Some Properties of Thermostable β-Galactosidase of *Bacillus coagulans*

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熱耐性이 强한 *Bacillus coagulans* 의 β-Galactosidase의 特性에 對하여

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ABSTRACT

A thermostable β-galactosidase (β-galactoside galactohydrolase, EC 3.2.1.23) was inducible in *Bacillus coagulans* by lactose and D-galactose. The enzyme was purified 87 fold, and the optimum temperature and pH for activity were determined to be 60°C and pH 7.5, respectively. Kinetic determinations at 55°C established a Km of 3.3 mM for the chromogenic substrate o-nitrophenyl β-D-galactopyranoside (ONPG). Galactose and lactose were competitive inhibitors with Ki of 6.1 mM and 4.9 mM, respectively. The enzyme was relatively thermostable. The crude enzyme was inactivated about 20% after 20 min of exposure at 60°C and the purified was about 50%. Maximal enzyme activity required Mn^{++}, and for the thermal stabilization Fe^{++} and Ca^{++} were necessary.

INTRODUCTION

β-D-Galactosidase (EC 3.2.1.23, β-galactoside galactohydrolase, lactase) has been found in numerous microorganisms, animals, and plants. The widespread occurrence of intestinal β-galactosidase in various animals is mainly related to the hydrolysis of the dietary lactose. The lysosomal β-galactosidase is a key enzyme in the degradation of glycolipids, mucopolysaccharide, and glycoproteins (Spiro, 1970). Milk and milk products have been considered a valuable source of high quality protein, but they contain high levels of lactose. A majority of the adults with low level of intestinal β-galactosidase exhibits resultant maldigestion of the lactose and subsequent osmotic and fermtative diarrhea (Kretschmer, 1972). Decreasing the levels of lactose in milk products is important in solving the lactose intolerance and can increase sweetness of milk. β-Galactosidase from microorganisms are considered most suitable for the industrial application (Ikura and

Horikoshi, 1979). The yeast and fungal β-galactosidases have been investigated widely in the modification of milk and milk products (Woychik et al., 1973; Sugiura et al., 1978), and the bacterial enzyme, like E. coli, largely in the genetic control mechanisms and the induction-repression effects of the protein synthesis (Llands and McFall, 1969; Wannier et al., 1978). Although the fungi produce high cell yields but low enzyme activity, some bacteria exhibit higher enzyme activity but lower cell yields (Wierzbicki and Kosikowski, 1973). This study presents the evidence for the induction of a thermostable β-galactosidase in a thermophile, B. coagulans. Data are also given for a partial purification and characterization of the enzyme, especially for its thermostability.

MATERIALS AND METHODS

1. Materials

o-Nitrophenyl-β-D-galactopyranoside(ONPG) was obtained from Tokyo Cacei Industry; Sephadex G 200 and DEAE-Sephadex A 50 from Pharmacia Fine Cheimcals; bovine serum albumin from Difco. All the other chemicals were of reagent grade.

2. Microorganisms

The organisms used in these studies were gram positive, sporeforming, L(+)-lactic acid producing B. coagulans (Kim et al., 1977). Lactobacillus bulgaricus and E. coli were used for comparison of the thermostability of β-galactosidase.

3. Culture

For the production of β-galactosidase, B. coagulans was grown in MRS medium(Sharpe, 1962), supplemented with 2% lactose instead of glucose, for 12 hrs at 45°C in the reciprocaly shaking incubator with 130~140 stroke per min. L. bulgaricus and E. coli were grown at 37°C on the same conditions.

4. Preparation of cell-free extract

Cell harvest and preparation of cell-free extract were followed by the method of Kim et al. (1977).

5. Assay of β-galactosidase

The enzyme activity was determined by the procedure of Lederberg (1950). An enzyme unit was defined as moles of ONP liberated from ONPG per min of reaction, and the specific activity is expressed as units per mg of protein. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

6. Purification of β-galactosidase

1) Nucleic acid precipitation

Protamine sulfate was added to the cell-free extract with the ratio of mg per ml (McFeters et al., 1967). The solution was held at 20°C for 15 min and centrifuged at 20,000×g for 20 min at 0°C.

2) Fractionation with ammonium sulfate

The supernatant after step 1) was salted out with ammonium sulfate (60~80%) at 0°C for 2 hrs. The centrifuged precipitate was dissolved in a small amount of 0.05 M potassium phosphate buffer (PPB), and dialyzed against PPB at 4°C for 1 day.

3) Column chromatography on Sephadex G 200

1 ml of dialyzed enzyme solution was applied on Sephadex G 200 column (2×30 cm) equilibrated with PPB and diluted with PPB at a flow rate of 8 ml/hr. 2 ml elutes were collected.

4) Column chromatography on DEAE-Sephadex A 50

The pooled peak fraction from the Sephadex G 200 eluation were layed on 2.0×25 cm column containing DEAE-Sephadex A 50. This material was eluted with a linear gradient of 0.0 M to 1.2 M NaCl in 0.05 M tris buffer at pH 7. 0.3 ml eluates were collected at the rate of 20 ml/hr.

RESULTS

1. Induction and catabolite repression of β-galactosidase synthesis

The β-galactosidase synthesis of *B. coagulans* was induced by galactose and lactose, but repressed by glucose, fructose, and maltose (Fig. 1). The noninduced basal level of enzyme indicates that enzyme synthesis increased nearly 25 fold and 50 fold in the presence of lactose and galactose, respectively. Enzyme activity was not almost detectable in cells growing in the presence of glucose, fructose, or maltose.
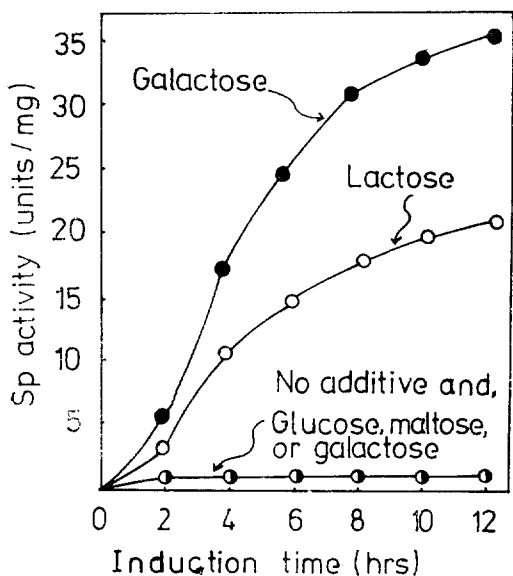

Fig. 1. Induction of β-galactosidase by various carbohydrates.

2. Purification of β-galactosidase

1.5 m*l* of dialyzed enzyme preparation, which was fractionated with ammonium sulfate, was layered on a 2×30 cm column containing Sephadex G 200. β-Galactosidase was appreciably purified through gel filtration as shown in Fig. 2. The pooled peak fraction from the Sephadex G 200 elution (fraction number 17~20) were applied on a 2×25 cm column containing DEAE-Sephadex A 50. Upon a linear gradient elution, as shown in Fig. 3, each 3 m*l* aliquot of eluent was collected. Purified β-galactosidase was obtained on fraction numbers 31 to 34. The overall purification and yield of activities are summerized in Table 1. A 87-fold overall purification was achieved with about 35% recovery of enzyme activity. The β-galactosidase purified by this procedure had a specific activity 1580 U/mg of protein.
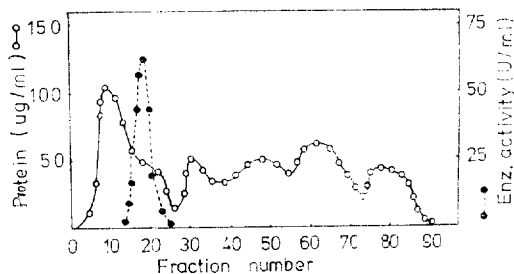

Fig. 2. Purification of β-galactosidase by column chromatography wi h Sephadex G 200.
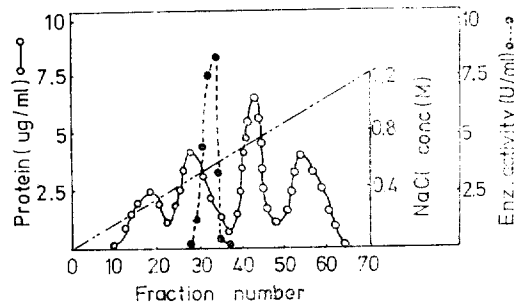

Fig. 3. Purification of β-galactosidase on DEAE-Sephadex A 50.

Table 1. Purification of β-Galactosidase

Step	Total vol. (m*l*)	Total units	Total protein (mg)	Specific activity (Units/mg)	Fold purified	Yield (%)
Crude	150	4421	244.5	18.1	1.0	100.0
Nucleic Acid Precipitate	145	4210	232.7	18.1	1.0	95.2
$(NH_4)_2SO_4$ Fractionation	15	4115	81.4	50.6	2.8	93.1
Sephadex G 200	10	2432	8.1	300.2	16.6	55.0
DEAE-Sephadex A 50	25	1553	1.0	1580.8	87.3	35.0

3. Effect of temperature on enzyme activity

Fig. 4 shows the relationship between temperature and β-gactosidase activity. The samples of enzyme were assayed for 10 min in ONPG solutions prepared in 0.05 M potassium phosphate buffer (pH 7.0) at each temperature indicated. Little activity was observed at temperature below 30°C or above 70°C, and optimal activity occurred at 60°C. However, ONPG thermal breakdown at 65°C and 70°C could be detected during the standard assay incubation time. From these data a temperature of 55°C was chosen as the standard assay temperature. Although this was not optimal, it had the virtue of providing a stable environment for the enzyme and ONPG.
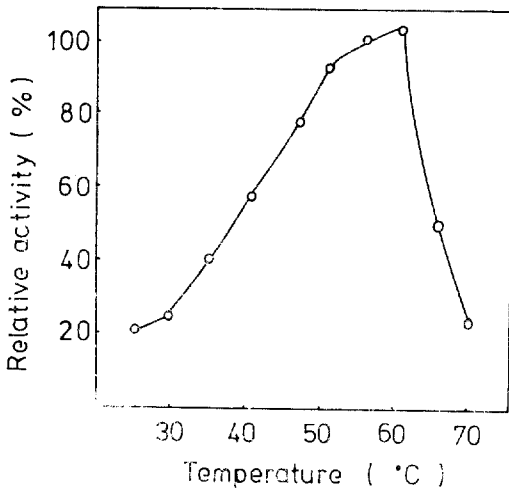

Fig. 5. Effect of pH on the activity of β-galactosidase.

5. Effect of divalent cation on enzyme activity

The effects of certain divalent cations on enzyme activity, when added to the assay mixture, are shown in Table 2. The assay was performed at 55°C, pH 7.5. Manganese activated the enzyme, however, the others had no an apparent effect.

Table 2. Effect of divalent cations on the activity of β-Galactosidase.

Cations	Concentrations (mM)	Relative activity(%)
None	—	100
Mn^{++}	1.0	130
Mg^{++}	1.0	99
Fe^{++}	1.0	103
Co^{++}	1.0	95
Ca^{++}	1.0	114
Zn^{++}	1.0	110

Fig. 4. Effect of temperature on the activity of β-galactosidase.

4. Effect of pH on enzyme activity

The pH dependence of β-galactosidase is shown in Fig. 5. The pH optimum is approximated 7.5 to 8.0 with 0.05 M potassium phosphate buffer. Under high pH conditions (above 8.0) enzyme activity was rapidly lost, and hydrolysis of ONPG occurred, so a pH of 7.5 was chosen for the standard assay. The enzyme hydrolysis of ONPG under acid conditions did not occur.
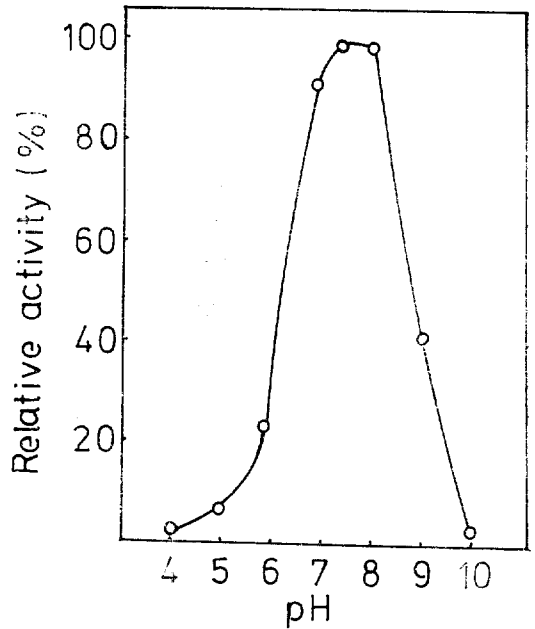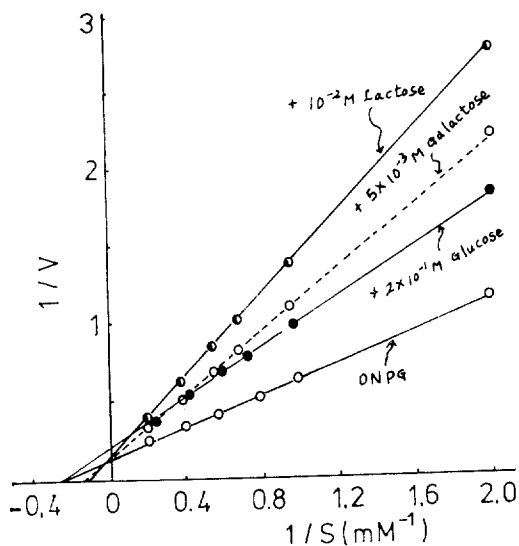
6. Kinetic studies

The effect of substrate concentrations on the velocity of the enzyme reaction was determined at 55 C°. Fig. 6 shows the data from low substrate concentrations plotted by the method

of Lineweaver and Burk. The Km value for ONPG was 3.3 mM. A series of sugars was tested as inhibitors of hydrolysis of ONPG by β-galactosidase. Lactose and galactose were competitive inhibitors of ONPG, and glucose was noncompetitive inhibitor. The Ki for lactose and galactose were 4.9 and 6.1 mM, respectively, as determined by the double-reciprocal methods of Dixon.

the β-galactosidase of *L. bulgaricis* and *E. coli*. Although the optimum temperature for the enzyme activity of *L. bulgaricus* was 55°C, it was 60% inactivated after 20min of exposed at 60°C. The mesophilic enzyme of *E. coli* showed approximately 80% loss of activity after 20 min of incubation at 50°C; after 20 min at 55°C, less than 1% activity remained.
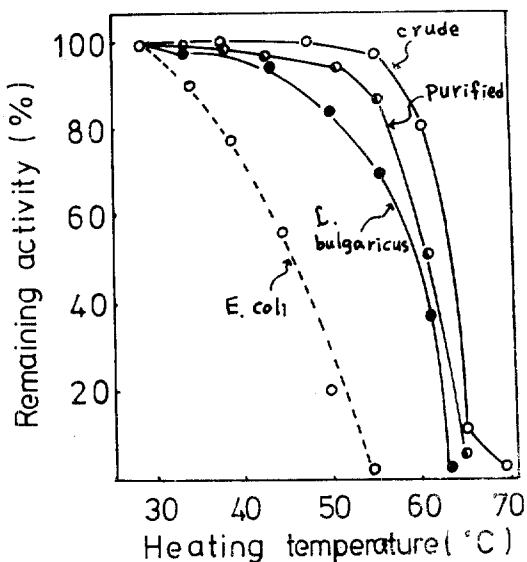

Fig. 6. Linewaever-Burk plots for the determination of the Michaelis constant for ONPG and the inhibitor constants for lactose, galactose, and glucose.

Fig. 7. Comparison of thermostability of β-galactosidase from *B. coagulans* (crude and purified), *L. bulgarious*, and *E. coli*.

7. Comparison of thermostability with other enzyme species.

The enzyme preparations from *B. coagulans*, *L. bulgaricus*, and *E. coli* were incubated at several temperatures for 20 min and assayed for residual activity. Fig. 7 shows the effect of temperature on the loss of enzyme activity. Temperatures in excess of 60°C rapidly distroyed enzyme activity. Incubation of the crude enzyme from *B. coagulans* at 60°C retained 80 % activity after 20 min. Comparatively, the purified resulted in a 50% loss of activity under same conditions. The thermostability of the enzyme was apparent when compared to

8. Effect of metal ions on the thermostability.

The enzyme preparation dissolved in 0.05 M potassium phosphate buffer (pH 7.5) was incubated with an equal volume of divalent cation solutions at 60°C. Same divalent cations were added to the assay mixture and the remaining activity was determined with same assay method. As shown in Table 3, ferrousion and calcium at concentration of 1.0 mM stabilized the enzyme to heat. Ferrous ion provided 85% thermostability and calcium 90% to the enzyme for up to 20 min when incubated at 60°C. In the presence of manganese and covalt, the enzyme was more labilized to heat than non-treated enzyme.

Table 3. Effect of divalent cations on the thermostability of β-Galactosidase.

Cations	Concentraions	Remaining activity(%)
None	—	51
Mn^{++}	1 mM	23
Mg^{++}	1 mM	57
Fe^{++}	1 mM	85
Co^{++}	1 mM	22
Ca^{++}	1 mM	90
Zn^{++}	1 mM	40

DISCUSSION

A thermostable β-galactosidase was induced by lactose and galactose in *B. coagulans*. The induction response to galactose was not without precedent, for galactose has been reported to be as effective as lactose in inducing β-galactosidase in *E. coil* (Llanes and McFall, 1969) and *Shigella sonnei* (Rickenberg, 1960), and it induced much more enzyme than lactose in *S. paradysenteriae* (Rickenberg, 1960) and *Thermus squaticus*(Ulrick *et al.*, 1972). The addition of glucose, fructose, and maltose had a repressive effect on the enzyme synthesis. Many studies have been involved in this effect, catabolite repression. Whereas most early studies of catabolite repression dealt with the permanent phase of catabolite repression, a much more severe type of repression refered to as transient repression(Tyler, 1967) occurs after addition of glucose to the growing culture. Whether or not transient repression and permanent catabolite repression are controlled by the same mechanism remains uncertain (Hamilton and Lo, 1978; Wanner *et al.*, 1978).

The optimal temperature for enzyme activity (60°C) was higher than that observed for *E. coli* (Lederberg, 1950), *B. subtilis* (Anema, 1964), and *Penicillium citrinnm* (Watanabe *et al.*, 1979), and similar to that of *Mucor pusillus* (Sorensen and Crison, 1974). The optimal pH was neutral (pH 7-8), as Blankenship and Wells (1974) found that most bacterial β-galactosidases are active at neutral pH.

The effect of divalent cations in enzymic hydrolysis has been controversial for a long time. The activation of the enzyme by Mn^{++} is in agreement with the effects of this cation on *T. aquaticus* (Ulrick *et al.*, 1972) and on *Kluyveromyces lactis* (Dickson *et al.*, 1979). For the thermostability of the enzyme, Fe^{++} and Ca^{++} were required. It was reported that highly purified and seven times recrystallized β-galactosidase shows to contain about 0.7% atoms of Ca^{++} per mole of 135,000 molecular weight (Wallenfels and Weil, 1972). It can not, therefore, be excluded that β-galactosidase is a metalloenzyme requiring divalent metal ions for maximal activity and stabilization.

Kinetic analysis of the purified β-galactosidase indicated a Km of 3.3 mM for ONPG. Anema (1964) found that β-galactosidase from *B. subtilis*, *E. coli*, and *B. megaterium* have a Km of 42 mM, 0.18 mM, and 0.16 mM, repectively. The Km value of 3.3 mM is about twice hihger than that for *T. aquaticus* (Ulrick *et al.*, 1972) and *K. lactis* (Dickson *et al.*, 1979). The Ki values of inhibitors for the enzyme showed considerable differences from those from various microorganisms (Ulrick *et al.*, 1972; Dickson *et al.*, 1979).

The crude β-galctosidase exhibited the unusual thermostability, similar to that of *M. pusillus*, and can be contrasted with the stability of the yeast, *K. flagilis* enzyme, which was rapidly inactivated attemperatures above 40°C (Sorensen and Crisan, 1974). They reported that the thermostability of the enzyme affords the substrate and product a degree of natural protection against the undesirable microbial contamination by permitting the enzymic hydrolysis to be carried out at elevated temperature. This might be of value in a process

using the thermostable *B. coagulans* β-galact-osidase to reduce the lactose content in milk. Many theories have been promulgated in attempts to explain the nature of life at high temperatures. The purified enzymes from thermophiles have revealed few physiochemical differences, except for the obvious thermal tolerance, when compared with mesophilic organisms. In addition, there is good evidence su-

ggesting the absence of protein-stabilizing factors in the intracelluar environment of thermophiles (Singleton *et al.*, 1969). Although the thermostable character of many isolated cellular components of thermophilic bacteria has been well documented, the regulation of the synthesis of these macromolecules has not been explored.

摘   要

*Bacillus coagulans*에서 β-galactosidase를 분리하여 87배가량 정제하였다. 이효소의 최적활성온도는 60°C, pH는 7.5였으며 Mn++의 첨가는 효소활성도를 크게 증가시켰다. 기질인 ONPG의 분해에 대한 Km값은 3.3mM 이고, galactose, lactose는 ONPG분해의 competitive inhibitor로 각각 6.1mM, 4.9mM의 Ki값을 가지며 glucose는 noncompetitive inhibitor로 작용하였다.

세균에서 분리된 다른 β-galactosidase에 비해 이 효소는 열내성이 높아 조효소의 경우는 60°C에서 20분간 열처리한 후 약 80%의 활성도를 갖고 있으며, 정제된 것은 약 50%를 잔유하고 있었다. Fe++와 Ca++의 첨가는 이 효소의 열내성을 더욱 증가시키는데 효과가 있었다.

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