Purification and Properties of α -D-Galactosidase Produced by *Corticium rolfii*

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An α -D-galactosidase was purified from the culture filtrate of *Corticium rolfii* IFO 6146 by a combination of QAE-Sephadex A-50 and SE-Sephadex C-50 chromatography. The purified enzyme was demonstrated to be free of other possibly interfering glycosidases and glycanases. The maximum activity of the enzyme towards *p*-nitrophenyl α -D-galactopyranoside was found to be at pH 2.5 to 4.5, and the enzyme was fairly active at pH 1.1 to 2.0. The enzyme was stable over a pH range 4.0 to 7.0 at 5°C for 72 hr and relatively unstable at pH 1.1 to 2.0 as compared with endo-polygalacturonase, α -L-arabinofuranosidase and β -D-galactosidase produced by *C. rolfii*. The enzymic activity was completely inhibited by Hg^{2+} and Ag^+ ions, respectively. K_m values were determined to be 0.16×10^{-3} M for *p*-nitrophenyl α -D-galactopyranoside and 0.26×10^{-3} M for *o*-nitrophenyl α -D-galactopyranoside. The values of $V_{\max}$ were also determined to be 26.6 μ moles and 28.6 μ moles per min per mg for *p*- and *o*-nitrophenyl α -D-galactopyranoside, respectively.

A variety of micro-organisms has been reported to produce α -D-galactosidase (α -D-galactoside galactohydrolase, EC 3. 2. 1. 22) and the typical organisms have been shown to be *Saccharomyces cerevisiae*,^{1,2)} *Aspergillus niger*,^{3,4)} *Streptomyces olivaceus*,⁵⁾ and *Mortierella vinacea*.^{6,7)} *Corticium rolfii* K2⁸⁾ was also found to be a good source of α -galactosidase and to produce 27.5 times as much enzyme as *A. niger* ATCC 10577. The enzyme produced by *C. rolfii* was shown to be optimal under acidic condition and tolerable at pH values 2 to 7 with crude enzyme preparation.⁸⁾ The α -galactosidases produced by *C. rolfii* were also found to be separated into three components by the chromatography on QAE-Sephadex A-50.^{7,8)} The purpose of the present publication is to describe the method for further purification and some properties of one purified component.

MATERIALS AND METHODS

Organism. The strain IFO 6146 of *C. rolfii* was used throughout the present work, and was cultured by the method described in the previous paper.⁸⁾

Purification of enzyme. The mycelium was removed by filtration and subsequent centrifugation, then $(\text{NH}_4)_2\text{SO}_4$ was added to the culture filtrate to 0.9 saturation. After the mixture had stood overnight, the resulting precipitate was collected by centrifugation and dissolved in deionized water. The solution was dialyzed for 24 hr each, first against deionized water and then against 0.01 M sodium phosphate buffer (pH 7.5). The dialyzed solution was applied to a column of QAE-Sephadex A-50 which had been equilibrated with the same buffer (pH 7.5). The enzyme was eluted by the step-wise addition of 0.1 and 0.2 M NaCl, successively, with constant 0.01 M sodium phosphate buffer (pH 7.0). Fractions rich in α -galactosidase eluted by the buffer containing 0.1 M NaCl, were combined and dialyzed for 24 hr against deionized water and for further 24 hr against 0.04 M citrate-phosphate buffer (pH 3.3). The enzyme solution was applied to a column of SE-Sephadex C-50

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which was previously equilibrated with the same buffer (pH 3.3). The enzyme was eluted by the step-wise addition of 0.04 M citrate-phosphate buffer, pH 3.3, pH 3.7, and pH 4.1, successively. Fractions rich in α -galactosidase, eluted at pH 4.1, were collected, dialyzed for 24 hr each, first against deionized water and then 0.01 M sodium phosphate buffer (pH 7.5). The second chromatography on QAE-Sephadex A-50 was performed as described above. Fractions rich in α -galactosidase were collected and dialyzed against 0.003 M sodium phosphate buffer (pH 6.0) and subjected to the test of enzyme properties. The solution was also concentrated by lyophilization, and the concentrated solution was used for Disc electrophoresis.

Substrates and other chemicals. *p*-Nitrophenyl α -D-galactopyranoside was used as substrate throughout the present work and *o*-form was also used in the kinetic study. The former galactoside was purchased from Pierce Chemicals Comp., and the latter from Sigma Chemicals Comp. The other chemicals tested for substrate specificity or inhibitory effect were purchased as follows: *p*-nitrophenyl α - and β -D-glucopyranosides (Koch-Light Laboratories); *p*-nitrophenyl β -D-galactopyranoside (Sigma Chemicals Comp.); *p*-nitrophenyl β -D-xylopyranoside and *p*-nitrophenyl α -L-fucopyranoside (Pierce Chemicals Comp.); *p*-nitrophenyl N-acetyl β -D-galactosaminide and *p*-nitrophenyl N-acetyl β -D-glucosaminide (Seikagaku Kogyo Comp.); phenyl β -L-arabinopyranoside (Ikagaku Sōgō Laboratories, Kyoto); melibiose, and stachyose (Sigma Chemicals Comp.); raffinose (E. Merk Comp.); *p*-chloromercuribenzoic acid sodium salt (Mann Research Laboratories).

Phenyl α -L-arabinofuranoside was prepared according to the method reported by Börjeson *et al.*⁹⁾ Acid soluble pectic acid was prepared by the method described in the previous paper.¹⁰⁾

Enzyme assay. A reaction mixture containing 0.8 ml of 1.0 mM *p*-nitrophenyl α -D-galactopyranoside, 0.8 ml of 0.1 M HCl-sodium citrate buffer (pH 2.5), 0.8 ml of enzyme solution and two drops of toluene was incubated at 30°C for 30 min. The reaction was stopped by the addition of 1 ml of 0.1 M Na₂CO₃ to 1 ml of reaction mixture. The *p*-nitrophenol released by the action of enzyme was determined by measuring the absorbance at 400 m μ by a Hitachi spectrophotometer (model 139). One unit of α -D-galactosidase was defined as the amount of enzyme which liberated 1 μ mole of nitrophenol per min under the above condi-

tions. The actions on the biological sugars were tested in a reaction mixture containing 1.0 ml of 10 mM substrate (pH 2.5), 3.0 ml of 0.1 M HCl-sodium citrate buffer (pH 2.5), 1.0 ml of enzyme solution, 0.25 ml of deionized water and 2 drops of toluene. The galactose released was determined by the method of Nelson-Somogyi.^{11,12)}

Electrophoresis of enzyme. Electrophoresis was carried out on polyacrylamide gel according to the procedure described by Davis.¹³⁾

Determination of protein. The amount of protein in the enzyme solution was calculated from the absorbance at 280 m μ with crystalline α -L-arabinofuranosidase which had been prepared from culture fluid of *A. niger* in our laboratory.¹⁴⁾

RESULTS

Purification of enzyme

The crude enzyme solution, precipitated with (NH₄)₂SO₄ and dialyzed against deionized water and 0.01 M sodium phosphate buffer (pH 7.5) before hand, was subjected to a column of QAE-Sephadex A-50. As already described in the previous paper,⁸⁾ the activity of α -galactosidase was recovered in three peaks, I, II and III. As shown in Fig. 1, most of the activity was eluted in peak I with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, and active fractions were combined and dialyzed. The dialyzed solution was applied to a column of SE-Sephadex C-50. As shown in Fig. 2, active fractions eluted with 0.04 M citrate-phosphate buffer (pH 4.1) were combined and dialyzed. The enzyme solution was subjected to the second column chromatography on QAE-Sephadex A-50. The result shown in Fig. 3 indicated that the activity curve of α -galactosidase was parallel to the amount of protein.

The results of the over all purification procedures are summarized in Table I and show the 95-fold increase in specific activity. For the purpose of electrophoretic analysis, the enzyme solution was dialyzed against

FIG. 1. Chromatography on QAE-Sephadex A-50 (I).

QAE-Sephadex A-50 was equilibrated with 0.01 M sodium phosphate buffer (pH 7.5) and packed in a column, 2.5 cm $\times$ 60 cm. The enzyme solution, 380 ml containing 1.9 g protein or 247 units of α -galactosidase, was poured onto the column. The enzyme was eluted with a step-wise addition of 0.1 and 0.2 M NaCl, successively, in 0.01 M sodium phosphate buffer (pH 7.0). Fractions 32~39 were combined. —, α -galactosidase activity; ---, β -galactosidase activity; ·····, protein.

FIG. 2. Chromatography on SE-Sephadex C-50.

SE-Sephadex C-50 was equilibrated with 0.04 M citrate-phosphate buffer (pH 3.3) and packed in a column, 2.5 cm $\times$ 40 cm. The enzyme solution, 160 ml containing 205 mg of protein or 96 units of α -galactosidase, was poured onto the column, which was washed with a step-wise addition of the same buffer having pH 3.3, pH 3.7 and pH 4.1, successively. Fractions 76~86 were combined. —, enzyme activity; ·····, protein.

FIG. 3. Chromatography on QAE-Sephadex A-50 (II).

QAE-Sephadex A-50 was equilibrated with 0.1 M sodium phosphate buffer (pH 7.5) and packed in a column, 2.0 cm $\times$ 35 cm. The enzyme solution, 110 ml containing 6.8 mg protein or 60.5 units of α -galactosidase, was poured onto the column. The enzyme was eluted with a linear gradient of 0~0.2 M NaCl in 0.01 M sodium phosphate buffer (pH 7.0). Fractions 40~48 were combined. —, enzyme activity; ·····, protein.

TABLE I. PURIFICATION OF α -GALACTOSIDASE

Step	Vol (ml)	Total protein (mg)	Total activity (units)	Yield (%)	Specific activity (units/mg)
(NH ₄) ₂ SO ₄ (0.4~0.7 sat.)	380	1900	247	100	0.13
QAE-Sephadex A-50 (I)	160	205	96	38.9	0.47
SE-Sephadex C-50	110	6.8	60.5	24.5	8.90
QAE-Sephadex A-50 (II)	90	2.7	33.3	13.5	12.30

FIG. 4. Disc Electrophoresis of α -Galactosidase on Polyacrylamide Gel.

The purified enzyme solution containing 90 μ g of protein was layered on a column of 7% polyacrylamide gel and allowed to migrate in Tris-glycine buffer (pH 8.3) for 3 hr. Protein was stained with amido black 10 B.

0.003 M sodium phosphate buffer (pH 6.0) and lyophilized for 5 hr. As shown in Fig. 4, the purified enzyme preparation was demonstrated to be almost one component indicated by band I but a faint band II was also detected besides the main enzyme protein.

Enzymic properties

Effect of enzyme concentration. The amount of *p*-nitrophenol produced was a linear function of enzyme concentration up to 3.3 μ g protein per ml of reaction medium under the conditions mentioned above.

Effect of pH activity of enzyme. The effect of pH on the activity of purified enzyme is shown in Fig. 5. The optimum range of pH

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

A reaction mixture containing 0.8 ml of 1.0 mM *p*-nitrophenyl α -D-galactopyranoside, 0.8 ml of buffer and 0.8 ml of enzyme solution (4.4 m units per ml) was incubated at 30°C for 30 min. 0.1 M HCl-sodium citrate buffer was used for pH 1.1 to 3.0; 0.1 M sodium citrate-0.2 M sodium phosphate buffer for pH 3.0 to 8.0.

values was 2.5 to 4.5 and the enzyme was fairly active at pH 1.5 to 2.0. The purified α -galactosidase was still active at very low pH value of 1.1.

Effect of pH on stability of enzyme. Stability of purified α -galactosidase at various pH values and 5°C for 72 hr was examined using 0.1 M HCl-sodium citrate buffer for pH 1.1 to

Fig. 6. Effect of pH on Stability of α -Galactosidase.

(I) At 5°C. One ml of enzyme solution (10 m units) and 1.0 ml of each buffer were used for acid stability. Each mixture was allowed to stand at 5°C for 72 hr. The procedure was described in the text. Residual activity was represented as per cent of untreated enzyme solution.

(II) At 30°C. Each mixture was allowed to stand at 30°C, 17.2 m units of enzyme was used.

3.0, 0.1 M sodium citrate-0.2 M sodium phosphate buffer for pH 3.0 to 8.0, 0.2 M Tris-0.1 M HCl buffer for pH 8.0 to 9.0 and 0.05 M Na_2CO_3 -0.1 M NaHCO_3 buffer for pH 9.0 to 11.0. One milliliter of the purified enzyme solution (pH 2.5) containing 10 m units of enzyme was mixed with 1 ml of each buffer. Toluene was added to the mixture and the solution was kept at 5°C for 72 hr. After incubation, the pH of each mixture was adjusted to 2.5 and deionized water was added to bring each solution to the same volume; 4 ml. The enzyme activity was then determined by the method mentioned above. The result shown in Fig. 6(I) indicated that purified α -galactosidase was relatively stable at pH 4.0 to 7.0.

Stability of the purified enzyme at low pH values and 30°C for 30 min to 20 hr was also examined using 0.1 M HCl-sodium citrate buffer. The amount of enzyme used here was 17.2 m units and the procedure was the same as described above. The temperature of incubation was adjusted to 30°C in place of low temperature, 5°C. As shown in Fig. 6(II), the purified enzyme was inactivated after 20 hr of incubation at pH 1.5 to 2.0, and 75% of enzyme activity was lost after 20 hr of incubation at pH 2.5.

These results evidently demonstrate that the stability of α -galactosidase at low pH is a lesser degree than that of α -L-arabinofuranosidase or endopolygalacturonase produced by *C. rolfii* which is characteristic of later enzyme.

Action of α -galactosidase on various galactosides. The various kinds of synthetic glycosides listed in Table II were examined with the purified enzyme solution and the highly purified α -galactosidase from *C. rolfii* was demonstrated to have no enzymic activity on these substrates except *p*-nitrophenyl α -D-galactopyranoside. The various kinds of biological sugars were also tested for the action of purified enzyme and the clear activity was demonstrated on melibiose, raffinose and stachyose, respectively.

TABLE II. ACTION OF α -GALACTOSIDASE ON VARIOUS KINDS OF GLYCOSIDES.

Glycoside	Concentration of enzyme (m units/ml of reaction mixture)	Incubation time (hr)	<i>p</i> -Nitrophenol or sugar produced
<i>p</i> -Nitrophenyl α -D-galactopyranoside	7.0	0.5	70 (m μ mole/ml of reaction mixture)
<i>p</i> -Nitrophenyl β -D-galactopyranoside	7.0	5	0
<i>p</i> -Nitrophenyl N-acetyl β -D-galactosaminide	7.0	5	0
<i>p</i> -Nitrophenyl N-acetyl β -D-glucosaminide	7.0	5	0
<i>p</i> -Nitrophenyl α -D-glucopyranoside	7.0	5	0
<i>p</i> -Nitrophenyl β -D-glucopyranoside	7.0	5	0
<i>p</i> -Nitrophenyl α -L-fucopyranoside	7.0	5	0
<i>p</i> -Nitrophenyl β -D-xylopyranoside	7.0	5	0
Phenyl α -L-arabinofuranoside	7.0	5	0
Phenyl β -L-arabinopyranoside	7.0	5	0
Acid soluble pectic acid ^{a)}	4.0	5	0 (μ g/ml)
Cellobiose	80.0	5	0
Gentibiose	80.0	5	0
Lactose	80.0	5	0
Maltose	80.0	5	0
Sucrose	80.0	5	0
Trehalose	80.0	5	0
Melibiose	80.0	5	16
Raffinose	80.0	5	170
Stachyose	80.0	5	20
Pectic acid ^{b)}	4.0	5	0 (viscosity decrease %)

^{a)} Activity was determined by increase of reducing value at pH 2.5 and 4.0.¹⁰⁾

^{b)} Activity was determined by measuring viscosity decrease at pH 4.0.¹⁰⁾

FIG. 7. Lineweaver-Burk Plots for *C. rolfsii* α -Galactosidase.

Assays were carried out at 30°C and pH 2.5 for 10 min. Concentration of enzyme protein was 0.17 μ g (I) and 0.50 μ g (II), respectively.

(I) *p*-Nitrophenyl α -D-galactopyranoside was used as substrate.

(II) *o*-Nitrophenyl α -D-galactopyranoside was used as substrate.

The enzymic product was paperchromatographically confirmed to be D-galactose in each test.

Michaelis constants were determined to be 0.16×10^{-3} M for *p*-nitrophenyl α -D-galactopyranoside and 0.26×10^{-3} M for *o*-nitrophenyl α -D-galactopyranoside. The values of $V_{\max}$ were also calculated to be 26.6μ moles for *p*-nitrophenyl α -D-galactopyranoside and 28.6μ moles per min per mg for *o*-nitrophenyl α -D-galactopyranoside, respectively. These constants were determined by the method of Lineweaver and Burk.¹⁵⁾ The results are shown in Fig. 7(I) and (II).

Inhibition studies. For studying the inhibitory effect of salts on the enzymic action, 0.8 ml of enzyme solution containing 4.4 m units of enzyme, 0.4 ml of 0.1 M sodium citrate-0.2 M sodium phosphate buffer (pH 4.0) and 0.4 ml of salt solution were mixed and the mixture was allowed to stand at 30°C for 20 min, to which was added 0.8 ml of 1.0 mM *p*-nitrophenyl α -D-galactopyranoside solution (pH 4.0) and the reaction was carried out at

30°C for 30 min. The enzymic activity was assayed and represented in Table III as per cent of the activity of mixture without salt. Among various metal ions tested, Hg^{2+} and Ag^+ were found to be potent inhibitor.

DISCUSSION

The presence of α -galactosidase has been reported in a variety of micro-organisms, animals and higher plants. Various micro-organisms have been shown to produce α -galactosidase having acidic pH optimum; pH 4.0 for *A. niger*,⁴⁾ 5.2 for *S. olivaceus*,⁵⁾ 4.0 to 6.0, and 3.0 to 5.0 for *M. vinacea*.⁷⁾ In 1969, one of the authors, Kaji, and Ichimi⁸⁾ have reported that *C. rolfsii* strain K2 produces acid α - and β -galactosidases which are shown to be stable and optimally active under acidic conditions using crude enzyme preparation. The results from the present work demonstrate that the highly purified α -galactosidase of *C. rolfsii* is most active at pH 2.5 to 4.5 towards *p*-nitrophenyl α -D-galactopyranoside and fairly active at lower pH values of 1.1 to 2.0. Generally speaking, pH curve of enzyme have a clear peak. On the contrary, the activity curve of this α -galactosidase was shown to be rather flat between pH 2.5 and 4.5. The results also show that the highly purified α -galactosidase from *C. rolfsii* is stable over a pH range, 4 to 7, but less stable at pH 1.1 to 2.0 as compared with the other acid glycosidases of *C. rolfsii*.

C. rolfsii is a oxalate-forming basidiomycete and the acidity of the culture fluid drops from pH 5.4 to 2.0~2.2 because of the production of oxalic acid. It is reasonable that this fungus produces acid glycosidases, glycanases and acid proteinase which are active at low pH and tolerable to hydrogen ions. These characters are able to be indicated not only in α -galactosidase but also α -L-arabinofuranosidase,^{16,17)} β -D-galactosidase,^{8,18)} endopolygalacturonase¹⁰⁾ and β -1, 3-D-glucanase.¹⁹⁾

TABLE III. EFFECT OF SALT ON ENZYMIC ACTION

Salt	Concentration (mM)	Relative activity (%)
MnSO ₄	1	100
Fe ₂ (SO ₄) ₃	1	90
FeSO ₄	1	95
CuSO ₄	1	100
ZnSO ₄	1	100
Mercuric acetate	1	0
Calcium acetate	1	100
CoCl ₂	1	100
AgNO ₃	1	0
NaCl	100	102
NaCl	10	101
KCl	10	100
<i>p</i> CMB ^{a)}	10 ⁻²	100
EDTA ^{b)}	1	100
None		100

^{a)} *p*-Chloromercuribenzoate

^{b)} Ethylenediamine tetraacetate

All of these enzymes have been already purified and their properties have been reported in the previous reports.

However, the degrees of activity at low pH and acid tolerance are found to vary among these enzymes as detailed in presented results. Endo-polygalactosidase, α -L-arabinofuranosidase and β -D-galactosidase are most active at very low pH, 1.5 to 2.5, and β -1,3-D-glucanase and α -D-galactosidase are active at higher pH values; e.g. the former is most active at pH 3.0 to 5.5 and the latter at pH 2.5 to 4.5.

Regard with acid tolerance, the stability of α -galactosidase is lower than the other glycosidase produced by *C. rolf sii*. Evidently, the purified α -galactosidase lost 64% of its activity at pH 2.0 at 5°C for 72 hr, the purified α -L-arabinofuranosidase¹⁶⁾ lost 17% under the same conditions, β -galactosidase in crude state⁸⁾ lost 6% at pH 2.0 at 2°C for 72 hr, and the purified endo-polygalacturonase¹⁰⁾ lost 6.5% at pH 1.5 and 20°C for 72 hr, respectively.

However, the highly purified α -galactosidase from *C. rolf sii* was demonstrated to be still active at pH 1.1, and three enzymes mentioned above except β -1,3-D-glucanase were also active at the same pH value. It is surprising that various enzymes produced by *C. rolf sii* have clear activity under strong acidic condition in which ordinary living organisms are not able to survive without being specially protected from hydrogen ions. It is concluded that α -galactosidase purified in the present work belongs to acid glycosidase and it is called acid α -D-galactosidase.

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