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Studies on β-Galactosidase. I. Purification and Properties of β-Galactosidase I and II from *Sclerotium tuliparum*[1]

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Acid β-galactosidase I and II (β-D-galactoside galactohydrolase, EC 3.2.1.23) from *Sclerotium tuliparum* were purified by column chromatography with DEAE-cellulose, SP-Sephadex C-50, Sephadex G-200 and by isoelectric focusing (pI, 4.5 and 4.4, respectively). The purified β-galactosidase I and II were homogeneous in disc electrophoresis.

The enzymes were most active at pH 2.0 and stable over a pH range from 3.0 to 6.0 at 37° for 3 hr. Optimum temperatures of β-galactosidase I and II were 53° and 47°, respectively, and the thermal stability of β-galactosidase I was slightly higher than that of β-galactosidase II. Both enzymes were completely inactivated by N-bromosuccinimide at 0.01 mM. K_m of β-galactosidase I and II were 1.4 mM and 1.2 mM for o-nitrophenyl β-D-galactopyranoside (ONPG) and 20 mM and 19 mM for lactose, respectively, and V_{max} of β-galactosidase I and II were 433 μmoles·min^{-1}·mg^{-1} and 480 μmoles·min^{-1}·mg^{-1} for ONPG and 139 μmoles·min^{-1}·mg^{-1} and 149 μmoles·min^{-1}·mg^{-1} for lactose, respectively.

β-Galactosidase (EC 3.2.1.23) is widely distributed in plant, moulds, yeasts, bacteria and animal organ.[3] Because of simplicity in measurement of its activity and of interests in its activity towards lactose, glycoproteins and glycolipids, many investigators have engaged in the studies on the enzyme, and the mechanism of protein synthesis and of its genetic control has been also elucidated by means of the studies on the enzyme. A great deal of articles has been presented on β-galactosidase from *Escherichia coli*. It was highly purified[4] and obtained in crystalline form[5] and its properties were researched in detail using crystalline enzyme and various glycosides as substrates.[6] Optimum pH of the bacterial β-galactosidase was shown to be 7.0 to 7.3.[4,7]

Acid β-galactosidase, having an optimum pH at 2.0 to 3.8, has been reported with *Aspergillus niger*[8] and *Corticum rolfsii*,[9,10] but little was known about occurrence of such β-galactosidases in other micro-organismus.

Previously, Sakaguchi[11] has reported the production of acid β-galactosidase from *Sclerotium tuliparum* and the properties of the crude enzyme. In this report, the authors describe the purification and some properties of *Sclerotium* β-galactosidase I and II.

1) This work forms part CXIV of "Studies on Enzyme" by M. Sugiura.
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Materials and Methods

Enzyme——The crude enzyme was obtained from the culture filtrate of *Sclerotium tuliparum* IFO 6168 by ethanol fractionation and gel-chromatography with the use of Sephadex G-75.[11]

Reagents——DEAE-cellulose was the product of Brown, SP-Sephadex C-50 and Sephadex G-200 were products obtained from Pharmacia Fine Chemicals A.B. *o*-Nitrophenyl β-D-galactopyranoside (ONPG) was used as substrate throughout the present work. It was purchased from Nakarai Chemicals Ltd. and lactose was from Wako Pure Chemicals Industries Ltd. *p*-Nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl α-D-galactopyranoside, *p*-nitrophenyl α-D-glucopyranoside, *p*-nitrophenyl α-L-fucopyranoside, *p*-nitrophenyl α-D-mannopyranoside, *p*-nitrophenyl β-D-xylopyranoside and *p*-nitrophenyl N-acetyl β-D-glucosaminide were purchased from Seikagaku Kogyo Co. Ltd. The other chemicals used were of reagent grades.

Enzyme Assay——A reaction mixture containing 2.0 ml of 10 mM ONPG, 2.0 ml of 0.1 M HCl–sodium acetate buffer (pH 2.0) and 1.0 ml of enzyme solution was incubated at 37° for 10 min. The reaction was stopped by the addition of 1.0 ml of 1M Na_2CO_3 to reaction mixture. The *o*-nitrophenol released by the action of enzyme was determined by measuring the absorbance at 420 nm by Hitachi spectrophotometer (model 101). One unit of β-galactosidase was defined as the amount of enzyme which liberated 1 μmole of *o*-nitrophenol per minute under the above conditions. $\varepsilon_{420\,nm}=4390$ for *o*-nitrophenol was used for calculation of enzyme unit. Activity toward lactose was measured as follows, a reaction mixture containing 2.0 ml of 10 mM lactose, 2.0 ml of HCl–sodium acetate buffer (pH 2.0) and 1.0 ml of enzyme solution was incubated for 10 min at 37° and then was kept in boiling water for 2 min to stop the reaction. Then 0.2 ml of the reaction mixture and 5 ml of Blood sugar color test reagent (Boehringer) were incubated at 25° for 1 hr and the absorbance at 430 nm was measured. One unit of the activity was defined as the amount of enzyme which liberated one μmole of D-glucose per minute under the above conditions.

Determination of Protein——Protein was assayed by the method of Lowry[12] using bovine serum albumin as standard.

Isoelectric Focusing and Disc Electrophoresis——Isoelectric focusing was carried out in accordance with the method of Vesterberg and Svensson.[13] A column of 110 ml capacity was used and ampholine covering a pH range from 3 to 5 was used at 1% concentration. A voltage of 750 V was supplied for 40 hr at 4°. Disc electrophoresis was performed according to the procedure of Nagai.[14] Protein was stained with Amido Schwarz 10B.

Results

Purification of β-Galactosidase I and II

The crude enzyme preparation (3.5 g) was dissolved in 300 ml of 0.01M sodium phosphate buffer (pH 7.0) and then the solution was centrifuged at 10000 rpm for 10 min at 0°. The clear supernatant solution was applied to a column of DEAE-cellulose equilibrated with 0.01M sodium phosphate buffer (pH 7.0) and the elution was carried out with linear gradient system of NaCl (0—0.3M) in the same buffer. The result is shown in Fig. 1. Crude enzyme preparation was separated into two fractions, *i.e.* β-galactosidase I and II. β-Galactosidase I and II obtained from DEAE-cellulose column chromatography were separately subjected to column chromatography with SP-Sephadex C-50 equilibrated with 0.05M HCl–Na_2HPO_4 buffer (pH 2.86) and the enzymes were eluted with linear gradient system of NaCl (0—0.2M). As shown in Fig. 2a and 2b, β-galactosidase I and II were eluted at a concentration of 0.10M and 0.08M NaCl, respectively. Since β-galactosidase I contained appreciable amount of impurities, the β-galactosidase I was applied to a column (2.80×80 cm) of Sephadex G-200 equilibrated with 0.05M HCl–Na_2HPO_4 buffer (pH 2.86) containing 0.1M NaCl to remove high molecular impurities. β-Galactosidase I and II thus obtained were concentrated with a ultrafiltration and were further purified with isoelectric focusing using ampholine covering a pH range from 3 to 5, separately. As shown in Fig. 2c and 2d, β-galactosidase I and II were electrofocused at pH 4.5 and 4.4, respectively. The active fractions were separately applied to a Sephadex G-200 column (2.80×80 cm) equilibrated with 0.05M HCl–Na_2HPO_4 buffer (pH 2.86) containing 0.1M NaCl to remove ampholine and sucrose.

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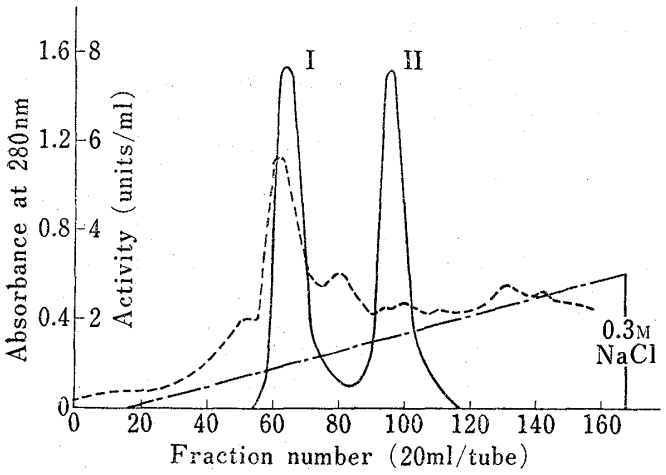

Fig. 1. Chromatography of Crude β-Galactosidase from *Sclerotium tuliparum* on DEAE-Cellulose Column

300 ml of enzyme solution (3.5 g of crude enzyme) was applied to a column (5×20 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). The elution was carried out with a linear gradient of NaCl (0—0.3 M) at a flow rate of 30 ml/hr.
———, activity; ------, absorbance at 280 nm; ————, concentration of NaCl with linear gradient elution.
I, β-galactosidase I: II, β-galactosidase II

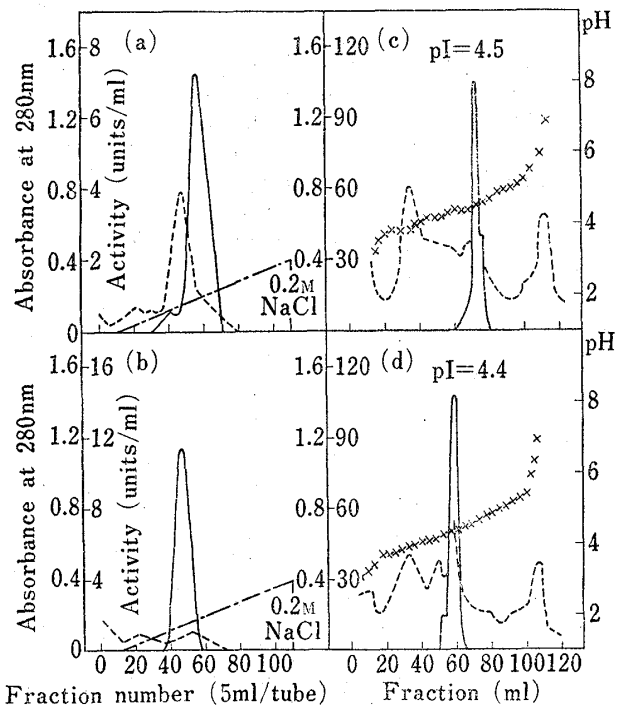

Fig. 2. Purification of β-Galactosidases I and II by Column Chromatography with SP-Sephadex C-50 (a and b) and Isoelectric Focusing (c and d)

(a) and (b): active fractions from DEAE-cellulose column chromatography in Fig. 1 were separately adsorbed onto SP-Sephadex C-50 column (2×22 cm) equilibrated with 0.05 M HCl-Na_2HPO_4 buffer (pH 2.86). The elution was carried out with a linear gradient of NaCl (0—0.2 M).
(c): β-galactosidase I from Sephadex G-200 gel-filtration and (d): β-galactosidase II from SP-Sephadex C-50 column (b) were separately subjected to isoelectric focusing. An ampholine was selected to cover a pH range from 3 to 5 and used at 1% concentration. Electrofocusing was performed for 40 hr with a potential of 750 V at 4°. The column volume was 110 ml.
(a) and (c): β-galactosidase I, (b) and (d): β-galactosidase II
———, activity; --------, absorbance at 280 nm; × × × ×, pH; ————, concentration of NaCl with linear gradient elution.

The results of the purification procedures are summarized in Table I. β-Galactosidase I and II were purified about 65-fold and 82-fold, respectively and the yield of activity of the purified enzymes was 5 to 8%.

Homogeneity

The homogeneity of purified β-galactosidase I and II was examined with disc electrophoresis. A homogeneous single protein band for both enzymes is shown in Fig. 3 and β-galactosidase II showed slightly large mobility toward cathode than that of β-galactosidase I.

Optimum pH and Optimum Temperature

The pH-activity profiles of β-galactosidase I and II are shown in Fig. 4a. These enzymes were most active at pH 2.0. Effect of temperature on activity of β-galactosidase I and II was determined at various temperatures and pH 2.0 for 15 min incubation as shown in Fig. 4b. β-Galactosidase I and II were most active at 53° and 47°, respectively.

Table I. Purification of β-Galactosidase I and II from *Sclerotium Tuliparum*

Step of purification	Activity (units)		Protein (mg)		Specific activity (units/mg)		Yield of activity (%)	
Crude enzyme	4920		1020		4.8		100	
	(I)	(II)	(I)	(II)	(I)	(II)	(I)	(II)
DEAE-cellulose	1720	1240	186	68	9.3	18	45	33
SP-Sephadex C-50	1190	768	28	4.3	43	179	32	20
Sephadex G-200	623	—	3.9	—	169	—	16	—
Sephadex G-200 after isoelectric focusing	252	406	0.8	1.1	312	369	5	8

(I): β-galactosidase I; (II): β-galactosidase II

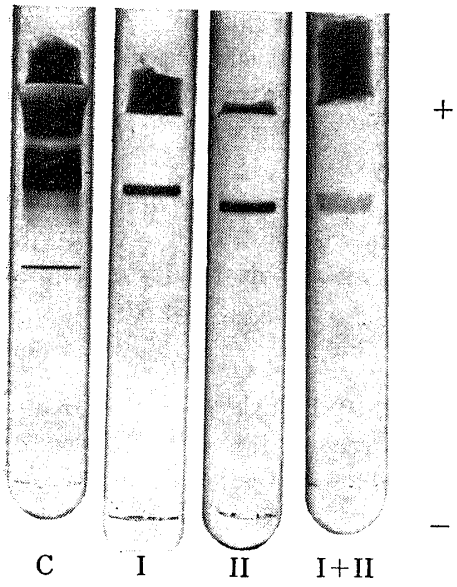

Fig. 3. Disc Electrophoretic Patterns of Crude and Purified β-Galactosidase I and II

About 100 μg of crude enzyme and 30 μg of purified enzymes were subjected to electrophoresis at pH 3.6. A current of 3mA/tube was supplied for 2.5 hr at 4°. Staining was carried out Amido Schwarz 10 B
C, crude enzyme; I, β-galactosidase I; II, β-galactosidase II; I+II, β-galactosidase I+β-galactosidase II.

Table II. Effect of Various Metal Ions and Reagents on Activity of β-Galactosidase I and II from *Sclerotium tuliparum*

Metal ion and reagent	Remaining activity (%) β-Galactosidase I	β-Galactosidase II
None	100	100
$HgCl_2$	81	99
$CaCl_2$	88	103
$MgCl_2$	73	108
$ZnCl_2$	91	113
$CuCl_2$	88	102
$CoCl_2$	89	99
$MnCl_2$	85	108
$FeCl_3$	102	121
EDTA	74	91
o-Phenanthroline	90	112
Cysteine	94	104
2-Mercaptoethanol	87	98
N-Bromosuccinimide[a]	0	0
Iodine	70	88
Iodoacetic acid	86	106
p-Chloromercuribenzoate[b]	104	113
Diisopropyl fluorophosphate	90	106

0.5 ml of the enzyme solution (10 μg) was incubated with 0.5 ml of 0.05 M citric acid–0.1 M Na_2HPO_4 buffer (pH 4.0) containing 2 mM metal ion or reagent for 30 min at 37°. The mixture was adjusted to pH 2.0 and diluted 50-fold with 0.1 M citric acid and the remaining activity was determined under standard conditions.
a) 0.01 mM, *b*) 0.1 mM

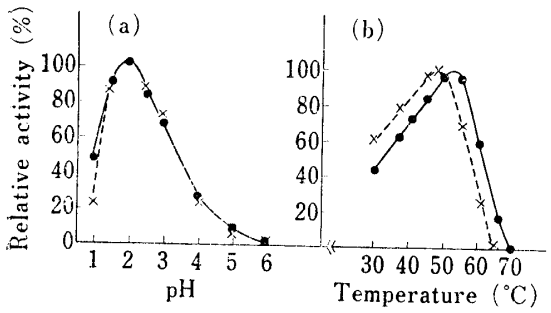

Fig. 4. Effect of pH (a) and Temperature (b) on Activity of β-Galactosidase I and II

(a): the reaction was carried out at various pH in a usual manner. 0.1 M HCl-sodium acetate buffer (pH 1—3) and 0.1 M citric acid–0.2 M Na_2HPO_4 buffer (pH 3—6) were used.
(b): the reaction was carried out at various temperatures for 15 min at pH 2.0 (0.1 M HCl – sodium acetate buffer) and the other conditions were the same as described in the materials and methods.
●——●, β-galactosidase I; ×------×, β-galactosidase II

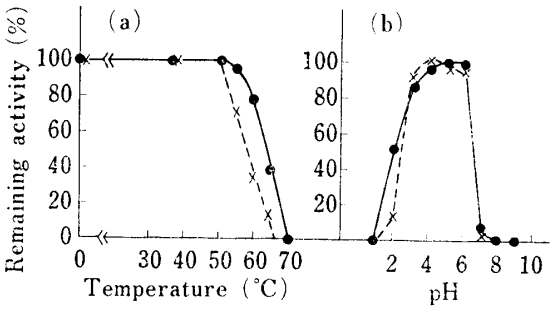

Fig. 5. Effect of Temperature (a) and pH (b) on Stability of β-Galactosidase I and II

(a): the enzyme was heated at various temperatures and pH 5.0 (0.05 M acetate buffer) for 30 min and then the solution was diluted 10-fold with cold 0.2 M HCl-sodium acetate buffer (pH 2.0) and the remaining activity was measured under standard conditions.
(b): the enzyme was incubated at various pH and 37° for 3 hr and then the solution was adjusted to pH 2.0 with 0.1 M citric acid and the remaining activity was determined under standard conditions. 0.1 M HCl-sodium acetate buffer (pH 1—3), 0.1 M citric acid–0.2 M Na_2HPO_4 buffer (pH 3—8) and 0.1 M $NaHCO_3$–Na_2CO_3 buffer (pH 9.0) were used.
●——●, β-galactosidase I; ×------×, β-galactosidase II

Thermal Stability and pH-Stability

The thermal stability of both enzymes was examined at pH 5.0 for 30 min. As shown in Fig. 5a, β-galactosidase I and II were stable up to 55° and 50°, respectively. pH-Stability of these enzymes was investigated at 37° for 3 hr. The result is shown in Fig. 5b. Both enzymes were stable in a pH range from 3.0 to 6.0.

Effect of Some Metal Ions and Reagents on the Enzyme Activity

The effect of metal ions and reagents on the enzymes was investigated as described in Table II. Both enzymes were not seriously affected by the metal ions. Among various reagents, β-galactosidase I and II were completely inactivated only by N-bromosuccinimide at 0.01 mM.

Kinetic Studies

K_m and V_{max} were determined by the method of Lineweaver and Burk[15] for ONPG and lactose as substrate. K_m values of β-galactosidase I and II were 1.4 mM and 1.2 mM for ONPG, and 20 mM and 19 mM for lactose, respectively. V_{max} values of β-galactosidase I and II were 433 μmoles·min^{-1}·mg^{-1} and 480 μmoles·min^{-1}·mg^{-1} for ONPG, and 139 μmoles·min^{-1}·mg^{-1} and 145 μmoles·min^{-1}·mg^{-1} for lactose, respectively.

Activity of β-Galactosidase I and II toward Various Glycosides

The result of activity of β-galactosidase I and II toward various synthetic glycosides is listed in Table III. Both enzymes were found to have high activity toward ONPG, but very low activity toward *p*-nitrophenyl β-D-galactopyranoside. These enzymes showed about 7% activity toward lactose as compared with that of ONPG. However, both enzymes did not show any activity toward *p*-nitrophenyl α-D-galactopyranoside and the other glycosides.

Table III. Activity of β-Galactosidase I and II from *Sclerotium tuliparum* toward Various Substrates

Substrate	Activity[a]	
	β-Galactosidase I	β-Galactosidase II
o-Nitrophenyl β-D-galactopyranoside	312	369
p-Nitrophenyl β-D-galactopyranoside	3.12	3.67
p-Nitrophenyl α-D-galactopyranoside	0	0
p-Nitrophenyl α-D-glucopyranoside	0	0
p-Nitrophenyl α-D-mannopyranoside	0	0
p-Nitrophenyl N-acetyl β-D-glucosaminide	0	0
p-Nitrophenyl α-L-fucopyranoside	0	0
p-Nitrophenyl β-D-xylopyranoside	0	0
Lactose	22.7	25.5

a) micromoles of products released per min per mg of protein at pH 2.0 (0.1 M HCl–sodium acetate buffer), 37° and 4 mM substrate

Discussion

β-Galactosidase I and II showed similarity in optimum pH, pH-stability, kinetic parameters and effect of metal ions and reagents, but they slightly differed in optimum temperature, thermal stability, affinity to DEAE-cellulose, isoelectric point and mobility in disc electrophoresis.

Both β-galactosidases were most active at pH 2.0. This property was similar to that of β-galactosidase from *Corticum rolfsii*, having an optimum pH at 2.0 to 2.5,[10] and of β-galacto-

15) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

sidase from *Asp. niger* which was most active at pH 3.8.[8] The *Sclerotium* β-galactosidases showed high activity toward ONPG, but low activity toward lactose. This character also resembled to that of *Corticum* β-galactosidase.[10] From the results, it can be considered that these enzymes are not lactase.[16]

As regards to animal β-galactosidase, Alpers had isolated two types of β-galactosidase from intestine and showed that one of them was a lysosomal acid β-galactosidase with a pH optimum of 3.0 and that the enzyme, which hydrolyzed synthetic substrate in higher rate than lactose, was relatively thermostable.[17] It is interesting that these properties of lysosomal acid β-galactosidase is similar to those of the *Sclerotium* β-galactosidases.

The *Sclerotium* β-galactosidases were not appreciably activated or inhibited by metal ions, metal-chelating reagents, reducing reagents and SH-reagents, but were completely inhibited by N-bromosuccinimide at 0.01 mM. *Corticum* β-galactosidase was fairly inactivated by Hg^{2+}, but was not affected by *p*-chloromercuribenzoate (PCMB) and Ag^{+}.[10] β-Galactosidase from *E. coli*[18] was activated by K^{+} and Na^{+}, and β-galactosidase from *Saccharomyces fragilis*[19] was also activated by K^{+}, Mn^{2+}, Mg^{2+} and Co^{2+}, but both enzymes were inhibited by Hg^{2+}, Ag^{+}, Cu^{2+} and PCMB.

Since *p*-nitrophenyl β-D-galactopyranoside was hydrolyzed by the *Sclerotium* β-galactosidase I and II at a rate of about 1% as compared with ONPG, it is considered that the site of the nitro-group on the aglycon is important to hydrolysis rate of the substrate. This problem will be elucidated in the light of kinetic studies using various synthetic substrates.

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