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Studies on β-Galactosidase. II. Purification of β-Galactosidase from *Macrophomina phaseoli* and Its Enzymatic Properties[1a,b]

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Acid β-galactosidase (EC 3.2.1.23) was purified from the culture filtrate of *Macrophomina phaseoli* by column chromatography using DEAE-cellulose and Sephadex G-200, and isoelectric focusing (pI, 3.6). The purified enzyme was homogeneous on disc electrophoresis.

The enzyme is considered to be a glycoprotein, because the mobility of the protein band coincided with that of sugar one in disc electrophoresis, and because sugar content was not varied before and after isoelectric focusing of the purified enzyme. The sugar content of the enzyme was estimated to be 12% with phenol-sulfuric acid method.

The enzyme was most active at pH 5.0 and 60°, and stable over a pH range from 4.0 to 8.0 and below 55°. The enzymatic activity was completely inactivated only by N-bromosuccinimide at 0.1 mM. K_m value was determined to be 0.45 mM for *o*-nitrophenyl β-D-galactopyranoside (ONPG) and 15 mM for lactose, and V_{max} was calculated to be 93.6 μmoles·min^{-1}·mg^{-1} for ONPG and 54.5 μmoles·min^{-1}·mg^{-1} for lactose.

β-Galactosidase (EC 3.2.1.23) is widely distributed in bacteria, yeasts, moulds, plants and animals. A great deal of articles has been presented on β-galactosidase from *Escherichia coli*.[3] In contrast to the enzyme from *E. coli*, the enzymatic properties of β-galactosidases from the other microorganismus are relatively unexplored. Most microbial enzymes have optimum pH in the range of pH 6.0—7.0,[4-10] and the presence of metal ions is likewise essential to full activities for the enzymes from *Saccharomyces fragilis*,[4] *S. lactis*,[5] *Diplococcus pneumoiae*,[6] and *Trichomonas foetus*,[7] as for those from *E. coli*.[11]

As regard to acid β-galactosidase, the enzymes from *Aspergillus niger*[12] and *Corticum rolfsii*[13] were purified and found to be most active at pH 2.0—4.0. The authors have also reported the purification and properties of β-galactosidase I and II from *Sclerotium tuliparum*, having an optimum pH at 2.0.[1a] Recently, Tanaka has reported the isolation and properties of acid β-galactosidase from *Asp. oryzae* which showed an optimum pH at 4.5—4.8.[14] However, there are few reports concerning such acid β-galactosidase from mould except for above enzymes.

1) *a*) Part I: M. Sugiura, M. Suzuki, M. Sasaki, and T. Shimomura, *Chem. Pharm. Bull.* (Tokyo), **24**, 788 (1976); *b*) This work forms part CXV of "Studies on Enzyme" by M. Sugiura.
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In this paper, we describe the purification and properties of acid β-galactosidase from *Macrophomina phaseoli*.

Materials and Methods

Enzyme——The crude enzyme preparation was a ethanol precipitate of the culture filtrate of *Macrophomina phaseoli* MO 421.

Enzyme Assay——Enzyme activity was determined at pH 5.0 (0.1 M acetate buffer) by the method as reported in the previous paper.[1a]

Disc Electrophoresis and Determination of Sugar——Disc electrophoresis was performed according to the procedure of Davis.[15] Protein was stained with Amido Schwarz 10B and sugar was detected with fuchsin-bisulfite stain after periodic acid oxidation (PAS).[16] Sugar was measured by the phenol-sulfuric acid method according to Dubois[17] using glucose as standard.

All other experimental methods and the reagents used were the same as described in the previous paper.[1a]

Results

Purification of β-Galactosidase

The crude enzyme preparation (2 g) was extracted with 50 ml of 0.01M sodium phosphate buffer (pH 7.0) and the solution was centrifuged at 10000 rpm for 10 min at 0°. The supernatant solution was loaded on a column of DEAE-cellulose equilibrated with 0.01M sodium phosphate buffer (pH 7.0) and the elution of the enzyme was carried out with linear gradient system of NaCl (0—0.23M) in the same buffer. The elution pattern of the enzyme is presented in Fig. 1a. The enzyme was eluted from DEAE-cellulose at 0.16M NaCl. The enzyme obtained from DEAE-cellulose chromatography was applied to a column (2.8×80 cm) of Sephadex G-200 equilibrated with 0.01M sodium phosphate buffer (pH 7.0) containing 0.1M NaCl. As shown in Fig. 1b, higher and lower molecular impurities were removed from the enzyme. The active fractions from Sephadex G-200 gel-filtration were further purified with isoelectric focusing using ampholine to give a pH range from 3 to 5. The result is shown in Fig. 1c. The enzyme was electrofocused at pH 3.6. The active fractions were applied to a column (2.8×80 cm) of Sephadex G-200 equilibrated with 0.01M sodium phosphate buffer (pH 7.0) containing 0.1M NaCl to remove ampholine and sucrose.

The results of the purification procedures are summarized in Table I. The β-galactosidase was purified about 84-fold and the yield of activity of the purified enzyme was 28%.

Homogeneity and Sugar Content

The homogeneity of purified β-galactosidase was examined with disc electrophoresis. A homogeneous single band for protein or sugar is shown in Fig. 2, where the protein band coincided with the sugar one. Sugar content of the purified β-galactosidase chromatographed on a Biogel P-150 column (1.5×40 cm) equilibrated with 0.01M sodium phosphate buffer (pH 7.0) containing 0.1M NaCl was determined with the phenol-sulfuric acid method. Sugar content of the enzyme was estimated to be 12% using glucose as standard. This value was agreed with that of the purified enzyme which was isoelectrofocused using ampholine to covering a pH range from 3 to 5 and then chromatographed on a Biogel P-150 column as mentioned above.

Optimum pH and Optimum Temperature

The pH-activity profile of the β-galactosidase is shown in Fig. 3a. The enzyme was most active at pH 5.0. Optimum temperature of β-galactosidase was determined by varing incuba-

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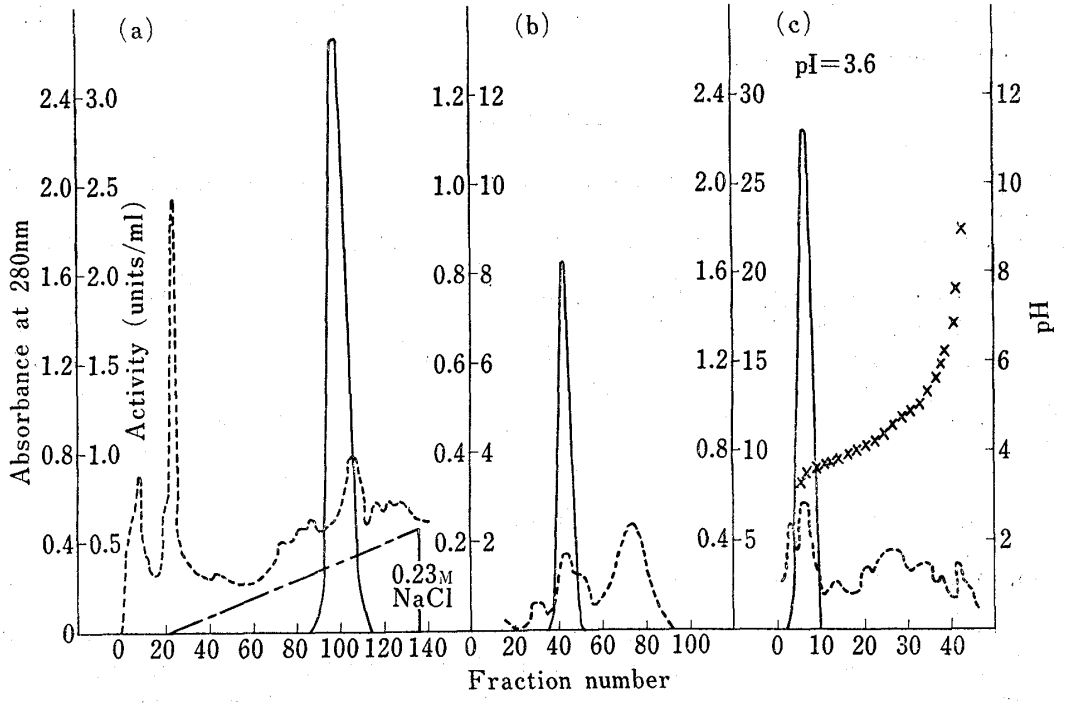

Fig. 1. Purification of β-Galactosidase from *Macrophomina phaseoli* by Column Chromatography with DEAE-Cellulose (a), Sephadex G-200 (b), and Isoelectric Focusing (c)

(a): 50 ml of enzyme solution (2 g of crude enzyme) was applied to a column (3.8×20 cm) of DEAE-cellulose equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). The elution was carried out with a linear gradient of NaCl (0—0.4 M, 1—1 liter) at a flow rate of 30 ml/hr and the fractions of 10 ml were collected.

(b): β-galactosidase from DEAE-cellulose (a) was concentrated with ultra filtration and applied to a column (2.8×80 cm) of Sephadex G-200 equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl and the fractions of 8 ml were collected.

(c): β-galactosidase from Sephadex G-200 (b) was concentrated with ultra filtration and subjected to isoelectric focusing. An ampholine was selected to give 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. After electrofocusing, the fractions of 2 ml were collected.

———: activity, ----------: absorbance at 280 nm, × × × ×: pH, ———·———: concentration of NaCl with linear gradient elution

TABLE I. Purification of β-Galactosidase from *Macrophomina phaseoli*

Step of purification	Activity (units)	Protein (mg)	Specific activity (units/mg)	Yield of activity (%)
Crude enzyme	392	362	1.08	100
DEAE-cellulose	244	21.8	11.2	62
Sephadex G-200	158	2.20	53.3	40
Sephadex G-200 after isoelectric focusing	110	1.21	90.6	28

tion temperature for 15 min at pH 5.0 as shown in Fig. 3b. The enzyme was most active at 60°.

pH-Stability and Thermal Stability

pH-Stability of the enzyme was investigated at 37° for 3 hr. The result is shown in Fig. 4a. The enzyme was stable in a pH range from 4.0 to 8.0. The thermal stability of the enzyme was examined at pH 5.0 for 30 min. As shown in Fig. 4b, β-galactosidase was stable up to 55°.

Effect of Some Metal Ions and Reagents on the Enzyme Activity

The effect of metal ions and reagents on the enzyme was investigated as described in Table II. The enzyme was slightly inactivated by Hg^{2+}, but was not affected by the other

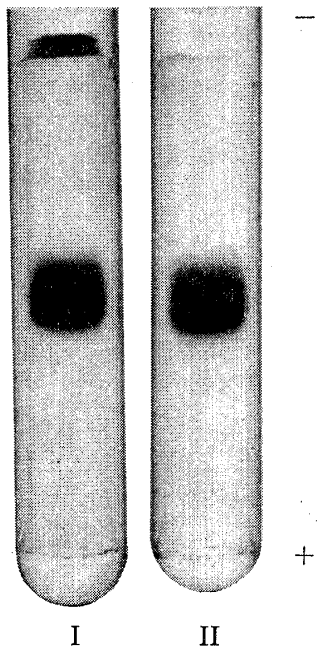

Fig. 2. Disc Electrophoretic Pattern of Purified β-Galactosidase

About 50μg of purified enzyme was subjected to electrophoresis at pH 9.4. A current of 4mA/tube was supplied for 80 min at 4°. Gel I was stained with Amido Schwarz 10 B to visualize the protein and gel II was treated with periodic acid followed by fuchsin-bisulfite to demonstrate the sugar.

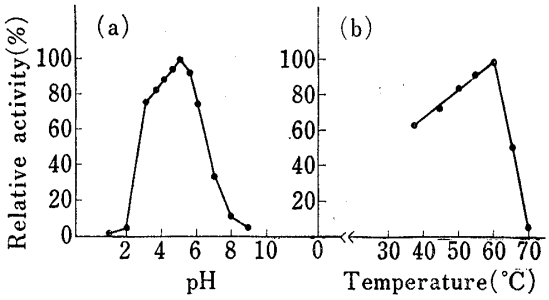

Fig. 3. Effect of pH (a) and Temperature (b) on Activity of β-Galactosidase

(a): The reaction was carried out at various pH in a usual manner. 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.
(b): The reaction was carried out at various temperatures and pH 5.0(0.1 M acetate buffer) for 15 min and the other conditions were the same as the enzyme assay.

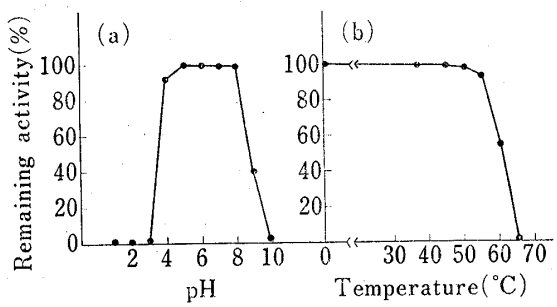

Fig. 4. Effect of pH (a) and Temperature (b) on Stability of β-Galactosidase

(a): The enzyme was incubated at various pH and 37° for 3 hr, and then the solution was adjusted to pH 5.0 with 0.2 M citrate buffer (pH 5.0) and the remaining activity was measured 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 and 10) were used.
(b): 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 acetate buffer (pH 5.0) and the remaining activity was determined by the usual method.

metal ions. Among various reagents, the β-galactosidase was completely inactivated only by N-bromosuccinimide at 0.1 mM.

Kinetic Studies

K_m and V_{max} of the enzyme were determined by the method of Lineweaver and Burk[18] for *o*-nitrophenyl β-D-galactopyranoside(ONPG) and lactose as substrate. K_m value of the β-galactosidase was 0.45 mM for ONPG and 15 mM for lactose. The V_{max} value was 93.6 μmoles·min^{-1}·mg^{-1} for ONPG and 54.5 μmoles·min^{-1}·mg^{-1} for lactose.

Activity of the β-Galactosidase toward Various Glycosides

Activity of the enzyme toward various synthetic glycosides is presented in Table III. The enzyme was found to have high activity toward ONPG and slightly lower activity toward *p*-nitrophenyl β-D-galactopyranoside than ONPG. This tendercy differed from that of *Sclerotium* β-galactosidase I and II.[1a] Toward lactose, the enzyme showed about 12% activity as compared with that of ONPG. However, the enzyme did not hydrolyze *p*-nitrophenyl α-D-galactopyranoside and the other glycosides.

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

Table II. Effect of Various Metal Ions and Reagents on Activity of β-Galactosidase from *Macrophomina phaseoli*

Metal ion	Remaining activity (%)	Reagent	Remaining activity (%)
None	100	EDTA	102
$HgCl_2$	81	*o*-phenanthroline	100
$CaCl_2$	100	cysteine	100
$MgCl_2$	100	2-mercaptoethanol	101
$ZnCl_2$	101	N-bromosuccinimide[a]	0
$CuCl_2$	101	iodine	99
$CoCl_2$	101	iodoacetic acid	99
$MnCl_2$	101	*p*-chloromercuribenzoate[a]	101
$FeCl_3$	97	diisopropyl fluorophosphate	99
NaCl	101		
KCl	100		

0.5 ml of the enzyme solution (30 μg) was incubated with 0.5 ml of 0.05 M citric acid–0.1 M Na_2HPO_4 buffer (pH 5.0) containing 2 mM metal ion or reagent for 30 min at 37°. The mixture was diluted 50-fold with 0.1 M acetate buffer (pH 5.0) and the remaining activity was determined under standard conditions.
a) 0.1 mM

Table III. Activity of β-Galactosidase from *Macrophomina phaseoli* toward Various Substrates

Substrate	Activity[a]
o-Nitrophenyl β-D-galactopyranoside	90
p-Nitrophenyl β-D-galactopyranoside	75
p-Nitrophenyl α-D-galactopyranoside	0
p-Nitrophenyl α-D-glucopyranoside	0
p-Nitrophenyl α-D-mannopyranoside	0
p-Nitrophenyl N-acetyl β-D-glucosaminide	0
p-Nitrophenyl α-L-fucopyranoside	0
p-Nitrophenyl β-D-xylopyranoside	0
Lactose	11.3

a) micromoles of products released per min per mg of protein at pH 5.0 (0.1 M acetate buffer), 37° and 4 mM substrate

Discussion

In this report, to know the enzymatic properties of the β-galactosidase from *Macrophomina phaseoli*, the enzyme was purified to homogeneous state by column chromatography with DEAE-cellulose and Sephadex G-200, and isoelectric focusing.

The purified enzyme is considered to be a glycoprotein because the mobility of the protein band is equal to that of the sugar in disc electrophoresis, and because the sugar content (12%) of the purified enzyme was constant before and after isoelectric focusing. This result is related to the fact that lysosomal acid β-galactosidase from human liver binds to concanavalin A-Sepharose.[19]

The purified β-galactosidase was most active at pH 5.0 and thermostable and hydrolyzed more preferably ONPG than lactose. These properties of the enzyme was similar to those of β-galactosidase from *Asp. oryzae*[14] in optimum pH, activity and K_m value for ONPG and effect of metal ions and the various reagents, but differed in stability and the activity toward lactose. Except for optimum pH, the *Macrophomina* β-galactosidase was also resembled to

19) C.W. Anthony and J.S. O'Brien, *Biochem. Biophys. Res. Commun.*, **56**, 193 (1974).

β-galactosidases from *Sclerotium tuliparum*[1a] and *Corticum rolfsii*,[13] showing an optimum pH at 2.0—2.5.

The β-galactosidase was almost unaffected by metal ions, but was completely inactivated only by N-bromosuccinimide at 0.1 mM. In contrast to these properties, β-galactosidase from *E. coli*[11] was activated by K^+ and Na^+, and β-galactosidase from *S. fragilis*[4] was also activated by K^+, Mn^{2+}, Mg^{2+} and Co^{2+}, but both enzymes were inactivated by Hg^{2+}, Ag^+, Cu^{2+} and *p*-chloromercuribenzoate.

From these results, it is suggested that the β-galactosidase from *Macrophomina phaseoli* is an acid β-galactosidase similar to those from *Sclerotium tuliparum*, *Corticum rolfsii*, *Asp. oryzae* and *Asp. niger*.

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