Purification and Enzymatic Properties of α -Galactosidase from *Pycnoporus cinnabarinus*

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α -Galactosidase (EC 3.2.1.22) from *Pycnoporus cinnabarinus* was purified by precipitation with ammonium sulfate, column chromatographies on DEAE-Sephadex A-50, Sephadex G-100, CM-Sephadex C-50, and SP-Sephadex C-50, and isoelectric focusing. The purified enzyme was homogeneous on polyacrylamide disc gel electrophoresis, and the molecular weight was estimated to be about 210,000 by gel filtration on Sephacryl S-200 and about 52,000 by sodium dodecyl sulfate gel electrophoresis. The enzyme exhibited the optimum pH at 5.0 and was stable between pH 3 and 9. The optimum temperature of the enzyme was 75°C. The enzyme was thermostable at pH 5.0 and completely lost its activity after heating at 90°C or at pH 3.5. The Michaelis constants were 0.31 mM for *p*-nitrophenyl α -D-galactoside, 0.80 mM for melibiose, 2.16 mM for raffinose, and 1.15 mM for stachyose. The α -galactosidase activity was strongly inhibited by Ag^+ , Hg^{++} , *p*-chloromercuribenzoate, galactose, and melibiose. The enzyme also seemed to catalyse a glycosyl-transfer reaction.

α -Galactosidase (EC 3.2.1.22) is widely distributed¹⁾ in nature and is known to be able to hydrolyse α -galactosyl linkages in oligo- and polysaccharides. Much research has been done to date on the purification of α -galactosidase from animals,^{2~5)} plants,^{6~14)} and microorganisms.^{15~23)} The homogeneous enzymes have been obtained mainly from plant sources,^{7,8,11~14)} and their activities against oligosaccharides and galactomannan have been studied. Microbial α -galactosidases have been purified from bacteria,^{15,16)} *Streptomyces* sp.,^{17~19)} and fungi,^{20~23)} but highly purified and homogeneous enzyme has been obtained only from *Mortierella vinacea*.²²⁾

α -Galactosidase is a useful tool not only in the determination of raffinose and the structural studies of carbohydrates, but in the beet sugar industry. Microbial α -galactosidases have also been applied to remove raffinose and stachyose in soymilk processing.^{24~27)} For this purpose, a more active and stable α -galactosidase preparation is required.

We previously found²⁸⁾ that *Pycnoporus cinnabarinus* produced various glycosidases, such

as α - and β -galactosidases, β -glucosidase, and β -*N*-acetylhexosaminidase, and α -galactosidase was most active among those enzymes. We have already reported the purification of the β -galactosidase²⁸⁾ and the β -*N*-acetylhexosaminidase,²⁹⁾ but the α -galactosidase had not been isolated. In this study, we purified this enzyme from *P. cinnabarinus* and found that the purified enzyme was more thermostable than enzymes reported in earlier studies. In this paper, we deal with the purification and enzymatic properties of this thermophilic α -galactosidase.

MATERIALS AND METHODS

Microorganisms and enzyme production. *Pycnoporus cinnabarinus* IFO 6139 was cultured by the procedure described in our previous paper,²⁹⁾ and the culture filtrate was used as starting material for enzyme purification.

Materials. *p*-Nitrophenyl α -D-galactoside (pNPGal) and other *p*-nitrophenyl glycosides were purchased from Koch-Light Laboratories. Melibiose was obtained from Difco Laboratories, raffinose from the Wako-Junyaku Kôgyo Co., and stachyose from the Sigma

Chemical Co. DEAE-Sephadex A-50, CM-Sephadex C-50, SP-Sephadex C-50, and Sephadex G-100 were Pharmacia products. Bio-Lyte, pH 3~5 and pH 3~10, was purchased from Bio-Rad Laboratories.

Enzyme assays. α -Galactosidase activity was assayed in a reaction mixture consisting of 0.2 ml of 4 mM pNPGal, 0.2 ml of 0.2 M Na_2HPO_4 -0.1 M citric acid buffer (pH 5.0), and 0.1 ml of enzyme solution. After incubation for 10 min at 37°C, the reaction was stopped by the addition of 2 ml of 0.2 M Na_2CO_3 , and the *p*-nitrophenol released was measured by reading the absorbance at 420 nm. One unit of enzyme activity was defined as the amount of enzyme which released one μmol of *p*-nitrophenol.

α -Galactosidase activity against melibiose, raffinose, and stachyose was assayed in a reaction mixture containing 0.1 M oligosaccharides instead of 4 mM pNPGal. After incubation for 10 min at 37°C, the reaction was stopped by boiling for 4 min. The activity against melibiose was assayed by estimating the amount of glucose produced by the use of glucose oxidase (Glucose C-Test Wako, the Wako Junyaku Kôgyo Co.) and the activity against raffinose and stachyose by estimating the amount of galactose released according to the method of Somogyi and Nelson.³⁰⁾

Protein determination. Protein was determined by the method of Hartree,³¹⁾ with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis. Polyacrylamide disc gel electrophoresis was carried out at pH 9.5 in 7.5% polyacrylamide gel by the procedure described by Davis.³²⁾ Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed in 7.5% polyacrylamide gel containing 0.1% SDS by the method of Webber and Osborn described by Hayashi and Ohba.³³⁾ The gels were stained with Coomassie brilliant blue R-250 and periodic acid-fuchsin reagent.

Estimation of molecular weight. The molecular weight of the enzyme was estimated by SDS-polyacrylamide gel

electrophoresis as described above, using BDH molecular weight markers which were a product from BDH Chemical Ltd., as molecular weight standards. The molecular weight was also estimated by gel filtration on a Sephacryl S-200 column according to the procedure of Andrews.³⁴⁾ The marker proteins used as molecular weight standards were ovalbumin (MW 45,000), bovine serum albumin (68,000), aldolase (158,000), catalase (240,000), and ferritin (450,000) which were from Boehringer Mannheim GmbH.

High-performance liquid chromatography (HPLC). A Waters Associates 6000A pump coupled to a U6K universal injector and a R-401 Differential Refractometer were used for HPLC. Sugars were separated on a Waters Associates μ -Bondapak carbohydrate column (3.9 mm $\times$ 30 cm) using a 80:20 mixture of acetonitrile and water as the mobile phase, at a flow rate of 1.5 ml/min, and detected by monitoring the refractive index at $\times 8$ attenuation. Acetonitrile and water were the special grade for HPLC, which were purchased from Katayama Kagaku Kôgyo Co.

RESULTS

Purification of α -galactosidase

The purification procedure is summarized in Table I. The culture filtrate (3.1 liters) which contained α - and β -galactosidase, β -glucosidase and β -*N*-acetylhexosaminidase, was brought to 75% saturation by adding solid ammonium sulfate. The precipitate was dissolved in deionized water and desalted by passing through a column (5.0 $\times$ 48 cm) of Sephadex G-25, which had been equilibrated with 0.02 M phosphate buffer (pH 6.0). The desalted enzyme solution (229 ml) was applied to a column (5.0 $\times$ 44 cm) of DEAE-Sephadex A-50, previously equilibrated with the same

TABLE I. PURIFICATION OF α -GALACTOSIDASE FROM *Pycnoporus cinnabarinus*

Step	Protein (mg)	Activity (units)	Yield (%)	Specific activity (units/P)	Purification (fold)
Ammonium sulfate precipitation	2,717	10,448	100	3.85	1
DEAE-Sephadex A-50	202	9,265	88.7	45.9	11.9
Sephadex G-100	101	6,837	65.4	67.7	17.6
CM-Sephadex C-50	37	4,955	47.4	134	34.8
SP-Sephadex C-50	14	3,492	33.4	249	64.7
Isoelectric focusing	6.6	1,462	14.0	222	57.7

buffer. The column was washed with 0.02 M phosphate buffer (pH 6.0) and the enzyme protein adsorbed was eluted with a linear gradient of NaCl from 0 to 0.8 M in the same buffer at a flow rate of 72 ml/hr. The α -galactosidase active fractions (225 ml) were pooled and concentrated by ultrafiltration with an Amicon membrane PM-10.

The concentrated enzyme solution (4.7 ml) was loaded on a column (2.6 $\times$ 80 cm) of Sephadex G-100 which had been equilibrated with 0.05 M citrate buffer (pH 4.0) and eluted with the same buffer at a flow rate of 20 ml/hr. The α -galactosidase active fractions (55 ml) which were pooled, were applied to a column (2.6 $\times$ 28 cm) of CM-Sephadex C-50 which had been equilibrated with 0.05 M citrate buffer (pH 4.0) and eluted with the same buffer at a flow rate of 20 ml/hr. The α -galactosidase active fractions (50 ml) which passed through the column were pooled and lyophilized. In this chromatography, α -galactosidase was separated from β -glucosidase and β -N-acetylhexosaminidase, which were adsorbed on the column. β -Galactosidase was weakly adsorbed on the column and it appeared in a wide range of fractions eluted after the peak of α -galactosidase activity.

The lyophilized enzyme was dissolved in deionized water and dialyzed against 0.04 M Na_2HPO_4 -0.02 M citric acid buffer (pH 3.2). The dialyzed enzyme solution (7.8 ml) was applied to a column (1.6 $\times$ 20 cm) of SP-Sephadex C-50 which had been equilibrated with the same buffer. After the column was washed with the same buffer, the enzyme was eluted with a pH gradient using equal volumes of 0.04 M Na_2HPO_4 -0.02 M citric acid buffer of pH 3.2 and 5.0, at a flow rate of 20 ml/hr. As shown in Fig. 1, α -galactosidase activity appeared in the protein peak eluted at about pH 3.5 and nearly paralleled the elution of protein. The α -galactosidase active fractions (24 ml) were pooled. This fraction was completely free from β -galactosidase, β -glucosidase, and β -N-acetylhexosaminidase, but it was contaminated with small amounts of impurities, which gave a slight sub-band in polyacrylamide disc

FIG. 1. Chromatography of α -Galactosidase on a SP-Sephadex C-50 Column.

The experimental details are described in the text. —●—, absorbance at 280 nm; —●—, pH; —○—, activity; —, fractions pooled.

FIG. 2. Separation of α -Galactosidase by Isoelectric Focusing.

Isoelectric focusing was carried out in a 110 ml LKB column with 500 V for 48 hr at 2°C. The other experimental details are described in the text. —●—, absorbance at 280 nm; —●—, pH; —○—, activity; —, fractions pooled.

gel electrophoresis.

Half of the α -galactosidase fraction was concentrated by ultrafiltration, dialyzed against 1% glycine and subjected to isoelectric focusing³⁵ in a 110 ml LKB column containing 1% Bio-Lyte solution, a 4:1 mixture of Bio-Lyte pH 3~5 and pH 3~10, with a stepwise sucrose gradient. The result is shown in Fig. 2. α -Galactosidase activi-

FIG. 3. Polyacrylamide Disc Gel Electrophoresis of Purified α -Galactosidase.

(A) Purified enzyme (30 μ g protein) was applied to a 7.5% polyacrylamide gel. The migration was downward toward the anode. Left; stained with Coomassie brilliant blue R-250. Right; stained with periodic acid-fuchsin. (B) Purified enzyme (5 μ g protein) was applied to a 7.5% SDS-polyacrylamide gel.

ty was found only in the protein peak migrating to about pH 3.5. The active fractions (7.5 ml) were pooled and used as the purified enzyme preparation, after passing through a column (2.5 $\times$ 22 cm) of Sephadex G-50, previously equilibrated with 0.02 M phosphate buffer (pH 6.0).

As Table 1 shows, α -galactosidase was purified about 57-fold with a recovery of 14%, starting from the ammonium sulfate precipitate of the culture filtrate.

Homogeneity

The polyacrylamide disc gel electrophoretic pattern of α -galactosidase is shown in Fig. 3A. This figure indicates that the purified enzyme is homogeneous and is glycoprotein in nature.

Molecular weight

The purified α -galactosidase migrated as a single band in SDS-polyacrylamide gel electrophoresis (Fig. 3B). Thus, the molecular weight of the enzyme was estimated to be about 52,000, as shown in Fig. 4A. On the

FIG. 4. Estimation of Molecular Weight of α -Galactosidase by SDS-Polyacrylamide Gel Electrophoresis (A) and Gel Filtration on Sephacryl S-200 (B).

—○—, marker proteins; (A), 1 to 6; monomer to hexamer (MW 14,300 to 85,800), (B), 1; ovalbumin, 2; bovine serum albumin, 3; aldolase, 4; catalase, 5; ferritin, —●—, α -galactosidase.

other hand, the molecular weight for α -galactosidase estimated by gel filtration on Sephacryl S-200 was about 210,000 (Fig. 4B). These results suggest that the enzyme might be composed of four identical subunits.

Effect of pH and temperature on activity

The effect of pH on α -galactosidase activity was examined using pNPGal and oligosaccharides as substrates. The pH-activity profile for the hydrolysis of pNPGal is shown in Fig. 5. The enzyme exhibited the maximum activity at about pH 5.0. The optimum pH's of α -galactosidase activity against melibiose, raffinose and stachyose were also found at pH 5.0.

The effect of temperature on α -galactosidase activity is shown in Fig. 6. The maximum activity was observed at 75°C.

Effects of pH and temperature on stability of the enzyme

The effect of pH on the stability of the

FIG. 5. Effect of pH on α -Galactosidase Activity.

The enzyme activities were assayed under standard condition in various buffers of pH 1.5 to 7.5. —○—, 0.1 M sodium citrate-0.1 N HCl buffer; —●—, 0.2 M Na_2HPO_4 -0.1 M citric acid buffer.

FIG. 6. Effect of Temperature on α -Galactosidase Activity.

The enzyme activities were assayed under standard condition at various temperatures from 30 to 95°C.

enzyme is shown in Fig. 7. The enzyme was incubated in buffers of various pH values for 2 hr at 37°C and the residual activity was assayed. The enzyme was stable between pH 3 and 9, and retained about 95% of its original activity.

The effect of temperature on the stability of the enzyme was examined at pH 5.0. The enzyme was incubated for 15 min at various temperatures from 30 to 90°C and the residual activity was assayed. The enzyme was thermostable and no loss in activity was found below 75°C, but it was inactivated at 80°C or

FIG. 7. Effect of pH on the Stability of α -Galactosidase.

The enzymes were kept for 2 hr at 37°C in various buffers of pH 2.0 to 10.5. pH 2.0~2.5, 0.1 M sodium citrate-0.1 N HCl buffer; pH 3.0~8.0, 0.2 M Na_2HPO_4 -0.1 M citric acid buffer; pH 9.0~10.5, 0.2 M boric acid plus 0.2 M KCl-0.2 M Na_2CO_3 buffer.

more and completely lost its activity at 90°C. Thus, the effect of pH on the thermal stability of the enzyme was investigated during incubation for 30 min. The results are shown in Fig. 8. The enzyme did not lose its activity during incubation for 30 min at 75°C, at pH 5.0 but it was rapidly inactivated after heating at pH 3.5 (Fig. 8A) or at 90°C (Fig. 8B). The thermal stability of the enzyme was further tested during the long incubation period of 8 hr at 75°C and pH 5.0. There was no loss in activity after incubation for 5 hr and about 70% of activity was recovered even after incubation for 8 hr.

Effect of substrate concentrations

The enzyme hydrolysed α -galactoside linkages of melibiose, raffinose and stachyose in addition to pNPGal. The effect of substrate concentrations on the velocity of hydrolysis of pNPGal and the oligosaccharides was investigated. The Michaelis constant (K_m) and the maximum velocity for each substrate were calculated from the Lineweaver-Burk plots shown in Fig. 9. The K_m values of α -galactosidase were 0.31, 0.80, 2.16, and 1.15 mM, and the maximum velocities were 630, 87, 106, and 123 $\mu\text{mol}/\text{min}/\text{mg}$ protein for pNPGal, melibiose, raffinose, and stachyose, respectively.

FIG. 8. Effects of Temperature and pH on the Stability of α -Galactosidase.

The enzymes were kept for 5–30 min at 75°C and different pH's (A), and at different temperatures and pH 5.0 (B).

FIG. 9. Lineweaver-Burk Plots for the Hydrolysis of pNPGal, Melibiose, Raffinose, and Stachyose.

The enzyme activities were assayed at substrate concentrations of 0.12–1.6 mM for pNPGal, 0.16–1.0 mM for melibiose, 1.6–10 mM for raffinose, and 0.80–10 mM for stachyose, under the standard condition.

Inhibition by metal ions and sugars

As Table II shows, the enzyme was strongly inhibited by metal ions such as Ag^+ and Hg^{++} , and PCMB. EDTA, L-cysteine and

TABLE II. EFFECTS OF METAL IONS AND SOME REAGENTS ON α -GALACTOSIDASE ACTIVITY

A mixture consisting of 0.1 ml of enzyme solution, 0.2 ml of 0.2 M Na_2HPO_4 –0.1 M citric acid buffer (pH 5.0), and 0.1 ml of metal ions or some reagents (final concentrations indicated) was incubated for 15 min at 37°C, and α -galactosidase activity was determined after adding 0.2 ml of 4 mM pNPGal. The activity was expressed as a percentage of the activity level in the absence of the compound.

Compounds	Concentration (mM)	Activity (%)
None		100
KCl	1	96
AgNO_3	1	0
AgNO_3	0.1	2
CaCl_2	1	102
MgSO_4	1	103
CoCl_2	1	104
CdCl_2	1	102
ZnSO_4	1	105
CuSO_4	1	105
HgCl_2	1	9
HgCl_2	0.1	13
EDTA	1	99
L-Cysteine	1	101
Mercaptoethanol	1	102
PCMB	0.1	18

EDTA, ethylenediamine tetraacetate; PCMB, *p*-chloromercuribenzoate.

mercaptoethanol did not affect the enzyme activity. The effects of sugars on α -galactosidase activity are summarized in Table III. Among the sugars tested, galactose and melibiose were powerful inhibitors. Glucose, mal-

TABLE III. EFFECTS OF VARIOUS SUGARS ON α -GALACTOSIDASE ACTIVITY

The mixture was the same as in Table II, except that 0.1 ml of sugars (final concentration, 10 mM) was used instead of metal ions.

Sugars (10 mM)	Activity (%)
None	100
Arabinose	96
Xylose	83
Glucose	54
Galactose	14
Maltose	55
Lactose	77
Sucrose	73
Melibiose	20
Raffinose	72
Stachyose	55

FIG. 10. Lineweaver-Burk Plots for the Hydrolysis of pNPGal in the Presence of Some Inhibitors.

The reaction mixture consisting of 0.2 ml of 0.30–1.2 mM pNPGal, 0.2 ml of 0.2 M Na_2HPO_4 –0.1 M citric acid (pH 5.0), 0.1 ml of inhibitors and 0.1 ml of enzyme solution, was incubated for 10 min at 37°C. —○—, no inhibitors; —●—, galactose (4 mM); —▲—, HgCl_2 (0.8 μM).

tose, and stachyose also markedly inhibit the enzyme activity, but raffinose showed weak inhibition. The Lineweaver-Burk plots for the hydrolysis of pNPGal in the presence of some inhibitors are shown in Fig. 10. The inhibition of α -galactosidase activity by galactose and Hg^{++} was of the noncompetitive type.

Glycosyltransfer activity of the enzyme

A reaction mixture which contained 0.2 ml of 0.2 M raffinose, 0.2 ml of 1/30 M phosphate

FIG. 11. Separation of the Products Formed in the Raffinose Digests by HPLC.

A reaction mixture consisting of 0.2 ml of 0.2 M raffinose, 0.2 ml of 1/30 M phosphate buffer (pH 5.5), and 0.1 ml of enzyme solution (0.164 units) was incubated for 3 and 24 hr at 37°C. The mixture was filtered through a 0.45 μm Millipore filter and 10 μl of the mixture was injected. Experimental details are described in MATERIALS AND METHODS. The positions of the standard sugars are given with arrows: a, galactose; b, sucrose; c, raffinose; d, stachyose.

buffer (pH 5.5), and 0.1 ml of enzyme solution was incubated for 3 or 24 hr at 37°C. The reaction products were separated by HPLC, as shown in Fig. 11. In addition to galactose and sucrose, new oligosaccharides were detected; the main product was an oligosaccharide with an elution time corresponding to stachyose. The amount of galactose produced during hydrolysis was less than that of sucrose. In the digests of melibiose and stachyose, new oligosaccharides were also produced in addition to the hydrolysis products. However,

no new oligosaccharides were detected in the reaction mixture which contained 0.01 M substrate instead of 0.2 M. These results suggest that the enzyme possibly catalyses a galactosyltransfer reaction at high substrate concentrations.

DISCUSSION

α -Galactosidase was purified from the culture filtrate of *P. cinnabarinus* to homogeneity by polyacrylamide disc gel electrophoresis and the purified enzyme was thermostable. Of the α -galactosidases isolated from other sources, only a few enzymes^{13,19,22)} are somewhat heat-stable, and among them the enzyme purified from *Vicia sativa*⁷⁾ retains about 84% of its activity after heating for 40 min at 75°C. However, to the best of our knowledge, thermal stability like that observed in this study has yet not been reported for a α -galactosidase. The specific activity of the α -galactosidase purified from *P. cinnabarinus* is comparable to that of the enzyme from *M. vinacea*,²²⁾ which is now commercially available. Thus, *P. cinnabarinus* is a useful source of microbial α -galactosidase.

In the present investigation, a homogeneous α -galactosidase preparation was obtained after 6 steps of purification and the yield of the enzyme was 14% (Table I). As reported previously, β -galactosidase²⁸⁾ and β -N-acetylhexosaminidase²⁹⁾ which were contained in the same culture filtrate of *P. cinnabarinus* were heat-labile and completely destroyed by heating at 60°C. It is, therefore, probable that heat treatment at 75°C will be effective in simplifying the purification procedure and increasing the yield of α -galactosidase. A rapid purification of the *P. cinnabarinus* α -galactosidase will be described elsewhere. As the purification in this study is started from the culture filtrate for chitinase production,²⁹⁾ the enzyme activity may be enhanced by the use of an optimal culture medium for α -galactosidase production as the starting material.

The existence of multimolecular forms of α -galactosidase has been reported in the enzymes

from various seeds.¹⁾ Dey *et al.*¹⁴⁾ have recently found that the α -galactosidase of *Lens culinaris* is separated by gel filtration on Sephadex G-100 into two forms, α -galactosidase I with a molecular weight of 160,000 and II with that of 40,000. No multiple forms appeared during purification of the α -galactosidase from *P. cinnabarinus*, although the enzyme was composed of four identical subunits (Fig. 4). The *P. cinnabarinus* α -galactosidase is an acidic protein, differing from the *L. culinaris* enzyme¹⁴⁾ and is a glycoprotein, as has been reported for the α -galactosidases purified from *L. culinaris*¹⁴⁾ and *M. vinacea*.²²⁾

The α -galactosidase of *P. cinnabarinus* has the maximum activity for pNPGal and oligosaccharides at about pH 5.0 and this value is similar to the optimum pH's measured for the enzymes from plants^{12,13)} and microorganisms.^{19,22)} The optimum temperature of the *P. cinnabarinus* enzyme is at 75°C and is the highest among the α -galactosidases described in earlier studies.^{13,19)} When the *P. cinnabarinus* enzyme reacts at the optimum temperature, its specific activity can be elevated about 8 times (Fig. 6). However, this enzyme is heat-labile at the acidic pH of 3.5, because its thermal stability is pH-dependent (Fig. 8). The K_m value of the *P. cinnabarinus* α -galactosidase for raffinose resembles that of the *M. vinacea* enzyme²²⁾ and is smaller than those of the α -galactosidases obtained from plant sources.^{7,11~14)} As the *P. cinnabarinus* enzyme is more active against raffinose and stachyose compared with the plant enzymes, this enzyme can be used to remove these oligosaccharides.

Further study of the physicochemical properties of and glycosyltransfer reaction of the *P. cinnabarinus* α -galactosidase is now in progress.

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