Protein O-mannosyltransferase A of *Aspergillus awamori* is involved in O-mannosylation of glucoamylase I

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Industrially important extracellular enzymes from filamentous fungi are often O-mannosylated. The structure and function of the *pmtA* (*AapmtA*) gene encoding the protein O-D-mannosyltransferase of *Aspergillus awamori* were characterized. The *AapmtA* disruptant, designated AaPMTA, was constructed by homologous recombination. The strain AaPMTA exhibited fragile cell morphology with respect to hyphal extension, as well as swollen hyphae formation and conidia formation in potato dextrose medium. Moreover, the *AapmtA* disruptant showed increased sensitivity to high temperature and Congo red. Thus, the AaPmtA protein is involved in the formation of the normal cell wall. The strain AaPMTA could grow well in liquid synthetic medium and secrete glucoamylase I (GAI-AaPMTA) to a similar extent to the wild-type strain (GAI-WT). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of the GAIs revealed that approximately 33 mannose moieties of GAI were absent in strain AaPMTA. This result indicates that the AaPmtA protein is responsible for the transfer of mannose to GAI. Structural analysis of the O-linked oligosaccharides of GAI also demonstrated that the *AapmtA* disruption resulted in a reduction of the amounts of O-linked oligosaccharides, such as D-mannose and α -1,2-mannotriose, in GAI-AaPMTA. However, the amount of α -1,2-mannobiose was comparable between GAI-WT and GAI-AaPMTA. The result suggests the presence of a compensatory mechanism in the synthetic pathway of O-mannosylation in *A. awamori*.

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INTRODUCTION

Protein glycosylation, which is a major post-translational modification, plays essential roles in eukaryotic cells from fungi to mammals (Burda & Aebi, 1999; Gemmill & Trimble, 1999). Compared with N-linked oligosaccharides in glycoproteins, which share a relatively common structure regardless of their origins, O-linked oligosaccharides in glycoproteins are diverse among the eukaryotic organisms with respect to both

their sugar components and the mode of sugar linkages (Haltiwanger & Lowe, 2004; Strahl-Bolsinger & Scheinost, 1999; Willer *et al.*, 2003). O-Mannosylation, which is commonly found in the glycoproteins of fungi, has been extensively studied in the budding yeast *Saccharomyces cerevisiae* (Gentzsch & Tanner, 1996; Lussier *et al.*, 1997; Strahl-Bolsinger & Scheinost, 1999). The initial reaction of mannose transfer to serine and threonine residues in proteins is catalysed by protein-O-D-mannosyltransferase (Pmt) in the endoplasmic reticulum, where dolichol phosphate (Dol-P)-Man is required as an immediate sugar donor (Gentzsch & Tanner, 1996). Subsequently, O-mannosylation is linearly elongated by up to seven mannose residues in the Golgi complex by mannosyltransferases (Mnts) that utilize GDP-mannose as the mannosyl donor for the elongation process. At least six Pmt-encoding genes (*PMT1*–*6*), three α -1,2-Mnt-encoding genes (*MNT1/KRE2*, *KTR1*, *KTR3*) and three α -1,3-Mnt-encoding genes (*MNN1*, *MNT2*, *MNT3*) are known to be involved in the complete reactions of O-mannosylation in *S. cerevisiae* (Lussier *et al.*, 1997, 1999; Romero *et al.*, 1999; Strahl-Bolsinger & Scheinost, 1999; Yip *et al.*, 1994).

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Abbreviations: CD, circular dichroism; GAI, glucoamylase I; GlcNAc, N-acetylglucosamine; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Pmt, protein O-D-mannosyltransferase; wt, wild-type.

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The Pmt family proteins can be classified into the PMT1, PMT2 and PMT4 subfamilies on the basis of their phylogeny (Girrbach *et al.*, 2000). The PMT1 subfamily proteins form a heteromeric complex with the PMT2 subfamily proteins, and PMT4 subfamily protein forms a homomeric complex (Girrbach & Strahl, 2003). Disruption of three different types of *pmt* genes results in the death of yeast cells (Gentsch & Tanner, 1996). As concerns the PMT family of filamentous fungi, Shaw & Momany (2002) reported that the mutation in the *swaA* gene that causes the abnormal hyphae development is identical to the *pmtA* (*AnpmtA*) mutation in *Aspergillus nidulans* (Momany *et al.*, 1999; Shaw & Momany, 2002). We have previously characterized the *AnpmtA* gene and shown that the mutant exhibits a fragile cell wall phenotype and alteration in the carbohydrate composition, and particularly a reduction in the skeletal polysaccharides in the cell wall (Oka *et al.*, 2004).

Pmts are widely present in the higher eukaryotic cells. It has been implicated that mutations of the human *pmt* (*POMT1*) gene give rise to Walker–Warburg syndrome, a severe congenital neuromuscular disorder (Beltran-Valero de Bernabe *et al.*, 2002; Willer *et al.*, 2003). Disruption of the *POMT1* gene of mice results in embryonic lethality (Willer *et al.*, 2004). It has been suggested that human Pomt1 also requires other Pmt family proteins, such as Pomt2, to exert its protein *O*-mannosyltransferase activity (Manya *et al.*, 2004).

The black koji mould *Aspergillus awamori* produces large amounts of extracellular hydrolases and organic acids and thus has been used for the production of enzyme and fermented foods. It is well known that in the presence of starch, *A. awamori* secretes large amounts of glucoamylase I (GAI), encoded by the *glaA* gene (Hayashida *et al.*, 1989). GAI is composed of three functional domains: the catalytic domain (CD), the Ser/Thr-rich domain (Gp-I), and the starch-binding domain (SBD). The Gp-I is suggested to hold a spatial distance between the CD and the SBD, and is required for the correct folding of this protein (Semimaru *et al.*, 1995; Goto *et al.*, 2004). The Gp-I domain is highly glycosylated with *O*-mannose-type oligosaccharides composed of mono-, di-, and trimannoses. The *O*-mannose-type glycosylation is found not only in GAI but also in other important hydrolases, for instance, cellulases from the filamentous fungus *Trichoderma reesei* (Hui *et al.*, 2002). However, little is known about the function and synthetic pathway of the *O*-mannose-type oligosaccharides in filamentous fungi, although it has been suggested that *O*-mannosylation is closely associated with secretion (Kruszewska *et al.*, 1999), intracellular and extracellular stability (Goto *et al.*, 1999; Harty *et al.*, 2001; Nakatsukasa *et al.*, 2004) and the localization of proteins (Bourdineaud *et al.*, 1998).

In this study, we characterized the *pmtA* gene of *A. awamori* (*AapmtA*) and constructed an *AapmtA* disruptant to analyse the role of this gene with respect to fungal cell morphology and the *O*-mannosylation of GAI in *A. awamori*. In addition,

we investigated the effect of the structure of the *O*-mannose-type oligosaccharide of GAI on the secretion and function of GAI using the *AapmtA* disruptant.

METHODS

Micro-organisms and growth conditions. *A. nidulans* strain P3 (*veA*⁺, *biA1*, *pmtA::argB*) used in this study was previously constructed as an *AnpmtA* disruptant (Oka *et al.*, 2004). Strains were grown on MM medium (1% glucose, 0.6% NaNO₃, 0.052% KCl, 0.052% MgSO₄·7H₂O, 0.152% KH₂PO₄ and Hunter's trace elements, pH 6.5) as described by Barratt *et al.* (1965). *A. awamori* var. *kawachi* was grown on Culture A medium (4% potato starch, 1% diammonium hydrogen citrate, 0.1% KH₂PO₄, 0.01% MgSO₄·7H₂O, 0.01% NaCl, 10 µg l⁻¹ each of FeSO₄, MnSO₄, CuSO₄ and CoSO₄) or potato dextrose (Becton Dickinson) medium supplemented with 2% agar as a solidifier (PDA). The growth experiments in submerged culture were done by inoculation with 2 × 10⁸ conidia into 100 ml Culture A medium in 500 ml culture flasks. The flasks were reciprocally shaken at 120 r.p.m. at 30 °C. Standard transformation procedures for *A. nidulans* and *A. awamori* were used (Yelton *et al.*, 1984). Plasmids were amplified in *Escherichia coli* JM109 or XL-1 Blue.

Isolation of the *AapmtA* gene. A genomic DNA library of *A. awamori* var. *kawachi* was constructed. Genomic DNA was partially digested with *Sau3A*I and the resultant DNA fragments of 40–50 kb were fractionated. The DNA fragments thus obtained were inserted into the *Bam*HI site of SuperCosI (Stratagene) and subjected to *in vitro* packaging using Gigapack III XL (Stratagene). *E. coli* transformants carrying the genomic DNA were analysed by colony hybridization. A partial region of the *AapmtA* gene was amplified by degenerate PCR with primers *AapmtA*-F [5'-GA(C/T)GT(A/C/G/T)-CA(C/T)CCIC(C/A/C/G/T)(C/T)T-3'] and *AapmtA*-R [5'-(A/G)TA-(A/G)CA(A/G)GT(A/T)AC(C/T)TG(C/T)TG(C/T)TG-3'] and was used as a hybridization probe. A clone carrying pSC-*AapmtA* showed a positive hybridization signal with the probe. The 7.2 kb *Ban*III–*Kpn*I fragment from pSC-*AapmtA* was inserted into the corresponding sites of pBluescript-II KS⁺ to yield pBS-*AapmtA* containing the entire *pmtA* gene. In order to isolate the cDNA of the *AapmtA* gene, RT-PCR was performed with a High Fidelity RNA PCR kit (Takara) using primers *AapmtA*-RT-F (5'-TTGGATCCGAAATGGCTGAAA-TTGGCTTTG-3') and *AapmtA*-RT-R (5'-GTTCTCTCGAGGTTAGC-GATTCCGCAAC-3').

Sequence analysis. To analyse the nucleotide and amino acid sequences, GENETYX-MAC (Genetyx Co., Tokyo, Japan) was used according to the supplier's protocol.

Complementation of the *pmtA* mutation of *A. nidulans* with *AapmtA*. A plasmid for expression of *AapmtA* was constructed as follows. PCR was performed to amplify the entire *AapmtA* gene using KOD DNA polymerase (Toyobo), primers *AapmtA*-expression-F (5'-AAAACCCGGGATCGCATTTCTTCAGAATCT-3') and *AapmtA*-expression-R (5'-AAAACCCGGGCTTGTGTTCAGAGTTGTCA-3'), and pSC-*AapmtA* as template DNA. The amplified 4.0 kb DNA fragment was digested with *Sma*I and inserted into the same site of pPTR-II (Takara) carrying the *ptrA* gene as a selective marker and the *AMA1* sequence, to yield pPTRII-*AapmtA*. Transformation of *A. nidulans* strain P3 was done with pPTRII-*AapmtA* and pPTR-II. Transformants were selected on MM medium supplemented with 0.1 µg pyrithiamine ml⁻¹ and 0.8 M NaCl. Three transformants were arbitrarily selected. The introduction of *AapmtA* into strain P3 was confirmed by Southern blot analysis. The 2.5 kb coding region of the *AapmtA* gene was amplified by PCR with primers *AapmtA*-RT-F and *AapmtA*-RT-R and also used as a probe.

Construction of the *AapmtA* disruptant. A plasmid, pBS- Δ AapmtA::hph, for disruption of the *AapmtA* gene was constructed as follows. The 4.0 kb *SpeI* and *HindIII* DNA fragment from pBS-hph (Goto *et al.*, 1997) was inserted into the middle of the *AapmtA* gene at the *AatII* site of pBS-AapmtA to yield pBS- Δ AapmtA::hph. The plasmid pBS- Δ AapmtA::hph was used for the transformation of *A. awamori* var. *kawachi*. The disruption of the *AapmtA* gene in the hygromycin B-resistant transformants was confirmed by Southern blot analysis. The 0.8 kb N-terminal region of the *AapmtA* gene was amplified by PCR with primers AapmtA-NF (5'-AGACTCTCA-ACAAAAGCTGG-3') and AapmtA-NR (5'-TCTTCGGGATACTTC-TCACC-3') and used as a probe.

Analysis of efficiency of conidiation. The efficiency of conidiation was determined as previously described (Oka *et al.*, 2004). Approximately 10^5 conidia were inoculated onto the PDA medium. After 2 weeks of cultivation to allow hyphal development at 30 °C, the formed conidia were collected by suspending mycelia in 5 ml of 0.01 % (w/v) Tween-20 solution. The number of conidia in the suspension was counted using a haemocytometer.

Analysis of of glucan and chitin content. Strains were grown on MM medium for 40 h. Analysis of the glucan and chitin content was done according to the methods described previously (Oka *et al.*, 2004).

Purification and assay of GAI. *A. awamori* wild-type (wt) and *pmtA*-disruptant strains were cultured for 48 h in 8 l of Culture A medium with 4 % starch to allow induction of *glaA* expression. Purification of GAI was done by affinity chromatography and gel filtration, as previously described (Goto *et al.*, 1999). The GAI activity was assayed using soluble starch as substrate. One unit of GAI activity was defined as the amount of enzyme that liberated 1 μ mol of reducing sugar from soluble starch in 1 min. The amounts of the reducing sugar were determined using glucose as a standard by the dinitrosalicylic acid (DNS) method (Goto *et al.*, 1997). Peptide-*N*-glycosidase F (Roche) was used to remove the *N*-glycosylated oligosaccharides of GAI. The reaction mixture contained 20 mM phosphate buffer, pH 8.6, 0.2 % (w/v) SDS, 1 % (w/v) 2-mercaptoethanol and 100 μ g GAI. After boiling for 3 min followed by the addition of NP-40 detergent (1 %, w/v) and peptide-*N*-glycosidase F (2 units), the mixture was incubated at 37 °C for 24 h. Endo H_f (BioLabs Inc.) was also used to remove the *N*-glycosylated oligosaccharides of GAI. The reaction mixture contained 2 mM Tris/HCl buffer, pH 7.5, 5 mM NaCl, 0.5 mM EDTA, Endo H_f (1000 units) and 0.1 μ g GAI. The mixture was incubated at 37 °C for 24 h.

Circular dichroism. Circular dichroism (CD) spectroscopy was performed with a Jasco J-720 spectrometer using 5.72 μ M GAI at 25 °C. The spectra were measured over a wavelength range of 190–250 nm at 0.2 nm resolution and band width 2.0 nm. The final spectra were the sum of four scans accumulated at a speed of 50 nm min⁻¹ with a response time of 2 s.

Analysis of O-linked oligosaccharides from GAI. Isolation of the O-linked oligosaccharides was done as described by Yoko-o *et al.* (1998), with some modifications. The O-linked oligosaccharides of GAI were prepared by hydrazinolysis (Hydrazinolysis Reagent C, Honen Co., Tokyo, Japan) using Hydracub S-206 (Honen) according to the manufacturer's protocol. GAI (500 μ g) was hydrolysed by hydrazine anhydride at 65 °C for 6 h. The liberated O-linked oligosaccharides from GAI were labelled with fluorescent *p*-aminobenzoic acid ethylester (ABEE) using an ABEE labelling kit (Honen) according to the manufacturer's protocol. Treatment of the O-linked oligosaccharides with α -mannosidase (jack bean, Sigma) was done according to the manufacturer's protocol. α -1,2-Mannobiose (M2) and α -1,2-mannotriose (M3) were synthesized by α -1,2-mannosyltransferase (Calbiochem). The synthesized M2 and M3, as well as M1, were

labelled with ABEE and used as a standard. The ABEE-labelled oligosaccharides were analysed by an HPLC apparatus equipped with a Honepak C18 column (4.6 $\times$ 75 mm) at a flow rate of 0.5 ml min⁻¹ with acetate solution (0.1 M ammonium acetate buffer, pH 4.0, 10.5 %, v/v, acetonitrile) at 25 °C (Kobayashi *et al.*, 2002). The structure of the oligosaccharides was determined by comparison of the retention times with the ABEE-labelled standard. As for α -mannotetraose (M4), the peak fraction corresponding to the deduced M4 was detected by an earlier retention time than that of M3. The same fraction was completely converted to M1 by treatment with α -mannosidase. Thus we identified this peak fraction as M4. The amount of the oligosaccharides was quantified from their fluorescence intensity. The analyses were done in triplicate independently.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis. The mass spectrometric analyses of GAI were done at Shimadzu Biotech, Japan, using an AXIMA-CFR MALDI-TOF MS (Shimadzu Co.).

Thermal stability of GAI. The thermal stability of GAI (82.5 milli-units) was determined. After GAI was incubated at temperatures ranging from 30 to 70 °C for 8 h, the remnant GAI activity was independently measured twice.

RESULTS

Analysis of the *pmtA* gene of *A. awamori*

We cloned the *pmtA* gene of *A. awamori* var. *kawachi* (*AapmtA*) from the genomic cosmid library. The 7.2 kb *BanIII*–*KpnI* DNA fragment obtained contains a 2223 bp ORF from which a 741 amino acid protein may be deduced with a putative molecular mass of 84.7 kDa. The nucleotide sequence of *AapmtA* was deposited in GenBank (accession no. AF396953). Four introns were present in the *AapmtA* gene, and all the intron–exon junctions follow the canonical GT–AG rules. The deduced protein sequence of *AapmtA* was compared to those of proteins from other organisms that had been characterized as protein O-D-mannosyltransferases (Pmts). AaPmtA showed an 80.4 % sequence identity with AnPmtA (accession no. AF225551), 48.5 % with *S. cerevisiae* Pmt2p (L05146), 48.5 % with *S. cerevisiae* Pmt3p (X83797), 36.8 % with *Candida albicans* Pmt1 (AF000232) and 33.1 % with *S. cerevisiae* Pmt1p (L19169). Analysis of the hydropathy profile suggests that AaPmtA is a protein with multiple transmembrane regions, as shown in ScPmt1p (data not shown) (Strahl-Bolsinger *et al.*, 1999). Pmt proteins can be classified into three major families, PMT1, PMT2 and PMT4 (Girrbach *et al.*, 2000). A phylogenetic analysis revealed that AaPmtA belongs to the PMT2 family (data not shown).

Complementarity of the *AapmtA* gene in the *A. nidulans pmtA* mutant

To examine whether *AapmtA* functions as a Pmt, the 4.0 kb DNA fragment carrying the entire *AapmtA* gene was introduced into the *pmtA*-disruptant strain P3 of *A. nidulans*. Introduction of the *AapmtA* gene in the selected transformants was confirmed by Southern blot analysis. The hybridized band of 4.0 kb in size including the *AapmtA* gene

was detected in strain P3 carrying pPTR-II-*AapmtA* (data not shown). The same band was not detected with strain P3 carrying pPTR-II alone. We have previously reported that the *pmtA*-disruptant P3 showed a reduced rate of colony formation due to deficient hyphal development (Oka *et al.*, 2004). In particular, strain P3 exhibited a severe growth repression at 42 °C. The introduction of *AapmtA* into strain P3 resulted in the recovery of colony formation not only at 30 °C but also at 42 °C (Fig. 1). This result indicates that the *AapmtA* protein has a coordinative function with the *AnpmtA* protein as a *Pmt* in *A. nidulans* strain P3.

Characterization of the *AapmtA* disruptant

The plasmid pBS- Δ *AapmtA*::*hph* was used for transformation of *A. awamori* var. *kawachi* to construct the disruptant of the *AapmtA* gene (Fig. 2A). We obtained approximately 800 hygromycin B-resistant transformants and found that a transformant designated *AapmtA* solely formed a small colony. Southern blot analysis revealed that the 7.0 kb *Bam*HI and the 6.3 kb *Apal* DNA fragments were hybridized with the probe in the wt strain (Fig. 2B), and the 3.8 kb *Bam*HI and the 8.0 kb *Apal* DNA fragments were hybridized in strain *AapmtA*, respectively, indicating that homologous double-crossover recombination occurs at the *AapmtA* locus in strain *AapmtA*.

The *AapmtA* disruption caused the inhibition of hyphal extension and conidia formation. We observed that strain *AapmtA* formed a fragile colony of approximately 30 % of the diameter of the wt strain after cultivation on PDA

Fig. 1. Complementation of the *AnpmtA*-disruptant P3 with the *AapmtA* gene. Colony formation of *AnpmtA*-disruptant P3 with or without the *AapmtA* gene. *A. nidulans* transformants were grown on MM agar medium for 3 days at 30 °C (upper panels) or 42 °C (lower panels). Left- and right-hand panels show the *AnpmtA*-disruptant P3 carrying pPTR-II and pPTR-II-*AapmtA*, respectively.

Fig. 2. Disruption of the *pmtA* gene in *A. awamori*. (A) Schematic representation of the *pmtA* disruption. The regions used as probes for Southern blot analysis are shown as bars. Abbreviations: A, *Apal*; B, *Bam*HI. (B) Southern blot analysis was carried out for the *Bam*HI-digested total DNA (lanes 1 and 2) and the *Apal*-digested total DNA (lanes 3 and 4) of the wt (lanes 2 and 4) and the *AapmtA*-disruptant strain *AapmtA* (lanes 1 and 3) using the probe indicated in the figure.

medium at 30 °C for 48 h. The growth defect was almost recovered on PDA medium in the presence of 0.8 M KCl (Fig. 3A). It has been reported that the *AnpmtA* mutation causes increased sensitivities to Calcofluor white (CW) and Congo red (CR) in *A. nidulans* (Momany *et al.*, 1999; Oka *et al.*, 2004). Thus, we examined the susceptibility of *A. awamori* to these drugs. When concentrations of 450 and 900 µg CR ml⁻¹ were used, strain *AapmtA* showed hypersensitivity compared to the wt strain. However, the hyphal extension of strain *AapmtA* was not affected significantly at 300 and 600 µg CW ml⁻¹. In addition, strain *AapmtA* could not grow on PDA at 42 °C. CR and CW sensitivities were also repressed by the addition of an

Fig. 3. (A) Increased sensitivity to anti-fungal reagents of strain AaPMTA (right panel) compared to the wt strain (left panel). Strains were grown in the presence of Calcofluor white (CW300, 300 $\mu\text{g ml}^{-1}$; CR600, 600 $\mu\text{g ml}^{-1}$) and Congo red (CR450, 450 $\mu\text{g ml}^{-1}$; CR900, 900 $\mu\text{g ml}^{-1}$) on PDA medium for 48 h at 30 °C. (B) The hyphal phenotypes of the wt strain and the *pmtA*-disruptant strain AaPMTA. (C) The wt strain (a) and strain AaPMTA (b–e) were grown in PD medium for 3 days. A moribund region near the centre of the colony was observed (e).

osmotic stabilizer (Fig. 3A). We determined the conidiation efficiency of strain AaPMTA and the wt strain. The formation of black conidia was remarkably repressed in strain AaPMTA in comparison with the wt strain on PDA medium (Fig. 3B). The efficiency of conidiation in strain AaPMTA was reduced to approximately 3.2 % compared with the wt strain and it was partially remediable by the addition of the osmostabilizer (data not shown). The structure of the hyphae in the PD liquid medium was observed by microscopy. The hyphae of the *A. awamori* wt strain were linearly grown [Fig. 3(C), image (a)]. In contrast, swollen hyphal formation (balloon formation) appeared in the apical or umbilical regions of the hyphae [Fig. 3(C), images (b)–(e)] in strain AaPMTA. Hyphae in the central

region of a colony were severely swollen in strain AaPMTA [Fig. 3(C), image (e)]. Abnormal branching of hyphae also appeared [Fig. 3(C), images (c) and (d)].

To clarify the difference in cell wall composition between the wt strain and strain AaPMTA, the cell wall from each strain was fractionated. The *AapmtA* disruptant showed a 27 % decrease in the alkali-insoluble fraction. The GlcNAc contents of the cell walls of the *AapmtA* disruptant were increased by 28 % compared with the wt strain. The total sugar content and the alkali-soluble fraction in the cell wall of the *AapmtA* disruptant were comparable to those of the wt strain (Table 1). The alkali-insoluble fraction is believed to be responsible for fungal cell wall rigidity (Fontaine

Table 1. Cell wall composition of *A. awamori* wild-type strain and *pmtA* mutant strain PMTA

The amount of total sugar is expressed as the amount of glucose equivalent. Values in parentheses are percentages based on the wild-type strain.

Strain	Total sugar [$\mu\text{g (mg cell wall)}^{-1}$]	Alkali-soluble fraction [$\mu\text{g (mg cell wall)}^{-1}$]	Alkali-insoluble fraction [$\mu\text{g (mg cell wall)}^{-1}$]	Total GlcNAc [$\mu\text{g (mg cell wall)}^{-1}$]
Wild-type	609 $\pm$ 92 (100)	307 $\pm$ 21 (100)	277 $\pm$ 30 (100)	126 $\pm$ 7 (100)
PMTA	580 $\pm$ 8 (95)	294 $\pm$ 31 (96)	202 $\pm$ 3 (73)	161 $\pm$ 11 (128)

et al., 2002). The reduction in the alkali-insoluble fraction indicates that the cell wall was weakened. The increase in GlcNAc content indicates that the loss of *AapmtA* induces a set of compensatory reactions to ensure cell integrity.

Structural analysis of GAI

In order to confirm the *in vivo* mannosylation activity toward the secretory protein GAI, GAIs from the wt strain (GAI-WT) and strain AaPMTA (GAI-AaPMTA) were purified and analysed by SDS-PAGE (Fig. 4A). The mobility of GAI-AaPMTA on SDS-PAGE was apparently greater than that of GAI-WT. The molecular masses of GAI-WT and GAI-AaPMTA were estimated to be 96.0 and 89.5 kDa, respectively. After treatment with peptide-*N*-glycosidase F or Endo H_f, GAI-WT and GAI-AaPMTA were completely de-*N*-glycosylated and designated GAI-WT-DENG and GAI-AaPMTA-DENG, respectively. The molecular masses of GAI-WT-DENG and GAI-AaPMTA-DENG were estimated to be 89.0 and 82.5 kDa, respectively. The difference in the molecular mass was estimated to be approximately 7 kDa between GAI and the de-*N*-glycosylated GAI, in both the wt and AaPMTA strains. Thus, the *N*-glycosylation was not affected by the *AapmtA* disruption. The results indicated that the amount of the *O*-linked oligosaccharides of GAI-AaPMTA decreased compared with that of GAI-WT. For a more detailed analysis, a MALDI-TOF MS analysis was done to determine their correct molecular masses. At the peak fraction, the molecular masses of GAI-WT, GAI-AaPMTA, GAI-WT-DENG and GAI-AaPMTA-DENG were determined to be 82 500, 76 500, 79 000 and 73 000 Da, respectively (Fig. 4B, C). The Endo H_f was detected as the peak of 71 500 Da (Fig. 4C). A variety of protein molecules were found in GAI-WT and GAI-AaPMTA due to the heterogeneity of the *O*-linked oligosaccharides (Fig. 4B). Thus, the distribution of the average molecular mass of GAI-AaPMTA and GAI-AaPMTA-DENG shifted 6000 Da lower than that of GAI-WT and GAI-WT-DENG, respectively. The molecular masses of GAI-DENGs from which the *N*-glycosylated oligosaccharides were removed by Endo H_f shifted approximately 3500 Da lower than those of the respective intact GAIs. This demonstrates that the *N*-glycosylation was not affected by the *AapmtA* disruption. Two *N*-glycosylation sites, Asn-182 and Asn-395 in GAI from *A. awamori*, were *N*-glycosylated (Chen *et al.*, 1994), and in general, *N*-linked oligosaccharides were composed of eight mannoses and two GlcNAcs, with a molecular mass of 1720 Da. Thus, the difference in the molecular masses (3500 Da) between GAIs and GAI-DENGs was reasonable. The putative molecular mass of the mature GAI without carbohydrates is calculated to be 65 765 Da. Taking this into account, approximately 13 200 and 7200 Da of mannose moieties was *O*-glycosidically linked to GAI-WT-DENG and GAI-AaPMTA-DENG, respectively. This result is further evidence that the AaPmtA protein catalyses the *O*-mannosylation of GAI. In addition, the MALDI-TOF MS analysis clearly revealed that GAI-AaPMTA is still mannosylated.

Fig. 4. (A) Immunoblotting of GAIs. Purified GAI-WT (lanes 2 and 3) and GAI-AaPMTA (lanes 4 and 5) were separated on 8% SDS-PAGE, and GAIs were detected by immunoblotting using anti-GAI antibody. Precision plus protein standards (Bio-Rad) were used as a molecular size marker (lanes 1 and 6). GAI was treated with peptide-*N*-glycosidase F (lanes 3 and 5). The molecular mass of GAI was estimated by an image analyser (Bio-profile V6.0) after SDS-PAGE. (B, C) MALDI-TOF MS spectrograms. The molecular masses of GAI-WT and GAI-AaPMTA (B), and GAI-WT-DENG and GAI-AaPMTA-DENG (C).

Structural analysis of the *O*-linked oligosaccharides of GAI

The *O*-linked oligosaccharides of GAI were isolated by hydrazinolysis from GAI-WT and GAI-AaPMTA and separated by HPLC (Fig. 5). Several peaks were detected in both GAIs, and some of them were identified as D-mannose (M1),

Fig. 5. O-Linked oligosaccharide profiles of GAI-WT and GAI-AaPMTA. Abbreviations: M1, D-mannose; M2, α -1,2-mannobiose; M3, α -1,2-mannotriose; M4, putative α -mannotetraose. Profiles show O-linked oligosaccharides released from GAI-WT (A), GAI-AaPMTA (B), GAI-WT treated with α -mannosidase (C) and GAI-AaPMTA treated with α -mannosidase (D).

α -1,2-mannobiose (M2), α -1,2-mannotriose (M3) and α -mannotetraose (M4). The ratios of M1, M2, M3 and M4 in GAI-WT were 38.6, 31.6, 26.7 and 3.2 %, respectively. The O-linked oligosaccharides from GAI-AaPMTA also yielded four peaks corresponding to M1, M2, M3 and M4, with ratios of 22.1, 51.8, 23.2 and 2.9 %, respectively. The α -mannosidase treatment resulted in the disappearance of M2, M3 and M4, and concomitantly the generation of M1. The amount of M1 generated is proportional to the total number of mannose residues O-glycosidically attached to GAI. Thus, the total mannose residues in GAI decreased to 59.4 % compared with that of GAI-WT. The amounts of M1, M3 and M4 in GAI-AaPMTA were significantly decreased to 34.1, 51.7 and 54.6 %, respectively, in comparison with those of GAI-WT. In contrast, unexpectedly, the amount of M2 in GAI-AaPMTA (97.4 %) was comparable with that of GAI-WT. This indicates that the *AapmtA* disruption causes not only a reduction in the amounts of the manno-oligosaccharides, but also an alteration of the O-linked oligosaccharide profiles.

Functional analysis of O-linked oligosaccharide of GAI

The growth of strain AaPMTA was reduced on PDA plate medium (Fig. 3A); however, strain AaPMTA could grow in the Culture A liquid medium at a rate similar to that of the wt strain. GAI-AaPMTA was secreted to the medium, and the amount of GAI-AaPMTA was comparable to that of GAI-WT (Fig. 6). It is well known that *A. awamori* produces

proteases that can degrade GAI to yield multiple forms of glucoamylase. The major bands with slower and faster mobilities on SDS-PAGE correspond to intact GAI and to truncated GAI that has lost its starch-binding domain,

Fig. 6. Secretion of GAI from *AapmtA*-disruptant AaPMTA and wt strains after cultivation in Culture A liquid medium. The growth experiments in a submerged culture were done by inoculation with 2×10^8 conidia into 100 ml Culture A medium in 500 ml culture flasks. Proteins in the culture filtrate of the wt strain (left-hand lane of each pair) and AaPMTA (right-hand lane of each pair) were separated on 7% SDS-PAGE, and GAI was detected by immunoblotting using anti-GAI antibody.

Fig. 7. (A) CD spectrum of GAI from the wt strain and strain AaPMTA. CD spectra of GAI were measured using a Jasco J-720 spectrometer at 25 °C. The solid and dashed lines show GAI-WT and GAI-AaPMTA, respectively. (B) Thermal stability of GAIs from the wt strain and strain AaPMTA. After GAI was incubated at each temperature for 8 h, the remnant GAI activity was independently measured twice. ■, GAI-WT; ▲, GAI-AaPMTA.

respectively. The ratios of truncated GAI to intact GAI from both the wt strain and the *AapmtA* disruptant increased as the time of cultivation increased.

GAI-WT and GAI-AaPMTA from the liquid cultures were purified and their enzymic properties determined. The specific activities of GAI-WT and GAI-AaPMTA toward soluble starch (means $\pm$ standard deviations) were 211 ± 7 units mg^{-1} and 132 ± 1 units mg^{-1} , respectively. The specific activity of GAI-AaPMTA decreased to 63 % compared with that of GAI-WT. In addition, analysis of the CD spectrum revealed that there is no apparent alteration in the secondary structure between GAI-WT and GAI-AaPMTA (Fig. 7A). The thermal stability of GAI was used to clarify the effect of the underglycosylation of the GAIs. The stability of GAI-AaPMTA decreased at temperatures higher than 45 °C, compared with that of GAI-WT (Fig. 7B). These results indicate that the *O*-linked oligosaccharides are involved in the stability of the GAI.

DISCUSSION

Currently, the function of *O*-mannosylation in the eukaryotic cell is of increasing interest. In mammals, α -dystroglycans, which contain *O*-mannose-type chains, play a role in binding to extracellular matrix ligands such as laminin, and an α -dystroglycan defect causes a neuromuscular disorder (Beltran-Valero de Bernabe *et al.*, 2002; Willer *et al.*, 2003). In *S. cerevisiae*, the lack of *O*-mannosylation causes the incorrect proteolytic processing of the WSC family and of Mid2p, which are the cell-surface sensor proteins responsible for cell wall integrity through the MAP kinase pathway, resulting in cell death (Lommel *et al.*, 2004). Moreover, *O*-mannosylation functions as a sorting determinant for the cell-surface delivery of a protein (Proszynski *et al.*, 2004).

In this study, we isolated the *pmtA* gene of *A. awamori* var. *kawachi* and constructed the disruptant of the *AapmtA* gene to analyse the function of the *O*-linked oligosaccharides in *A. awamori*. The heterologous expression of the *AapmtA* gene in the *pmtA* disruptant of *A. nidulans* could recover the rate of colony formation and the temperature sensitivity of this strain (Fig. 1). Therefore, AaPmtA functions as a protein *O*-mannosyltransferase, as previously demonstrated with AnPmtA (Shaw & Momany, 2002; Oka *et al.*, 2004). The *AapmtA* disruption produced critical damage to the cell; in particular, it caused a severe inhibition of hyphal extension and conidia formation. Thus, AaPmtA plays crucial roles in the morphogenesis of *A. awamori*. The formation of a balloon structure in hyphae was also observed with a high frequency. These phenotypic alterations in *A. awamori* were to some extent similar to those of the *pmtA* disruptant of *A. nidulans* strain P3. However, strain AaPMTA exhibited more severe phenotypes than the *AnpmtA* disruptant. Thus, *A. awamori* largely relies on the *O*-mannosylation by *pmtA*. In *A. nidulans*, the cell walls in the *pmtA* disruptant change to an incorrect structure due to the reduction of the skeletal polysaccharides of the β -glucans and the increase in the chitin content (Oka *et al.*, 2004). Thus it is suggested that similar phenomena could occur in the *A. awamori* strain AaPMTA.

GAI is an important industrial enzyme and is produced in large quantities from *A. awamori* var. *kawachi*. In the last few decades, much effort has gone into the study of GAI for industrial applications, including the enzymic properties, the function of domains and amino acids, and the 3D structure (Sauer *et al.*, 2000). However, little attention has been paid to the function and the synthetic pathway of the *O*-linked oligosaccharides of GAI. Disruption of the *AapmtA* gene resulted in the underglycosylation of GAI. The total amount of mannose residues of GAI-AaPMTA decreased to 55 % of that of GAI-WT. However, the

O-linked oligosaccharides still remained in GAI-AaPMTA. Recently, genome-sequencing projects have been completed for filamentous fungi such as *A. nidulans*, *Aspergillus fumigatus*, *Neurospora crassa* and *Fusarium graminearum*. BLAST search analysis reveals that these fungi have three distinct *pmt* orthologues belong to the PMT1, PMT2 and PMT4 families, indicating that additional AaPmtA isozymes participate in the synthesis of the O-mannose oligosaccharides in *A. awamori*.

According to the results of MALDI-TOF MS, a 6000 Da difference in the major molecular mass of GAI was observed between the wt strain and the *AapmtA* disruptant, whereby the corresponding 33 mannose residues are expected to be absent in GAI-AaPMTA. Previously, we reported that *A. awamori* GAI could not be a preferred substrate for AnPmtA, because the expression of the *A. awamori glaA* gene in *A. nidulans* strain P3 resulted in a slight decrease in the molecular mass of GAI (Oka *et al.*, 2004). Gentzsch & Tanner (1996) reported that in *S. cerevisiae*, Pmt proteins have distinct substrate specificities for proteins including Ser or Thr. Therefore, it is suggested that substrate recognition is different between AaPmtA and AnPmtA, although these proteins share the high sequence identity of 80.4%. Intriguingly, according to the structural analysis of the oligosaccharides of GAI, the amount of M2 from GAI-AaPMTA was comparable with that of GAI-WT, even though those of M1, M3 and M4 were reduced in GAI-AaPMTA (Fig. 5). The Gp-I region is composed of the successive sequence of Thr and Ser. Thus it is possible that the decrease in mannose residues linked to GAI-AaPMTA in the ER might allow GAI-AaPMTA to be a better substrate for the addition of the remaining O-linked mannoses by α -1,2-mannosyltransferase in the Golgi apparatus. The possibility that regulation mechanisms exist for O-glycosylation cannot be excluded. Janik *et al.* (2003) reported that the level of expression of *dpm1*, which encodes mannosylphosphodolichol synthase, was elevated by overexpression of the *mpg1* gene, which encodes GTP:mannose-1-phosphate guanylyltransferase in *T. reesei*. Thus, loss of *pmtA* may lead to alteration of the activities of other Pmt isozymes or mannosyltransferases to compensate for the loss of the O-mannosylation.

According to other reports, the O-linked oligosaccharides of GAI are mostly composed of mannose moieties ranging in size from one to three with linkages of α -1,2, α -1,3 and α -1,6, including not only a linear form but also a branched structure (Gunnarsson *et al.*, 1984; Pazur *et al.*, 1980). Moreover, the O-linked oligosaccharides contain glucose and galactofuranose moieties in addition to mannose moieties in the same protein (Gunnarsson *et al.*, 1984; Wallis *et al.*, 1999). As judged by the comparison with the retention times of authentic manno-oligosaccharide standards in chromatographic analysis, the main structures of the O-linked oligosaccharides of GAI were identified as Man-O-(Ser/Thr), Man- α -1,2-Man-O-(Ser/Thr) and Man- α -1,2-Man- α -1,2-Man-O-(Ser/Thr). This result indicates that the structure of the oligosaccharides of

GAI is composed mainly of α -1,2-linked mannose in the strains and under the cultivation conditions used. However, we also found the presence of the minor peaks in the chromatograms (Fig. 5A, B). Furthermore, some of them appear to be sensitive to jack bean α -mannosidase, suggesting that they could represent other O-linked oligomannose structures (Fig. 5C, D). In contrast, some of them appear to be resistant to jack bean α -mannosidase, suggesting that they could represent the terminal-glucose or galactofuranose oligosaccharides. This result implies that the structure of the oligosaccharides of GAI can be altered depending on the strains and the cultivation conditions used.

Little is known about the relationship between O-glycosylation and the secretion of proteins in filamentous fungi. Kruszewska *et al.* (1999) reported that the overexpression of the *S. cerevisiae DPM1* gene, encoding mannosylphosphodolichol synthase, in *T. reesei* results in an increased level of protein secretion and abnormal cell ultrastructure. In the present study, secretion of GAI from the *AapmtA*-disruptant AaPMTA was comparable with that from the wt strain in the Culture A liquid medium (Fig. 6). Thus the *AapmtA* disruption does not affect the secretion of GAI when fungal cells grow properly. Filamentous fungi are widely used for the production of homologous and heterologous proteins. However, when heterologous genes were expressed in fungi, the proteins that were not O-mannosylated in the original host were O-mannosylated in fungi (Mochizuki *et al.*, 2001). It is possible that an adventitious O-mannose residue was diminished or eliminated by expressing heterologous proteins in strain AaPMTA without decreasing the productivity.

A number of proteins secreted into the culture and localized in the cell wall are O-mannosylated (Mrsa & Tanner, 1999; Woo *et al.*, 2003). However, the functions of the O-mannosylation remain to be elucidated. We are currently attempting to determine the function of the O-mannosylation in secretory traffic using the *pmtA* disruptant.

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