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SHORT COMMUNICATION

Filamentous fungus *Aspergillus oryzae* has two types of α-1,2-mannosidases, one of which is a microsomal enzyme that removes a single mannose residue from $Man_9GlcNAc_2$

Takashi Yoshida[1]*, Yoji Kato[2], Yoshihiro Asada[1] and Tasuku Nakajima[3]

[1]Faculty of Agriculture and Life Science, [2]Faculty of Education, Hirosaki University, 3 Bunkyo, Hirosaki, Aomori 036-8561, Japan, [3]Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiya, Aoba, Sendai 981-8555, Japan

α-Mannosidase activities towards high-mannose oligosaccharides were examined with a detergent-solubilized microsomal preparation from a filamentous fungus, *Aspergillus oryzae*. In the enzymatic reaction, the pyridylaminated substrate $Man_9GlcNAc_2$-PA was trimmed to $Man_8GlcNAc_2$-PA which lacked one α-1,2-mannose residue at the nonreducing terminus of the middle branch (Man8B isomer), and this mannooligosaccharide remained predominant through the overall reaction. Trimming was optimal at pH 7.0 in PIPES buffer in the presence of calcium ion and kifunensine was inhibitory with IC_{50} below 0.1 μM. These results suggest that the activity is the same type as was previously observed with human and yeast endoplasmic reticulum (ER) α-mannosidases. Considering these results together with previous data on a fungal α-1,2-mannosidase that trimmed $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ (Ichishima, E., *et al.* (1999) *Biochem J*, 339: 589–597), the filamentous fungi appear to have two types of α-1,2-mannosidases, each of which acts differently on *N*-linked mannooligosaccharides.

Keywords: **α-Mannosidase, endoplasmic reticulum, fungi, *Aspergillus***

Abbreviations: **Man, mannopyranose; GlcNAc, *N*-acetylglucosamine; PA, pyridylaminated; PIPES, piperazine-*N,N′*-bis(2-ethanesulfonic acid); HPLC, high performance liquid chromatography; ER, endoplasmic reticulum; KF, kifunensine.**

Introduction

In the biosynthesis of glycoproteins with *N*-linked oligosaccharides, $Glc_3Man_9GlcNAc_2$ is transferred to nascent polypeptide chains in the endoplasmic reticulum (ER) [1,2]. In mammalian cells, several steps of trimming are initiated with the removal of three glucose residues by α-glucosidases. Subsequently $Man_9GlcNAc_2$ is trimmed to $Man_8GlcNAc_2$ by ER α-mannosidase, the first α-1,2-specific mannosidase [3,4]. The remaining three α-1,2-mannose residues in $Man_8GlcNAc_2$ are removed in the Golgi by several Golgi α-1,2-mannosidases (IA, lB and IC) [2,5–7]. Both ER and Golgi α-1,2-mannosidases are specific for the α-1,2-mannosidic linkage, but their modes of action are quite different. ER α-mannosidase clips only the terminal α-1,2-mannose residue at the middle branch of $Man_9GlcNAc_2$, making $Man_8GlcNAc_2$ isomer B (Man8B isomer). No further clipping occurs on the remaining three α-1,2-mannose residues in $Man_8GlcNAc_2$. Golgi α-1,2-mannosidases act on $Man_9GlcNAc_2$ to produce mannooligosaccharides ranging in size from $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ [8]. Man8A and Man8C isomers, but not Man8B, are the trimming intermediates which are finally trimmed to $Man_5GlcNAc_2$ [5].

Fungal α-1,2-mannosidases have been purified from *Aspergillus* and *Penicillium* [9,10]. The gene encoding each enzyme has been cloned [11,12] and overexpressed in a fungal host-vector system [13,14]. The fungal protein sequences had about 30% identity to the mammalian

*To whom correspondence should be addressed:
E-mail: ytakashi@cc.hirosaki-u.ac.jp

α-1,2-mannosidases and removed all four α-1,2-linked mannose residues from $Man_9GlcNAc_2$ to make Man_5-$GlcNAc_2$ [13,14]. They therefore resemble the Golgi α-1,2-mannosidases in mammalian cells. In a recent paper, three α-1,2-mannosidase-related genes have been identified in *Aspergillus* suggesting that gene duplication occurred in fungal evolution [15]. *Saccharomyces cerevisiae* is known to have another type of α-1,2-mannosidase, ER α-mannosidase [1]. The *Saccharomyces* enzyme makes the Man8B isomer from $Man_9GlcNAc_2$ [1]. Contrary to *Aspergillus*, a Golgi-type α-1,2-mannosidase that makes $Man_5GlcNAc_2$ from $Man_9GlcNAc_2$ has yet to be found in *S. cerevisiae*. It has long been uncertain whether these lower eukaryotes have α-1,2-mannosidases localized separately in the ER and Golgi compartments as is the case in mammalian cells, or whether they use fewer enzymes for the trimming of α-1,2-linked mannooligosaccharides.

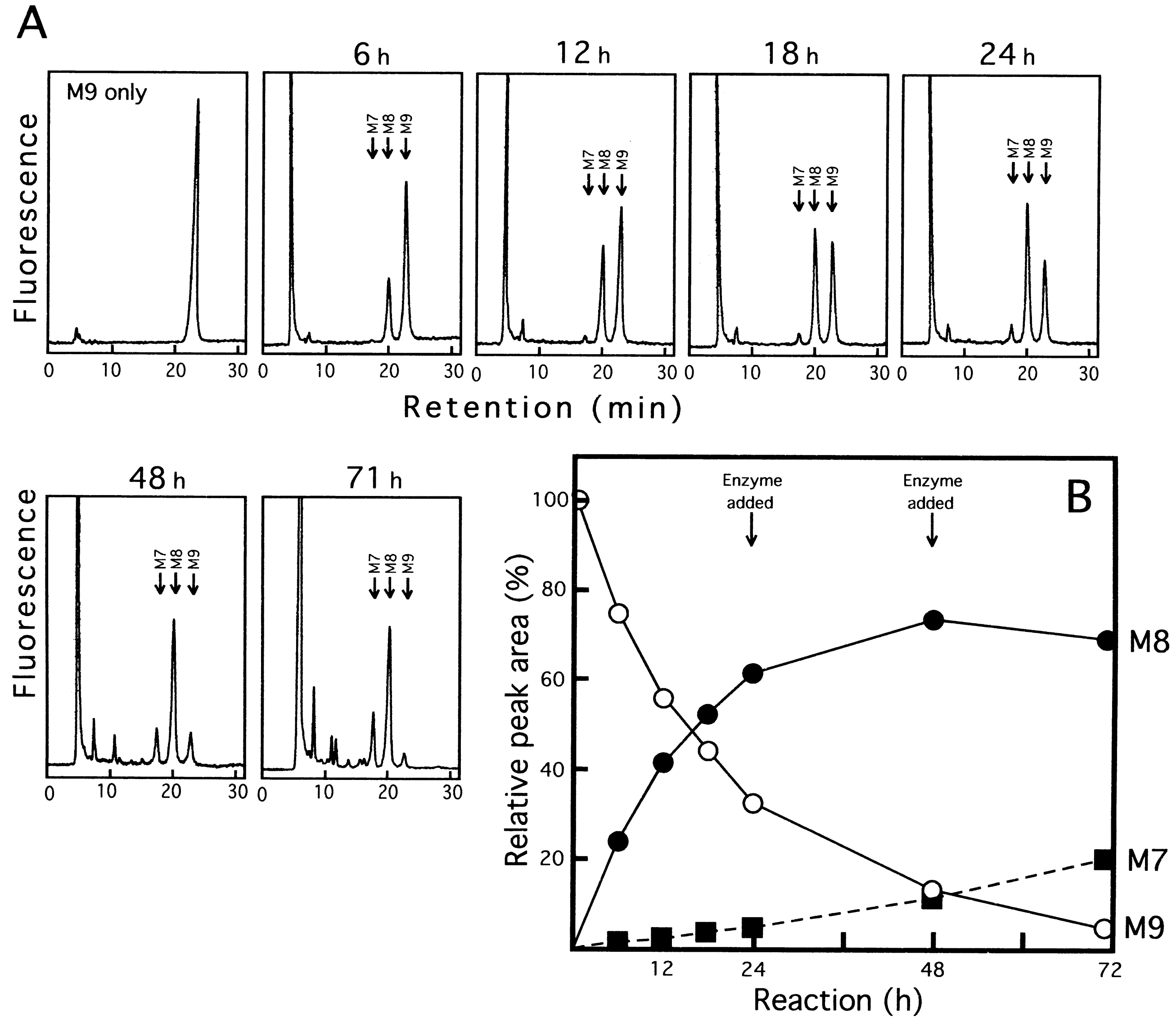

Figure 1. Time course of trimming of $Man_9GlcNAc_2$-PA by a microsomal fraction from *A. oryzae*. **A**: Mannooligosaccharides analyzed by HPLC. Reaction mixtures (*Materials and Methods*) were incubated at 30°C for 6 to 71 h. For prolonged reaction, 10 μg of microsomal protein was supplied at 24 h and 48 h of incubation. PA-mannooligosaccharides were separated by molecular size using an amide column (Palpak type N, 4.6 × 250 mm, Takara Co., Japan). The solvent contained 0.5 M acetic acid/triethylamine buffer, pH 7.3, acetonitrile, and water (8:55:37 (v/v)). Flow was at 0.6 ml/min, and fluorescence was monitored at 315 nm for excitation and at 380 nm for emission. Peaks eluted before 12 min were impurities in the microsomal preparation. In the upper left panel, only $Man_9GlcNAc_2$-PA (2 pmol) was loaded. M9, $Man_9GlcNAc_2$-PA; M8, $Man_8GlcNAc_2$-PA; M7, $Man_7GlcNAc_2$-PA. **B**: Relative areas of M9, M8, and M7 in panels A were plotted against time.

In this report, we describe a second α-1,2-mannosidase activity in the microsomal preparation from *Aspergillus oryzae*. The Man8B isomer was dominant in the trimming of $Man_9GlcNAc_2$ by this enzyme, suggesting that filamentous fungi have two types of α-1,2-mannosidases each of which has a different mode of action on high mannose oligosaccharide. It might also mean potential heterogeneity in the glycochain processing systems among lower eukaryotes.

Materials and methods

Fungal cells were grown in DPY medium (2% dextrin, 1% peptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.05% $MgSO_4.7H_20$, pH 5.5) at 30°C for 44 h. Mycelia were harvested by filtration and ground in an ice-chilled mortar in 50 mM PIPES buffer, pH 7.0, containing 25% sucrose and sea sand. After removing cellular debris by centrifugation at 4°C, 10 000 × g for 15 min, the supernatant was centrifuged at 4°C, 100 000 × g for 1 h. Microsomal pellets were suspended in the same buffer and centrifuged again. The second pellets were suspended in 50 mM PIPES buffer, pH 7.0, containing 30% glycerol and stored at −80°C. Frozen microsomal pellets were thawed in ice, solubilized in 1% Triton X-100, then centrifuged at 4°C, 10 000 × g for 5 min. Cleared supernatants were used for assays. Assay mixtures containing 100 mM PIPES, pH 7.0, 10 mM $CaCl_2$, 10 pmol of $Man_9GlcNAc_2$-PA, and microsomal extracts (10 μg protein) in a total volume of 10 μl were incubated at 30°C. Mixtures were filtered through a centrifugal membrane-filter (0.2 μm), then analyzed on HPLC. PA-mannooligosaccharides were monitored with a Hitachi model F-1080 fluorescent detector. Standard PA-mannooligosaccharides ($Man_{9-5}GlcNAc_2$-PA including Man8A, 8B, and 8C isomers) were purchased from Takara Co. (Japan). Kifunensine (KIF) was from Toronto Research Chemicals (Canada). Protein was determined by the method of Lowry *et al.* [16] with a standard of bovine serum albumin.

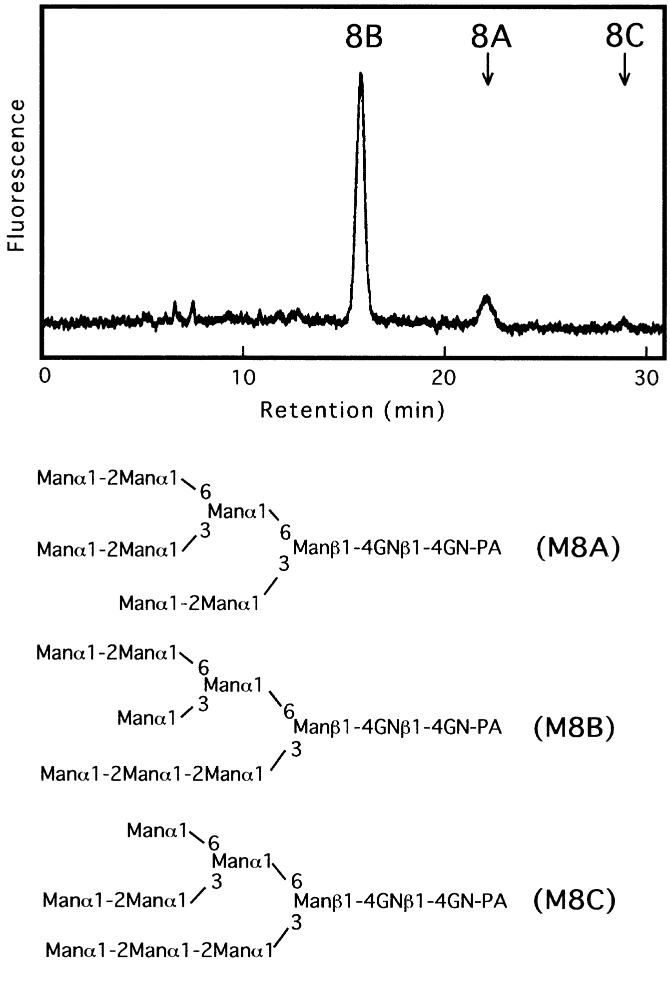

Figure 2. Isomers of $Man_8GlcNAc_2$-PA were analyzed on HPLC. A peak of $Man_8GlcNAc_2$-PA separated in the first HPLC (12 h-reaction in Figure 1A) was recovered, concentrated, then loaded on to the second HPLC with an ODS column (Palpak type R, 4.6 × 260 mm, Takara, Japan). The solvent contained 0.1 M acetic acid-triethylamine, pH 4.0, and 0.025% (v/v) 1-butanol. Flow rate was 0.6 ml/min, and fluorescence was monitored at 315 nm for excitation and 400 nm for emission.

Results and discussion

Microsomal protein extracts from *A. oryzae* were incubated with a fluorescent substrate, $Man_9GlcNAc_2$-PA, then the time course of trimming was monitored by HPLC (Figure 1). In the early part of the incubation, a peak of $Man_8GlcNAc_2$-PA appeared while $Man_9GlcNAc_2$-PA decreased (Figure 1A, 6–12 h). As the incubation proceeded, $Man_8GlcNAc_2$-PA became predominant but $Man_7GlcNAc_2$-PA did not increase much (Figure 1A, 18–24 h). We supplied the microsomal extract at 24 and 48 h of incubation, but the ratio of $Man_8GlcNAc_2$-PA in the reaction remained at 73% after 48 h and 69% after 71 h of incubation (Figure 1B). $Man_8GlcNAc_2$-PA increased to 61% of total mannooligosaccharides within the first 24 h, whereas the increase of $Man_7GlcNAc_2$-PA in the next 48 h (24 to 71 h) was 15% (Figure 1B). The data suggested the presence of activity that effectively trimmed $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$. The peak of $Man_8GlcNAc_2$-PA separated by HPLC (Figure 1) was recovered and analyzed under different HPLC conditions (Figure 2). We found that the majority (85%) of $Man_8GlcNAc_2$-PA was Man8B isomer (Figure 2). The trimming of $Man_9GlcNAc_2$ by the fungal microsomal mannosidase was optimal at pH 7.0 in 100 mM PIPES buffer with 41% of the activity observed at pH 5.5 and 18% at pH 8.0 (data not shown). In the presence of 10 mM EDTA, trimming was completely inhibited. The addition of Ca^{2+} restored activity (data not shown). Kifunensine (KF), a potent inhibitor of mammalian α-1,2-mannosidases [17,18], showed a drastic inhibition of fungal microsomal mannosidase with IC_{50} between 50 and 100 nM (Figure 3).

In the trimming of $Man_9GlcNAc_2$ by another fungal α-1,2-mannosidase [10], activity was optimal at lower pH of 4.5–5.5

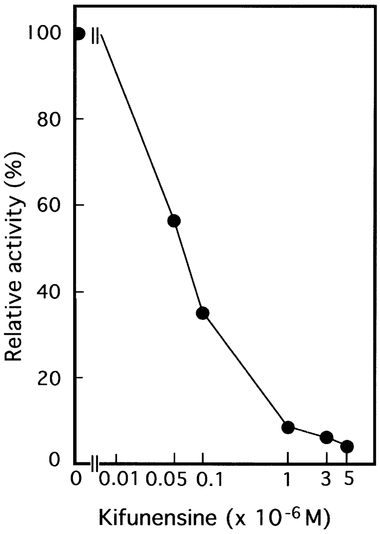

Figure 3. Effect of kifunensine on microsomal α-mannosidase. $Man_9GlcNAc_2$-PA was treated with microsomal extracts from *A. oryzae* for 13 h in the presence or absence of kifunensine, then the relative amount of undigested Man9 was determined using HPLC (Palpak type N column) (Materials and Methods). Decrease of Man9 in the absence of KIF was taken for 100% activity.

and $Man_8GlcNAc_2$ did not accumulate during the enzymatic reaction [13]. In this case, $Man_7GlcNAc_2$ transiently increased, and was trimmed finally to $Man_5GlcNAc_2$ [13]. In initial trimming of $Man_9GlcNAc_2$ by that enzyme, isomers Man8A and 8C but not Man8B were observed as intermediates [14]. Considered together with previous data, the fungal microsomal preparation appears to contain another mannosidase that can make the Man8B isomer from $Man_9GlcNAc_2$ at a neutral pH, and has sensitivity to kifunensine. These properties are similar to those of yeast and human ER α-mannosidases [1,3,4]. At present the fungus *Aspergillus* may be unique among lower eukaryotes in having two types of α-1,2-mannosidases, one of which acts on $Man_9GlcNAc_2$ similarly to yeast and human ER α-1,2-mannosidases while the other resembles mammalian Golgi α-1,2-mannosidases. Although localization of these enzymes in fungal cells is yet to be determined, each mannosidase is presumed to act differently on a high-mannosyl oligosaccharide in the *N*-glycochain processing pathway.

Acknowledgments

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