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A 1,2-α-D-mannosidase from a *Bacillus* sp.: purification, characterization, and mode of action †,‡

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ABSTRACT

A 1,2-α-D-mannosidase was purified to homogeneity from the culture supernatant of *Bacillus* sp. M-90, which was isolated from soil by enrichment culture on baker's yeast mannan. The purified enzyme had M_r 380000 Da, and was comprised of two apparently identical 190000 Da subunits. It had a neutral optimum pH (7.0) and an isoelectric point of 3.6. The enzyme was highly specific for α1,2-linked D-mannose oligosaccharides. An *N*-linked high-mannose type oligosaccharide, $Man_9GlcNAc_2$, was a good substrate, yielding $Man_5GlcNAc_2$, and the α1,2-linked side chains of *Saccharomyces cerevisiae* mannan were also specifically hydrolyzed by the enzyme. *p*-Nitrophenyl α-D-mannopyranoside and 1,2-α-D-mannobiitol were not hydrolyzed at all. Calcium ion, 1-deoxymannojirimycin, and swainsonine had no effect on the enzyme, but the activity was completely inhibited by EDTA. The mode of action on α1,2-linked mannotetraose indicated that the enzyme is an exo-1,2-α-D-mannanase.

INTRODUCTION

Among the various known α-D-mannosidases (EC 3.2.1.24), those with high specificity for 1,2-α-D-mannosidic linkages are especially important, not only for the study of the processing, secretion, and sorting of glycoproteins[1–5], but also for the analysis of the structure of high-mannose type sugar chains[6,7].

Previously, we reported some fungal 1,2-α-D-mannosidases (*Aspergillus*[8], *Basidiomycete*[9], and *Penicillium*[10]) which hydrolyze the α1,2-linked side chains of yeast mannan as well as *N*-linked high-mannose type oligosaccharides. Now, to obtain a highly active, specific, and stable 1,2-α-D-mannosidase, we surveyed soil bacteria and found a new enzyme of this group in one isolate. Several bacterial α-D-mannosidases or α-D-mannanases that act on yeast mannan[11–15] have been reported.

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‡ Dedicated to Professor C.E. Ballou.

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In this paper, we describe the purification, characterization, and mode of action of the 1,2-α-D-mannosidase from *Bacillus* sp. M-90.

EXPERIMENTAL

Materials.—Mannans from *Saccharomyces cerevisiae* wild type and its mutant were obtained from laboratory collections[16,17] that were originally donated by Dr. C.E. Ballou, University of California at Berkeley. The mannooligosaccharides Man(α1-2)Man, Man(α1-3)Man, Man(α1-6)Man, Man(α1-2)Man(α1-2)Man(α1-2)Man, Man(α1-3)Man(α1-2)Man(α1-2)Man, Man(α1-2)Man(α1-2)Man-ol (4,2-α-D-mannotriitol'), and Man(α1-2)Man-ol were also prepared in the course of previous work[12,16,18]. Pyridylaminated *N*-linked high-mannose type oligosaccharides, namely $Man_9GlcNAc_2$-PA [*N*-(2-pyridyl)oligosaccharide glycamines], $Man_6GlcNAc_2$-PA, $Man_5GlcNAc_2$-PA, and $Man_3GlcNAc_2$-PA, were purchased from Takara Biochemicals Co. All other materials were available from usual commercial sources.

Microorganism and enzyme production.—The medium for isolating 1,2-α-D-mannosidase-producing bacteria contained 0.2% of *S. cerevisiae* mannan as a sole carbon source in a mixture containing 0.002% $FeSO_4$, 0.04% $MgSO_4 \cdot 7H_2O$, 0.006% $CaCl_2 \cdot 2H_2O$, 0.75% K_2HPO_4, 0.23% KH_2PO_4, and 0.1% yeast extract in 1 L of water at pH 7.0. A few drops of aqueous extract of humic soil was incubated in the medium for 24 h at 30°C. After several transfers, the 1,2-α-D-mannosidase activity of the culture filtrate was assayed, and a strain (M-90) that was found to produce the highest activity was characterized according to Bergy's Manual[19]. The bacterium is a Gram-positive, aerobic rod with several peritrichous flagellae, and it forms an endospore, indicating that it is a *Bacillus*.

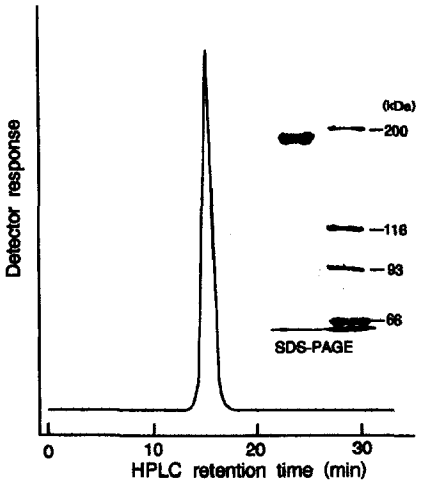

Fig. 1. Analysis of the purity of 1,2-α-D-mannosidase by HPLC and SDS-PAGE.

TABLE I

Purification of 1,2-α-D-mannosidase

Purification step	Total protein (mg)	Total activity (μkat)	Specific activity (mkat/kg)	Purification (fold)	Yield (%)
Culture filtrate	93	1.3	14	1.0	100
DEAE-Toyopearl 650S (1st)	8.7	1.0	120	8.6	81
DEAE-Toyopearl 650S (2nd)	6.0	0.9	140	10	66
Toyopearl HW-55F	2.4	0.6	250	18	47
Hydroxyapatite	1.2	0.3	250	18	23

The *Bacillus* sp. M-90 was grown in the mannan-salts medium at 30°C for 48 h with shaking. At this time, the production of 1,2-α-D-mannosidase had reached a maximum. The steps of the enzyme purification were carried out below 4°C.

Enzyme assays.—The assay mixture for 1,2-α-D-mannosidase contained 200 μg of either 1,2-α-D-mannotriitol or *S. cerevisiae* wild-type mannan, 0.1 mL of 0.4 M phosphate buffer, pH 7.0, 0.01–0.1 mL of enzyme solution, and distilled water to 1 mL. The mixture was incubated at 40°C for 10 min, then the reducing sugar was measured by the method of Nelson–Somogyi[20]. One unit (16.67 nkat) of enzyme is defined as the amount that liberates 1 μmol of reducing sugar equivalent, expressed as mannose, per min under the standard conditions.

General methods.—Gel electrophoresis was preformed in 7.5% polyacrylamide at pH 8.9 according to Davis[21], and SDS gel electrophoresis was carried out by the method of Laemmli[22]. Gel electrofocusing for determination of the isoelectric point (p*I*) of the enzyme was performed by the method of Wringley[23], and gel filtration for the determination of the molecular weight was done on a Shodex

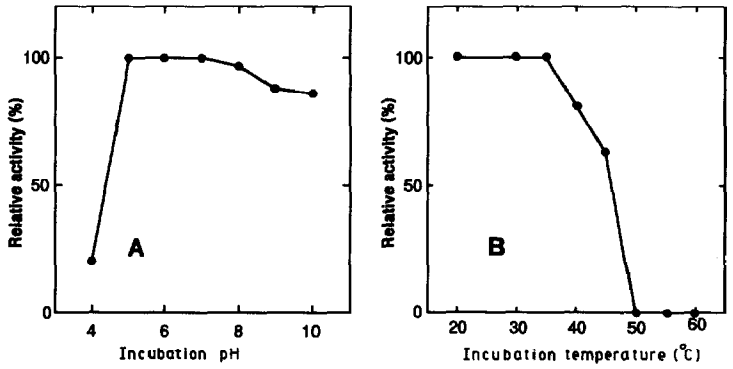

Fig. 2. Effect of pH and temperature on the stability of 1,2-α-D-mannosidase. A, pH stability; the enzyme was incubated at 4°C for 24 h in 0.05 M Britton–Robinson universal buffer at various pH, then the remaining activity was assayed under the standard conditions. B, thermostability; the enzyme was heated at various temperatures for 10 min in 0.05 M phosphate buffer, pH 7.0. After rapid cooling, the remaining activity was assayed under the standard conditions.

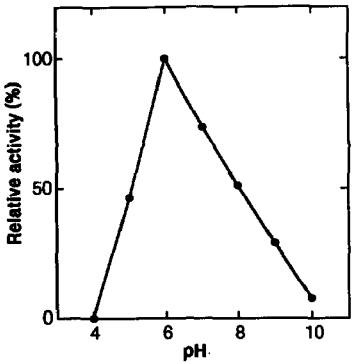

Fig. 3. pH Optimum of the 1,2-α-D-mannosidase. The enzyme activity was assayed under the standard conditions except that Britton–Robinson universal buffer[29] at various pH was used.

WS-803F column with a Waters M-45 chromatograph. Separation of pyridylaminated sugar derivatives was accomplished on a TSKgel Amide-80 column with a Shimadzu LC-6A chromatograph equipped with a fluorescence detector. Total carbohydrate was determined by the method of Dubois et al.[24] and protein concentration was estimated by the method of Lowry et al.[25]. Gas phase hydrolysis of enzyme protein was carried out in 7 N HCl containing 10% CF_3CO_2H and 0.1% phenol at 155°C for 45 min according to the method of Tsugita et al.[26]. The amino acids were analyzed with a Beckman SYSTEM GOLD. The N-terminal amino acid sequence was determined with an Applied Biosystems 477A gas-phase sequencer equipped with a PTH analyzer. ^1H NMR spectra of the samples were recorded at 400 MHz on a Jeol GSX 400 spectrometer at 50°C.

TABLE II

Effect of metal ions and various reagents on the activity of 1,2-α-D-mannosidase

Reagent (1 mM)	Relative activity (%)
None	100
$CdCl_2$	100
$CoCl_2$	100
$FeCl_2$	100
$CaCl_2$	100
$MgCl_2$	93
$ZnCl_2$	93
$MnCl_2$	83
$CuCl_2$	61
$NiCl_2$	48
$HgCl_2$	10
EDTA	0
N-Ethylmaleimide	100
1-Deoxymannojirimycin	100
Swainsonine	100

TABLE III

Amino acid composition of 1,2-α-D-mannosidase

Amino acid [a]	Residues per 380000 Da (nearest integer)
Asp	432
Glu	314
Ser	253
Thr	296
Arg	123
Gly	386
Ala	484
Pro	160
Tyr	226
Val	279
Met	14
Ile	114
Leu	238
Phe	139
Cys	[b]
Lys	111
His	45

[a] Tryptophan was not determined. [b] Not detected

RESULTS AND DISCUSSION

Purification of 1,2-α-D-mannosidase.—The purification steps are summarized in Table I. The enzyme, purified 18-fold over the culture filtrate, was obtained in 23% yield with a specific activity of 250 mkat per kg protein. The purified enzyme

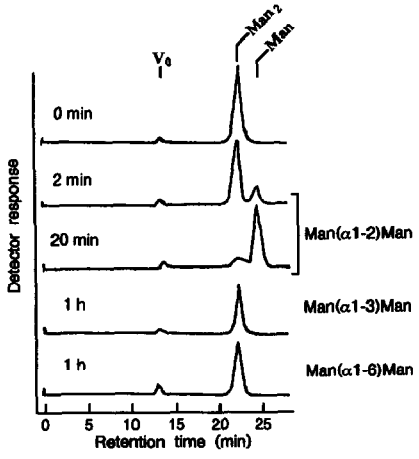

Fig. 4. Susceptibility of α-linked mannobioses to the 1,2-α-D-mannosidase. The hydrolysis products were separated on a gel filtration column of TSKgel G2000PW with a Waters HPLC. Time of incubation is indicated above each trace.

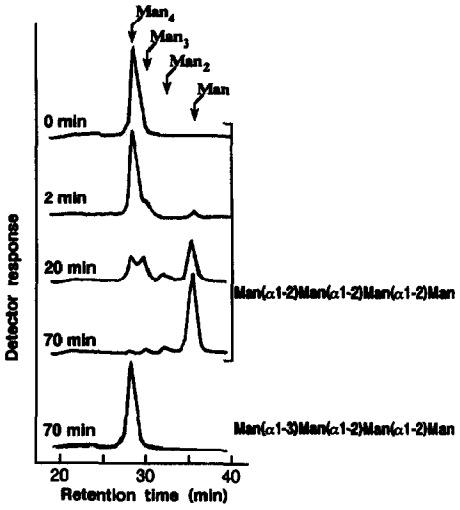

Fig. 5. Time course of hydrolysis of 1,2-α-D-mannotetraose followed by separation on TSK gel G2000PW.

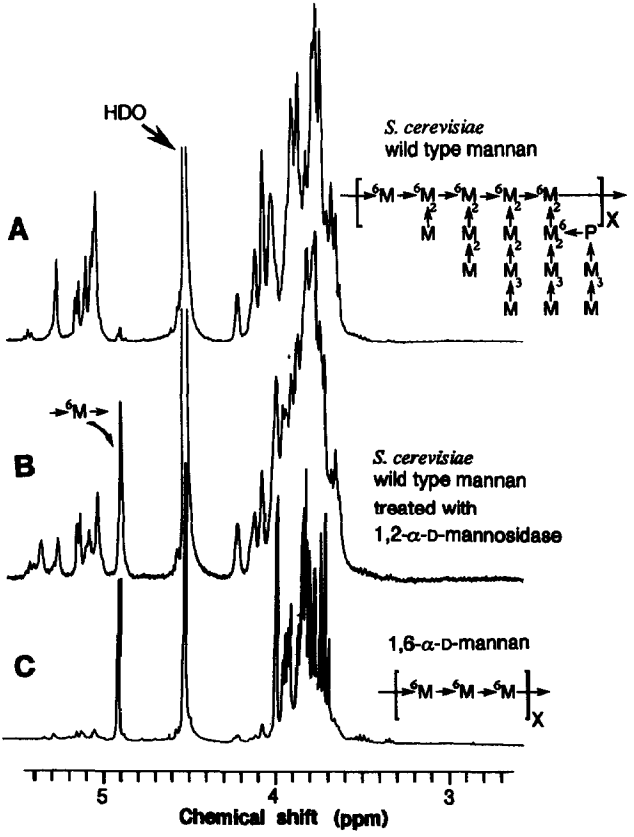

Fig. 6. ^{1}H NMR spectra of the native and the enzyme-digested mannans from *Saccharomyces cerevisiae* wild type. A 1,6-α-D-mannan was used as a control.

gave a single protein band on SDS-PAGE, and HPLC analysis showed a single symmetrical protein peak (Fig. 1). The 1,2-α-D-mannosidase activity comigrated with the protein both on PAGE (nondenaturing) and HPLC.

General properties.—The effect of pH on the stability of the purified enzyme is shown in Fig. 2A. The enzyme was stable in the pH range 5 to 8. The purified enzyme was stable at 4°C for at least 10 months in 0.1 M potassium phosphate buffer at pH 7.0, and to over 35°C on heating for 10 min, but it was rapidly denatured at temperatures over 45°C (Fig. 2B). The highest activity of the enzyme was found at pH 6.0 (Fig. 3) under the standard conditions. The effects of various inhibitors and divalent cations on the enzyme activity are shown in Table II. The activity was inhibitied significantly by Hg, Ni, and Cu. EDTA caused complete inhibition of the activity while a mannose analogue, 1-deoxymannojirimycin[27] and swainsonine[28], an inhibitor of glycoprotein processing mannosidases, showed no effect.

Physical and chemical properties.—The molecular weight of the enzyme was estimated as approximately 190000 by SDS-PAGE (Fig. 1) and 380000 by gel filtration on HPLC. These results indicate that the enzyme protein is a dimer of

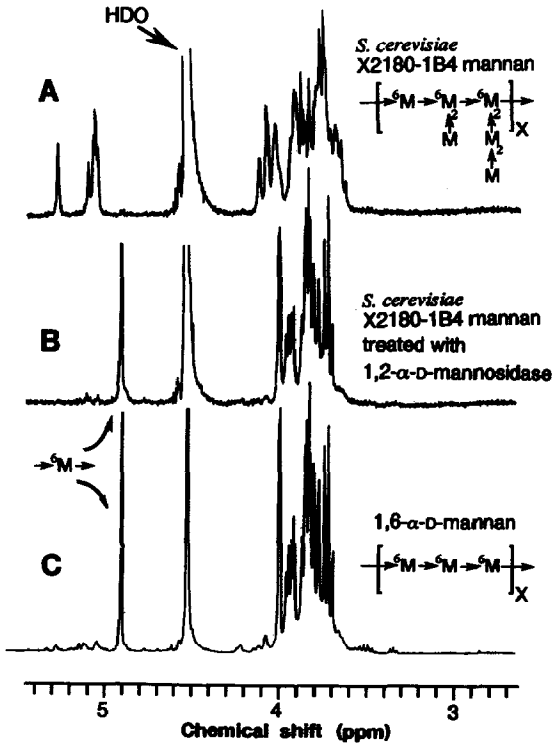

Fig. 7. ^{1}H NMR spectra of the mutant mannan and the residue produced by the mannosidase treatment. A 1,6-α-D-mannan was used as a control.

apparently identical subunits of M_r 190kDa. The N-terminal amino acid sequence (residues 1 to 7) was determined to be Ala-Ala-Gly-Gly-Val-Phe-Ala and the isoelectric point (p*I*) was found to be 3.6. In the amino acid composition of the enzyme (Table III), the prominent amino acids are Ala, Asp, Gly, and Glu. The high proportion of Asp and Glu accounts for the acidic character of the enzyme, as revealed by its low p*I*.

Mode of action.—Fig. 4 shows the HPLC analysis of the products formed by the action of the enzyme on 1,2-α-, 1,3-α-, and 1,6-α-D-mannobioses. The enzyme hydrolyzed only 1,2-α-D-mannobiose. *p*-Nitrophenyl α-D-mannoside was not cleaved by this enzyme. Reduced 1,2-α-D-mannobiose and α1,2-linked mannotetraose containing an α1,3-linked nonreducing terminal mannose residue [Man(α1-3)Man(α1-2)Man(α1-2)Man] (Fig. 5) were not hydrolyzed at all.

The product distribution at different times during the hydrolysis of 1,2-α-D-mannotetraose by the enzyme is shown in Fig. 5. In the early stage, nonreducing terminal mannose was removed from the mannotetraose to give mannotriose. Then, mannotriose was hydrolyzed to mannobiose and mannose. Finally, the α1,2-linked mannotetraose was completely hydrolyzed, and only mannose was produced as a final product. These results suggest that the mannosidase is an

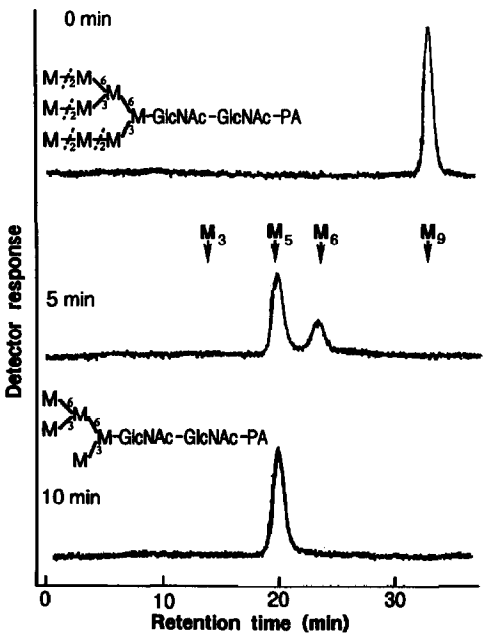

Fig. 8. Size-fractionation HPLC of the mannosidase digests of $Man_9GlcNAc_2$. The arrows mark the elution positions of the reference oligosaccharides $Man_9GlnNAc_2$-PA (M_9); $Man_6GlcNAc_2$-PA (M_6); $Man_5GlcNAc_2$-PA (M_5); and $Man_3GlcNAc_2$-PA(M_3). Time of incubation is indicated above each trace.

exo-type enzyme with strict specificity for the α1,2-mannosidic linkage. This conclusion is supported by the following additional data.

The enzyme acted on the α1,2-linked side chains of yeast mannan. ^{1}H NMR spectra of the *S. cerevisiae* mannan showed that the signal at 4.9 ppm, assigned to H-1 of α1,6-linked mannose, was markedly enhanced after digestion by the enzyme, while signals at 5.3 and 5.1 ppm that were derived from α1,2-mannosidic linkages were reduced in intensity (Fig. 6). When a mutant mannan that has exclusively α1,2-mannosyl side chains along an α1,6-mannosyl backbone was used a substrate, the enzyme completely removed the side chains and an unbranched 1,6-linked mannan was produced (Fig. 7). Finally, an *N*-linked high-mannose type oligosaccharide, $Man_9GlcNAc_2$, was hydrolyzed to yield $Man_5GlcNAc_2$, indicating that the enzyme specifically removed α1,2-linked mannosyl residues (Fig. 8). This result shows that the *Bacillus* mannosidase has the same substrate specificity for $Man_9GlcNAc_2$ as Golgi mannosidase IA[4].

These properties of the *Bacillus* 1,2-α-D-mannosidase should be useful for structural studies of high-mannose type sugar chains as well as for the elucidation of the biological functions of glycoproteins.

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