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Characteristic Features of an α-Galactosidase from *Penicillium purpurogenum*

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A α-galactosidase (α-D-galactoside galactohydrolase; EC 3.2.1.22) was purified from the culture filtrate of *Penicillium purpurogenum* by DEAE-cellulose column chromatography, gel filtration of Bio gel p-100, and subsequent SP-Sephadex C-25 chromatography. The final preparation thus obtained showed a single band on polyacrylamide disc-gel and SDS-polyacrylamide gel electrophoresis. The molecular weight and isoelectric point were determined to be 63,000 and pH 4.0 by SDS-polyacrylamide gel electrophoresis and isoelectric focusing, respectively. The galactosidase exhibited maximum activity at pH 4.5 and 55°C, and was stable between pH 2 and 5, and also stable up to 40°C. The enzyme activity was not affected considerably by treatment with other metal compounds except mercuric chloride and silver nitrate. Copra galactomannan was finally hydrolyzed to galactose, mannose and mannobiose through the sequential actions of the purified galactosidase and mannanase from the same strain. The enzyme hydrolyzed melibiose and raffinose, but not lactose.

α-Galactosidase have been found to be widely distributed in nature (13) but it is only comparatively recently that they have been purified to apparent homogeneity (2, 3, 4, 12). Consequently little is known about their kinetics, the situation being further complicated by the lack of readily available, naturally occuring oligosaccharides and polysacchardies as substrate. Moreover, as techniques of purification and identification have improved, the existence of more than one α-galactosidase has been shown in the coffee bean (9), vicia faba (broad bean) (3), coconut (1) and, for separation of the α-galactosidase and chitinase (8). Otherwise, this strain from *Penicillium purpurogenum* accompany by β-mannanase. And so, the authors tried to sequential actions of α-galactosidase and β-mannanase on copra galactomannan.

Since the present paper deals with purification of galactosidase from *Penicillium purpurogenum* and some chemical and enzymatic properties of the purified enzyme.

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Key words: *Penicillium purpurogenum*, α-galactosidase, copra galactomannan

MATERIALS AND METHODS

Brown Copra Meal (BCM)

BCM, which is a by-product of oil extraction from copra, was kindly supplied by Fuji oil Co., Ltd. (Osaka, Japan). BCM contained 47.7% total sugar, which was analyzed according to the method reported previously (5), and was found to be composed of 66.0% mannose, 5.8% galactose, 22.5% glucose, 4.0% arabinose and 1.7% xylose.

Copra Galactomannan and β-1,4-Manno-oligosaccharides

The ratio of mannose to galactose of the galactomannan was 14:1. The substrate were prepared by the method described in previous papers (6, 11).

Preparation of the Crude Enzyme and Determination of Galactosidase Activity

The galactosidase from *Penicillium purpurogenum*, which was isolated from soil, was prepared by submerged culturing, in medium containing BCM as a carbon source, according to the procedure described in the preceding paper (5). The resultant culture filtrate was dialyzed against distilled water, and then used as the enzyme

source for purification.

Galactosidase activity was assayed by spectrophotometric measurement of p-nitrophenol relased from p-nitro-phenyl α-D-galactopyranoside (PNPGal; Wako Pure Chemical Ind., Japan). The assay mixture contained 0.5 m*l* of 2 mM PNPGal 0.4 m*l* of McIlvaine buffer solution (pH 4.5) and 0.1 m*l* of enzyme solution. The reaction was performed at 55℃ for 10 min, and stopped by adding 1.0 m*l* of 0.2 M sodium carbonate. One unit of α-galactosidase activity was defined as the amount corresponding to the release of 1 μmol of p-nitrophenol per min, measured at 400 nm.

Purification of Galactosidase

All the procedures were carried out in a chromato-chamber at about 4℃, as described in the RESULTS section.

Determination of Protein

The protein concentration was determined by measuring the absorbance at 280 nm, assuming that the absorbance at 280 nm at the concentration of 1 mg per m*l* is 1.0.

Gel Electrophoresis

Polyacrylamide disc-gel electrophoresis was performed at pH 9.5 on 7% polyacrylamide gel. The enzyme was loaded onto the gel and then electrophoresis was carried out with a current of 3 mA per gel for 3 h at room temperature.After the electrophoresis, the protein in the gel was stained with 0.05% Coomassie Brilliant Blue R-250 (Sigma Chemical Co., Missouri, U.S.A.).

Molecular Weight Determination

The molecular weight of the enzyme was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, using molecular weight makers (BDH chemicals Ltd., Poole London). SDS-polyacrylamide gel electrophoresis was carried out in 0.2% SDS-0.2 M sodium phosphate buffer solution (pH 7.2) at 6 mA per gel according to the method of Weber and Osborn (7).

Isoelectric Focusing

Isoelectric focusing was carried out using Servalyt (Serva Fein-biochemica, Heidelberg) in the pH range of 2~4. The density gradient in an electrofocusing column was formed with sucrose. Electrofocusing was performed at 900 V and 2℃ for 24 h.

Examination of the Effects of Various Compounds on the Galactosidase Activity

The enzyme solution containing a test compound (1.0×10^{-3} M) was pre-incubated at 20℃ for 1 h. After the pre-incubation,the remaining activity was determined by the above method. Monoiodoacetic acid, N-bromosuccinimide, sodium dodecyl sulfate and EDTA were added to the reaction mixture for the enzyme assay (1.0×10^{-3} M) without pre-incubation with the enzyme, and the activity was assayed by the above method.

Sugar Chromatographies

Papar chromatography: The paper chromatography was carried out by the ascending method on Toyo-roshi No. 51 filter paper with a solvent system of 1-butanol: pyridine:water (6:4:3, v/v). Sugar on the paper were detected by the silver nitrate dip method (14).

Thin-layer chromatography: Thin-layer chromatography was performed on a plate of Merck TLC plate silica gel 60 with a solvent system of 1-propanol:nitromethane:water (5:2:3, v/v). The sugars on the plate were detected by heating at 110℃ for 10 min after spraying with 50% sulfuric acid.

RESULTS

Purification of Galactosidase

DEAE-cellulose column chromatography: The enzyme source was dialyzed against a 0.02 M potassium phosphate buffer solution (pH 6.). The enzyme solution was applied to a column (3×25 cm) of DEAE-celluose (Whatman Ltd., Maidstone, Kent, England) equilibrated with the same buffer solution. Elution was then carried out with the a linear gradient of sodium chloride, from zero to 0.1 M, at the flow rate of 16 m*l* per h. The elution profile is shown in Fig. 1. The active fractions, No. 170-185, were combined to minimize contamination by β-mannanase.

Gel filtration on Bio-gel p-100 column chromatography: The pooled galactosidase fractions (No. 170-185) eluted from the DEAE-cellulose column were dialyzed against a 0.02 M phosphate buffer solution (pH 6.0) containing 0.1 M sodium chloride. The concentrated enzyme solution was then applied to the column (4.5×90 cm) and eluted with the same buffer solution at the flow rate of 15 m*l* per h (Fig. 2). The active fractions,

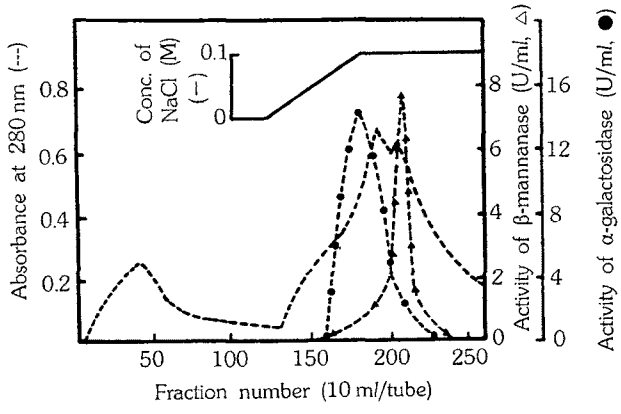

Fig. 1. Chromatography of the galactosidase on a column of DEAE-cellulose.

Fraction No. 170-185 were pooled for the following purification step.

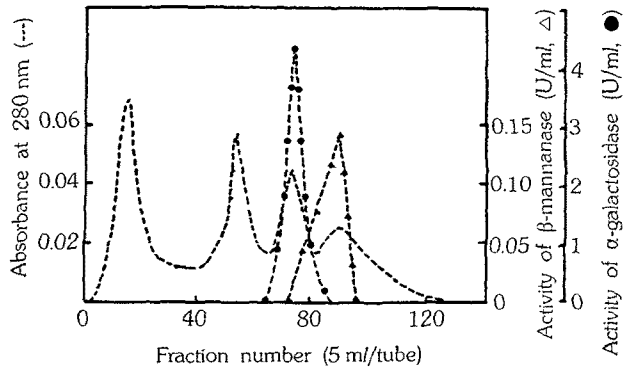

Fig. 2. Chromatography of the galactosidase on a column of Bio-gel p-100.
Fraction No. 68-79 were pooled for the following purification step.

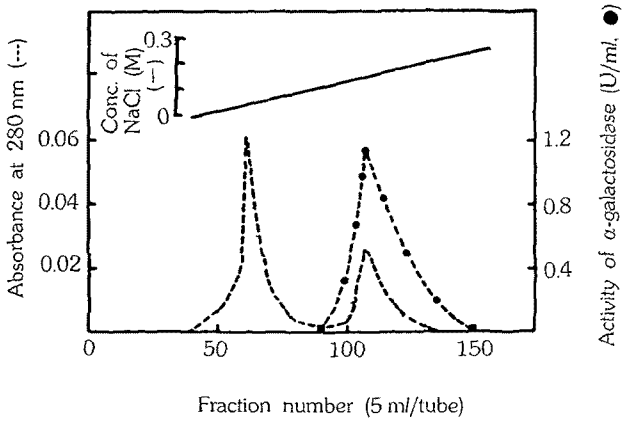

Fig. 3. Chromatography of the galactosidase on a column of SP-Sephadex C-25.
Fraction No. 100-130 were pooled for studying the characteristics of the purified galactosidase.

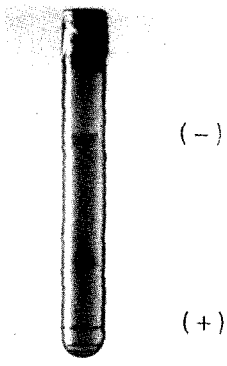

Fig. 4. Polyacrylamide disc-gel electrophoresis of the purified galactosidase.

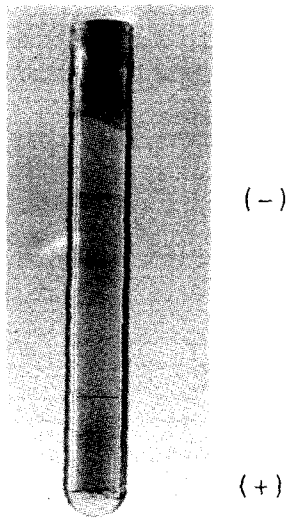

Fig. 5. SDS-polyacrylamide gel electrophoresis of the purified galactosidase.

No. 68-79, were combined.

SP-Sephadex C-25 column chromatography: The pooled galactosidase fraction eluted from the Bio-gel p-100 column were dialyzed against 0.01 M sodium citrate-0.01 N hydrochloride (pH 3.0). The dialyzed enzyme solution was applied to a SP-Sephadex C-25 column (1.5×10 cm) equilibrated with the same buffer solution at the flow rate of 10 ml per h, and then elution was carried out with a linear gradient of sodium chloride, from zero to 0.3 M (Fig. 3). Main fraction No. 100-130, free from β-mannanase activity, were combined. The purified galactosidase, thus obtained, gave a single band on polyacrylamide disc-gel electrophoresis and SDS-polyacrylamide gel electrophoresis, respectively (Fig. 4 and Fig. 5).

Table 1 summarizes the increases in the specific activity of galactosidase and the yield during the purifaction.

Table 1. Summary of purification of galactosidase from *Penicillum purpurogenum*

Steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Crude extract	4200	3264	1.28	100
DEAE-cellulose column chromatography	3616	188.4	19.19	86.9
Gel filtration on Bio gel p-100	3441	57.8	59.57	81.9
SP-Sephadex C-25 column chromatography	2528	10.8	234.09	60.4

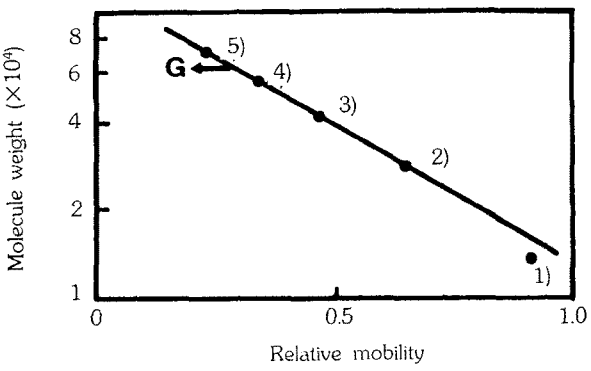

Fig. 6. Estimation of molecular weight of the galactosidase by SDS-polyacrylamide gel electrophoresis.
1) m.w. marker monomer (14,300); 2) dimer (28,600); 3) trimer (42,900); 4) tetramer (57,200);5) pentamer (71,500).

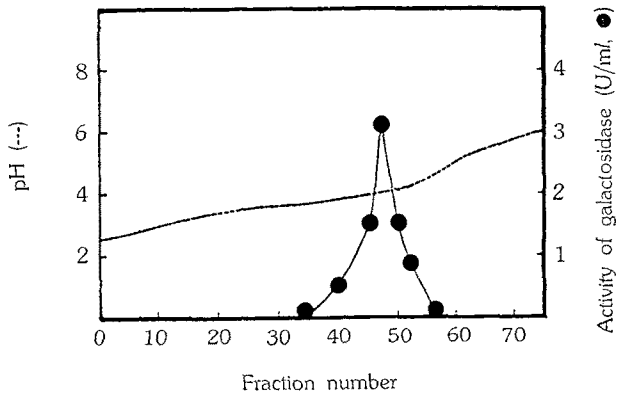

Fig. 7. Isoelectric focusing of the purified galactosidase.

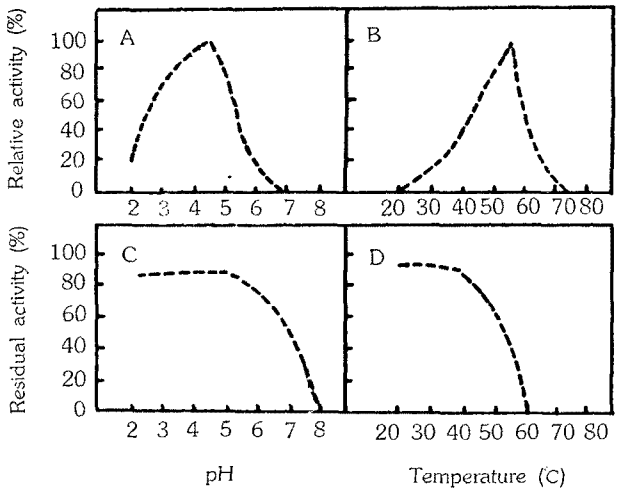

Fig. 8. Effects of pH and temperature on the purified galactosidase.
(A) Optimum pH. McIlvaine buffer solution with a pH range of 2.1~8.0 was used. The reaction mixture contained 0.1 ml of enzyme, 0.5 ml of 2 mM p-nitrophenyl-α-D-galactopyranoside (PNPG) and 0.4 ml of buffer solution. The enzyme reaction were carried out under the condition 55℃ for 10 min. (B) Optimum temperautre. The enzyme reactions were carried out at the indicated temperatures for 10 min. (C) pH stability. Mixutres of the enzyme solution and McIlvane buffer solution were stood at 40℃ for 1 h. Then the residual activity was determined under standard condition. (d) Thermal stability. The enzyme solution was stood at indicated temperatures for 1 h. Then, the residual activity was determined under standard condition.

Molecular weight and isoelectric point: The molecular weight of the purified galactosidase was estimated to be 63,000 by SDS-polyacrylamide gel electrophoresis (Fig. 6). The result of isoelectric focusing is shown in Fig. 7.

The purified galactosidase was found to be an acidic protein with an isoelectric point of 4.0.

Enzyme Properties

Effects of pH and temperature on the activity of the purified galactosidase: The galactosidase activity was measured at various pHs, from 2 to 8, using a McIlvaine buffer solution. The optimum pH for the activity was found to be around 4.5 (Fig. 8A). The enzyme activity was measured at various temperature, from 20℃ to 80℃. The optimum temperature for the activity was around 55℃ (Fig. 8B).

Effect of pH and temperature on the stability of the purified galactosidase: The enzyme solution was maintained at various pH, ranging from 2 to 8, using the McIlvaine buffer solution, for 1 h at 40℃, and then the remaining activity was assayed. The enzyme was stable between pH 2 and 5 (Fig. 8C). The enzyme was treated in a water bath at various temperatures, from 20℃ to 80℃, for 1 h at pH 4.5 in the McIlvaine buffer solution, and then the remaining activity was assayed. the enzyme was stable up to 40℃, but 30% of the original activity was lost on the same treatment at 50℃ and the enzyme was completely inactivated by the same treatment at 60℃ (Fig. 8D).

Effects of various compounds on the activity of the purified galactosidase: The effects of various compounds (1 mM) on the activity of the purified galactosidase are shown in Table 2. The enzyme was inhibited by treatment with mercuric chloride or silver nitrate. The enzyme activity was not affected considerably by treatment with other metal compounds.

Sequential actions of α-galactosidase and β-mannanase on copra galactomannan: It was reported in the preceding paper (5) that the mannanase system of *Penicillium purpurogenum* hydrolyzed copra galactomannan to monosaccharides and oligosaccharides at the early stage of the reaction, and monosaccharides and

Table 2. Effects of various compounds on galactosidase. Concentration of the compound, 1.0×10^{-3} M

Compound	Relative activity(%)	Compound	Relative activity(%)
None	100	$HgCl_2$	10
NaCl	94	$FeCl_2$	87
KCl	95	$FeCl_3$	98
NH_4Cl	103	$CoCl_2$	85
$MgCl_2$	86	$NiCl_2$	85
$CaCl_2$	98	$CdCl_2$	88
$BaCl_2$	90	$ZnCl_2$	89
$AlCl_3$	114	$Pb(CH_3COO)_2$	105
$SnCl_2$	100	N-Bromosuccinimide	99
$CuSO_4$	83	Iodoacetic acid	105
$AgNO_3$	5	Sodium lauryl sulfate	83
$MnCl_2$	78	EDTA	105

mannobiose at the final stage. Thus, this experiment was carried out to elucidate the mechanism by which the enzyme system produces monosaccharides and mannobiose as final products from copra galactomannan. A 10 ml purified mannanase solution was added to 200 mg of copra galactomannan in an L-shape tube. The reaction was then carried out at 50℃ for 14 h on a Monod shaker. The reaction mixture was heated in a boiling water for 5 min to inactive the enzyme, and then centrifuged to remove the very small amounts of insoluble materials from the hydrolysate. A small quantity of the supernatuant (M) was subjected to paper chromatography for characterization of the hydrolysis products. As shown in Fig. 9(M), the mannanase yielded mannose and mannooligesaccharades (presumably containing galactomanno-oligosaccharides) from copra galactomannan, but not galactose.

Subsequently, α-galactosidase was added to the supernatant (M) obtained above, and the reaction was carried out at 50℃ for 9 h. After the same treatment as that described above, a small quantity of the resultant hydrolysate (M-G) was subjected to paper chromatography. As shown in Fig. 9 (M-G), the α-galactosidase reaction led to the appearance of galactose with the disappearance of an oligosaccharide. Mannanase was again added to the above hydrolysate (M-G), and the reaction was carried out under the same conditions, that is, pH 5.0, 50℃ and 14 h, as described above (this hydrolysate is desingnated as M-G-M). As shown in Fig. 9 (M-G-M), an oligosaccharide disappeared through the mannanase action. α-Galactosidase was again added to the above hydrolysate (M-G-M). As shown in Fig. 9 (M-G-M-G), copra galactomannan was finally to decomposed to mannose, galactose and mannobiose through the sequential actions of α-galactosidase and β-mannanase.

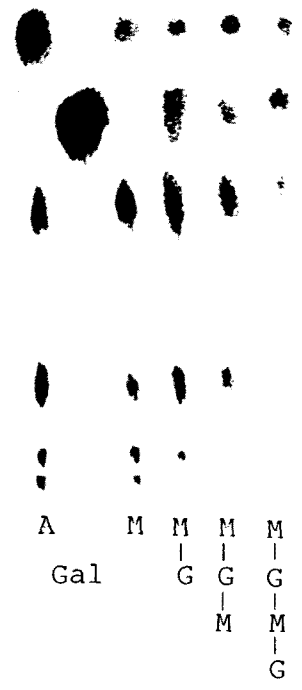

Fig. 9. Paper chromatogram of hydrolysates obtained by alternating action of the purified mannanase and the purified galactosidase on copra galactomannan.
A, authentic mannose, mannobiose, mannotriose, mannotetraose and mannopentaose from top to bottom; Gal, authentic galactose, M, β-mannanase action; G-α-galactosidase action.

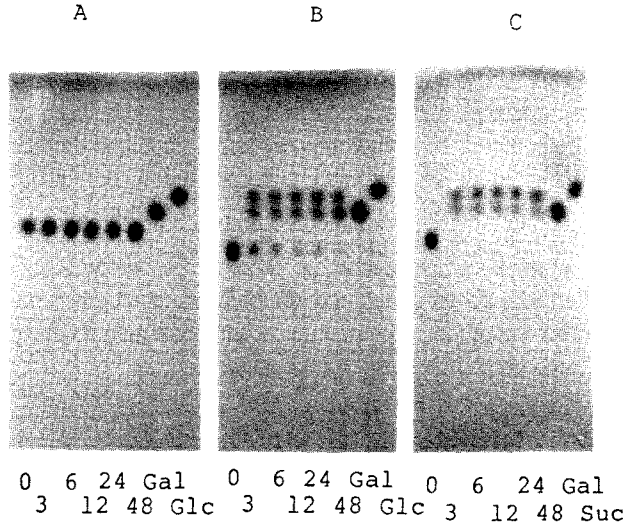

Fig. 10. Thin-layer chromatograms of hydrolysates with the galactosidase.
A; Lactose, B; Melibiose, C; Raffinose, Gal; galactose, Glc; glucose, Suc; sucrose.

Action pattern of the α-galactosidase: The thin-layer chromatographies which were obtained with the lactose, melibiose and raffinose after 48 h incubation with the enzyme are presented in Fig. 10. This figure are presented the results, showing that the galactosidase dose not attack lactose. However, melibiose and raffinose were hydrolyzed to produce galactose and glucose, and galactose and sucrose.

DISCUSSION

The authors isolated *Penicillium purpurogenum* from soil, which produces a strong galactosidase. It appears that this strain produces one kind of galactosidase, are shown in Fig. 1.

Copra galactomannan was finally hydrolyzed through the sequential actions of galactosidase and mannanase to galactose, mannose and mannobiose, through the following mechanism: 1) The mannanase hydrolyzed the galactomannan to mannose, manno-oligosaccharides and mixed oligosaccharides (galactomanno-oligosaccharides) composed of galactose and mannose. 2) The α-galactosidase removed galactose from the resultant galactomanno-oligosaccharides to produce galactose and manno-oligosaccharide. 3) The manno-oligosaccharides were finally hydrolyzed to mannose and mannobiose.

The α-galactosidase from germinated seeds of Vicia sative (10) has been shown to hydrolyse raffinose, stachyose and galactinol but not melibiose or galactomannans. Lens esculenta contains an α-galactosidase in the dormant seed which hydrolyses raffinose, melibiose to a lesser extent, but not galactomannan (4). Otherwise, the α-galactosidase from *Penicillium purpurogenum* hydrolyse melibiose, raffinose and galactomannan but not lactose.

All of the naturally occuring α-D-galacto-pyranosides are hydrolyzed by α-galactosidases. The velocity of hydrolyesis seems to be reduced by increase in the "D-galactosidic" chain-length. And also, a free reducing group in the sugar molecule reduces the rate of hydrolysis; for example, melibiose is hydrolyzed less rapidly than raffinose (Fig. 10).

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