

PURIFICATION AND SOME PROPERTIES OF A NOVEL β -MANNOSIDASE PRODUCED BY A BACTERIUM ISOLATED FROM SOIL

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土壌より分離した一細菌の生産する β -マンノシダーゼの精製とその諸性質

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A gram positive bacterium isolated from soil was found to produce β -mannosidase that act on O- β -D-mannopyranosyl-(1 \rightarrow 2)-D-mannose in a cell-free extract. The enzyme was partially purified from the cell-free extract to remove other glycosidase activities. The optimum pH and temperature for the activity to β -mannosidase was pH 6.5, and 40°C, respectively. The enzyme was completely inhibited by Cu^{2+} and Hg^{2+} , and strongly inhibited by the addition of mannose. The enzyme almost completely hydrolyzed O- β -D-mannopyranosyl-(1 \rightarrow 2)-D-mannose to mannose, but did not act on p-nitrophenyl- β -D-mannoside or O- β -D-mannopyranosyl-(1 \rightarrow 2)-D-mannitol.

土壌より分離したグラム陽性一細菌が β -1, 2-マンノビオースを分解する β -マンノシダーゼをその細胞内に生産することを見出した。本酵素をその細胞内抽出液より部分精製して、他のグリコシダーゼ活性を除いた。本酵素の至適pHは6.5, 至適温度は40°Cであった。また本酵素活性は、 Cu^{2+} , Hg^{2+} イオンにより完全に阻害され、反応生成物であるマンノースによっても強く阻害された。本酵素は β -1, 2-マンノビオースをマンノースにはほぼ完全に分解したが、p-ニトロフェニル- β -D-マンノシドや β -1, 2-マンノシルマンニトールには全く作用しなかった。

Introduction

β -Mannosidase (EC 3.2.1.25) has been purified from various sources such as snail⁽¹⁾, hen oviduct⁽²⁾, mushroom^(3,4), pineapple⁽⁵⁾, *Aspergillus niger*^(6,7), and also from the liver of *Turbo cornutus*⁽⁸⁾. Because of its strict substrate specificity β -mannosidase is used as an indispensable tool for studies on glycoprotein structure. β -Mannosidic linkage is known to occur in plant mannans⁽⁹⁾ and plant gums⁽¹⁰⁾. In general, the mannose of mannose-containing polysaccharides in plants has the configuration of β -1,4-linkage. Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4GlcNAc-Asn is also a common sequence in the core region of asparagine-linked oligosaccharides of various glycoproteins.

Recently, we obtained a disaccharides from the polysaccharides of *Fusarium* sp. M7-1 by digestion with a bacterium enzyme preparation and identified it as O- β -D-mannopyranosyl-(1 \rightarrow 2)-D-mannose (abbreviated

as $\text{Man } \beta 1 \rightarrow 2\text{Man}$ ⁽¹⁾. This is the first report to our knowledge on the isolation of $\text{Man } \beta 1 \rightarrow 2\text{Man}$ and there have been no reports of the substrate specificity of β -Mannosidases for $\text{Man } \beta 1 \rightarrow 2\text{Man}$.

This paper describes the unique β -mannosidase from a gram positive bacterium isolated from soil.

Materials and Methods

Microorganisms and cultivation The bacterium strain FO 1-2A was used throughout this investigation. The bacterium was isolated from a soil sample in Osaka city by an enrichment culture method using $\text{Man } \beta 1 \rightarrow 2\text{Man}$ as the sole carbon source. The maintenance medium contained 0.5% peptone, 0.5% yeast extract, 0.5% NaCl and 2% agar (pH 7.0). The bacterium was transferred from a slant culture to 5 ml of the medium in a test tube, and the seed culture was incubated at 28°C, for 22 hr, with shaking. Each culture was then transferred to a 500 ml shakeflask containing 150 ml of the medium and cultivation was carried out at 28°C on a reciprocal shaker.

Preparation of the cell-free extract Bacterial cells were washed two times with 10 mM potassium phosphate buffer (pH 7.0), resuspended in the same buffer and then disrupted with an ultrasonic oscillator (Nihon Seiki Co. 15KHz, 15min). The supernatant obtained by centrifugation was dialyzed against 10 mM potassium phosphate buffer (pH 7.0) and used as the crude enzyme preparation.

Partial purification of β -mannosidase from FO 1-2A Stain Unless otherwise specified, all operations concerning enzyme purification were carried out at about 5°C.

Step 1. Ammonium sulfate fractionation To the dialyzed crude extract, solid ammonium sulfate was added to 30% saturation. After stirring for 30 min, the mixture was centrifuged. To the supernatant, solid ammonium sulfate was added to 50% saturation. The mixture was stirred for 30 min and then centrifuged. The resulting precipitate was dissolved in a minimal volume of 10 mM potassium phosphate buffer containing 1 mM 2-mercaptoethanol, pH 7.0, and dialyzed against the same buffer for 12 hr.

Step 2, 1st DEAE-Toyopearl 650M column chromatography The dialyzed solution was applied to a DEAE-Toyopearl 650M column (3.0×40cm) previously equilibrated with 10 mM potassium phosphate buffer containing 1 mM 2-mercaptoethanol, pH 7.0. After the column was washed with the same buffer, it was eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. The active fractions were pooled and then precipitated by ammonium sulfate.

Step 3. 2nd DEAE-Toyopearl 650M column chromatography The concentrated enzyme was applied to a second DEAE-Toyopearl column (1.5×30cm) previously equilibrated with 10 mM phosphate buffer containing 2-mercaptoethanol. After the column was washed with the same buffer, it was eluted a linear gradient of 0 to 0.3 M NaCl in the above buffer. The most active fractions were pooled.

Preparation of $\text{Man } \beta 1 \rightarrow 2\text{Man}$ $\text{Man } \beta 1 \rightarrow 2\text{Man}$ was isolated from a polysaccharide of *Fusarium* sp. M7-1 by the previously described method⁽¹⁾. O- β -D-Mannopyranosyl-(1→2)-D-mannitol ($\text{Man } \beta 1 \rightarrow 2\text{Man-ol}$) was prepared by the NaBH_4 reduction of $\text{Man } \beta 1 \rightarrow 2\text{Man}$.

Materials Yeast mannan and gum guar were obtained from Sigma Chemicals Co. p-Nitrophenyl derivatives of sugars were also purchased from Seikagaku Kogyo Co. All other chemicals used were of the

highest grade commercially available.

Enzyme assay. The reaction mixture was composed of 600 nmol of Man β 1 \rightarrow 2Man, 30 μ mol of potassium phosphate buffer (pH 7.0) and the enzyme solution in a total volume of 100 μ l. After incubation for an appropriate time, fifty microliters of the reaction mixture were analyzed by HPLC and the levels of Man β 1 \rightarrow 2Man and mannose were determined. One unit is defined as the amount of enzyme that degrades 1 μ mol of Man β 1 \rightarrow 2Man per min at 37 $^{\circ}$ C under the assay conditions. p-Nitrophenyl-glycoside hydrolyzing activity was assayed with the appropriate p-nitrophenyl glycosides. Levels of p-nitrophenol released were determined from absorbance at 400 nm (molar extinction coefficient, 17,700). One unit of enzyme activity was defined as the amount which released 1 μ mol of p-nitrophenol per min.

Analytical methods. HPLC for saccharide analysis was carried out with a Hitachi model L6000 chromatograph equipped with a Shodex RI SE-31. For the separation of manno-
biose and mannose, a column of Shim-Pack ION S-802 (eluted with deionized water at a flow rate of 1 ml per min at 60 $^{\circ}$ C) was employed. Protein was determined by the method of Lowry *et al.* with crystalline egg albumin as the standard⁽¹²⁾. The protein content, by column chromatography, was determined by measuring the absorbance at 280 nm.

Results

Culture conditions for the production of β -mannosidase from FO 1-2A strain. A soil microorganism which was capable of growing on Man β 1 \rightarrow 2Man was isolated by enrichment culture techniques. The β -mannosidase-producing bacterium FO 1-2A was gram-positive and did not form spores. Analysis of culture filtrates and harvested cells, which had been disrupted by sonication, showed that all β -mannosidase activity was intracellular.

The effect of various carbon sources on the production of β -mannosidase is shown in Table 1. All the carbon sources repressed the formation of β -mannosidase.

Stabilization of β -mannosidase. The β -mannosidase from FO 1-2A was very unstable and in a 50 mM potassium phosphate buffer (pH 7.0) at 5 $^{\circ}$ C for 24 hr, the enzyme lost more than 90% of its original activity. Therefore, we examined ways to stabilize the enzyme activity with the addition of various chemicals to the solution. The addition of 1 mM 2-mercaptoethanol temporarily stabilized the enzyme, but 3 days later, the enzyme activity had decreased to below 30% of that at the start (Fig. 1). Moreover, when

Table 1. Partial Purification of β -1,2-Mannosidase from FO 1-2A

Step	Total Protein (mg)	Total activity (munits)	Specific activity (munits/mg)	Yield (%)	p-Np* β -Man (munits)
Cell-Free Extract	13,590	232	0.017	100	50
Ammonium sulfate (30-50% Satn.)	2,560	155	0.06	67	10
1st DEAE-Toyopearl	360	110	0.30	47	—
2nd DEAE-Toyopearl	33	46	1.36	20	—

*Hydrolyzing activity toward p-Nitrophenyl- β -mannoside

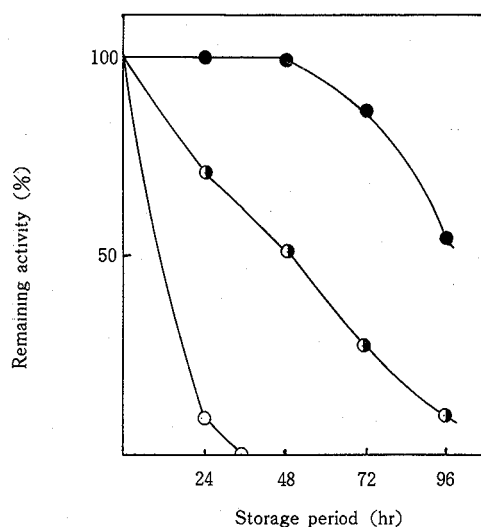


Fig. 1. Effect of 2-Mercaptoethanol and N₂ Substitution on β -Mannosidase Stability.

2-Mercaptoethanol was added to FO 1-2A cell-free extracts, then the air in the test tubes was substituted with N₂ gas.

Samples were preserved at 5°C and the remaining activities were assayed at intervals ●, none; ◐, addition of 2-mercaptoethanol; ○, addition of 2-mercaptoethanol and N₂ substituted.

substitution with N₂ gas was employed, the crude enzyme solution was stable for 3 days in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM 2-mercaptoethanol (Fig 1).

Properties of β -Mannosidase. The overall purification and yield of β -Mannosidase are summarized in Table 2. Throughout the procedure, an 80-fold purification was attained with a recovery of about 20% from the cell-free extract. The partially purified β -mannosidase was practically free from all other glycosidase tested (p-nitrophenyl- α -or- β -D-glucopyranoside, - α -or- β -D-galactopyranoside, - α -or- β -L-fucopyranoside, - α -or- β -D-mannopyranoside, - β -D-xylopyranoside, - β -D-glucuronoside, and -2-acetamide-2-deoxy- β -D-glucopyranoside as substrates).

Effects of pH and temperature on enzyme activity and stability. The optimum pH of the enzyme was

Table 2. Effects of Various Carbon Sources on Enzyme Production.

Additon to Medium	Conc.(%)	Enzyme activity(%)
None		100
Glucose	0.5	34
Yeast mannan	0.1	30
Gum guar	0.1	22
Mannose	0.3	12
Konjak mannan	0.1	12

FO 1-2A was grown on medium containing 0.5% peptone, 0.5% Yeast extract, 0.5% NaCl, and a carbon source at the concentration indicated. After culturing for 1 day, the cells were harvested and β -1,2-mannosidase of the cell free extracts was determined.

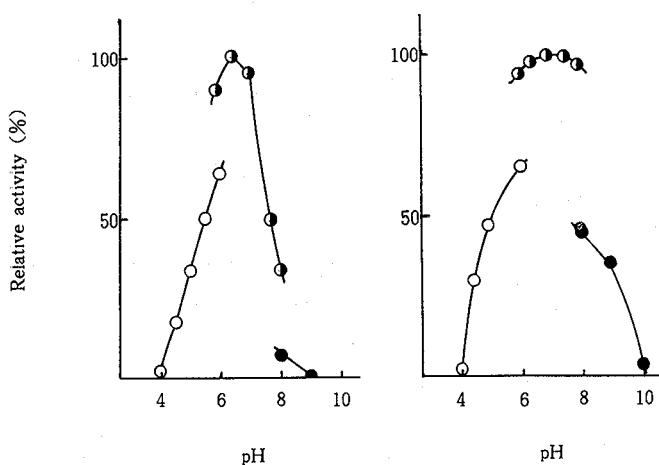


Fig 2. Effects of pH on the Activity and Stability of the β -Mannosidase from FO 1-2A.

(a) Effect of pH on the activity. The enzyme was assayed in 100 mM buffers of various pHs under standard conditions.

(b) The enzyme was pretreated in 50 mM buffers of various pHs at 5°C for 24 hr, then the remaining activity was assayed at pH 7.0. Buffers used: ○, sodium acetate-acetic acid; ●, potassium phosphate; ●, glycine-NaOH.

Table 3. Effects of Various Chemicals on Enzyme Activity

Compound	Conc. (%) (mM)	Enzyme Activity (%)
None		100
MgSO ₄	1	74
MnSO ₄	1	88
ZnSO ₄	1	66
CoSO ₄	1	82
HgCl ₂	1	0
CuSO ₂	1	0
EDTA	1	47
Glucose	10	100
Galactose	10	82
N-acetylglucosamine	10	100
N-acetylgalactosamine	10	110
Chitobiose	10	100
Mannose	10	24
Yeast mannan	15 mg/ml	100

The enzyme was preincubated at 37°C for 10 min in 30 mM potassium phosphate buffer (pH 7.0) with various compounds and the residual activity was assayed under standard conditions.

found to be 6.0 to 7.0. The enzyme was stable in the pH range of 6.0-8.0 when kept at 5°C for 24 hr (Fig. 2). The enzyme showed the highest activity at 40°C under the standard assay conditions. Its thermal stability was examined by heating it at various temperatures for 10 min, at pH 7.0. The enzyme was found to be stable up to 40°C.

Effects of various compounds on the enzyme activity. The effects of various metal ions and sugars on the enzyme activity were examined by incubating the enzyme with the compounds at 37°C and then directly assaying the activity (Table 3). The enzyme was completely inactivated by treatments with Hg^{2+} and Cu^{2+} . And the enzyme activity was greatly inhibited by the addition of mannose. Other sugars such as glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine, and yeast mannan did not inhibit the enzyme activity when used in concentrations of 10 mM.

Substrate specificity The rate of hydrolysis with respect to the $\text{Man } \beta 1 \rightarrow 2\text{Man}$ concentration was examined. The apparent Michaelis-Menten constant of the enzyme, calculated from a Lineweaver-Burk plot, was 12.8 mM for $\text{Man } \beta 1 \rightarrow 2\text{Man}$.

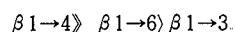
The activities of the enzyme were investigated with $\text{Man } \beta 1 \rightarrow 2\text{Man}$ and $\text{Man } \beta 1 \rightarrow 2\text{Man-ol}$. About 4 μ mol of $\text{Man } \beta 1 \rightarrow 2\text{Man}$ and $\text{Man } \beta 1 \rightarrow 2\text{Man-ol}$ was incubated at 37°C with 8 munits of the enzyme in 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM 2-mercaptoethanol (total volume, 500 μ l). $\text{Man } \beta 1 \rightarrow 2\text{Man}$ was hydrolyzed almost completely by the enzyme within the 12 hr incubation. In contrast, $\text{Man } \beta 1 \rightarrow 2\text{Man-ol}$ was not hydrolyzed.

Discussion

Gorin and his co-workers reported a methanol-assimilating yeast, *Pichia pastoris*, and were able to elaborate on a mannan containing both α -1,2 and β -1,2 linkages in the branching moieties⁽¹³⁾. They also had a similar main-chain as demonstrated by proton magnetic resonance spectra studies⁽¹³⁾.

We obtained the $\text{Man } \beta 1 \rightarrow 2\text{Man}$ from acidic polysaccharides of *Fusarium* sp. M7-1⁽¹¹⁾. Moreover, all the *Fusarium* strains that we examined produced similar acidic polysaccharides containing $\text{Man } \beta 1 \rightarrow 2\text{Man}$ ⁽¹⁴⁾. Therefore, β -1,2-linked mannose-containing polysaccharides may be widely distributed among yeasts and molds.

We examined the action of commercial β -mannosidase preparations (*Achatina fulica* and *Turbo cornutus*) on $\text{Man } \beta 1 \rightarrow 2\text{Man}$. Although they could act on $\text{Man } \beta 1 \rightarrow 2\text{Man}$, the hydrolysis rates were very slow (manuscript in preparation). β -Mannosidases from *Achatina fulica*, *Turbo cornutus* and *Aspergillus niger* appeared to be specific for the mannosyl β -1,4 linkages. For example, the hydrolysis rate of three β -mannosyl-N-acetylglucosamine by *Aspergillus niger* β -mannosidase is in the following order,



To search for a better source of β -1,2 specific mannosidase, we used an enrichment culture technique in which culture were grown on $\text{Man } \beta 1 \rightarrow 2\text{Man}$ as a sole carbon source. All the soil microorganisms which were capable of growing on $\text{Man } \beta 1 \rightarrow 2\text{Man}$, showed that the β -mannosidase activities were within the cells (data not shown).

Because of its unstability, we could not purify the enzyme of FO 1-2A to homogeneity. But the partially purified β -mannosidase was free all other glycosidases tested. We compared the enzymatic properties of the β -mannosidase with those of the other β -mannosidases. The β -mannosidases from other sources showed strong activities on p-nitrophenyl- β -D-mannopyranoside, while the β -mannosidase from FO 1-2A did not. The β -mannosidase from other sources had the highest activities at acidic pH values. However, the optimum pH for the FO 1-2A enzyme was around 7.0. The β -mannosidase from FO 1-2A was considerably inhibited by the addition of mannose, not by the addition of other monosaccharides. Bouquelet reported that the β -mannosidase activity of *Aspergillus niger* was decreased to half of the original by the addition of 20 mM mannose to the reaction mixture⁶⁾.

The β -mannosidase from FO 1-2A did not hydrolyze Man β 1 \rightarrow 2Man-ol. In addition, this enzyme could not produce mannose from *Fusarium* sp. M7-1 polysaccharide containing Man β 1 \rightarrow 2Man (data not shown). Therefore, this enzyme has strict aglycon specificities and seems to be highly specific for Man β 1 \rightarrow 2Man. Further studies on the substrate specificity of the new β -1,2 specific mannosidase from FO 1-2A are currently being investigated.

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(Received October 31, 1988)