Overproduction of 1,2-α-Mannosidase, a Glycochain Processing Enzyme, by Aspergillus oryzae

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Received August 22, 1997

A recombinant strain of Aspergillus oryzae has been constructed in which 1,2-α-mannosidase, an intracellular glycochain processing enzyme with specificity toward 1,2-α-mannosidic linkages, has been overexpressed. For the construction, the N-terminal signal-encoding sequence of the 1,2-α-mannosidase gene (msdC) from Penicillium citrinum was replaced with that of the aspergillopepsin I signal, and the fused gene was inserted between amyl promotor-terminator elements in the expression plasmid pTAPM1. A transformant of A. oryzae (the strain PM-1) secreted a great deal of heterogeneous 1,2-α-mannosidase into the culture media, which was purified by CM ion-exchange chromatography. Approximately 21 mg of the purified enzyme was obtained per liter of culture. N-terminal amino acid analysis indicated that the signal peptide was removed from the secreted enzyme. The Penicillium 1,2-α-mannosidase expressed in A. oryzae did not show any notable difference from the enzyme from P. citrinum in such properties as $M_r$, specific activity, CD spectra, or kinetic parameters. Man$_2$GlcNAc$_2$ accumulated temporarily during the degradation of Man$_4$GlcNAc$_2$ to Man$_2$GlcNAc$_2$ by fungal 1,2-α-mannosidase.

Key words: α-mannosidase; expression; fungi; Penicillium; Aspergillus

Three kinds of fungi, Aspergillus, Pycnoporus, and Penicillium, produce 1,2-α-mannosidases (EC 3.2.1.113). These mannosidases specifically remove 1,2-α-linked mannose residues at the non-reducing end of mannoooligosaccharides including high-mannose type glycochains. To date we have cloned two 1,2-α-mannosidase genes from filamentous fungi, Aspergillus saitoi (now designated Aspergillus phoenicis) and Penicillium citrinum. These fungal 1,2-α-mannosidases had a hydrophobic signal peptide in their N-terminal regions, and had characteristic amino acid sequence motifs which were common in Class 1 α-mannosidases such as yeast endoplasmic reticulum (ER) 1,2-α-mannosidase and mammalian Golgi α-mannosidase. Most of these Class 1 α-mannosidases are localized in ER or Golgi bodies and take an essential part in the early processing of Asn-linked glycochains. In yeast, trimming of one residue of 1,2-α-linked mannose from Man$_n$GlcNAc$_2$ by ER α-mannosidase is an important step that leads to the subsequent hyper-mannosylation, while in mammalian cells Golgi α-mannosidase I removes all four 1,2-α-linked mannoses to make Man$_n$GlcNAc$_2$. This is subsequently converted to the “core” structure, GlcNAc(Man)$_n$GlcNAc$_2$, to which various glycosyltransferases add GlcNAc, Gal, and/or NANA residues to form complex oligosaccharides. Fungal 1,2-α-mannosidases have a similar specificity with mammalian enzymes rather than a yeast enzyme. Looking into the mechanisms of catalytic action by 1,2-α-mannosidase is a matter of interest at least from the point of view that a minute difference in the substrate specificity of the enzymes might deeply concern the differences in the glycosylation between yeast and fungi.

Apart from the enzymological interest, the nature of fungal 1,2-α-mannosidases that cleave all the 1,2-α-mannosidic linkages in Man$_n$GlcNAc$_2$ to form Man$_n$GlcNAc$_2$ has been used to analyze the carbohydrate structure in glycoproteins. In spite of the potential availability of the enzyme in analytical fields, its low productivity in fungi has been a barrier to its widespread use.

We felt that composing an efficient system for the expression of fungal 1,2-α-mannosidase gene was essential both for study of the catalytic structure of the enzyme and for its application to analytical fields. In our preliminary work on the expression of fungal 1,2-α-mannosidase gene in S. cerevisiae, all the experiments in which the intact signal peptide of mannosidase was used proved unsuccessful (Inoue of this laboratory, unpublished data). We have not yet identified the cause of the problem, but it seemed necessary to replace the original signal peptide with that of other secreted proteins. When the DNA coding the catalytic part of Aspergillus 1,2-α-mannosidase (msdS) was joined to the signal sequence of aspergillopepsin I and expressed in S. cerevisiae under the control of GAPDH promoter, 1,2-α-mannosidase was secreted in the culture medium.

This system was available for the site-directed mutagenesis experiment by which we could identify five of catalyti-
cally crucial acidic amino acids. When we had to use the homogeneous preparation of the mutant enzyme, however, it was not practical to do the several steps of purification for the amount in the yeast culture. Furthermore, fungal 1,2-α-mannosidase expressed in yeast was hyper-glycosylated and we had to treat it with N-glycanase to have a single M, of the enzyme. These problems of low productivity and hyper-glycosylation were not solved when msdS with the α-factor signal peptide was expressed in yeast under the control of Gal1 promoter (Sadaie et al. of this laboratory, unpublished data). We also examined E. coli as the host for the expression of fungal 1,2-α-mannosidases. In this case a protein of the expected M, (53 k) was observed in the total cell-free lysate on immunoblotting, but the most part of mannosidase became insoluble and the soluble form of 1,2-α-mannosidase was little. Moreover the specific activity of the enzyme purified from E. coli was less than 1/40 of the original enzyme from fungi (Ikariishi of this laboratory, unpublished data). These preliminary works reminded us of the importance of the appropriate glycosylation and correct folding in the expression of heterogeneous proteins as well as the design of the signal peptide.

Several species of Aspergillus have long been used in food production in Japan and are known to secrete enzymes in quantity. In this paper we describe the expression of Penicillium 1,2-α-mannosidase gene in A. oryzae and analysis of the enzyme which it secretes.

Materials and Methods

Materials. O-Methyl mannobiose were purchased from Sigma (St. Louis, U.S.A). Total RNA was extracted from mycelia of P. citrinum by the guanidine thiocyanate-phenol-chloroform procedure and poly(A) + RNA was isolated using an Oligotex-Dt30 (Takara Shuzo). The first-strand DNA was produced with RNaseH-free reverse transcriptase (Gibco BRL). A. oryzae M-2-3, an arginine auxotroph (arg B), and a plasmid pTAE3 (7.6 kb) which contained arg B gene of A. nidulans were generously provided by Dr. Katsuji Gomi in the National Research Institute of Brewing (Hiroshima, Japan). Plasmid pTAE3N was made by inserting an oligonucleotide containing Not I site at Eco R I site in pTAE3. Pyridylaminated high-mannose type oligosaccharides (Manα2-GlcNAcβ-PA) were purchased from Takara Biomedic Co., Japan.

Construction of the expression plasmid for 1,2-α-mannosidase. DNA of Penicillium 1,2-α-mannosidase gene (msdC) lacking its signal encoding sequence (nucleotides 1 to 105) was obtained by the polymerase chain reaction (PCR) technique using the primers GAGggatccAGTAACCAAGCCAAAAGC and TGAagcgccgc-GTCTACTGCGGGAAGCG (BamHI I and Not I sites are indicated in small letters, respectively) with the first-strand DNA as a template. An approximately 1.44 kb PCR product was joined to the 3' BamHI I end of aseppilopepsin's signal coding sequence (70 bp of DNA coding responding to nucleotides 10 to 60 in apnS). The DNA of total 1.51-kb fragment was inserted to the Not I site in pTAE3N, thus the expression plasmid pTAPM1 was constructed (Fig. 1).

Fungal transformation. Transformation of A. oryzae was done basically by the method of Gomi et al. In our experiment arg B was used as the selectable marker. In brief, protoplasts were prepared by treating the fresh (48 h cultured) mycelia of A. oryzae M-2-3 with 1% of Novozyme 234 at 30°C for 2 h in the hypertonic solution (0.67 M NaCl and 0.27 M CaCl2). After filtration and centrifugation, DNA of pTAPM1 was added to the protoplasts, then left at room temperature for 30 min in the presence of 50% PEG4000. After centrifugation, the suspension of protoplasts was mixed with the hyper-CD (Czapek-Doxy) soft agar (1.2 M sorbitol, 0.3% NaNO3, 0.2% KCl, 0.1% KH2PO4, 0.05% MgSO4-7H2O, 0.002% FeSO4-7H2O, 2% glucose, and 0.6% agar, pH 5.5) and spread onto a hyper-CD plate (hyper-CD containing 2% agar). To obtain transformants with a homokaryon state, the conidia were selected four times on a CD plate (in this plate sorbitol was omitted). For the measurement of 1,2-α-mannosidase activity in the culture supernatant, the isolated transformants were cultured in the DYP medium (2% dextrin, 1% polypeptide, 0.5% yeast extract, 0.5% KH2PO4, and 0.05% MgSO4-7H2O, pH 5.5) at 30°C. Culture filtrates were dialyzed against 50 mM sodium acetate buffer, pH 5.0, then α-mannosidase activity toward Manα1-2Man-OMe was measured (described later).

Genomic PCR. Mycelia of A. oryzae from a 5-ml DYP culture were lyophilized. The dried mycelia were ground in a mortar to which sea sand was added. Genomic DNA was extracted from the powdered cells basically by the method described by Boel et al. PCR was done using Primer 1 (AATATGCTTCCCTCATGAC-GAG/170-192) and Primer 2 (GGGTGAACCTCAG-
TGGTGTCAC/1492–1514) (numbers indicate the positions of the primers in *msdC* cDNA) with the genome DNA as a template.

In quantitative analysis, Primer 3 (AATATGCTTCCCTCATGACGAG/170–192 in *msdC*) and Primer 4 (CAACAGACCTCCAGACAAAATT/1053–1075 in *msdC*) were used to amplify a part (906 bp) of the 1,2-α-mannosidase gene. Primer 5 (TATACAGACCCATCAATCATG-21–3 in *nucS*) and Primer 6 (CTGACTCGCAATCAAAC1933–954 in *nucS*) were used to amplify a part (975 bp) of the nuclease S1 gene. Reaction mixtures contained approximately 210 ng of the genome DNA from *A. oryzae* PM-1, 100 pmol each of the primers (3 and 4, or 5 and 6), 250 μmol dNTP, 50 mM KCl, 2 mM MgCl₂, 1 mM 2-mercaptoethanol, and Taq polymerase (1 unit, Takara) in 50 μl of TAPS buffer, pH 9.3. In this experiment the two genes were amplified in the separate tubes. PCR was done with the following thermal program; 40 sec at 94°C, 40 sec at 62°C, and 1 min at 72°C. We preliminarily confirmed that only an 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. After PCR, 40 μl of the mixture was added to 2 ml of TE containing Hoechst 33258 (100 ng/ml) and 0.2 M NaCl, then fluorescence was measured at Ex 365 nm and Em 460 nm. In this method the fluorescence by Hoechst 33258 was proportional to the amount of dsDNA up to at least 5 × 10⁻⁸ g. A PCR mixture which did not contain the enzyme was used as a blank.

Production and purification of *Penicillium* 1,2-α-mannosidase expressed in *A. oryzae*. The transformant of *A. oryzae* was cultured in 2 liters of DPY medium at 30°C for 3 days and the mycelia were separated by filtration (Toyo filter paper, No. 2). The following operations were done below 4°C unless otherwise noted. The filtrate was 75% saturated with (NH₄)₂SO₄, and centrifuged for 20 min at 10,000 × g. The precipitate was dissolved in 20 mM sodium acetate buffer, pH 5.0, and dialyzed in the same buffer (this step was for the crude fraction). The solution was then dialyzed in 50 mM sodium acetate buffer, pH 4.0, and put on a column of CM Toyopearl 650 M (1.8 × 11 cm) equilibrated in 50 mM sodium acetate buffer, pH 4.0. Most of the colored components were eluted in the flow-through fraction. After the column was washed with the same buffer, the enzyme was eluted in an increasing gradient of pH formed by 250 ml of 50 mM sodium acetate buffer, pH 4.0 and 250 ml of 200 mM sodium acetate buffer, pH 6.0. The 1,2-α-mannosidase eluted in the initial one third of the gradient.

Assays. 1,2-α-Mannosidase activity was measured by the published method with Manα1-2Man-O-Me as substrate. For the calculation of kinetic parameters, 50 μl of the reaction mixture containing 50 mM sodium acetate, pH 5.0, 195 ng of enzyme, Manα1-2Man-O-Me was incubated at 30°C for 10 min. One katal of 1,2-α-mannosidase was defined as the amount of enzyme required to liberate 1 mol of mannose from methyl mannosiose per s at 30°C and pH 5.0.

Protein concentrations were measured by the method of Lowry et al. with BSA as a standard.

PAGE and immunoblotting. PAGE was done by the method of Davis, and SDS-PAGE by the method of Laemmli. The intensity of the protein band was measured using a ScanJet 4c scanner (Hewlett Packard) and a BioMax 1D image analysis system (Kodak). Immunoblotting was done as described previously. Antibody was raised in a rabbit against *Penicillium* 1,2-α-mannosidase purified from *P. citrinum*.

N-Terminal sequence analysis. N-Terminal amino acid analysis was done using an Applied Biosystems (ABI) 473A protein sequenator with an ABI 610A data analysis system.

CD Measurements. Samples were dialyzed in 20 mM sodium acetate buffer, pH 5.0, then diluted in the same buffer to 0.2 mg protein/ml by adjusting OD₂₈₀ to 0.2. The CD measurements were done as described previously by Yamaguchi et al. using a Jasco J-700 spectropolarimeter. The contents of α-helix and β-structure of the enzyme were calculated by the SSE-338 program given by Yang et al.

HPCL analyses. An amide column (TSKgel Amide 80, 4 × 250 mm) was run in 3% acetic acid/triethylamine/water, pH 7.3, containing a decreasing gradient of acetonitrile (80% to 48% in 50 min) at a flow rate of 0.7 ml/min. Fluorescence was monitored at an excitation of 310 nm and emission 380 nm. In separate the isomers of Man-GlcNAc-PA, an ODS column (Cica-Merck, LiChrospher RP-18, 4 × 250 mm) was run in 100 mM acetic acid/0.025% n-butanol/triethylamine/water, pH 4.0, at a flow rate of 0.6 ml/min. Fluorescence was monitored at an excitation of 320 nm and emission 400 nm.

Results

Expression of *Penicillium* 1,2-α-mannosidase gene in *A. oryzae*

After the transfection of *A. oryzae* M-2-3 with the expression plasmid pTAPM1, a few colonies grew on a CD plate. The transformants were selected four times on the minimal plate to obtain the conidia of homokaryons. Finally, a single transformant was selected (strain PM-1). To check that PM-1 was not an "abortive" transformant, PCR was done with its genomic DNA. By using Primers 1 and 2, which were complementary to the regions near the 5' or 3' end of the *msdC* gene, respectively, a single DNA of 1.35 kb was amplified (data not shown). The same size of DNA was made with the template of plasmid pTAPM1, while no DNA fragment was amplified after the PCR when the DNA from M-2-3 was the template. These results indicated that the *msdC* gene had been integrated into the genome of *A. oryzae* PM-1.

*A. oryzae* is known to have a single copy of the nuclease S1 gene (*nucS*) in its genome. To estimate the
copy number of msdc in the strain PM-1, we monitored the amplification of msdc and nucS during PCR (Fig. 2). In our experiment a linearity of the exponential increase in the amount of DNA was kept for all both genes till the cycles reached 20, while in further cycles the linearity was lost (Fig. 2). The ratio of DNA amplified from the two genes (msdc/nucS; g/g) at cycles 12, 14, 16, and 20 were 2.3, 2.2, 1.9, and 1.8, respectively. When counterbalancing a minute difference between the sizes of amplified DNA (906 bp in msdc while 975 bp in nucS) the values could be modified to 2.5, 2.3, 2.0, and 1.9, respectively (these meant a molar ratio between the two of amplified DNA). From these data we supposed that the strain PM-1 would have two copies of msdc in its genome.

When the proteins in the culture supernatants from strains M-2-3 and PM-1 were compared on SDS-PAGE, a protein of M. approx. 53 k was shown to have increased in the latter strain (Fig. 3A). On the immunoblotting, this protein cross-reacted with the anti-Penicillium 1,2-α-mannosidase antibody (Fig. 3B). Though a similar size of protein was also observed in the supernatant from M-2-3 on SDS-PAGE, this protein did not cross-react with the antibody at all. The result showed that the protein of Penicillium 1,2-α-mannosidase was secreted in the culture supernatant of the transformant.

Purification and characterization of the expressed 1,2-α-mannosidase

A. oryzae strain PM-1 was cultured in DPY medium and the protein in the culture filtrate was precipitated with ammonium sulfate, then put onto a CM-Toyopearl column. We obtained a homogeneous preparation of 1,2-α-mannosidase by this single step of column chromatography (Fig. 4A). Approximately 64 mg of pure enzyme was obtained from 3 liters of the culture filtrate (Table 1). The specific activity of the expressed enzyme

![Fig. 2](image-url)

**Fig. 2.** Measurements of PCR Products.

Partial DNA of msdc (closed circle) or nucS (open circle) was amplified with the template of genomic DNA from A. oryzae PM-1. At the indicated intervals the amount of DNA in each reaction was measured by the fluorogenic methods as described in Materials and Methods, and the increase was plotted. Amplified DNA was insufficient to measure below the 12th cycle.

![Fig. 3](image-url)

**Fig. 3.** Immunoblotting Analysis of the Culture Filtrate from Aspergillus oryzae M-2-3 and PM-1 Strains.

Culture filtrate (0.5 ml each) from A. oryzae PM-1 strain (lane 1) and M-2-3 strain (lane 2) were precipitated with TCA, then separated on SDS-PAGE. Proteins were detected by staining with CBB (A) or by immunoblotting using anti-Penicillium 1,2-α-mannosidase antibody (B). The positions of molecular-mass markers are indicated at the right side of each gel.

![Fig. 4](image-url)

**Fig. 4.** PAGE (A) and SDS-PAGE (B) of Penicillium 1,2-α-Mannosidase Expressed in A. oryzae.

In gel A, the crude fraction (10 µl, lane 1) or CM TOYOPEARL purified enzyme (5 µl, lane 2) was separated on PAGE in the native condition at pH 8.9. The dark and open triangles show the positions of 1,2-α-mannosidase and Taka-amylase A, respectively. In gel B, Penicillium 1,2-α-mannosidase purified from P. citrinum (lane 1) or from A. oryzae PM-1 (lane 2) was separated on SDS-PAGE. The positions of molecular-mass markers are indicated at the left side of the gel. About 4 µg of the proteins were put on. Gels were stained with CBB in both experiments.
was 54 mkat/kg toward Manα1-2ManOMe, which was comparable to that of the original enzyme purified from *P. citrinum* (49 mkat/kg). In N-terminal amino acid analysis, the initial 15 residues of the secreted 1,2-α-mannosidase was GSSNQAKADAVKEAF, which was identical to that of the original enzyme from *P. citrinum* except that the expressed enzyme had extra Gly-Ser at its N-terminus. The Gly-Ser was a translation of the *BamHI* sequence that was used to join the aspergillopepsin I signal-encoding sequence to *msdC*. The result indicated that the aspergillopepsin I signal peptide had been removed. In N-terminal amino acid sequencing of the faster moving protein (shown with an open triangle in Fig. 4A), the sequence ATPADWRSQSIYFLLTD was obtained. This meant that the protein corresponded to Taka-amyrase A. When comparing the intensity of the protein bands in the gel, relative ratio of the two proteins (mannosidase/Taka-amyrase A) was calculated to be 2.2. This suggested that the amount of the secreted mannosidase in the culture of PM-1 strain was approximately twice that of Taka-amyrase A. The enzyme expressed in *A. oryzae* showed a similar *M*<sub>r</sub> with the original enzyme on SDS-PAGE (Fig. 4B). In CD spectra measurement, the expressed enzyme showed patterns almost identical to the original enzyme from *P. citrinum* (Fig. 5). These data suggested that the two proteins, one expressed in *A. oryzae* and another from the original *P. citrinum*, did not have a notable difference in the secondary structure.

The purified enzyme cleaved Manα1-2ManOMe but Manα1-3ManOMe and Manα1-ManOMe were not substrates (data not shown). The *K*<sub>m</sub>, *k*<sub>cat</sub>, and *k*<sub>cat</sub>/*K*<sub>m</sub> on the hydrolysis of Manα1-2ManOMe by the enzyme expressed in *A. oryzae* were determined as 4.4 × 10<sup>-4</sup> (5.2 × 10<sup>-4</sup>) M, 2.1(2.5) s<sup>-1</sup>, and 4.8 × 10<sup>9</sup> (4.9 × 10<sup>8</sup>) M<sup>-1</sup>s<sup>-1</sup>, respectively. The values in parentheses are the parameters obtained with the original enzyme from *P. citrinum*. Respectively, the degradation of Man<sub>3</sub>GlcNAc<sub>2</sub>-PA by the expressed enzyme was monitored by varying the reaction period. When the S/E ratio (substrate/enzyme; mol/mol) was 1.0, all the substrates were changed to Man<sub>3</sub>GlcNAc<sub>2</sub>-PA after the reaction at 30°C for 3 h (Fig. 6). No further degradation occurred. Several intermediates were observed, however, when the S/E ratio was raised to 25. Under these conditions, the degradation of Man<sub>3</sub>GlcNAc<sub>2</sub>-PA seemed to be retarded at the hydrolysis of Man<sub>7</sub>GlcNAc<sub>2</sub>-PA before all four 1,2-α-linked mannose residues were removed (Fig. 6). The peak of Man<sub>7</sub>GlcNAc<sub>2</sub>-PA

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**Table 1. Purification of *Penicillium* 1,2-α-Mannosidase Expressed in *Aspergillus oryzae* PM-1 Strain.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (10&lt;sup&gt;3&lt;/sup&gt; x katal)</th>
<th>Specific activity (10&lt;sup&gt;3&lt;/sup&gt; x katal/kg)</th>
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<tbody>
<tr>
<td>Crude enzyme</td>
<td>380.0</td>
<td>42.5</td>
<td>1.1</td>
</tr>
<tr>
<td>CM Toyopearl fraction</td>
<td>63.8</td>
<td>34.5</td>
<td>5.4</td>
</tr>
</tbody>
</table>

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**Fig. 5. CD Analysis of 1,2-α-Mannosidase.**

CD spectra of *Penicillium* 1,2-α-mannosidase purified from *P. citrinum* (straight line) or from *A. oryzae* PM-1 (dotted line) was measured as described in Materials and Methods.

**Fig. 6. Degradation of High Mannose Type Oligosaccharide by *Penicillium* 1,2-α-Mannosidase.**

The reaction mixture (50 μl) containing 1,2-α-mannosidase purified from *A. oryzae* PM-1 and 100 pmol of Man<sub>3</sub>GlcNAc<sub>2</sub>-PA in 30 μl sodium acetate buffer, pH 5.0, was incubated at 30°C. At regular intervals, the reaction product (10 μl) was analyzed on HPLC (TSKgel Amide-80 column) as described in Materials and Methods. Arrows indicate the positions of Man<sub>3</sub>GlcNAc<sub>2</sub>-PA. S/E represents the substrate/enzyme ratio (mol/mol) in the reaction mixture.
in the reaction mixture after 4 h digestion (S/E of 25) was recovered and analysed further by HPLC using an ODS column. On the second HPLC, a single peak corresponding to M7D appeared while no other peak was observed (chromatogram not shown).

\[
\text{Man}_{1-2} \text{Man}_{1-2} \text{Man}_{1-2} \text{GlcNAc}_{2-4} \text{GlcNAc}_{2-4}
\]  
(M7D)

When the enzyme purified from \textit{P. citrinum} was used to digest \textit{Man}_{9}\textit{GlcNAc}_{2-PA}, we obtained the similar result. In the case also \textit{Man}_{9}\textit{GlcNAc}_{2-PA} was the major intermediate during the digestion which finally reached the accumulation of \textit{Man}_{9}\textit{GlcNAc}_{2-PA} (data not shown). These results indicated that the enzyme expressed in \textit{A. oryzae} was also specific toward \textit{1,2-\alpha}-mannosidic linkage and \textit{Man}_{9}\textit{GlcNAc}_{2} (type M7D) was a predominant intermediate in the degradation of high-mannose type oligosaccharide as true of the original enzyme.

**Discussion**

In this work we chose \textit{Aspergillus} as a host for the expression of \textit{Penicillium} 1,2-\alpha-mannosidase and a signal of aspergillopepsin I for the secretion. On immunoblotting it was shown that transformed \textit{A. oryzae} (strain PM-1) secreted \textit{Penicillium} 1,2-\alpha-mannosidase (Fig. 3). The yield of 1,2-\alpha-mannosidase by the strain PM-1 was 21 mg (as a purified enzyme) per liter of culture, a remarkable value compared to the case where 1.2 mg of mannosidase was purified from 30 g of the dried \textit{Penicillium} culture product. On SDS-PAGE of 1,2-\alpha-mannosidase expressed in \textit{A. oryzae}, \textit{M}_{9} was shown to be almost the same as the enzyme from \textit{Penicillium} (Fig. 4B). This suggested that no extra glycosylation occurred in the expressed enzyme as in the case of yeast, though we have not analyzed the detailed structure of the glycochain.

The CD spectra of the expressed and original enzymes were almost identical. The relative ratio (\%) of \alpha-helix, \beta-sheet, turn, and random coil were estimated to be 41, 25, 23, and 13, respectively. This, together with the prediction according to the parameters by Chou and Fasman, suggested that \textit{Penicillium} 1,2-\alpha-mannosidase was an \alpha/\beta type protein. This was in contrast to the case of aspergillopepsin I in which \beta-structure was predominant.

In the degradation of high-mannose type oligosaccharide by a pig liver microsomal 1,2-\alpha-mannosidase (\textit{Man}_{9}-\textit{mannosidase}), three 1,2-\alpha-linked mannosides were removed to give \textit{Man}_{9}\textit{GlcNAc}_{2}-PA. Bause et al. predicted that the enzyme was localized in ER. As to the degradation of \textit{Man}_{9}\textit{GlcNAc}_{2}-PA by \textit{Penicillium} 1,2-\alpha-mannosidase expressed in \textit{A. oryzae}, all the substrate was finally changed to \textit{Man}_{9}\textit{GlcNAc}_{2}-PA (Fig. 6), which meant that it resembled mammalian Golgi resident \alpha-mannosidase I. At the lower enzyme concentration (S/E = 25), however, the degradation was retarded at the step of hydrolysis on \textit{Man}_{9}\textit{GlcNAc}_{2}-PA. This phenomenon was repeated when the original enzyme from \textit{P. citrinum} was used. In our past experiment in which \textit{Aspergillus} 1,2-\alpha-mannosidase expressed in \textit{S. cerevisiae} was used, an accumulation of the intermediate, \textit{Man}_{9}\textit{GlcNAc}_{2}-PA, was also observed during the degradation of \textit{Man}_{9}\textit{GlcNAc}_{2}-PA to \textit{Man}_{9}\textit{GlcNAc}_{2}-PA (unpublished data). We supposed that \textit{Man}_{9}\textit{GlcNAc}_{2} (type M7D) would be a major intermediate in the processing of N-linked glycochain by fungal 1,2-\alpha-mannosidases.

The overexpression system constructed in this study opens up a way for the crystallographic study of \alpha-mannosidase on which no data is available. Further analysis of the enzymological structure will shed more light on the mechanism of the catalytic reaction by 1,2-\alpha-mannosidase.

**Acknowledgments**

We are deeply grateful to Drs. Katsuya Gomi and Byung Rho Lee of the National Research Institute of Brewing (Japan) for kindly providing \textit{A. oryzae} strain M-2-3 and a plasmid pTAex3 as well as for their technical advice.

**References**

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