Molecular and enzymic properties of recombinant 1,2-α-mannosidase from *Aspergillus saitoi* overexpressed in *Aspergillus oryzae* cells

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For the construction of an overexpression system of the intracellular 1,2-α-mannosidase (EC 3.2.1.113) gene (*msdS*) from *Aspergillus saitoi* (now designated *Aspergillus phoenicis*), the N-terminal signal sequence of the gene was replaced with that of the aspergillopepsin I (EC 3.4.23.18) gene (*apnS*) signal, one of the same strains as described previously. Then the fused 1,2-α-mannosidase gene (f-*msdS*) was inserted into the *Not*I site between P-No8142 and T-*agdA* in the plasmid pNAN 8142 (9.5 kbp) and thus the *Aspergillus oryzae* expression plasmid pNAN-AM1 (11.2 kbp) was constructed. The fused f-*msdS* gene has been overexpressed in a transformant *A. oryzae* niaD AM1 cell. The recombinant enzyme expressed in *A. oryzae* cells was purified to homogeneity in two steps. The system is capable of making as much as about 320 mg of the enzyme/litre of culture. The recombinant enzyme has activity with methyl-2-*O*-α-D-mannopyranosyl α-D-mannopyranoside at pH 5.0, while no activity was determined with methyl-3-*O*-α-D-mannopyranosyl α-D-mannopyranoside or methyl-6-*O*-α-D-mannopyranosyl α-D-mannopyranoside. The substrate specificity of the enzyme was analysed by using pyridylaminated (PA)-oligomannose-type sugar chains, $Man_{9-6}(GlcNAc)_2$-PA (Man is mannose; GlcNAc is *N*-acetylglucosamine). The enzyme hydrolysed $Man_8GlcNAc_2$-PA (type 'M8A') fastest, and 'M6C' {Manα1-3[Manα1-2Manα1-3(Manα1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-PA} slowest, among the PA-sugar chains. Molecular-mass values of the enzyme were determined to be 63 kDa by SDS/PAGE and 65 kDa by gel filtration on Superose 12 respectively. The p*I* value of the enzyme was 4.6. The N-terminal amino acid sequence of the enzyme was GSTQSRADAIKAAFSHAWDGYLQY, and sequence analysis indicated that the signal peptide from *apnS* gene was removed. The molar absorption coefficient, ε, at 280 nm was determined as 91 539 $M^{-1} \cdot cm^{-1}$. Contents of the secondary structure (α-helix, β-structure and the remainder of the enzyme) by far-UV CD determination were about 55, 38 and 7% respectively. The melting temperature, T_m, of the enzyme was 71 °C by differential scanning calorimetry. The calorimetric enthalpy, ΔH_{cal}, of the enzyme was calculated as 13.3 kJ·kg of protein^{-1}. Determination of 1 g-atom of Ca^{2+}/mol of enzyme was performed by atomic-absorption spectrophotometry.

Key words: *Aspergillus oryzae*, *Aspergillus saitoi*, 1,2-α-mannosidase, overexpression, recombinant α-mannosidase.

INTRODUCTION

The importance of α-mannosidase (EC 3.2.1.24) in the processing system of glycoproteins in higher eukaryotic cells is well known [1–4]. In mammalian cells, 1,2-α-mannosidases play an essential role in the early steps of *N*-linked oligosaccharide maturation [5]. In the Enzyme Nomenclature Recommendations [6], three types of α-mannosidases, namely α-D-mannoside mannohydrolase (EC 3.2.1.24), 1,2-α-mannosyl-oligosaccharide α-D-mannohydrolase (EC 3.2.1.113) and 1,3-(1,6)-mannosyl-oligosaccharide α-D-mannohydrolase (EC 3.2.1.114), are given as systematic names.

Jelinek-Kelly and Herscovics [7] first purified the yeast (*Saccharomyces cerevisiae*) specific processing α1,2-mannosidase that trims $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$. The processing α1,2-mannosidase, which removes one specific mannose residue from $Man_9GlcNAc_2$ to form $Man_8GlcNAc_2$, contains five cysteine residues, three of which are conserved [8]. Camirand et al. cloned the specific α1,2-mannosidase gene *MNS1* [9]. Herscovics et al. [10] observed that the yeast α1,2-mannosidase Mns1p is 35% identical in amino acid sequence to a mammalian α1,2-mannosidase cDNA (IA) which was being cloned by Lal et al. [11]. This similarity demonstrated the existence of an α-

Abbreviations used: DSC, differential scanning calorimetry; Man, mannose; OMe, methyl ester; GlcNAc, N-acetylglucosamine); -PA, pyridylaminated; M9A, Manα1-2Manα1-2Manα1-3[Manα1-2Manα1-3(Manα1-2Manα1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-PA; M8A, Manα1-2Manα1-2Manα1-3[Manα1-3(Manα1-2Manα1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-PA; M8B, Manα1-2Manα1-3[Manα1-2Manα1-3(Manα1-2Manα1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-PA; M8C, Manα1-2Manα1-2Manα1-3[Manα1-2Manα1-3(Manα1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-PA; M7A, Manα1-2Manα1-3[Manα1-3(Manα1-2Manα1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-PA; M7B, Manα1-2Manα1-2Manα1-3[Manα1-3(Manα1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-PA; M7D, Manα1-2Manα1-3[Manα1-2Manα1-3(Manα1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-PA; M6A, Manα1-3[Manα1-3(Manα1-2Manα1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-PA; M6B, Manα1-2Manα1-3[Manα1-3(Manα1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-PA; M6C, Manα1-3[Manα1-2Manα1-3(Manα1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-PA; M5A, Manα1-3[Manα1-3(Manα1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-PA; Manα1-2Man-OMe, methyl 2-*O*-α-D-mannopyranosyl α-D-mannopyranoside or α-D-Man-[1→2]-α-D-Man-1→OMe; Manα1-3Man-OMe, methyl-3-*O*-α-D-mannopyranosyl α-D-mannopyranoside or α-D-Man-[1→3]-α-D-Man-1→OMe; Manα1-6Man-OMe, methyl-6-*O*-α-D-mannopyranosyl α-D-mannopyranoside or α-D-Man-[1→6]-α-D-Man-1→OMe.

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mannosidase family that has been conserved through eukaryotic evolution. In contrast with the yeast α1,2-mannosidase [7], the two mouse α1,2-mannosidases IA and IB localize in the Golgi when expressed in cells in culture, and both can remove up to four α1,2-linked mannose residues from $Man_9GlcNAc$ [12]. On the basis of sequence similarity, Moremen et al. designated this family of enzymes Class I α-mannosidases [4].

We have described the purification to homogeneity of α-D-mannosidases from fungi *Aspergillus saitoi* (*A. phoenicis*) [13,14], *Penicillium citrinum* [15,16] and *Pycnoporus coccineus* [17] specifically capable of releasing α(1-2)-linked mannose residues from oligosaccharides derived from asparagine-linked glycans of glycoproteins. Ballou [18] isolated and characterized. the exo-α-1$\rightarrow$2-mannosidase from *A. saitoi* and examined its properties.

The predicted amino acid sequence of the 1,2-α-D-mannosidase from *A. saitoi* [19] is 70, 26 and 35% identical with those of *P. citrinum* 1,2-α-D-mannosidase [20], the yeast (*S. cerevisiae*) Man_9-specific α-mannosidase [7,8] and mouse Golgi 1,2-α-mannosidase [9] respectively. The amino acid sequences of several peptide regions, including aspartic acid and glutamic acid, in mannosidases from the fungi *A. saitoi* [19], *P. citrinum* [20] *S. cerevisiae* [7] and mammals (mouse) [9] bear striking similarities to each other. This finding may indicate that the mannosidases are coded for by evolutionarily related genes at the enzymic level. Site-directed mutagenesis of the fused 1,2-α-D-mannosidase gene (f-*msdS*) on the expression vector, pGAM-1 [19], was performed previously to determine the functional role of catalytic residues in the 1,2-α-D-mannosidase from *A. saitoi* expressed in yeast cells [21]. Replacement of Asp^{269} (the numbering is according to that used in a previous paper [19]) by glutamic acid and of the residues Glu^{273}, Glu^{411}, Glu^{414} and Glu^{474} to Asp altered the drastic decrease of specific activities with Man-α1-2Man-OMe and pyridylaminated $Man_9GlcNAc_2$ ($Man_9GlcNAc_2$-PA) as substrates and shifted the optimal pH of the mutant enzymes. Although we constructed an expression system for 1,2-α-mannosidase in yeast cells with expression vector pGAM-1 [21], that yeast expression system had very low efficiency. As discussed below, we have been working on enzymes and genes of intracellular 1,2-α-mannosidases, and describe here a highly efficient overexpression system of 1,2-α-D-mannosidase fusion gene (f-*msdS*) in *A. oryzae* cells as well as determination of the molecular and enzymic properties of the recombinant enzyme.

EXPERIMENTAL

Materials

A. saitoi (now designated *A. phoenicis* [18]) A.T.C.C. 14332 was used as a source of cDNAs of 1,2-α-D-mannosidase (3.2.1.113) gene (*msdS*, D49827) [19] and aspergillopepsin I (EC 3.4.23.18) gene (*apnS*, D25318) (22-24). *Escherichia coli* DH5α [*supE*44 Δ*lacU*169(ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1] was used for plasmid isolation and cloning. *A. oryzae* niaD, which is a nitrate reductase (EC 1.6.6.1)-gene (*niaD*)-deficient mutant derived from the wild-type strain RIB40 [25] and a high-level expression vector plasmid pNAN8142 (9.5 kb) [26], which contained α-glucosidase teminator T-*agdA* (D45179) [27], were used for transformation experiments. Plasmid pNAN-AM1 was made by inserting an oligonucleotide containing *Not*I site at the *Eco*RI site in pNAN8142 and using it for the transformation of *A. oryzae*. Czapek-Dox medium, consisting of 0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4,7H_2O$, 0.002% $FeSO_4,7H_2O$ and a 2% carbon source, adjusted to pH 5.5, was used as the minimum medium. Lysing enzyme was purchased from Sigma (St. Louis, MO, U.S.A.). Recombinant N-glycosidase F (lot 1 365 169) from *Flavobacterium meningosepticum* expressed in *E. coli* cells was purchased from Boehringer Mannheim, Tokyo, Japan. Nonidet P40 was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Manα1-2Man-OMe, Manα1-3Man-OMe and Manα1-6Man-OMe were purchased from Sigma. High-mannose type oligosaccharides ($Man_{9-5}GlcNAc_2$-PA) were purchased from Takara Shuzo Co. (Kyoto, Japan).

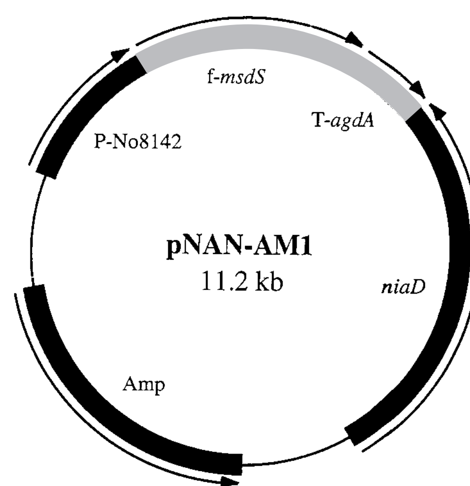

Figure 1 Plasmid pNAN-AM1

The fusion gene was constructed with the signal sequence *Not*1–*Bam*H1 fragment of aspergillopepsin I gene, *apnS*, and the *Bam*HI–*Not*1 fragment of *msdS* as reported [22]. The DNA of total 1.8 kb *Not*1 fragment from *msdS* fusion gene was inserted into the *Not*1 site between T-*agdA* and P-No8142 in pNAN8142 (9.5 kb); thus the expression plasmid pNAN-AM1 (11.2 kb) was constructed.

Construction of the expression plasmid for 1,2-α-D-mannosidase

For the construction of the fused gene (f-*msdS*), the N-terminal signal sequence of the 1,2-α-mannosidase gene (*msdS*) from *A. saitoi* was replaced with that of the aspergillopepsin I gene (*apnS*) signal gene as described in [19]. The fused gene f-*msdS* was conjugated with the signal sequence *Not*I–*Bam*HI fragment of aspergillopepsin I gene, *apnS*, and the the *Bam*HI–*Not*I fragment of the *msdS* gene, but lacked its signal-encoding sequence (nucleotides 1–105). The DNA of total 1.7 kb *Not*I fragment for f-*msdS* fusion gene was inserted into the *Not* I site between P-No8142 and T-*agdA* in pNAN8142 (9.5 kbp) [26], and thus the *A. oryzae* expression plasmid pNAN-AM1 (11.2 kbp) was constructed (Figure 1).

Fungal transformation

The f-*msdS* gene from *A. saitoi* has been overexpressed from a transformant *A. oryzae* niaD AM1 cell. The transformation of *A. oryzae* was performed basically according to the method of Gomi et al. [28]. In our experiment the nitrate reductase gene (*niaD*) was employed as the selectable marker. In brief, protoplasts were prepared by treating the 48h-cultured fresh mycelia of *A. oryzae* niaD with 0.5% of lysing enzyme (Sigma) at 30 °C for 2 h in a hypertonic solution (0.67 M NaCl/0.27 M $CaCl_2$). After filtration and centrifugation, the DNA of pNAN-AM1 was added to the protoplast, then the mixture was left at room temperature for 30 min in the presence of 50% poly(ethylene glycol) 4000. After centrifugation, the suspension of protoplast was mixed with hyper-Czapek–Dox soft agar (1.2 M sorbitol,

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0.3 % $NaNO_3$, 0.2 % KCl, 0.1 % KH_2PO_4, 0.05 % $MgSO_4$,$7H_2O$, 0.002 % $FeSO_4$,$7H_2O$, 2 % glucose and 0.6 % agar, pH 5.5) and spread out on to a hyper-Czapek-Dox plate (hyper-Czapek-Dox containing 2 % agar). To obtain transformants in a homokaryotic state, the conidia were selected four times on a Czapek-Dox plate (on this plate sorbitol was omitted). 1,2-α-D-Mannosidase activity in the culture supernatant was determined by culturing transformants in DPY medium (2 % dextrin, 1 % polypeptone, 0.5 % yeast extract, 0.5 % KH_2PO_4, and 0.05 % $MgSO_4$,$7H_2O$, pH 5.5) at 30 °C for 72 h. Culture filtrates were dialysed against 50 mM sodium acetate buffer, pH 5.0, then 1,2-α-D-mannosidase activity toward Manα1-2Man-OMe was measured at pH 5.0 as described in [21].

Genomic PCR

Mycelia of *A. oryzae* niaD AM1 from a 5 ml DPY (2 % dextrin/1 % polypeptone/0.5 % yeast extract/0.5 % K_2PO_4/ 0.05 % $MgSO_4$,H_2O) culture were freeze-dried. The dried mycelia were ground in a mortar to which sea sand was added. Genome DNA was extracted from the powdered cells basically by the method described by Boel et al. [29]. PCR was done using Primer 1 (ATGGTCGTCTTCAGCAAAACCGCTG/288–313 in *apnS* cDNA [22]) and Primer 2 (GATCCAATCCCGGTATGTT-TCCTG/1273–1296 in *msdS* cDNA [19]) was used to amplify part (1257 bp) of the 1,2-α-mannosidase fusion gene. Primer 3 (TATACAGACCATTCATCGATCATG/−21–3 in *nucS*) and Primer 4 (CTGACTCGCAATCAGATCCAAC/933–954 in *nucS*) were used to amplify part (975 bp) of the *A. oryzae* nuclease S_1 (EC 3.1.30.1) gene [30]. Reaction mixtures contained approx. 250 ng of the genomic DNA from *A. oryzae* niaD AM1, 100 pmol each of the primers (1 and 2, or 3 and 4), 200 μmol of dNTP, 50 mM KCl, 2 mM $MgCl_2$, 1mM 2-mercaptoethanol and *Taq* polymerase (1.25 units; Takara Shuzo Co., Kyoto) in 50 μl of Taps buffer, pH 9.3. In this experiment the two genes were amplified in separate tubes. PCR was done with the following thermal program: 40 s at 94 °C, 40 s at 62 °C and 1 min at 72 °C. We preliminarily confirmed that only the expected size of DNA was amplified in each PCR. The amount of the amplified DNA was measured basically by the method described by Teare et al. [31]. After PCR, 40 μl of the mixture was added to 2 ml of TE buffer (10 mM Tris/HCl, pH 7.5/1 mM EDTA) containing Hoechst 33258 [2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole, Sigma Chemical Co.] ($100\ ng \cdot ml^{-1}$) and 0.2 M NaCl, then fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 460 nm. In this method the fluorescence by Hoechst 33258 was proportional to the amount of dsDNA up to at least 1×10^{-6} g. A PCR mixture which did not contain the enzyme was used as a blank.

Production and purification of *Aspergillus* 1,2-α-D-mannosidase expressed in *A. oryzae*

The transformant strain AM1 of *A. oryzae* niaD with an expression plasmid pNAN-AM1 was cultured in 50 ml of DPY medium in a Sakaguchi flask at 30 °C for 3 days and the mycelia were separated by filtration (Toyo filter paper no. 2). The following operations were carried out below 4 °C unless otherwise noted. The filtrate was 75 %-saturated with $(NH_4)_2SO_4$ at pH 5.0, and centrifuged for 20 min at 27700 ***g***. The precipitate was dissolved in 20 ml of 10 mM sodium acetate buffer, pH 5.0. The solution was dialysed in 10 mM sodium acetate buffer, pH 4.0, and then applied to a column of a cation-exchange Resource S (1.6 cm × 3 cm, Pharmacia Biotech Inc.) equilibrated in 10 mM sodium acetate buffer, pH 4.0. Elution was performed with 0.8 M NaCl in this buffer at the same pH. The active enzyme fraction eluted from the Resource S column was dialysed against MilliQ water and then freeze-dried.

Enzyme assay

The transformants were grown in 50 ml of Czapek–Dox-P medium containing 2 % glucose as a carbon source at 30 °C for 3 days. A 2 ml portion of culture broth was filtered through Toyo filter paper no. 2, then dialysed at 4 °C against 10 mM acetate buffer, pH 5.0. The dialysed enzyme solution was assayed for 1,2-α-D-mannosidase activity at pH 5.0 with Manα1-2Man-OMe, Manα1-3Man-OMe and Manα1-6Man-OMe for specificity determination. The mannose from Manα1-2Man-OMe released by the enzymic reaction was stained by the Somogyi–Nelson method; 1 kat of 1,2-α-D-mannosidase activity was defined as the amount of enzyme required to liberate 1 mol of mannose from Manα1-2Man-OMe/s at 30 °C and pH 5.0.

HPLC analysis of PA-oligomannose-type sugar chains

Portions (300 ng) of the recombinant 1,2-α-mannosidases were incubated with 100 pmol of PA-oligosaccharides in 0.12 M sodium acetate buffer, pH 5.0, at 30 °C. The assay was stopped by boiling and the sample was filtered using an Ultrafree-MC centrifugal filter unit (0.22 μm-pore-size low-binding Durapore; Millipore Corp., Bedford, MA, U.S.A.). The filtrates were analysed by both HPLC with a Hitachi F-1050 fluorescence spectrophotometer, using a TSKgel ODS-$80T_M$ column (4.6 mm × 150 mm, Tosoh Corp., Tokyo, Japan), and with a Shimadzu RF-$10A_{XL}$ fluorescence spectrophotometer, using a TSKgel Amide-80 column (4.6 mm × 250 mm, Tosoh Corp.). The solvent and elution conditions used were described by Kondo et al. [32].

Deglycosylation

The purified and freeze-dried 1,2-α-D-mannosidase preparation (33 μg) was dissolved in 53.2 μl of 0.2 M sodium phosphate buffer, pH 8.6, containing 0.5 % SDS and 0.1 % 2-mercaptoethnanol. The heat-denaturation of the preparation was performed at 100 °C for 5 min. A 10 μl portion of the mixture, with 10 μl of 0.2 M sodium phosphate buffer, pH 8.6, and 5 μl of 7.5 % Nonidet P40 was added with 15 μl (3 units) of recombinant *Flavobacterium* N-glycosidase F dissolved in 100 mM sodium phosphate buffer, pH 7.2, containing 25 mM EDTA, 5mM sodium azide and 50 % (v/v) glycerol; after deglycosylation at 37 °C for 5 days, the deglycosylation reaction was stopped by heat treatment at 100 °C for 5 min, then the proteins were detected by SDS/PAGE.

Protein concentrations

Protein concentations were usually measured by the method of Lowry et al. [33] with BSA as a standard protein. The protein concentration of the purified and freeze-dried preparation of the enzyme was also determined by the molar absorption coefficient, ϵ, at 280 nm obtained by the previously described method [34].

Differential scanning calorimetry (DSC)

The freeze-dried 1,2-α-D-mannosidase was dissolved in distilled water. A 50 μl portion of the solution including 640 μg of the enzyme was sealed in a silver DSC cell. DSC was carried out four times using a DSC apparatus (Seiko SSC-560U) coupled with a thermal analysis data system (Seiko DS III) under the following

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conditions: temperature program of heating rate, 2 °C·min^{-1}; sensitivity, 100 μV; atmosphere, He, 40 ml·min^{-1}; data sampling rate, 0.5 s·data set^{-1}. Sample quantity in the cell was determined by DSC. The characteristic temperatures of the onset (T_o), the peak (T_p), and the recovery (T_r) in each DSC curve were recorded as described [35], and the melting enthalpy, ΔH_{cal}, was calculated from its peak area with correction for sample quantity.

CD measurements

Samples were dialysed in 20 mM sodium acetate buffer, pH 5.0, then diluted in the same buffer to 0.2 mg of protein/ml by adjusing the A_{280} to 0.2. The CD measurements were performed as described previously [36] using a Jasco J-720 spectropolarimeter. The path-length of optical cuvette was 1 mm for the measurement of the far- and near-UV regions. Contents of α-helix and β-structure of the enzyme were calculated according to the CONTIN Circular Dichroism Diskette.

Atomic-absorption-spectrophotometric analysis

Calcium analysis of the recombinant 1,2-α-mannosidase was done by atomic-absorption spectrophotometry using a Shimadzu model AA-660 (P/N206-1000-02) apparatus.

N-terminal sequence analysis

N-terminal amino-acid-sequence analysis was performed using an Applied Biosystems (ABi) 473 protein sequencer with an ABi 610A data-analysis system.

PAGE and SDS/PAGE

PAGE was carried out by the method of Davis [37] and SDS/PAGE by the method of Laemmli [38]. Gels were fixed and stained for 8 h in 0.25 % (w/v) Coomassie Brilliant Blue R-250, 50 % methanol, 10 % acetic acid solution and then destained with 40 % methanol/7 % acetic acid solution.

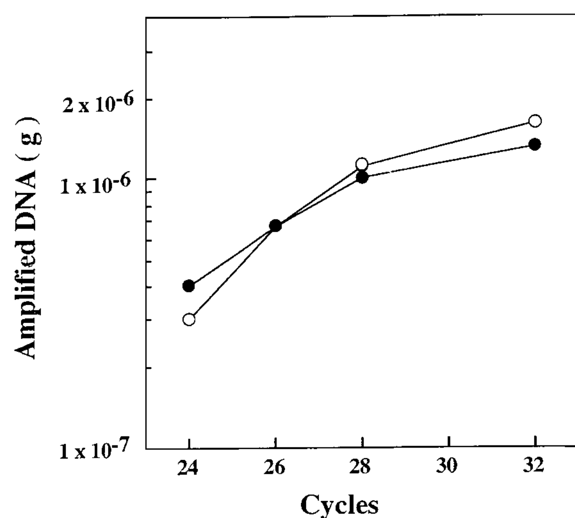

Figure 2 Measurements of PCR products

Partial DNA of *msdS* (●) or *nucS* (○) was amplified with the template of genomic DNA from *A. oryzae*. At the indicated intervals the amount of DNA in each reaction was measured by the fluorogenic method described in the Experimental section, and the increase was plotted. Amplified DNA was insufficient to measure below 24 h.

Gel filtration on Superose 12 with FPLC

For gel filtration on Superose 12, FPLC (Pharmacia Biotech, Sweden) was used to determine the molecular mass of the recombinant enzyme.

RESULTS

Expression of *A. saitoi* 1,2-α-D-mannosidase gene (*msdS*) in *A. oryzae*

A nitrate reductase gene, *niaD*, from a deficient mutant strain of *A. oryzae* niaD which includes an expression plasmid pNAN-AM1 (11.2 kb) (Figure 1) with this gene has been constructed in which the fused 1,2-α-mannosidase gene, f-*msdS*, has been overexpressed.

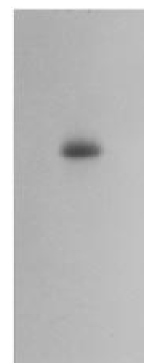

Figure 3 PAGE of the recombinant 1,2-α-D-mannosidase from *A. saitoi* overexpressed in *A. oryzae* cells

Gels were stained with Coomassie Brilliant Blue R-250 in both experiments.

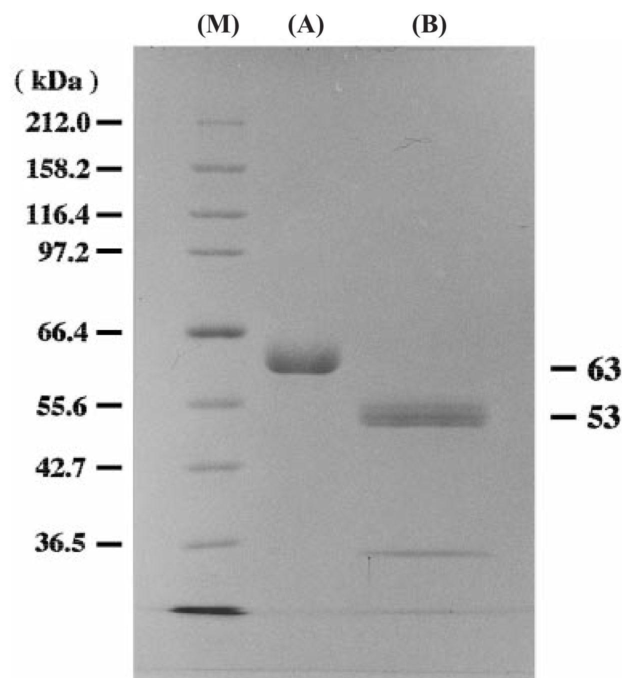

Figure 4 SDS/PAGE of the recombinant 1,2-α-D-mannosidase with or without N-glycosidase F from *F. meningosepticum*

(**M**) Molecular-mass markers (**A**) Without *F. meningosepticum* N-glycosidase. (**B**) With *F. meningosepticum* N-glycosidase F.

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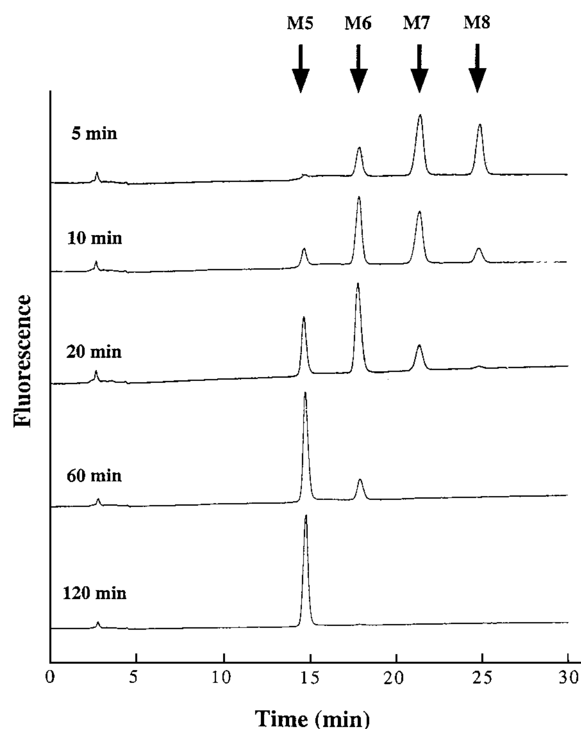

Figure 5 Degradation of high-mannose type of PA-oligosaccharide, $Man_8GlcNAc_2$-PA, by the recombinant 1,2-α-mannosidase from *A. saitoi* overexpressed in *A. oryzae* cells

The reaction mixture (50 μl) containing 1,2-α-mannosidase purified from *A. oryzae* niaD AM1 and 100 pmol of $Man_8GlcNAc_2$-PA in 1 mM sodium acetate buffer, pH 5.0, was incubated at 30 °C. The filtrates were analysed by both HPLC with a Hitachi F-1050 fluorescence spectrophotometer, using a TSKgek ODS-80TM column (4.6 mm × 150 mm; Tosoh Corp.) and with a Shimadzu RF-10AXL fluorescence spectrophotometer using a TSK gel Amide-80 column (4.6 mm × 250 mm; Tosoh Corp.). The solvent and elution conditions used were as described by Kondo et al. [32]. The arrows indicate the position of $Man_{8-5}GlcNAc_2$-PA.

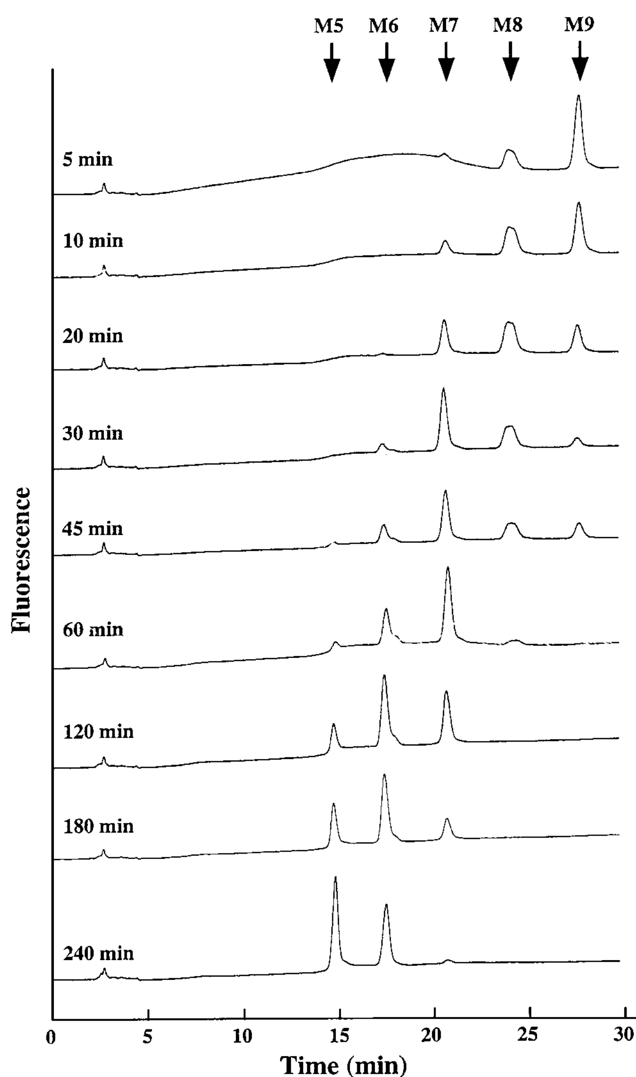

Figure 6 Degradation of high-mannose type PA-oligosaccharide, $Man_9GlcNAc_2$-PA, by the recombinant 1,2-α-mannosidase from *A. saitoi* expressed in *A. oryzae* cells

The reaction mixture (50 μl) containing 1,2-α-mannosidase purified from *A. oryzae* niaD AM1 and 100 pmol of $Man_9GlcNAc_2$-PA in 1 mM sodium acetate buffer, pH 5.0, was incubated at 30 °C. HPLC conditions were as described in the Experimental section. Arrows indicate the position of $Man_{9-5}GlcNAc_2$-PA.

After the transformation of *A. oryzae* niaD with the expression plasmid pNAN-AM1 (11.2 kb), a few colonies were grown on the minimal plate to obtain the conidia of homokaryon. The conidia of the homokaryons were selected four times on the minimal plate to obtain the condition of homokaryons. Finally, a single transformant was selected (strain AM1). To check that AM1 was not an 'abortive' transformant [39], PCR was performed with its genomic DNA. Using primers 1 and 2, which were complementary to the regions near the 5′-ends of the *apnS* and the *msdS* genes respectively, a single DNA of 1.3 kb was amplified (results not shown). The same size of DNA was made with the template of plasmid of pNAN-AM1, while no DNA fragment was amplified after the PCR when the DNA from the AM1 was the template. These results indicated that the *msdS* gene had been integrated into the genome of the *A. oryzae* AM1 strain.

A. oryzae is known to have a single copy of nuclease S_1 gene (*nucS*) in its genome [30]. To estimate the copy number of f-*msdS* in the strain AM1, we monitored the amplification of f-*msdS* and *nucS* during PCR (Figure 2). In our experiment, linearity of the exponential increase in the amount of DNA was maintained for both genes until the cycles reached 24–28. The relative ratio of DNA amplified from the two genes f-*msdS* and *nucS* at cycles 24, 26, 28 and 32 were 1.5, 1.0, 0.9 and 0.8 respectively. When counterbalancing a minute difference between the size of amplified DNA (1257 bp in f-*msdS* but 957 bp in *nucS*) the values were modified to 1.2, 0.8, 0.7 and 0.6 respectively (meaning a molar ratio between the two amplified DNA). From these data we assumed that the AM1 strain would have a single copy of the f-*msdS* gene in its genome.

The fused 1,2-α-mannosidase gene (f-*msdS*) from *A. saitoi* in the expression plasmid pNAN-AM1 has been overexpressed from a transformant *A. oryzae* niaD AM1 cell. *A. oryzae* strain AM1 was cultured in DPY medium at 30 °C, and the recombinant enzyme in the cultured filtrate was precipitated with $(NH_4)_2SO_4$ at 75% saturation, then put on to a cation-exchange Resource S column. Native PAGE indicated a homogeneous preparation of the recombinant 1,2-α-mannosidase (Figure 3). On SDS/PAGE a protein of molecular mass approx. 63 kDa for the recombinant enzyme is shown in Figure 4. Approx. 320 mg of pure enzyme was obtained from 1 litre of the culture filtrate.

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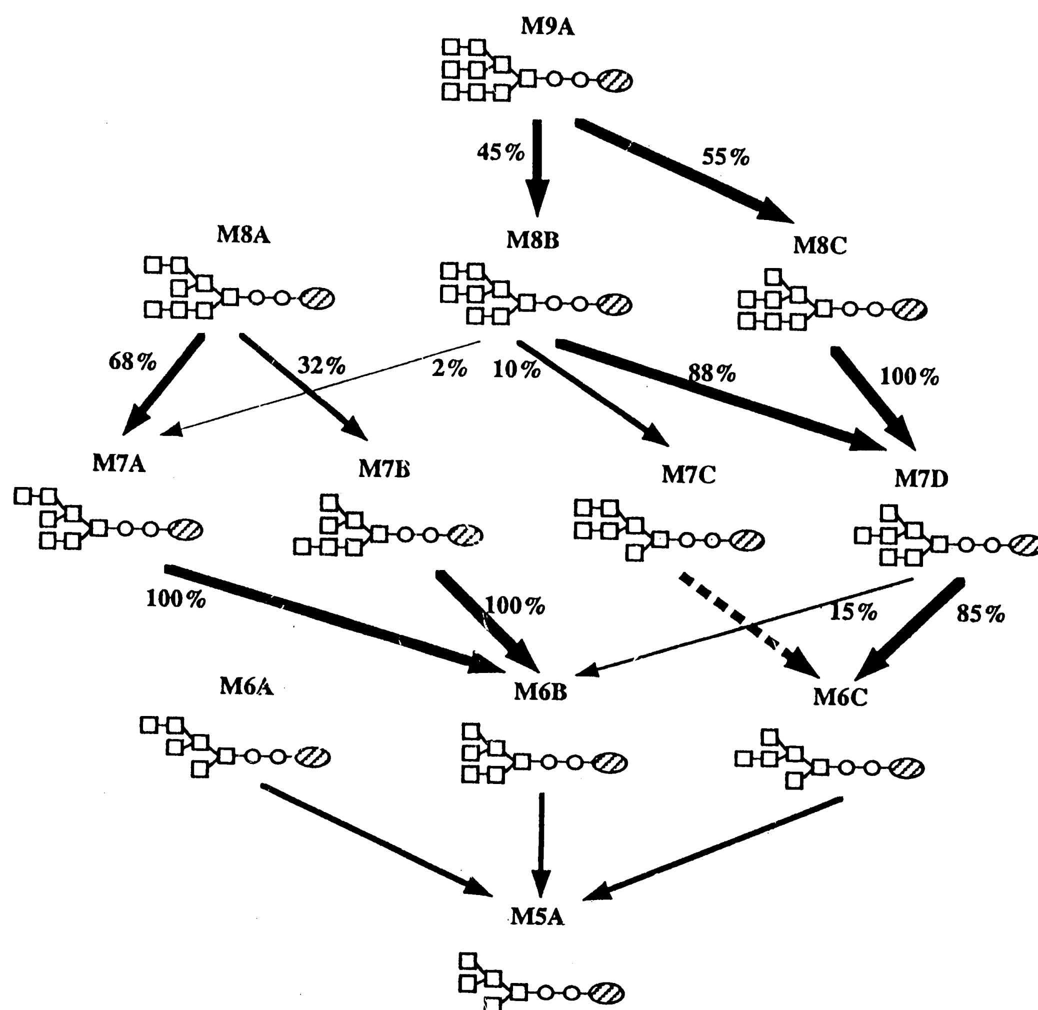
Table 1 Relative activities of the recombinant 1,2-α-mannosidase from *A. saitoi* overexpressed in *A. oryzae* cells towards fluorescent PA-oligomannose-type sugars

Oligosaccharide	Relative activity (%)
M9A	100
M8A	235
M8B	77
M8C	73
M7A	81
M7B	41
M7D	24
M6B	39
M6C	5

Specificity of recombinant *A. saitoi* 1,2-α-D-mannosidase overexpressed in *A. oryzae* cells

The recombinant enzyme expressed in *A. oryzae* niaD AM1 cleaved specifically to Manα1-2Man-OMe, but Manα1-3Man-OMe and Manα1-6Man-OMe were not substrates. The released mannose was stained by the Somogyi–Nelson method. The specific activity of the expressed recombinant enzyme was 48 mkat·kg of protein^{-1} toward Manα1-2Man-OMe, which was comparable with that of the recombinant 1,2-α-mannosidase from *P. citrinum* (54 mkat·kg^{-1}) [40]. When $Man_8GlcNAc_2$-PA (type M8A) was used as substrate of the recombinant 1,2-α-mannosidase, rapid conversion into $Man_5GlcNAc_2$-PA was observed (Figure 5). When the S/E ratio (substrate/enzyme; mol/mol) was 25, all the substrates were changed on a 'core' fragment, $Man_5GlcNAc_2$-PA (type M5A), after the reaction at pH 5.0 and 30 °C for 3 h (Figure 5). No further degradation occurred. The HPLC profiles demonstrated that the enzyme has strict specificity for the hydrolysis of 1,2-α-mannosidic bond (Figure 5). In contrast, the recombinant enzyme cleaved $Man_9GlcNAc_2$-PA to $Man_7GlcNAc_2$-PA and $Man_6GlcNAc_2$-PA and more slowly to $Man_5GlcNAc_2$-PA (Figure 6). When the S/E ratio was also 25, several intermediates were observed in the hydrolysis of $Man_9GlcNAc_2$-PA. The relative activities of the recombinant enzyme towards several PA-oligomannose-type sugar chains are shown in Table 1. The relative rate of M8A hydrolysis by the enzyme was about 2.4-fold higher than that of M9A hydrolysis (because of their complexity, these carbohydrate structures are defined only in the abbreviations footnote). The



Scheme 1 Possible courses of hydrolyses of $Man_8GlcNAc_2$-PA and $Man_9GlcNAc_2$-PA with the recombinant 1,2-α-mannosidase from *A. saitoi* overexpressed in *A. oryzae* cells

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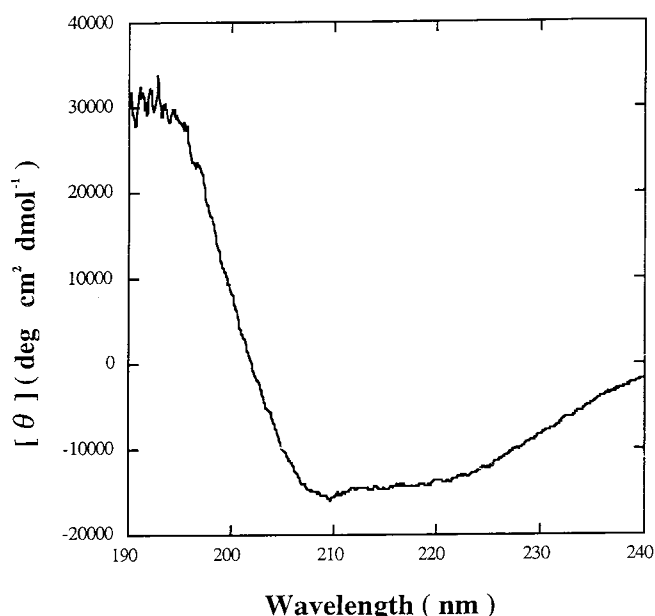

Figure 7 CD analysis of the recombinant 1,2-α-D-ammosidase from *A. saitoi* overexpressed in *A. oryzae* cells

relative rate values of M8B and M8C hydrolyses were 77 and 73 % respectively. These results indicated that the best substrate for the recombinant enzyme was M8A, and the poorest substrate for the enzyme was M6C (Table 1). When M7A, M7B and M7D were examined for cleavage, more mannose was released with M7A and M7B (Table 1). These results suggested that M7D has less affinity for the recombinant 1,2-α-mannosidase than those of M7A and M7B. The relative rate for M6C hydrolysis was only 5 % that of the M9A hydrolysis. From these results, the probable course of the hydrolyses of $Man_9GlcNAc_2$-PA and $Man_8GlcNAc_2$-PA (type M8A) to Man_5GlcNAc2-PA with the recombinant 1,2-α-mannosidase from *A. saitoi* can be speculated upon and is summarized in Scheme 1. These results indicated that the recombinant enzyme expressed in *A. oryzae* niaD AM1 cells was specific toward 1,2-α-mannosidic linkage and that $Man_7GlcNAc_2$-PA (type M7D) and $Man_6GlcNAc_2$-PA (type M6C) were predominant intermediates in the degradation of high-mannose-type oligosaccharide as is true of the original enzyme.

Molecular properties of recombinant *A. saitoi* 1,2-α-D-mannosidase overexpressed in *A. oryzae* cells

The molecular mass of the enzyme was determined to be 63 kDa by SDS/PAGE and 65 kDa by gel chromatography on Superose 12 (results not shown) respectively. Deglycosylation of the recombinant 1,2-α-mannosidase was performed with a recombinant *Flavobacterium* N-glycosidase F at pH 8.6 and 37 °C for 5 days (Figure 4). The molecular mass of the deglycosylated 1,2-α-mannosidase was 53 kDa. The result indicated that *N*-glycosides accounted for 20 % of the enzyme's molecular mass.

The N-terminal amino acid sequence of the enzyme was found to be predominantly GSTQSRADAIKAAFSHAWDGYLQY and a minor sequence of AGSTQ........DGYLQ was also identified. The aspergillopepsin I signal peptide had been removed, making it identical with that of the original enzyme from

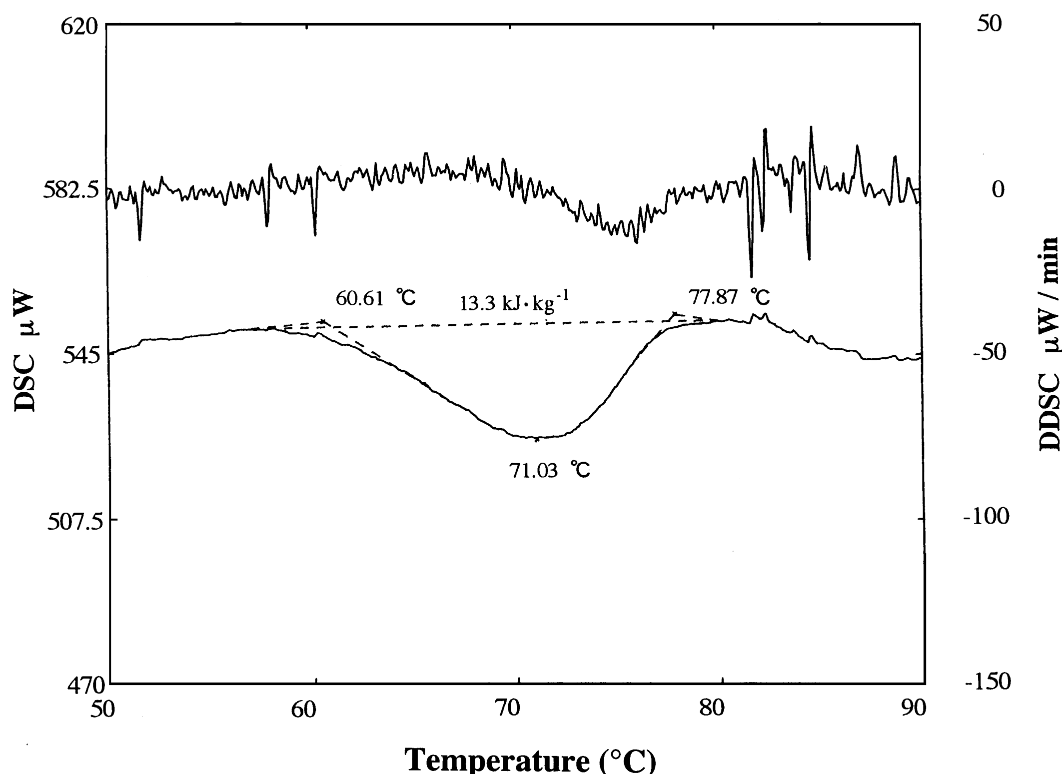

Figure 8 Determination of melting temperature, T_m, of the recombinant 1,2-α-D-mannosidase from *A. saitoi* by DSC

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Table 2 Summarized properties of the recombinant 1,2-α-D-mannosidase from *A. saitoi* overexpressed in *A. oryzae* cells

Property	Method or symbol	Value
Nucleotide sequence	cDNA of the *msdS* gene	D49827
Molecular mass	SDS/PAGE	63 kDa
	SDS/PAGE (deglycosylated)	53 kDa
	Gel filtration	65 kDa
	Deduced from nucleotide sequence	52156.7 Da
Total amino acid residues		477
N-terminal sequence	Protein sequencer	GSTQSRADAIKAAFSHAWGYLQY
Isoelectric point	pI	4.6
Molar absorption coefficient	ϵ_{280}	91539 $M^{-1} \cdot cm^{-1}$
Melting temperature	T_m	71 °C
Calorimetric enthalpy	ΔH_{cal}	13.3 $kJ \cdot kg^{-1}$
Specific activity	Manα1-2Man-OMe at pH 5.0	48 $mkat \cdot kg^{-1}$
Specificity	1,2-α-Mannosyl-oligosaccharide, α-D-Mannohydrolase	

A. saitoi [19], except that the expressed enzyme had extra Gly-Ser or Ala-Gly-Ser at its N-terminus. The Gly-Ser or Ala-Gly-Ser was a translation product of the *Bam*HI sequence used to join the aspergillopepsin I signal-encoding sequence to *msdS*. These results suggest that the recombinant enzyme has the same amino acid sequence as deduced from the nucleotide sequence [19]. The number of amino acid residues of the recombinant 1,2-α-D-mannosidase was assumed to be 477 by the predicted amino acid sequence described previously [19].

The molar absorption coefficient (ϵ_{280}) of the enzyme was determined to be 91539 $M^{-1} \cdot cm^{-1}$ by the method of Gill and von Hippel [34].

Contents of the secondary structure (α-helix, β-structure and the remainder of the enzyme) by CD determination shown in Figure 7 were about 55, 38 and 7 % respectively.

The melting temperature, T_m, onset temperature, T_o, and conclusion temperature, T_c, of the recombinant 1,2-α-D-mannosidase were 71.0, 60.6 and 77.9 °C respectively by DSC (Figure 8). The calorimetric enthalpy, ΔH_{cal}, of the enzyme was calculated as 13.3 $kJ \cdot kg^{-1}$ of enzyme using the Seiko SSC-560U DSC apparatus.

The pI value of the recombinant enzyme was determined from a plot of pH versus relative mobility to be 4.6.

Determination of 8.59 μmol of calcium/7.55 μmol of the recombinant 1,2-α-mannosidase was by atomic-absorption spectrophotometry. The results showed that the enzyme contained 1 g-atom of Ca^{2+}/mol.

Summarized properties of the recombinant 1,2-α-mannosidase from *A. saitoi* overexpressed in *A. oryzae* cells are shown in Table 2.

DISCUSSION

A classification of glycosyl hydrolases (EC 3.2.-.-) based on amino acid sequence similarities was proposed a few years ago [41]. In 1996 there were over 950 sequences of glycosyl hydrolases in the databanks (EMBL/GenBank and SWISS-PROT) [42]. Several sequences not fitting the existing families allow the definition of new families (designated 46–57) [42]. α-Mannosidase in family 47 of the sequence-based classification of glycosyl hydrolases was proposed in 1996 [42]. In the classification, no further detailed classification of α-mannosidases was proposed.

We earlier showed that the *N*-terminal of the purified 1,2-α-mannosidase from *P. citrinum* was at Ser[36] [15]. The molecular mass of the predicted protein lacking Met[1]–Lys[35] was calculated at 52777 [20]. These results suggested that catalytic cleavage of the peptide bond, Lys[35]–Ser[36] may occur as a result of action by a trypsin-like protease. In N-terminal amino acid analysis, the initial 15 residues of the secreted 1,2-α-mannosidase were GSSNQAKADAVKEAF, which was identical with that of the original enzyme from *P. citrinum*, except that the expressed enzyme had an extra Gly-Ser at its *N*-terminus. The Gly-Ser was a translation product of the *Bam*HI sequence that was used to join the *apnS* signal-encoding sequence to *msdC* [40]. The aspergillopepsin I signal peptide had been removed.

Yoshida et al. described overproduction of 1,2-α-mannosidase from *P. citrinum*, a glycochain processing enzyme expressed in *A. oryzae* [40]. The specific activity of the expressed enzyme was 54 $mkat \cdot kg$ of $protein^{-1}$ towards Manα1-2Man-OMe, which was comparable with that of the original enzyme purified from *P. citrinum* (49 $mkat \cdot kg^{-1}$) [15]. Approx. 320 mg of the purified recombinant 1,2-α-mannosidase from *A. saitoi* was obtained/litre of the culture filtrate in *A. oryzae* niaD AM1. The yield of the recombinant 1,2-α-mannosidase obtained in the present paper was a great deal higher than that of the enzyme from *P. citrinum* expressed in *A. oryzae* PM-1 [40].

The recombinant enzyme from *A. saitoi* expressed in *A. oryzae* had strict specificity toward Manα1-2Man-OMe. Manα1-3Man-OMe and Manα1-6ManOMe were not hydrolysed by the enzyme. We found that the best substrate of the recombinant 1,2-α-mannosidase from *A. saitoi* was $Man_8GlcNAc_2$-PA (type M8A) for PA-oligomannose-type sugar chains as shown in Table 1. When the recombinant 1,2-α-mannosidase from *A. saitoi* was used to digest $Man_9GlcNAc_2$-PA, we obtained results similar to those of the recombinant enzyme from *P. citrinum* which was expressed in *A. oryzae* [40]. In the present case $Man_7GlcNAc_2$-PA and Man_6GlcNAc2-PA were also the major intermediates during the digestion of $Man_9GlcNAc_2$-PA hydrolysis which finally equalled the accumulation of $Man_5GlcNAc_2$-PA. The present results showed that the poorest substrate was M6C for the 1,2-α-mannosidase, and it was assumed that the Manα1-2 residue in M6C interfered with the hydrolysis of other Manα1-2 residues. Hamagashira et al. reported similar results that the non-hydrolysable Manα1-2 residue in M9A and the Manα1-2 residue in M6C, interfered with hydrolysis of other Manα1-2 residues [43], whereas Lal et al. reported that the α-mannosidases IA and IB in murine Golgi membranes prefer the M8B isomer generated by a complementary mannosidase that removes a single mannose from $Man_9GlcNAc_2$ [44]. The present results suggest that the substrate specificities of the present 1,2-α-

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mannosidase from *A. saitoi* and yeast specific processing α1,2-mannosidase [10] seem to be complementary, since the former enzyme cleaves the rest of three Manα1-2-residues from the M8A structure produced by the latter.

The overexpression system of intracellular 1,2-α-mannosidase constructed in the present study identified several molecular properties of the enzyme: molecular mass, N- and C-terminal sequences, total amino acid residues, pI, molar absorption coefficient at 280 nm, melting temperature and calorimetric enthalpy as shown in Table 2. Further detailed structural studies require production of two-dimensional or three-dimensional crystal electron or X-ray diffraction analysis.

The *msdS* cDNA was expressed in *S. cerevisiae* YPH250 (*MTA*a, *ura*3, *trp*1, *his*3, *leu*2) with the yeast expression plasmid pG-3 (*Trp*1, 2 μm ori) [45] under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter and phosphoglycerate kinase terminator [19,22]. Transformation was accomplished as described [46]. After seed cultivation of the yeast cells in the complete minimum tryptophan drop-out (CMTrp. do) medium for 48 h the activity of the recombinant enzyme was expressed in yeast cells [19]. The results indicated that the corresponding cDNA region of N-terminal amino acid residues from 1 to 37 of 1,2-α-mannosidase from *A. saitoi* made no important contribution to expression of the enzyme, and in fact it is possible that it inhibited the expression of the enzyme. The recombinant protein cross-reacted with the anti-(*Aspergillus* 1,2-α-mannosidase) antibody [21]. It was therefore concluded that the expression product of the fusion f-*msdS* gene in the yeast cells is an active 1,2-α-mannosidase of *A. saitoi*. We have described here a new expression system of 1,2-α-mannosidase from *A. saitoi* using *A. oryzae* niaD AM1 cells with the high expression plasmid, pNAN-AM1, which includes the fused 1,2-α-mannosidase gene (f-*msdS*). Furthermore, in 1998 we constructed an expression vector of 1,2-α-mannosidase gene of *A. saitoi* with the 'HDEL' endoplasmic-reticulum retention/retrieval tag and expressed it in *S. cerevisiae* [47]. According to *in vitro* 1,2-α-mannosidase assay and Western-blot analysis, it was successfully localized in the endoplasmic reticulum. This is the first report that *S. cerevisiae* has been bred to produce $Man_5GlcNAc_2$-oligosaccharide, the intermediate to turn into hybrid-type as well as complex-type sugar chains.

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