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Studies on the Production of β-Galactosidase by *Lactobacillus sporogenes*

— Characterization of β-Galactosidase —

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*Lactobacillus sporgenes*에 의한 β-Galactosidase생산에 관한연구

-β-Galactosidase의 효소학적 성질-

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Extracellular β-galactosidase was prepared from a culture of *Lactobacillus sporogenes*, a spore-forming lactic acid bacterium. The enzyme functioned optimally at pH 6.8 and at 60°C o-nitrophenyl-β-D-galactopyranoside (ONPG) in 0.05M sodium phosphate buffer. The activation energy of the enzymatic hydrolysis of ONPG was about 16,000 cal/mole below 50°C and 11,300 cal/mole above the temperature. It was fairly stable over a pH range from 4.0 to 8.0 losing only less than 30% of its activity after heating at 60°C and pH 6.8 for 3 hours. Metal ions showed no significant effect on the enzyme actvity, whereas L-cysteine exerted a slight stimulatory effect at the concentration of 10mM. The Km values were 1.48mM for ONPG and 64.5mM for lactose. Hydrolysis of ONPG by the enzyme was product-inhibited by galactose (K_i = 13.3mM, competitive inhibition) and by glucose (K_i = 11.4mM, uncompetitive type). The enzyme activity was also noncompetitively inhibited in the presence of lactose (K_i = 17.8mM).

Lactose is, as well recognized, an important component of milk, imparting characteristic flavor and texture to milk and milk products as well as contributing to their nutritive value. On the other hand, it also presents problems such as lactose crystallization[1] in condensed and frozen milk products, pollution[2] when untreated whey is discharged, and lactose intolerance[3] prevailing in non-Caucasian children and adults. These factors have led many researchers to survey microorganisms with the purpose of selecting organisms capable of producing high levels of β-galactosidase (lactase, E. C. 3.2.1. 23), the enzyme that hydrolyzes lactose into its component monosaccharide units. Many bacteria[4,5], yearsts[6,7] and molds[8,9] have been reported as β-galactosidase producers.

Lactobacillus sporogenes was found to be a particularly promising microorganism for the production of β-galactosidase[10]. It not only yields extracellularly large quantities of β-galactosidase but its β-galactosidase possesses characteristics (pH and temperature optima, etc.) suitable for its applications in food industries. In the previous paper[10] the authors already described the cultural conditions optimum for the production of β-galactosidase from *L. sporogenes*. This study deals with certain physical and chemical character istics of the crude β-galactosidase produced by the organism.

Materials and Methods

Microorganism and media

All experiments were done with *Lactobacillus sporogenes*, a spore-forming lactic acid bacterium producing a considerable amount of extracellular β-galactosidase.

The strain was maintained on Peptone-Glucose-Yeast extract agar medium. For the enzyme production the following medium was employed as previously described; 1.0% lactose, 1.5% peptone, 0.2% ammonium sulfate, 0.8% ammonium phosphate (diabsic), 1.0%sodium acetate, 0.05% potassium chloride and 0.001% ferric chloride in distilled water. The pH of the medium was ajusted to 7.0 prior to autoclaving.

β-Galactosidase production

The organism was grown under the conditions previously established[10]. After culture cells were removed by centrifugation at 10,000 rpm for 10 minutes and the supernatant thus obtained was used as a crude enzyme solution.

Assay of β-galactosidase

β-Galactosidase activity was determined with o-nitro phenyl-β-d-galactopyranoside (ONPG) as a chromogenic substrate by a modification of the procedure of Lederberg[11], in which colorless substrate ONPG is hydrolyzed to form galactose and yellow o-nitrophenol (ONP). The reaction was carried out at 60°C for 10 minutes in reaction mixtures containing 0.5ml of enzyme solution and 2.0ml of 5mM ONPG in 0.05M sodium phosphate buffer, pH 6.8. The reactions were terminated by chilling the reaction mixtures, followed by addition of 2.5ml of 1M sodium carbonate. The absorbancy at 420nm was measured and o-nitro phenol released was determined from an ONP standard curve.

When lactose was used as the substrate, activity was measured by a modified method of Jasewicz, L. and A.E. Wasserman method[12]. The procedure consists of two steps; hydrolysis of lactose by the enzyme and determination of glucose in the hydrolyzed product.

Hydrolysis of lactose:

A reaction mixture containing 2.0ml of 0.5M lactose solution in 0.05M sodium phosphate buffer (pH 6.8) and 0.5ml of the crude enzyme solution was incubated at 60°C for 30 minutes. The reaction was stopped by heating the tubes containing the mixtures in a boiling water bath for 5 minutes.

Glucose determination:

The amount of glucose liberated during lactose hydrolysis was estimated with Glucostat reagent obtained from Worthington Biochemical Corp., Freehold, N.J.

An enzyme unit of β-galactosidase is defined as the amount of enzyme needed to liberated 1 μ mole/ of o-nitrophenol from ONPG or glucose from lactose per minute under the conditions described above.

Enzyme characterization

Optimal pH and temperature for β-galactosidase activity were determined with reaction mixture containing 0.5ml of enzyme solution and 2.0ml of 5mM ONPG in 0.05M sodium phosphate buffer at pH 6.8.

Thermal stability of the crude enzyme was determined by measuring the remaining activity after holding the enzyme for various time intervals indicated in Fig. 5 at 50-70°C in 0.05M sodium phosphate buffer of pH 6.8.

The effect of pH on enzyme stability was studied by incubating enzyme in various buffers for 3 hours at 37°C over a pH range 4.0-9.0.

Kinetics properties of the enzyme.

Mixtures of 2.0ml of various concentrations of ONPG or lactose solutions and 0.5ml of crude enzyme solution were incubated at 60°C for the desired time. After incubation, concentrations of o-nitrophenol or glucose liberated were determined as described in the enzyme assay. The data were plotted by the method described by Lineweaver and Burk[13]. Respective K*m* and Vmax values for ONPG and lactose were graphically determined. The K*i* values for sugars such as glucose, galactose and lactose with ONPG as the substrate were also calculated graphically by the method of Lineweaver-Burk secondary plotting[13].

Results and Discussions

Optimum pH and temperature

The effects of pH and temperature on β-galactosidase activity with ONPG as the substrate are shown in Fig. 1 and 2. Peak activity was observed at pH 6.8 in 0.05M sodium phosphate buffer as also described in Table 1. At the pH of milk (pH 6.6), more than 95% of the maximum activity was found to be expressed and this makes the enzyme be suitable for dairy products applications[14]. The highest hydrolysis rate of ONPG was obtained at 60°C under the standard assay conditions. The Arrhenius plot in Fig. 3 shows a break in the slope at 50°C possibly caused by conformational change of the enzyme. The activation energy of 11,300 cal/mole in the 50°C to 60°C range indicates that the reaction proceeds more easily than in the 40°C to 50°C range where the energy for activation was 16,000 cal/mole.

This value is smaller than that reported for *Bacillus stearothermophilus* β-galactosidase[15] but it is larger than those for *E.coli*[16] or *Streptococcus thermophilus* β-galactosidase[4].

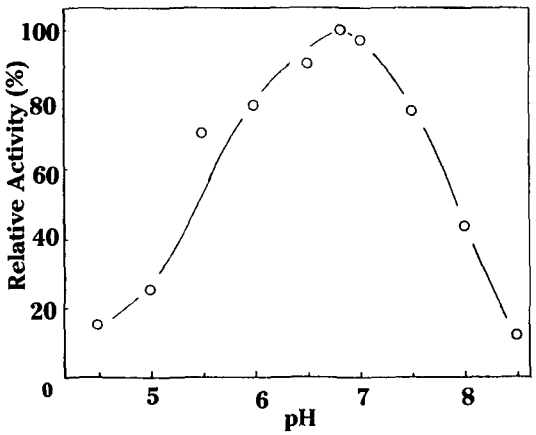

Fig. 1. Effect of pH on the β-galactosidase activity.

The reactions were carried out under the standard conditions as described in the text using the following buffers:
pH 4.0-5.5, 0.1 M McIlvaine buffer;
pH 6.0-7.5, 0.1 M Sodium phosphate buffer;
pH 8.0-8.5, 0.1 M Tris-HCl buffer;

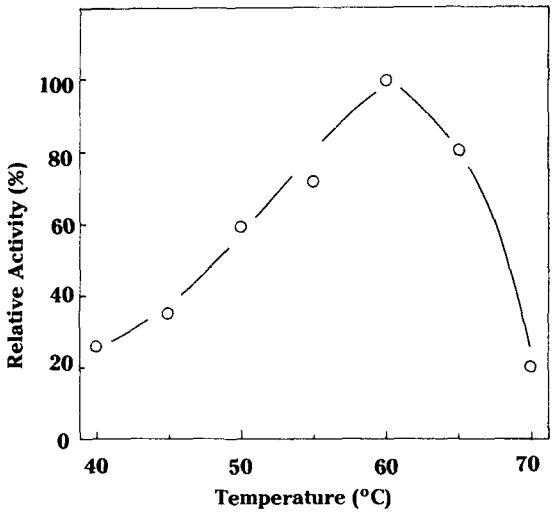

Fig. 2. Influence of temperature on the β-galactosidase activity

Table 1. Effect of various Buffers on the Activity of β-Galactosidase from *L. sporogenes*

Assay buffer solution	Activity (U/ml)	Relative activity (%)
0.05 M Sodium phosphate buffer	41.20	100
0.05 M Tris buffer	24.31	59
0.05 M Tris + NaCl buffer	22.61	55
0.05 M McIlvaine buffer	35.43	86
0.05 M Potassium phosphate buffer	41.20	100
0.01 M Sodium phosphate buffer	38.26	90
0.02 M Sodium phosphate buffer	40.66	96
0.05 M Sodium phosphate buffer	42.46	100
0.10 M Sodium phosphate buffer	39.04	92
0.20 M Sodium phosphate buffer	34.65	82

Effect of metal ions and reducing agents

As shown in Table 2, no remarkable influence of the cations examined could be detected in the standard reaction mixture. The enzyme was only slightly activated by barium and to an even lesser extent by magnesium ion; this activation was not similar to those of many other β-galactosidase of bacterial origin showing great stimulation with sodium ion.[14]

Activation of the β-galactosidases by reducing agents, especially by L-cysteine (10mM) could be recognized as seen in Table 3. This suggests that the enzyme is of the sulfhydryl type and then a reducing environment is necessary for its optimal activity.

Enzyme stability

The enzyme was essentially stable over the pH range between 4.0 and 8.0 as indicated in Fig. 4. The enzyme appears to retain its full activity over a wider pH range than *E.coli* β-galactosidase whose stability decreased sharply below pH 6.0 and more gradually above pH 8.0 at 40°C[17]. On the other hand, it is a contrast to that of *Streptococcus thermophilus* whose activity was reported to be stable at the rather alkaline pH ange (pH 7-10)[4].

Thermal inactivation of the enzyme was examined at three different temperatures as indicated in Fig. 5-A. Data

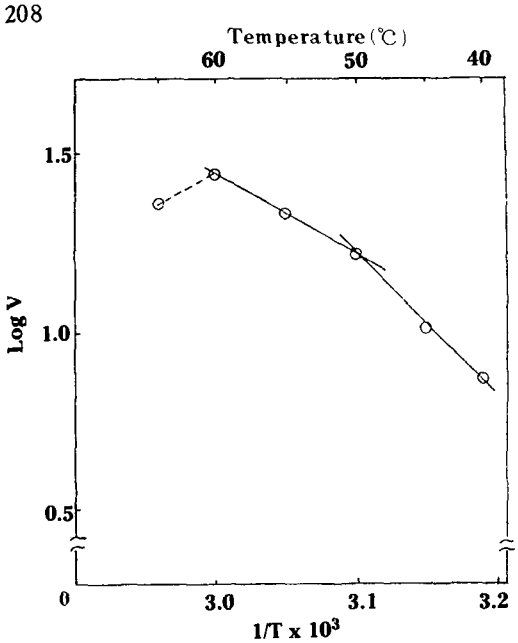

Fig. 3. Arrhenius Plot of Enzyme Activity against Temperature

Activity was measured using ONPG as a substrate over the temperature range of 40°C to 70°C under the conditions as stated in the assay.

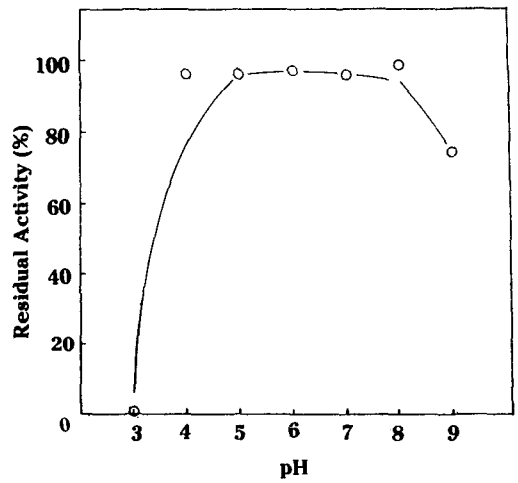

Fig. 4. Influence of pH on the Stability of the β-Galactosidase from *L. sporosenes*

The buffers used were:
pH 3.0-4.0, 0.1 M McIlvaine buffer;
pH 5.0-7.0, 0.1 M Sodium phosphate buffer;
pH 8.0-9.0, 0.1 M Tris-HCl buffer.

were not presented but an exposure to 50°C caused less than 10% loss of activity of the enzyme even after 3 hours. At 55°C the inactivation was about 17%, whereas at 60°C the inactivation was less than 30% after 3 hours exposure as shown in Fig. 5-B. *B. megaterium*[18], *Str. thermophilus*[4], and *B. subtilis* β-galactosidase[19] and it may meet the high temperature specification for its commercial applications.

Table 2. Effect of Minerals on the β-Galactosidase Activity

Minerals	Concentration	Activity (U/ml)	Relative activity (%)
None	—	39.30	100
KCl	1 mM	37.81	96.2
NaCl	1 mM	39.32	97.5
$BaCl_2$	1 mM	37.37	95.1
$MnSO_4$	1 mM	40.56	103.2
$MgSO_4$	1 mM	41.30	105.1
$ZnSo_4$	1 mM	33.72	85.8

Table 3. Affect of SH-containing Compounds on the β-Galactosidase activity

SH-Containing compounds	Concen.	Activity (U/ml)	Relative activity (%)
None	—	39.09	100
L-Cysteine	0.5 mM	39.09	100
L-Cysteine	1 mM	40.26	103
L-Cysteine	5 mM	41.75	106.8
L-Cysteine	10 mM	45.58	116.6
L-Cysteine	20 mM	39.87	102.0
L-Cysteine	50 mM	2.95	7.5
β-Mercaptoethanol	0.5 mM	38.76	99.2
β-Mercaptoethanol	1 mM	40.01	102.4
β-Mercaptoethanol	5 mM	41.20	105.4
β-Mercaptoethanol	10 mM	38.60	98.7
β-Mercaptoethanol	20 mM	36.90	94.4
β-Mercaptoethanol	50 mM	24.40	62.4

Enzyme kinetics

A Lineweaver-Burk plot for tests performed at pH 6.8 and 60°C allowed the calculation of K_m values equal to 1.48mM and 64.5mM for ONPG and lactose, respectively (Fig. 6). The values of Vmax were also determined in this experiments to be 38.5 μ mole/min/ml for ONPG and 1.43 μ mole/min/ml for lactose. The enzyme activity to ONPG was estimated to be about 27 times higher than that to lactose. The ratio of β-galactosidase activities measured with ONPG and lactose as the substrate (ONPG/lactose ratio)

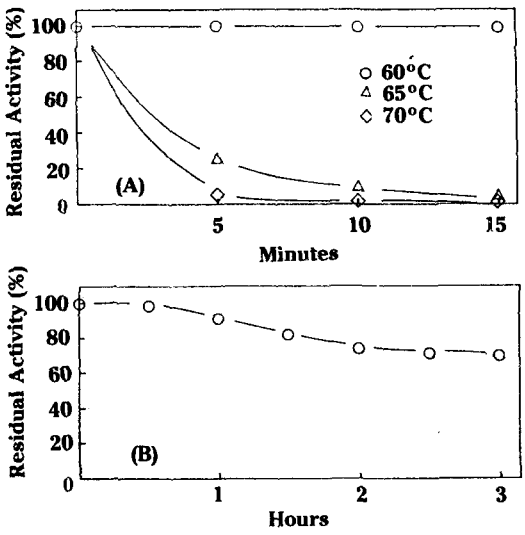

Fig. 5. Thermostability of the β-Galactosidase from *L. sporogenes*.

(A) Effect of various temperatures on the β-galactosidase stability.

(B) Effect of heating time on the stability at 60°C.

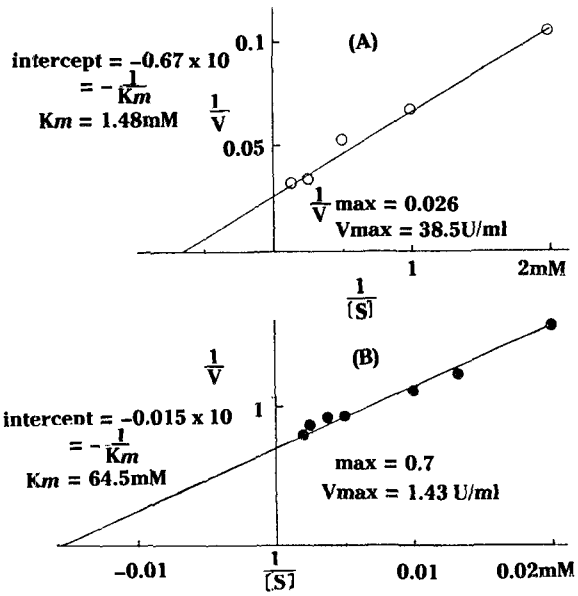

Fig. 6. Lineweaver-Burk Plot for the Determination of the Michaelis Constant for ONPG and lactose

(A) Double reciprocal plot for the hydrolysis of ONPG,

(B) Double reciprocal plot for the hydrolysis of lactose.

was reported to vary with the strains; ONPG/lactose ratio of *Str. thermophilus* was 4.72, while that of *L. helveticus* was 62.56[20].

A comparison of inhibition rates of the ONPG hydrolysis exerted by sugars is shown in Table 4. Mean while, Lineweaver-Burk plots of the inhibition by lactose indicated that lactose was unusually found to be a non-competitive type inhibitor for ONPG substrate as illustrated in Fig. 7. The *Ki* value for lactose was calculated to be 17.8mM. Generally, β-galactosidase from other microbial source such as those from *B. subtilis*[19] and *Asp. oryzae*[21], were reported to be competitively inhibited

Table 4. Effect of various Sugars on the β-Galactosidase Activity.

Sugars	Concen.	Activity (U/ml)	Relative activity (%)
None	—	40.94	100
Lactose	5 mM	26.73	65.3
Lactose	10 mM	22.6	55.2
Galactose	5 mM	30.3	74.0
Galactose	10 mM	26.37	64.4
Glucose	5 mM	29.68	72.5
Glucose	10 mM	21.04	51.4
Melibiose	5 mM	27.76	67.8
Melibiose	10 mM	20.43	49.9

The reactions were performed for 10 minutes at 60°C in the standard reaction mixture described in the enzyme assay.

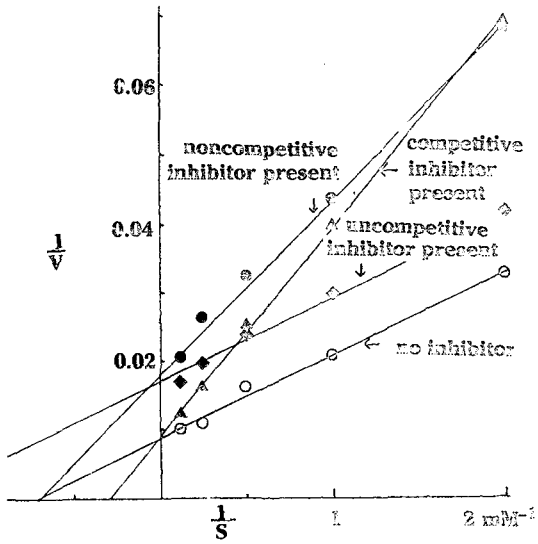

Fig. 7. Lineweaver-Burk plot of the Effect of varying Inhibitor Concentrations on Reaction Velocity of *L. sporogenes* β-Galactosidase.

Velocity was recorded as μmole of ONPG hydrolyzed per minute at 60°C. Symbols: ○, no inhibitor; ●, 20mM lactose; ▲, 20mM galactose; ◆, 10mM glucose.

by lactose. Galactose was a competitive inhibitor with K*i* value of 13.3mM whereas glucose as an uncompetitive inhibitor with K*i* of 11.4mM.

요 약

유포자 유산균인 *Lactobacillus sporogenes*에의한 extracellular β-galactosidase의 생산에 관한 연구에 이어 본 효소의 효소학적 일반성질을 조사하여 다음과 같은 결과를 얻었다.

L. sporogenes β-glactosidase는 o-nitrophenyl-β-d-glaactopyranoside(ONPG)를 가수분해 할때에 0.05M-sodium phosphate buffer를 사용하여 pH6.8과 반응온도 60℃에서 가장 높은 효소활성을 나타냈으며 또한 이 효소반응의 activation enery는 50~60℃에서는 11,300cal/mole, 50℃이하에서는 16,000cal/mol이었다. 효소활성에 미치는 금속 이온의 효과는 거의없었으나 L-cysteine 10 mM 첨가로 뚜렷한 첨가 효과를 나타내므로서 sulfhydryl enzyme의 특징을 보였다.

한편 lactose와 ONPG에 대한 Michaelis constant가 각각 6.45×10^{-2}M, 1.48×10^{-3}M을 나타냄으로서 본 효소는 ONPG에 훨씬 큰 친화성을 나타냈다. 또 ONPG분해반응은 lactose, galactose, glucose등의 당류첨가에 의해 그 반응속도가 현저히 감소되며 lactose는 특이하게 noncompetitive저해 양식을 나타내었으며 K*i*값은 17.8mM galactose는 K*i*값이 13.3mM, competitive inhibition; glucose는 11.4mM의 K*i*값에 uncompetitive inhibition을 나타냈다.

특히 본 효소는 중성부의 pH와 60℃에서 180분간 가열후에도 70%이상의 효소활성을 나타냄으로서 상당히 높은 열안정을 나타냄과 동시에 균체외 효소라는 장점도 아울러 가지고 있어 식품공업에의 그 이용 가능성이 매우 높다고 하겠다.

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