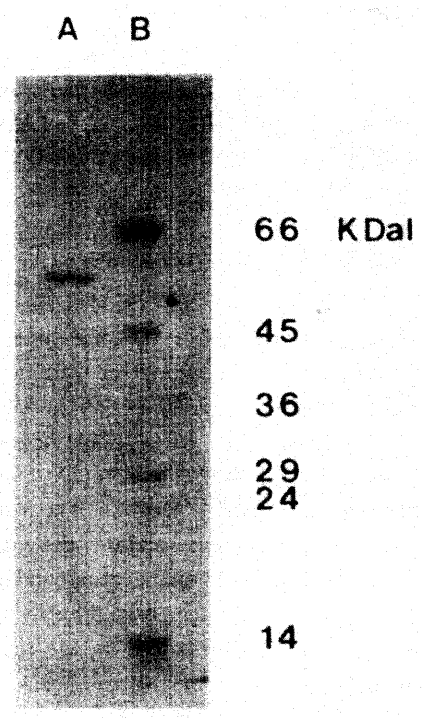

Fig. 1 SDS-polyacrylamide (10 %, w/v) gel electrophoresis of the purified β-glucosidase. Purified enzyme was run with standard marker proteins to enable to determine the molecular weight of the enzyme. Standard marker proteins are as follows: Albumin, bovine (Mr 66,000); Albumin, egg (Mr 45,000); Glyceraldehyde 3-phosphate dehydrogenase (Mr 36,000); Carbonic anhydrase (Mr 29,000); Trypsinogen (Mr 24,000); Trypsin inhibitor (Mr 21,100); α-lactalbumin (Mr 14,200).

Table 2. Substrate specificity of the purified β-glucosidase

substrates (10 mM)	enzyme activity (1,000 x U/mg protein)
p-nitrophenyl β-D-glucopyranoside	6.87
p-nitrophenyl β-D-cellobioside	0.06
cellobiose	1.04
lactose	0.52
maltose	0
carboxymethylcellulose	0

4. Kinetic Constants

The Km values for the purified enzyme were determined from Lineweaver-Burk plots. The calculated values for Km were 1.15 mM for p-nitrophenyl β-D-glucopyranoside, and 38.53 mM for cellobiose (data not shown).

5. Effects of pH and temperature

The activities of β-glucosidase on both substrates (p-nitrophenyl β-D-glucopyranoside and cellobiose) were measured at various pHs in McIlvaine buffer (pH 5.0 - 8.0) and McIlvaine-NaOH buffer (pH 8.0 - 10.0). Maximum activities on both substrates were observed at pH between 7.0 and 8.0 (Fig. 2). The optimal temperature determined under the assay conditions was observed to be at around 35℃. However, the optimum temperature was raised to 40℃ when DTT was added to the reaction mixture (Fig. 3).

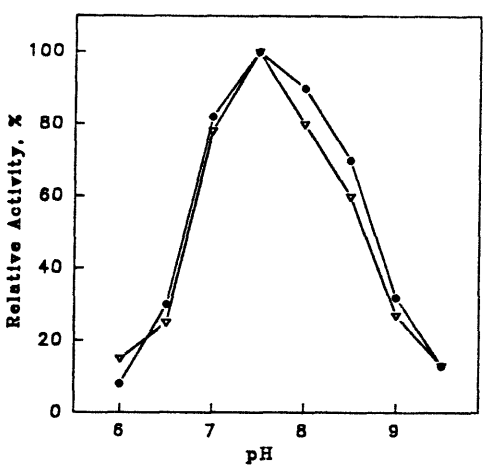

Fig. 2 Effect of pH on the hydrolysis of cellobiose (◁) and PNPG (●).

6. Effect of glucose on enzyme activity

When glucose, the end product of cellobiose hydrolysis reaction, was added to the reaction mixtures, PNPG hydrolyzing activity was reduced as the concentrations of glucose increased (Fig. 4).

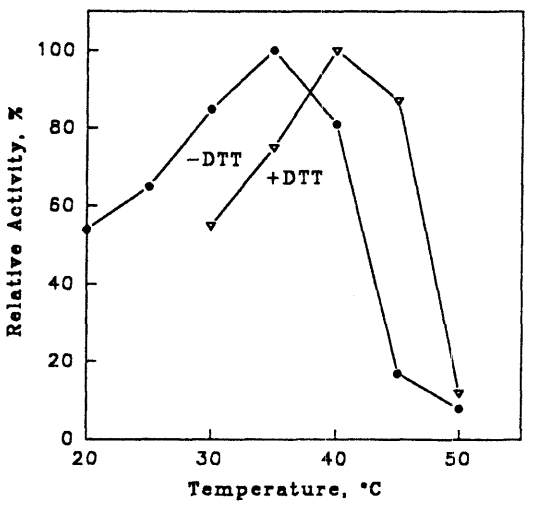

Fig. 3 Effect of temperature on the β-glucosidase activity. Reaction solution was incubated for 30 min under the tested temperature before the activity was determined. For the effect of dithiothreitol (DTT), 2.5 uM of DTT was added.

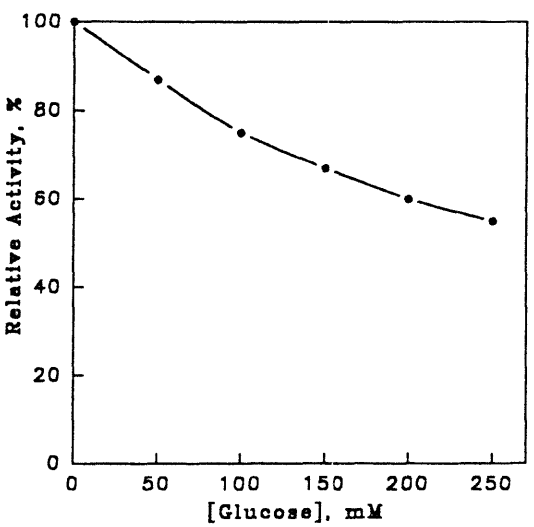

Fig. 4 Effect of glucose on the β-glucosidase activity.

7. Effects of chemicals on enzyme activity

The enzyme solutions were mixed with 50 mM phosphate buffer (pH 7.0) containing the test reagents at the final concentration of 5

mM and preincubated at 30 °C for 30 min. As shown in Table 3, Mn^{2+}. K^{+} did not affect the enzyme activity significantly. But certain heavy metals, notably Fe^{2+}, Hg^{2+}, Cu^{2+}, had a very inhibitory effect. Sulfhydryl group is known to react with heavy metal ions such as lead, iron, copper.[8,9] In addition to this, interestingly, DTT (dithiothreitol) which prevent disulfide formation increase enzyme activity substantially. This suggest that -SH group may be involved in the β-glucosidase enzyme reaction.

Table 3. Effects of chemicals on the β-glucosidase activity

compounds (5 mM)	relative activity, %
control	100
$FeSO_4 \cdot 7H_2O$	13.8
$HgSO_4$	4.8
$CuSO_4 \cdot 5H_2O$	7.3
$CoCl_2$	63.3
$MnCl_2 \cdot 4H_2O$	94.8
KCl	101.0
EDTA	45.0
DTT	147.2

Acknowledgement

This study was supported by a research grant from Pai Chai University.

References

1. Alexander, J.K. Methods Enzymol. 1972, 28; 299.
2. Ayers, W.A. J.Bacteriol. 1958, 76;515.
3. Palmer, R.E., and Anderson, R.L. J. Biol. Chem. 1972, 247; 3415.
4. Palmer, R.E., and Anderson, R.L. J. Biol. Chem. 1972, 247; 3420.
5. Kim, H.K., Pack, M.Y. Enzyme Microb. Technol. 1989 11(5) 313-316.
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. J. Biol. Chem. 1951, 193;265.
7. Laemmli, U.K. Nature, 1970, 227:680.
8. Scopes, R. Protein Purification-Principles and Practice. Springer-Verlag, 1982, p.195-197
9. Cooper, T.G. The Tools of Biochemistry. John Wiley & Sons, 1977, p. 365-367.