Induction and Purification of *Aspergillus oryzae* 1,2- α -Mannosidase

Kazuhito TANIMOTO, Tohoru KATSURAGI and Haruki YAMAGUCHI*

Department of Agricultural Chemistry, College of Agriculture,
University of Osaka Prefecture, Sakai,
Osaka 591, Japan

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The culture conditions favoring the production and the subsequent purification of *Aspergillus oryzae* 1,2- α -mannosidase were investigated. Baker's yeast mannan as the carbon source in liquid culture was found to induce 1,2- α -mannosidase production but to depress protein and other glycosidase productions. Better enzyme production was achieved by developing a suitable cultivation medium containing baker's yeast mannan and a nitrogen source in proper concentrations. From the culture product thus obtained, 1,2- α -mannosidase could be readily purified by the affinity chromatographic method previously developed [Tanimoto *et al.*, *J. Biochem.*, 99, 601 (1986)], without any other chromatographic step.

1,2- α -Mannosidase, which specifically cleaves α -D-Man-(1 $\rightarrow$ 2)-D-Man linkages in high mannose-type oligosaccharide chains, has previously been purified and characterized from the enzyme products of *Aspergillus saitoi*^{1,2)} and *Aspergillus oryzae*.³⁾ Since then, 1,2- α -mannosidase has become a convenient tool for characterization of asparagine-linked oligosaccharides.^{4~16)} However, the content of this enzyme in these enzyme products is very small and consequently, its purification is time-consuming and laborious. In addition, the enzyme product Taka-diastase, which has been used as the only source of *A. oryzae* 1,2- α -mannosidase, is not commercially available.

To improve this situation, we investigated the culture conditions favoring the production and the subsequent purification of *A. oryzae* 1,2- α -mannosidase and found that baker's yeast mannan as the carbon source in liquid culture afforded an enzyme product having a high specific activity of 1,2- α -mannosidase. This paper also describes the purification and characterization of the 1,2- α -mannosidase thus induced.

Materials and Methods

Materials. Spores of a strain of *A. oryzae* were obtained from Higuchi Moyashi Co., Osaka. Yeast mannan was extracted from compressed baker's yeast (*Saccharomyces cerevisiae*; Oriental Yeast Industry Co., Ltd., Tokyo) as described previously¹⁷⁾ and purified by passing through a Chelex 100 column. Mannan-gel was prepared from the purified mannan exactly as described previously.¹⁷⁾ Soluble starch, collodion, and 2-aminopyridine were obtained from Wako Pure Chem. Co. and bovine serum albumin was a product of Seikagaku Kogyo Co. Chelex 100 was from Bio-Rad. *p*-Nitrophenyl α -D-mannoside, *p*-nitrophenyl β -D-galactoside, and *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide were purchased from Sigma Chem. Co., Ltd. Ovalbumin glycopeptide GP-IV was prepared as described by Conchie and Strachan¹⁸⁾ and converted to the pyridylamino derivative.¹⁰⁾

Crude enzyme preparation. Crude enzyme extracts were routinely prepared at regular times from culture broths at 0~4°C. The mycelia separated from a culture broth (10 ml) by filtration were washed with 0.15 M NaCl, then dispersed in 50 mM acetate buffer (pH 5.0, 10 ml). After being exposed to 20 kHz sonic irradiation for 3 min with external cooling in an ice-water bath, the turbid solution was centrifuged. The supernatant was dialyzed against 3 changes of the same buffer in a collodion bag for 1 day, then concentrated to about 1 ml by ultrafiltration. The resulting concentrate was analyzed for both enzyme activities and protein content.

* To whom correspondence should be addressed.

Enzyme assay. The 1,2- α -mannosidase activity was assayed using the pyridylamino derivative of ovalbumin glycopeptide GP-IV as substrate. An enzyme solution (3 μ l) was mixed with a 2% substrate solution (10 μ l) and incubated at 37°C for 2 hr. After being heated at 100°C for 2 min, the solution was evaporated to dryness. The residue was dissolved in water (10 μ l) and an aliquot of the solution was analyzed by HPLC. A Shimadzu model LC-6A chromatograph with a Rheodyne model 7125 injector and a Shimadzu model RF-530 fluorescence spectrometer were used. Pyridylamino derivatives of glycopeptides were separated on a reverse phase column (Cosmosil 5C18-P, 150 $\times$ 4.6 mm) with 0.1 M ammonium acetate buffer (pH 4.0) containing 2% 1-butanol at a flow rate of 1.5 ml per min. An excitation wavelength of 320 nm and an emission wavelength of 400 nm were used. One unit of the enzyme was defined as the amount which hydrolyzed 1 μ mol of the substrate per minute.*

Amylase, sucrase, and other glycosidase activities were assayed as reported previously³⁾ and protease activity was estimated as described by Oda and Murao.¹⁹⁾

Analytical methods. Protein was measured by the method of Lowry *et al.*²⁰⁾ with bovine serum albumin as a standard, and the carbohydrate content of a culture filtrate was estimated by the orcinol-sulfuric acid method.²¹⁾ Mycelial growth was estimated by weighing dried mycelia in the following way. The mycelia obtained from a part (10 ml) of a culture broth by filtration was washed with 0.15 M NaCl on a glass filter, dried at 110°C for 20 min on a sheet of aluminium foil of known weight, and then weighed on a chemical balance.

Polyacrylamide disc gel electrophoresis was done in 7.5% polyacrylamide gels (pH 4.3) by the method of

Davis.²²⁾ Protein was stained with Coomassie brilliant blue G-250.

Cultivation. *A. oryzae* mycelia were grown in submerged culture with various carbohydrates and nitrogen sources. The basal medium contained the following in 1 l of water (% w/v): carbohydrate, 2.0; nitrogen source, 0.5; yeast extract, 0.3; MgSO₄·7H₂O, 0.05; KH₂PO₄, 0.05. The pH was adjusted to pH 5.0 by adding 1 M HCl and fermentation was done at 30°C with shaking (110 rpm) in 500 ml shake flasks containing 200 ml of medium.

Purification and characterization of 1,2- α -mannosidase. Affinity purification of 1,2- α -mannosidase was done on a mannan-gel column exactly as described previously.¹⁷⁾ Characterization of 1,2- α -mannosidase was also done as described previously.³⁾

Results

Production of 1,2- α -mannosidase

The influence of several kinds of carbon sources on 1,2- α -mannosidase production was examined (Fig. 1A). Among the carbon sources tested, baker's yeast mannan gave a high level of the intracellular 1,2- α -mannosidase activity. When cultured with any other carbon source, only a low level of 1,2- α -mannosidase activity was detected in the culture product, although the protein production was excellent compared to that observed for the mannan medium. These results are summarized in

Table I. GLYCOSIDASE AND PROTEIN PRODUCTION BY *A. oryzae* IN THE PRESENCE OF DIFFERENT CARBOHYDRATE SOURCES

	Carbohydrate source			
	Glucose	Starch	Mannose	Mannan
1,2- α -Mannosidase (munits)	27	42	2.0	200
α -Mannosidase (munits)	1.6	0.02	— ^a	0.02
β -Galactosidase (units)	1.0	0.1	ND ^b	0.1
β -N-Acetylhexosaminidase (units)	6.7	2.4	ND ^b	1.8
Amylase (units)	136	163	ND ^b	90
Sucrase (units)	33	44	ND ^b	24
Protein (mg)	31	36	32	8.1

After 4 days' cultivation, the culture products were assayed as described in the text. Cultivation conditions are described in Fig. 1. Values were obtained from 1 l culture.

^a Not detected. ^b Not determined.

* One unit defined here corresponded to about 2.5 units of the enzyme amount previously defined using α -D-Man-(1 $\rightarrow$ 2)-D-Man as substrate.¹⁷⁾

Table I. It is clear that the poor protein production in the mannan medium was favorable for an increase in the specific activity of 1,2- α -mannosidase in the culture product. The features of the protein production were consistent with those of the mycelial growth and the carbohydrate consumption in the culture media (Figs. 1B and 1C). Mannan as the carbon source appeared to be reluctantly consumed and this might be also the case for the mannan/glucose mixture carbon source. The effects of the mannan concentration on the 1,2- α -mannosidase production were examined (Fig. 2). The enzyme activity increased in proportion to the concentration of mannan added to the medium up to 1.5%. However, raising its concentration beyond 2% resulted in a decrease of all the enzymes, including 1,2- α -

mannosidase.

The yeast extract in the medium was also examined for its effect on *A. oryzae* cultivation (not illustrated). Raising the yeast extract concentration beyond 0.3% increased considerably the protein production and promoted the mycelial growth. In contrast no significant increase in 1,2- α -mannosidase activity was observed with its increasing concentration, resulting in a lower specific activity of 1,2- α -mannosidase in the culture product.

The influence of the nitrogen source on the 1,2- α -mannosidase production was also studied by replacing peptone with other inorganic sources, potassium nitrate, urea, and ammonium sulfate in varying concentrations (not illustrated). The maximum production of 1,2- α -mannosidase was found with 0.5%

Fig. 1. (A) Effects of Various Carbon Sources on *A. oryzae* 1,2- α -Mannosidase Production. (B) Effects of Various Carbon Sources on *A. oryzae* Mycelial Growth. (C) Carbohydrate Consumption by *A. oryzae*.

The medium contained 2% carbohydrate, 0.5% peptone, 0.3% yeast extract, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.05% KH_2PO_4 . ●, mannan; ○, glucose; ◐, mannose; ●, starch; ◐, glucose-mannan (1:1, w/w). Detailed procedures are described in the text.

ammonium sulfate.

Purification of 1,2- α -mannosidase

An attempt was made to purify the 1,2- α -mannosidase produced under the most favorable conditions. After 4 days of cultivation, the mycelia separated from the culture broth (1 l) by filtration was suspended in 20 mM acetate buffer (pH 5.0, 500 ml) containing 2.0 M NaCl and exposed to 20 kHz sonic irradiation for 5 min with external cooling in an ice-water bath. The resulting turbid solution was centrifuged and the clear supernatant was dialyzed

against the same buffer for 2 days, then concentrated to about 1 ml by ultrafiltration.

The concentrate (9.5 mg protein) was chromatographed on a mannan-gel column. As shown in Fig. 3, 1,2- α -mannosidase was retarded on the column and clearly separated from the main protein peak. However, when the active fractions indicated by a bar were combined and concentrated to a small volume, traces of α -amylase and β -N-acetylhexosaminidase activities were still detected in the concentrate. By repeating this chromatography under the same conditions these enzyme contaminants were completely removed without appreciable loss of the 1,2- α -mannosidase (Table II). The enzyme preparation thus purified was also free from protease activity and showed a single band on disc gel electrophoresis.

Fig. 2. Effects of Mannan Concentration on *A. oryzae* 1,2- α -Mannosidase Production.

The medium contained 0.5% ammonium sulfate, 0.3% yeast extract, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% KH_2PO_4 , and various amounts of baker's yeast mannan. After 4 days on a reciprocal shaker at 30°C, the enzymes in the culture products were assayed.

Fig. 3. The First Affinity Chromatography on Yeast Mannan-gel of the 1,2- α -Mannosidase Crude Extract.

●, 1,2- α -mannosidase; ---, 280 nm absorbance. Detailed procedures are described in the text.

Table II. PURIFICATION OF *A. oryzae* 1,2- α -MANNOSIDASE IN LIQUID CULTURE

Step	1,2- α -Mannosidase			Amylase (units)	Sucrase (units)	β -N-Acetyl- hexosaminidase (units)	Total protein (mg)
	Total activity (units)	Specific activity (units/mg)	Yield (%)				
Crude enzyme	0.22	0.023	100	100	26.5	20.5	9.5
1st Affinity chromatography	0.19	4.5	86	+ ^a	— ^b	+ ^a	0.042
2nd Affinity chromatography	0.16	5.5	73	— ^b	— ^b	— ^b	0.029

For the assay procedures see the text. Values were obtained from 1 l culture.

^a Trace activity. ^b Not detected.

Properties of 1,2- α -mannosidase

This 1,2- α -mannosidase was compared in some properties with the 1,2- α -mannosidase previously purified from the enzyme product "Taka-diaxase" of *A. oryzae*. However, there were no appreciable differences in their substrate specificities, optimum pH, or stability.

Discussion

A. oryzae 1,2- α -mannosidase was found for the first time as an extracellular enzyme in a culture product on wheat bran.³⁾ As a preliminary to the use of yeast mannan as a carbon source, therefore, *A. oryzae* was first cultivated with yeast mannan on wheat bran in solid culture. However, no stimulation effect on the production of extracellular 1,2- α -mannosidase was observed for yeast mannan. Because of some technical difficulties in extracting the enzyme from the mycelia grown in this solid culture, the level of the intracellular 1,2- α -mannosidase activity could not be found.

When yeast mannan was used as the carbon source in liquid culture, a significant increase in the intracellular 1,2- α -mannosidase activity was observed, though no detectable amount of the extracellular 1,2- α -mannosidase was found in culture filtrate. The reason for the lack of extracellular 1,2- α -mannosidase in the liquid culture product remains unclear.

The lowering of the enzyme production in the culture medium containing over 2% of yeast mannan appeared to be due to the increase in the viscosity of the medium.

An attempt was made to raise the enzyme level by growing the mycelia in a medium containing both mannan and glucose as the carbon sources, because yeast mannan did not seem to be an effective carbon source for the mycelial growth. The mixture carbon source, however, gave an unexpectedly low level of 1,2- α -mannosidase activity. This result, when considered with the feature of the carbohydrate consumption in the mannan/glucose medium (Fig. 1C), suggests that mannan was scarcely utilized by *A. oryzae* in the presence of glucose.

Since the *A. oryzae* 1,2- α -mannosidase previously characterized was a product in solid culture,³⁾ we felt much interest in comparing the properties of these two enzymes. However, no appreciable differences were found between them, suggesting that they may be virtually the same enzyme.

Our inducing method for 1,2- α -mannosidase production has several advantages. Yeast mannan can be readily and abundantly prepared from baker's yeast and it is also commercially available at a reasonable price. Further, it should be noted that mannan as the carbon source depresses the production of proteins other than 1,2- α -mannosidase. It is obvious that this effect of mannan makes it much easier to purify the 1,2- α -mannosidase from the culture broth. The proposed cultural method, when combined with the affinity purification procedure using mannan-gel^{1,7)} may be expected to make this enzyme a more convenient reagent.

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