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Production, Purification, and Characterization of α-Galactosidase from *Monascus pilosus*

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A *Monascus pilosus* strain was selected for production of intracellular α-galactosidase. Optimum conditions for mycelial growth and enzyme induction were determined. Galactose was one of the best enzyme inducers. The enzyme was purified by ammonium sulfate precipitation, gel filtration, and ion exchange chromatography and was demonstrated to be homogeneous by slab gel electrophoresis. The molecular weight of this enzyme, estimated by gel filtration, was about 150,000. The optimum conditions for the enzyme reaction was pH 4.5 to 5.0 at 55°C. The purified enzyme was stable at 55°C or below and in buffer at pH 3 to 8. The activity was inhibited by mercury, silver, and copper ions. The kinetics of this enzyme, with *p*-nitrophenyl-α-D-galactoside as substrate, was determined: K_m was about 0.8 mM, and V_{max} was 39 μmol/min per mg of protein. Enzymatic hydrolysis of melibiose, raffinose, and stachyose was analyzed by thin-layer chromatography.

α-Galactosidase (α-D-galactoside galactohydrolase; EC 3.2.1.22) catalyzes the disruption of the α-D-galactosidic bond of both simple and complex oligo- and polysaccharides (7). α-Galactosidase may have great potential in various applications. Soybeans and other legumes contain the raffinose family of oligosaccharides, e.g., 1% raffinose, 4% stachyose, and a trace of verbascose in soybeans (16). α-Galactosidase is not secreted in humans, and thus the presence of these oligosaccharides could hinder digestion and cause flatulence, since they are utilized by the gas-generating intestinal microorganisms. α-Galactosidase can be used to clear these oligosaccharides and upgrade the nutrition of legume food (26). In the sugar industry, this enzyme can also degrade the raffinose in molasses and thus increase the yield of crystallized sugar (20). Type B erythrocytes, which contain 3-*O*-α-D-galactopyranoside, can be transformed into type O erythrocytes by exposure to α-galactosidase (27). Fabry's disease of humans is due to a deficiency of thermolabile lysosomal α-galactosidase A (27). α-Galactosidase may be used in the future for such medical purposes as enzymotherapy.

Monascus sp. is the mold traditionally used in oriental countries in the production of food colorants and as a fermentation starter in red rice wine (28). Since the 1930s, a number of enzymes produced from *Monascus* spp. have been studied. The glucoamylase which was commercially produced in mainland China was demonstrated to have two active fractions (8). The acid protease was also purified, and its properties were determined. This enzyme may be used in food fermentation to enhance flavor (13). A *Monascus* strain isolated from soil was demonstrated to produce α-galactosidase, and the optimum conditions for enzyme production by that strain was determined (15). However, enzyme purification and characterization of the purified enzyme have not been published. This study reports on the screening of production strains of various *Monascus* cultures and the production, purification, and characterization of the purified α-galactosidase from a selected strain.

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MATERIALS AND METHODS

Microorganisms. A total of 104 cultures of *Monascus* spp. were obtained mainly from the Wine Research Institute, Taipei, Taiwan, and the Institute for Microbial Resources, Taichung, Taiwan, Republic of China, in addition to our own stocks. Most of the *Monascus* stocks of the American Type Culture Collection and the Institute for Fermentation in Osaka were included (14, 19). Some of these cultures were isolated from local wine fermentation starters (19). The cultures were stored on malt extract agar slants.

Chemicals. *p*-Nitrophenyl-α-D-galactoside, D-galactose, melibiose, raffinose, stachyose, and Coomassie brilliant blue G250 were purchased from the Sigma Chemical Co. Sephadex G-100 and CM-Sephadex C-50 were obtained from Pharmacia Fine Chemicals. Silica gel 60 F254 thin layers precoated on aluminum sheets were E. Merck AG products. Gel filtration standards were purchased from Bio-Rad Laboratories.

Culturing of microorganisms and induction of enzyme. Erlenmeyer flasks (250 ml), each containing 25 ml of growth medium controlled at different initial pHs and temperatures, were used to determine the optimum conditions for mycelial growth. The shaking speed was 150 rpm for all flasks. The growth medium consisted of 20 g of glucose, 5 g of NH_4NO_3, 5 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of yeast extract, and 1 liter of distilled water (15). The acidity was adjusted by addition of 1 M HCl or 1 M NaOH.

α-Galactosidase production was induced by addition of 25 ml of induction medium to the flask after the growth period. The induction medium consisted of 5 g of galactose, 5 g of NH_4NO_3, 5 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of yeast extract, and 1 liter of distilled water (15). Four mycelial disks, 0.7 cm in diameter, cut from the fast-growing mycelium on agar medium were used as inoculum for each flask.

For fermentor culture, 4 liters of growth medium was added to the 10-liter vessel of a Mitsuwa KJ10 bench-top fermentor. The enzyme activity, dissolved oxygen, pH value, and dry weight of mycelium of the culture were monitored. After a growth period of 5 days, 2 liters of

triple-strength induction medium was added. Four-day-old shaking-flask culture was used as the inoculum for the fermentor.

Enzyme assay. α-Galactosidase activity was determined by the rate of hydrolysis of *p*-nitrophenyl-α-D-galactoside at 40°C for 20 min (15). The A_{405} was measured with a Bausch & Lomb Spectronic 20 spectrophotometer. Enzyme solution treated at 100°C for 3 min was used as the blank.

One unit of α-galactosidase activity is defined as the amount of enzyme which liberates 1 μmol of *p*-nitrophenol per min under the given conditions. The specific activity is expressed in units of enzyme activity per milligram of protein.

Protein, glucose, and dry-weight determination. The amounts of protein were determined by the Coomassie brilliant blue G250 dye–protein-binding method modified from the method of Sedmak and Grossberg (23). The assay involved the addition of 2.5 ml of the standardized G250 dye solution to 2.5 ml of protein solution, immediate mixing, and determination of the A_{620} against a 1:1 mixture of acetate buffer and dye solution. The standard curve was determined by using bovine serum albumin as the standard protein.

The glucose concentration was determined by the colorimetric glucose tolerance test (Sigma). Five milliliters of *o*-toluidine reagent was added to 0.1 ml of culture broth or standard glucose solution, and the mixture was heated at 100°C for 10 min and cooled to room temperature. The A_{630} was determined according to the instructions of the manufacturer (Sigma manual no. 635).

The dry weight of the mycelium was determined after it had been dried to constant weight in an oven at 80°C.

Enzyme purification. (i) Extraction. *Monascus* strain 81 was cultured and induced to produce α-galactosidase. The mycelium was harvested, homogenized with a double volume (wt/vol) of 0.1 M acetate buffer (pH 4.5), in a blender at high speed for 2 min at 4°C, and extracted for 15 min at 4°C, and the cellular debris was removed by filtration with suction and centrifugation (8,000 × *g* for 20 min at 4°C). The clear supernatant was stored at 4°C and used in further purification.

(ii) Ammonium sulfate precipitation. The crude enzyme extract from (i) was adjusted to 50% saturation with ammonium sulfate, with gentle stirring at 4°C overnight. The solution was centrifuged to remove precipitated material. The supernatant was then adjusted to 100% saturation with ammonium sulfate, and the precipitate was suspended in 0.1 M acetate buffer (pH 4.5) and dialyzed against the same buffer for 48 h to remove the salt.

(iii) Gel filtration chromatography. Sephadex G-100 was used for molecular sieve chromatography. Five milliliters of the enzyme solution from (ii) was applied to a Sephadex G-100 column (2.6 by 70 cm), which had been equilibrated with 0.1 M acetate buffer (pH 4.5). The column was eluted with the same buffer at a flow rate of 60 ml/h. Every 5-ml fraction was examined at 280 nm for protein content and assayed for enzyme activity under standard conditions. Fractions which were shown to have enzyme activity were pooled and concentrated by lyophilization.

(iv) CM-Sephadex C-50 chromatography. One milliliter of the desalted and concentrated enzyme solution from (iii) was dialyzed against 0.01 M citrate buffer (pH 4.0). The dialysate was applied to a column of CM-Sephadex C-50 (2.3 by 20 cm) previously equilibrated with the citrate buffer. The column was eluted with the same buffer until the first protein peak was observed, and then linear gradient elution was carried out with 300 ml of 0.01 M citrate buffer (pH 4.0) and 300 ml of 0.10 M citrate buffer (pH 6.0). Fractions of 5 ml each were collected, and those with enzyme activity were pooled.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was performed with 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate by a modification of the method of Studier (24). Electrophoresis was performed at 10 mA for 5 h. After electrophoresis, the gel slab was stained for 30 min with Coomassie brilliant blue G250 solution, which consisted of 0.1% Coomassie brilliant blue G250, 3.6% 5-sulfosalicylic acid, 12% trichloroacetic acid, and 30% methanol. The gel was then destained electrophoretically for 2 h with a destaining solution containing 10% ethyl acetate, 5% acetic acid, and 7% ethanol.

Molecular weight determination. The molecular weight of the enzyme was estimated by gel filtration on a Sephadex G-100 column by the procedure of Andrews (2). The gel filtration standard components are thyroglobulin (molecular weight, 670,000), immunoglobulin G (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B_{12} (1,350). Gel filtration conditions were the same as described in (iii).

Enzyme hydrolysis of oligosaccharides. A mixture of 0.2 ml of purified enzyme solution, which contained 0.5 U of α-galactosidase activity, and 0.2 ml of 250 mM melibiose, raffinose, or stachyose in 0.1 M acetate buffer (pH 4.5), was incubated at 40°C for various periods. The hydrolysates of sugars were immediately analyzed qualitatively by thin-layer chromatography (5, 10).

About 2 μl of the treated samples was applied to analytical silica gel thin layers and continuously developed four times at room temperature in a saturated chamber containing the solvent system ethyl acetate-acetic acid-water (3:1:1, vol/vol/vol). After the final development was completed, the thin-layer chromatography plates were sprayed with 2% diphenylamine in acetone–2% aniline in acetone–85% phosphoric acid (5:5:1, vol/vol/vol) and placed on a hot plate at 90 to 100°C. Glucose, galactose, fructose, and sucrose were used as references.

RESULTS

Screening of production strain. For conciseness, some of the less significant experimental data on the screening of strains and determination of optimum conditions are not presented in detail.

All the strains were screened in shaking-flask culture and induced to produce α-galactosidase by galactose treatment. This screening process was repeated twice for highly productive strains. Strain 81, which was originally isolated from local fermentation starter, was selected for fast mycelial growth and more consistent enzyme production ability. This strain has fast mycelial growth, like typical *Monascus pilosus* and *M. ruber*, and heavy pigmentation with conidial and cleistothecium formation, similar to *M. purpureus*, but it has larger ascospores than other *Monascus* species. According to a recently revised taxonomy scheme (12), the strain was classified as an *M. pilosus* strain with large ascospores and extraordinarily heavy pigmentation. There was no evidence to show that the α-galactosidase productivity is species related.

Optimum conditions for mycelial growth and enzyme production. The optimum growth conditions for strain 81 in the growth medium were determined. An initial pH of 4 to 4.5 and incubation temperatures of 30 and 35°C were found to be optimal. Therefore, pH 4.5 and 30°C were taken as optimal for further studies.

TABLE 1. α-Galactosidase production by *Monascus* strain 81 at different times

Induction time (h)	Dry wt (mg)	A_{405}[a]	Enzyme activity (U/ml of extract)
2	70.7	0.46 ± 0.19	8.65
6	65.0	0.57 ± 0.16	11.05
10	73.6	0.68 ± 0.21	12.85
14	72.7	0.73 ± 0.14	13.90
18	73.5	0.60 ± 0.13	11.35

[a] Mean of four replicate determinations ± standard deviation.

The residual glucose concentration was determined during shaking-flask culture. Under optimum conditions, the glucose concentration dropped to zero after 4 days of incubation and maximum mycelial mass was obtained on day 5. For shaking-flask culture, we allowed 4 days to obtain nearly maximum mycelial growth for enzyme production; this could probably prevent the inhibition by glucose of α-galactosidase production (15).

The production of enzyme at different induction times was determined by using galactose as the induction agent. No significant difference was noticed (Table 1), and 14 h was arbitrarily chosen as the induction period for further study. Some of the sugars (0.5%) containing α-galactoside bonds were examined for enzyme induction activity. Lactose and glucose were also compared. Galactose was a good enzyme inducer, and other sugars containing α-galactoside bonds, such as melibiose, stachyose, and raffinose, also showed considerable induction ability (Table 2). Cultures in induction medium containing no induction sugar or in a medium without induction medium added at all were used as controls. Some enzyme activity was still obtained with the controls (Table 2).

Jar fermentor procedure. In the bench-top jar fermentor procedure, the same growth and induction media used for the shaking-flask culture were also used. The optimum conditions for mycelial growth during the growth phase were pH 4.5, aeration of 1 vvm, and stirrer speed of 250 rpm. The residue glucose concentration during the growth phase was close to zero at day 5 for 2.5 and 5% inocula. For 10% inocula, glucose was exhausted by day 4. A period of 5 days was used in fermentor culture for mycelial growth with a 5% inoculum. During enzyme induction, the optimum conditions were pH 4.5, aeration of 1 vvm, and stirrer speed of 300 rpm for 16 to 18 h. Increasing the inoculum size from 2.5 to 10% slightly increased the final cell mass of the mycelium; however, the enzyme activities were similar. The dissolved oxygen concentration dropped as the mold grew and remained around 3 to 4 mg/liter during the enzyme induction period.

TABLE 2. Induction of α-galactosidase in *Monascus* strain 81 by different carbon sources

Carbon source[a]	A_{405}[b]	Enzyme activity (U/ml of extract)
D-Galactose	0.73 ± 0.02[c]	13.75
D-Galactose pentaacetate	0.65 ± 0.11[d]	12.40
D-Glucose	0.33 ± 0.11	6.20
Lactose	0.21 ± 0.12	3.80
Melibiose	0.66 ± 0.19[d]	12.55
Stachyose	0.69 ± 0.08[c]	13.10
Raffinose	0.63 ± 0.12[d]	11.90
Gum guar	0.59 ± 0.31	11.05
No-carbon control	0.40 ± 0.18	7.45
No induction medium	0.33 ± 0.25	6.60

[a] Concentration of sugar, 5 g/liter.
[b] Mean of four replicate determinations ± standard deviation.
[c] Significant at $P = 0.05$ compared with no-carbon control.
[d] Significant at $P = 0.10$ compared with no-carbon control.

TABLE 3. Purification of *Monascus* α-galactosidase

Purification step	Activity (U)	Amt of protein (mg)	Yield (%)	Sp act (U/mg)	Purification (fold)
Crude extract	32.76	21.31	100	1.54	1.00
Ammonium sulfate precipitation	20.48	5.40	62.25	3.79	2.46
Sephadex G-100	15.68	0.56	47.86	28	18.18
CM-Sephadex C-50	10.50	0.06	32.05	175	113.64

Enzyme purification. The purification of α-galactosidase from *M. pilosus* is summarized in Table 3. α-Galactosidase was purified approximately 114-fold over the crude homogenate and was obtained in 32% yield. Enzyme collected from CM-Sephadex C-50 chromatography was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was shown to be homogeneous protein. The molecular weight of the purified α-galactosidase estimated by gel filtration on Sephadex G-100 was about 150,000.

Effect of temperature on enzyme activity and stability. The enzyme activities were assayed under the standard conditions (pH 4.5 for 20 min) at various temperatures. Figure 1 shows the temperature-activity profile for α-galactosidase. The enzyme showed maximum activity at 55°C. The purified enzyme solutions were incubated for 20 min at pH 4.5 at various temperatures, and the residual activities were assayed under standard conditions. The purified enzyme was heat stable up to 55°C but was quickly inactivated at higher temperatures (Fig. 2).

Effect of pH on enzyme activity and stability. The enzyme activities were assayed at 40°C for 20 min in citrate-phosphate and acetate buffers at pH 2.5 to 7.5. The pH-activity profile (Fig. 3) indicates that the optimum pH was 4.5 to 5.0. The enzyme was kept for 2 h at 30°C or for 24 h at 4°C in the citrate-phosphate buffer at pH 2.0 to 8.0, and the residual activity was assayed under standard conditions. The enzyme was stable between pH 3 and 8 (Fig. 4).

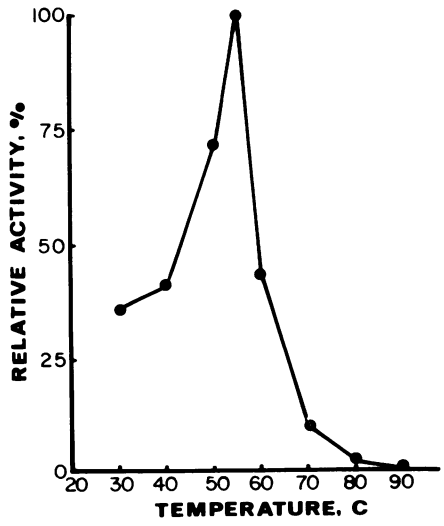

FIG. 1. Effect of temperature on the activity of purified *Monascus* α-galactosidase.

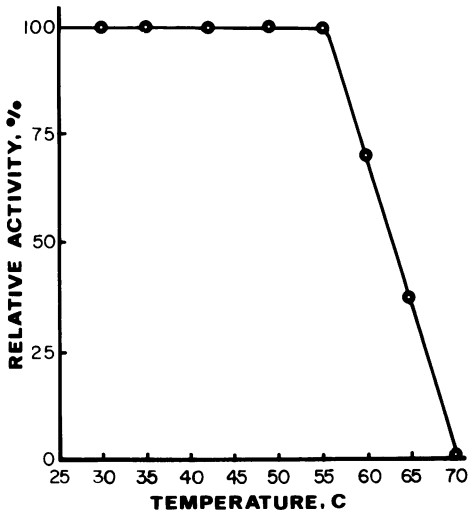

FIG. 2. Effect of temperature on the stability of purified *Monascus* α-galactosidase.

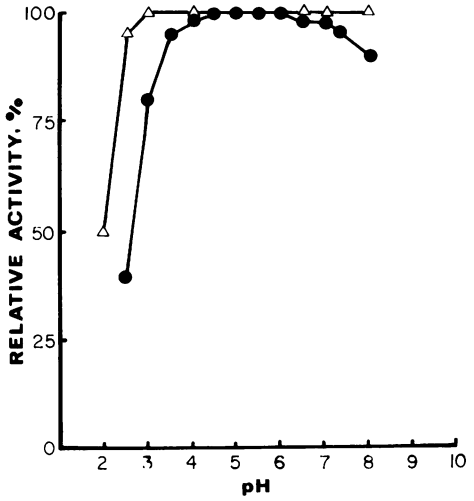

FIG. 4. Effect of pH on the stability of purified *Monascus* α-galactosidase incubated at 4°C for 24 h (△) or at 30°C for 2 h (●).

Effect of metallic ions and some reagents on the activity of α-galactosidase. A mixture consisting of 0.1 ml of the properly diluted enzyme solution and 0.1 ml of metallic ions or some reagents (final concentrations indicated) was incubated for 20 min at room temperature, and enzyme activity was then assayed under standard conditions. The enzyme was strongly inhibited by metallic ions such as Ag^+, Hg^{2+}, and Cu^{2+}, while EDTA, mercaptoethanol, and L-cysteine did not affect enzyme activity (Table 4).

Effect of substrate concentration (enzyme kinetics). The effect of substrate concentration on the rate of hydrolysis of *p*-nitrophenyl-α-D-galactoside was investigated. The concentrations of *p*-nitrophenyl-α-D-galactoside ranged from 0.05 to 10 mM, and the enzyme assay was done by the standard assay procedure. The apparent Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) for the enzyme were calculated from the Lineweaver-Burk plot to be about 39 μmol/min per mg and 0.8 mM, respectively. No significant inhibition effect was observed at high substrate concentrations.

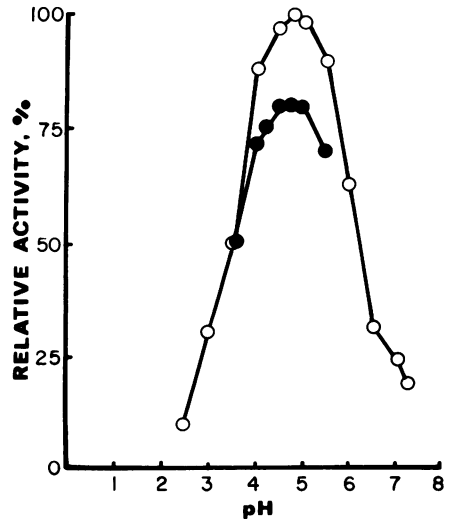

FIG. 3. Effect of pH on the activity of purified *Monascus* α-galactosidase in citrate-phosphate buffer (○) and acetate buffer (●).

Enzymatic hydrolysis of oligosaccharides. Hydrolysis of melibiose, raffinose, and stachyose was examined by thin-layer chromatography. Raffinose and melibiose were digested for 20 min and 1, 3, and 24 h. The amount of raffinose decreased, while the spots of sucrose and galactose became more apparent. Melibiose was also hydrolyzed to form glucose and galactose. In a further study, melibiose at 40 mM was completely hydrolyzed to form raffinose, sucrose, and galactose.

DISCUSSION

The optimum conditions for mycelial growth and enzyme production by *Monascus* strain 81 were similar to those reported by Imanaka et al. (15). α-Galactosidase was also found mostly mycelium bound. Sugars with α-galactoside bonds (melibiose, raffinose, and stachyose) and galactose all showed good enzyme-inducing ability, as occurred in a number of microorganisms (9, 11, 16). Lactose, which is an α-galactosidase inducer in actinomycetes (25), is usually not effective in the previous mentioned organisms.

TABLE 4. Effects of metallic ions and some reagents on the activity of *Monascus* α-galactosidase

Compound	Concn (mM)	Relative enzyme activity (%)
$HgCl_2$	1	2
$HgCl_2$	0.1	7
$AgNO_3$	1	2
$AgNO_3$	0.1	6
$CuSO_4$	1	31
$FeCl_3$	1	65
$CoCl_2$	1	85
$ZnCl_2$	1	89
EDTA	1	94
$FeSO_4$	1	95
None		100
Mercaptoethanol	1	100
$MgSO_4$	1	104
$Pb(NO_3)_2$	1	102
KCl	1	104
Cysteine	1	109

α-Galactosidase activity was detected for a number of microorganisms. However, purification to homogeneity and characterization of this enzyme have only been done for a few organisms (27). The α-galactosidase from *M. pilosus* was purified to homogeneity by conventional biochemical procedures. Recently, the method of choice has been affinity chromatography. This new method, involving the use of Sepharose or concanavalin A in conjunction with α-D-galactopyranosylamine as the absorbent, has greatly accelerated the purification of α-galactosidase (3, 27). The molecular weight of α-galactosidase vary with the different sources: *Escherichia coli*, 329,000 (22); *Saccharomyces carlsbergensis*, 300,000 (27); *Aspergillus niger*, 45,000 (1). Many of the α-galactosidases produced in legumes (6, 7), bacteria (11, 22), humans (18), and fungi (21) are made up of monomers or more than one active fraction. The molecular weight of the α-galactosidase from *Monascus* spp. as determined by the preliminary gel filtration method was about 150,000. No other active fraction has been detected.

Although a number of α-galactosidases have been studied, only those from *Mortierella vinacea* are available commerically (17, 21). A commercial enzyme should be stable and fast-reacting during reaction, should have low transferase activity, and should be produced by organisms free of toxicity. *Monascus* spp. have been widely used in food fermentation in the Orient for hundred of years (28). They are generally recognized as safe by many investigators; nevertheless, detailed toxicity studies must be conducted before a final conclusion is reached.

The properties of the α-galactosidase from *M. pilosus* and other sources are compared. This enzyme is fairly heat stable. Deactivation occurs at temperatures above 55°C, which is also the optimum reaction temperature (Fig. 1 and 2). α-Galactosidase from *Pycnoporus cinnabarinus* has an optimum reaction temperature of 75°C and is also stable at this temperature (21). The optimum temperature for most α-galactosidases is in the range of 37 to 40°C (27).

Most α-galactosidases are stable over a broad range of acidity. Generally, bacterial α-galactosidase has a pH optimum in the range of 6.0 to 7.5 (11, 16), while the pH optimum of the fungal and yeast α-galactosidase is about 3.5 to 5.0 (21, 27). The pH optimum of the *Monascus* α-galactosidase as determined with two buffer systems was close to 5 (Fig. 3). These properties are similar to those determined with crude enzyme extract (15).

α-Galactosidase from fungi (21), legumes (7), and sugar cane (4) are usually inhibited by silver and mercury ions competitively or noncompetitively. *Monascus* α-galactosidase was also strongly inhibited by silver and mercury ions, as well as by copper and iron ions to some extent. The inhibition by silver ions may be attributed to their reaction with the carboxyl group or histidine residues, and that by mercury ions may be attributed to their binding of the thiol group of the enzyme (4, 7). Bacterial α-galactosidases are also inhibited by lead, zinc, cobalt, magnesium, and nickel ions (27). The activity of α-galactosidase is also commonly inhibited by the substrate *p*-nitrophenyl-α-D-galactoside at high concentration but not by melibiose or raffinose (7, 27). D-Galactose is generally a strong inhibitor in a number of organisms.

Melibiose, raffinose, and stachyose are also commonly used as substrates in examination of the α-galactosidase activity. Melibiose is hydrolyzed to galactose and glucose, raffinose is hydrolyzed to galactose and sucrose, while stachyose is hydrolyzed to galactose and sucrose, with raffinose as the intermediate compound. As analyzed by thin-layer chromatography, the *Monascus* α-galactosidase also hydrolyzed these substrates to their basic components. No new oligosaccharide was detected. Therefore, the transferase activity of this enzyme was absent or extremely low in this study. The α-galactosidase of higher thermal stability from *P. cinnabarinus* also shows apparent transferase activity, as examined by high-performance liquid chromatography (21).

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