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

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Industry important extracellular enzymes from filamentous fungi are often O-mannosylated. The structure and function of the pmtA (AapmtA) gene encoding the protein O-β-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 GAI revealed that approximately 33% of the amounts of O-linked oligosaccharides 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 β-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.

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-β-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 mannooligosyltransferases (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-β-mannosyltransferase; wt, wild-type.

The GenBank/EMBL/DDBJ accession number for the AapmtA sequence reported in this paper is AF396953.
The Pmt family proteins can be classified into the PMT1, PMT2 and PMT4 subfamilies on the basis of their phylogeny (Girrbach et al., 2003). Disruption of three different types of pmt genes results in the death of yeast cells (Gentzsch & 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 (AapmtA) mutation in Aspergillus nidulans (Momany et al., 1999; Shaw & Momany, 2002). We have previously characterized the AapmtA 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 et al., 2003). Disruption of the POMT1 gene of mice results in embryonic lethality (Willer et al., 2004). It has been suggested that human Pmt1 also requires other Pmt family proteins, such as Pmt2, 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 (Seminari 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 (Kruszewskia 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+, biaA, pmtA::argB) used in this study was previously constructed as an AapmtA 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 x 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 Sau3AI and the resultant DNA fragments of 40–50 kb were fractionated. The DNA fragments thus obtained were inserted into the BambHI site of SuperCos1 (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)/G/CA(G/T)/G/CA(C/T)/G/CA(G/T)/G/CA(C/T)/G/CA(C/T)/G/CA(G/T)/G/CA(G/T)/G/CA(C/T)) and AapmtA-R (5'-AA(GT)/A(A/G)CA(A/G)/GT/AT(C/T)/TG(C/T)/TG(C/T)/TG(C/T)/TG(C/T)/TG(C/T)/TG(C/T)/TG(C/T)/TG(C/T)) and was used as a hybridization probe. A clone carrying pSC-AapmtA showed a positive hybridization signal with the probe. The 7.2 kb BanIII–KpnI 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'-TGAGATGCGAAATGCTGCAGAATGGTCCCTGCTGAGC-3') and AapmtA-RT-R (5'-ATTTTGTTCCTAGAGGTACG-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 BanIII–KpnI 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'-TGAGATGCGAAATGCTGCAGAATGGTCCCTGCTGAGC-3') and AapmtA-RT-R (5'-ATTTTGTTCCTAGAGGTACG-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'-AAAACCCGGGATCCATTCTCAGAATCT-3') and AapmtA-expression-R (5'-AAAAACCCGGTCTGTTCACAAAATTTGTCAC-3') and pSC-AapmtA as template DNA. The amplified 4.0 kb DNA fragment was digested with SmaI and inserted into the same site of pPTR-II (Takara) carrying the ptrA gene as a selective marker and the AMAI sequence, to yield pPTR-II-AapmtA. Transformation of A. nidulans strain P3 was done with pPTR-II-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-AapmtA::hph, for disruption of the AapmtA gene was constructed as follows. The 4.0 kb Spd 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-AaAapmtA::hph. The plasmid pBS-AaAapmtA::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′-AGACTCTCAGCACAAAGCTGG-3′) and AapmtA-NR (5′-TCTTCGGGATACCTTC-TACC-3′) and used as a probe.

Analysis of efficiency of conidiation. The efficiency of conidia was determined as the amount of enzyme that liberated 1 mgl-1,2-Mannobiose (M2) and 0 mgl-1,2-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 β-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 (105146), 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

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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-AaapmtA::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 BamHI and the 6.3 kb Apai DNA fragments were hybridized with the probe in the wt strain (Fig. 2B), and the 3.8 kb BamHI and the 8.0 kb Apai 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 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

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**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, Apai; B, BamHI. (B) Southern blot analysis was carried out for the BamHI-digested total DNA (lanes 1 and 2) and the Apai-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.
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

![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 µg ml⁻¹; CR600, 600 µg ml⁻¹) and Congo red (CR450, 450 µg ml⁻¹; CR900, 900 µg 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).

<table>
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<tr>
<th>Strain</th>
<th>Total sugar [µg (mg cell wall)⁻¹]</th>
<th>Alkali-soluble fraction [µg (mg cell wall)⁻¹]</th>
<th>Alkali-insoluble fraction [µg (mg cell wall)⁻¹]</th>
<th>Total GlcNAc [µg (mg cell wall)⁻¹]</th>
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<td>Wild-type</td>
<td>609 ± 92 (100)</td>
<td>307 ± 21 (100)</td>
<td>277 ± 30 (100)</td>
<td>126 ± 7 (100)</td>
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<tr>
<td>PMTA</td>
<td>580 ± 8 (95)</td>
<td>294 ± 31 (96)</td>
<td>202 ± 3 (73)</td>
<td>161 ± 11 (128)</td>
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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, GAI s 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 \(H_\varepsilon\), 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 \(H_\varepsilon\) 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 \(H_\varepsilon\) shifted approximately 3500 Da lower than those of the respective intact GAI s. 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 GlcNAc s, with a molecular mass of 1720 Da. Thus, the difference in the molecular masses (3500 Da) between GAI 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 and GAI-AaPMTA-DENG is still mannosylated.

**Fig. 4.** (A) Immunoblotting of GAI s. Purified GAI-WT (lanes 2 and 3) and GAI-AaPMTA (lanes 4 and 5) were separated on 8% SDS-PAGE, and GAI s 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 GAI s, and some of them were identified as \(D\)-mannose (M1),
α-1,2-mannobiose (M2), α-1,2-mannotriose (M3) and putative 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. 5. O-Linked oligosaccharide profiles of GAI-WT and GAI-AaPMTA. Abbreviations: M1, α-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).](http://mic.sgmjournals.org)

![Fig. 6. Secretion of GAIs 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 2×10⁶ 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.](http://mic.sgmjournals.org)
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 GAI 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 ± 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 GAI-WT and GAI-AaPMTA (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 Aspergillus 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 isoenzymes 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-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 z-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 z-1,2, z-1,3 and z-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-z-1,2-Man-O-(Ser/Thr) and Man-z-1,2-Man-z-1,2-Man-O-(Ser/Thr). This result indicates that the structure of the oligosaccharides of GAI is composed mainly of z-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 z-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 z-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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**REFERENCES**


