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Effect of a *PMR1* Disruption on the Processing of Heterologous Glycoproteins Secreted in the Yeast *Saccharomyces cerevisiae*

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Abstract The *Saccharomyces cerevisiae PMR1* gene encodes a Ca^{2+}-ATPase localized in the Golgi. We have investigated the effects of *PMR1* disruption in *S. cerevisiae* on the glycosylation and secretion of three heterologous glycoproteins, human α_1-antitrypsin (α_1-AT), human antithrombin III (ATHIII), and *Aspergillus niger* glucose oxidase (GOD). The *pmr1* null mutant strain secreted larger amounts of ATHIII and GOD proteins per a unit cell mass than the wild type strain. Despite a lower growth rate of the *pmr1* mutant, two-fold higher level of human ATHIII was detected in the culture supernatant from the *pmr1* mutant compared to that of the wild-type strain. The *pmr1* mutant strain secreted α_1-AT and the GOD proteins mostly as core-glycosylated forms, in contrast to the hyperglycosylated proteins secreted in the wild-type strain. Furthermore, the core-glycosylated forms secreted in the *pmr1* mutant migrated slightly faster on SDS-PAGE than those secreted in the *mnn9* deletion mutant and the wild type strains. Analysis of the recombinant GOD with anti-α1,3-mannose antibody revealed that GOD secreted in the *pmr1* mutant did not have terminal α1,3-linked mannoses unlike those secreted in the *mnn9* mutant and the wild type strains. The present results indicate that the *pmr1* mutant, with the super-secretion phenotype, is useful as a host system to produce recombinant glycoproteins lacking high-mannose outer chains.

Keywords: *PMR1*, *Saccharomyces cerevisiae*, secretion, glycosylation, heterologous protein.

INTRODUCTION

Yeasts have proven to be an attractive host system for the secretory production of proteins originated from higher eukaryotic cells [1]. As a microbial eukaryotic system, yeasts secrete and process glycoproteins in much the same way as mammalian cells do. Proteins entering the secretory pathway adopt their final tertiary structure in the endoplasmic reticulum (ER), where N- and O-linked glycans are attached to glycoproteins. They are further processed in the Golgi apparatus and then targeted to various organelles, becoming plasma membrane components, or are secreted outside the cell.

To improve the secretion capacity of yeasts for heterologous proteins, a number of research groups have attempted to develop yeast mutants with altered secretion properties [2,3]. One good example is the *ssc1* mutant of the traditional yeast *Saccharomyces cerevisiae*, which has been isolated as a mutant strain with the increased secretion of bovine prochymosin [4]. Interestingly, *SSC1* was later shown to be identical to *PMR1*, a gene coding for a P-type Ca^{2+}-ATPase, suggesting that the increased secretion resulted from the defect of the Ca^{2+} pump in the secretory pathway [5]. Intensive genetic and biochemical studies have provided the evidence that Pmr1p is a Ca^{2+}-transporting ATPase in the Golgi of *S. cerevisiae*, where it deeply influences the glycosylation and secretory processes [6,7].

As expected from the implication of Pmr1p in intracellular transport of Ca^{2+} and Mn^{2+} [8], *pmr1* mutant strains display some pleiotropic phenotypes that can be usefully exploited for the production of recombinant proteins. Mutation in the *PMR1* gene has been shown to increase secretion of some other heterologous proteins other than prochymosin, such as human prourokinase and bovine growth hormone, that are poorly secreted by the wild type [5,9-11]. The strains carrying the *pmr1*Δ allele have also shown a reduced level of hyperglycosylation of the secreted proteins [5,8,12]. Interestingly, it has been reported that invertase secreted from the *pmr1* deletion mutant did not reveal the α1,3-Man epitope although an α1,3-mannosyltransferase was active in microsomal extracts from the mutant strain [13]. More recently, a *pmr1* mutant was shown to be unable to degrade CpY*, a misfolded soluble ER protein [8], suggesting that loss of *PMR1* function diminishes the export of malfolded proteins, trapped in the ER, into the cytosol for ubiquitination

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and subsequent degradation by proteasomes. These observations suggest that *pmr1* mutant strains would be superior to the wild-type strain as a host system for the secretion of nonnative proteins, especially such as heterologous glycoproteins.

As a part of our studies to improve the quality as well as the quantity of recombinant glycoproteins secreted in yeasts, we have investigated the effect of a *PMR1* disruption on the secretion and glycosylation of heterologous proteins in *S. cerevisiae*. Human α_1-antitrypsin (α_1-AT), human antithrombin III (ATHIII) and *Aspergillus niger* glucose oxidase (GOD), which undergo N-linked glycosylation during the secretion process, were used as the reporter proteins in the present study.

MATERIALS AND METHODS

Yeast Strains

The *mnn9*Δ, *mnn1*Δ, and *pmr1*Δ strains used in this study were deletion mutant strains derived from *S. cerevisiae* L3262a (*MATa ura3-52 leu2-3,112 his4-34*). Construction of the *MNN9* and the *PMR1* deletion strains were described previously [14,15]. An *mnn1* deletion strain was constructed by transforming L3262a with the linear DNA fragment of the *MNN1* gene disrupted with the *LEU2* gene (*mnn1*Δ*::LEU2*). The 1.6 kb *NdeI*/*SalI* fragment corresponding to the internal coding region of the *MNN1* gene [16] was deleted and replaced by the 2.0 kb *HpaI*/*SalI* *LEU2* gene fragment derived from YEp351 [17], generating the *mnn1*Δ*::LEU2* DNA fragment.

Yeast Transformation, Media, and Culture Conditions

Yeast transformation was performed by the DMSO-lithium acetate method [18]. YP medium and minimal medium supplemented with the relevant amino acids, bases, glucose, or galactose were used for culturing yeast [19]. To induce the expression of recombinant proteins from the *GAL10* promoter, transformants were precultured overnight in a synthetic complete medium without uracil, transferred to flasks containing 25 mL YPDG medium (1% yeast extract, 2% peptone, 1% dextrose and 1% galactose), and then cultured for 2 days at 30°C.

Construction of Yeast Expression Vectors for ATHIII and GOD

The vector pYInuAT for the expression of human α_1-AT under the control of the *GAL10* promoter and *GAL7* terminator has been previously described [20]. The vector pYInuATHIII, expressing the human ATHIII fused with the inulinase signal sequence (Fig. 1), was constructed by exchanging the 1.2 kb *BglII*/*SalI* α_1-AT cDNA fragment in pYInuAT with the 1.35 kb *BglII*/*SalI* ATHIII cDNA fragment. The ATHIII cDNA was amplified by polymerase chain reaction (PCR) from pUC19-ATHIII (provided by O. J. Yoo, KAIST). To express the hexahistidine-tagged GOD, a 130-bp DNA fragment coding for the C-terminus of GOD tagged with 6 residues of histidine was obtained by PCR from the GOD expression vector pGalGO2 (provided by J. K. Jung, KRIBB). The PCR product was treated with *AlwNI*/*XhoI* and then exchanged with the 130-bp *AlwNI*/*SalI* fragment coding for the GOD C-terminus in pGalGO2, generating pYGOD-His. The secretion of recombinant GOD in *S. cerevisiae* was directed with the *A. niger* α-amylase signal sequence [21].

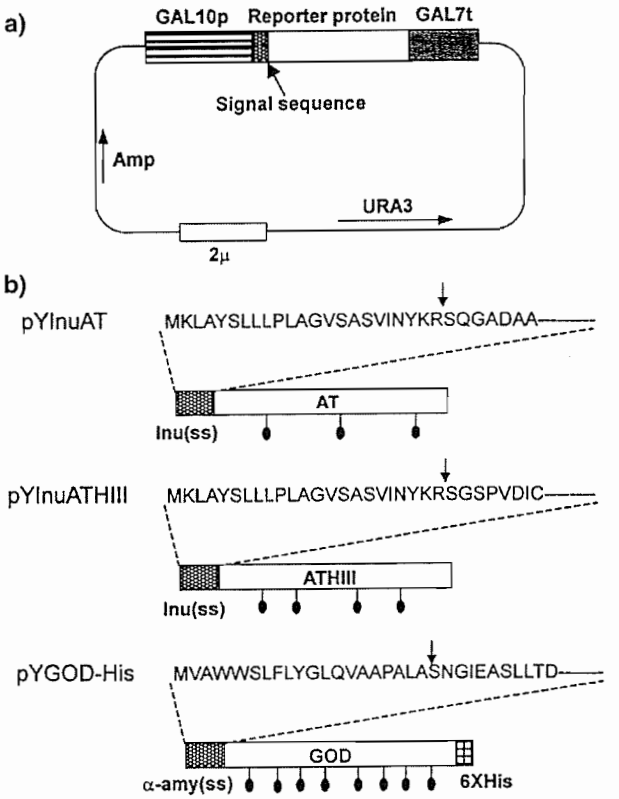

Fig. 1. Expression vectors of α_1-AT, ATHIII, and GOD for *S. cerevisiae*. (a) Generic plasmid base for the expression of recombinant proteins in *S. cerevisiae*. The expression vectors contain the α_1-AT coding sequence fused with the inulinase signal sequence (pYInuAT), the ATHIII coding sequence fused with the inulinase signal sequence (pYInuATHIII), or the GOD coding sequence fused with the α-amylase signal sequence (pYGOD-His) between the *GAL10* promoter and the *GAL7* terminator. (b) The amino acid sequences of the inulinase and the α-amylase signal sequences used to direct the secretion of human α_1-AT, ATHIII, or *A. niger* GOD-His in yeast are shown. Arrows indicate the cleavage site of signal sequence.

Immunoblot Analysis

Total yeast cell lysates (total intracellular fraction)

for immunoblot analysis were obtained as described previously [22]. For the analysis of secreted α_1-AT and ATHIII, the culture supernatants (extracellular fraction) of transformants were concentrated with the treatment of trichloroacetic acid (Sigma). The immunoblot analysis of secreted GOD, except the GOD secreted in the *mnn9* strain, was carried out directly without concentration. The polyclonal antibodies raised against human α_1-AT and human ATHIII were purchased from Sigma. The polyclonal antibody against *A. niger* GOD was from Accurate. Rabbit anti-α1,3-Man antibody was provided by R. Schekman (UC Berkeley).

Northern Blot Analysis

Yeast total RNA was prepared by the hot phenol extraction method of Elion and Warner [23], fractionated on a 1.2% formaldehyde-agarose gel, and then blotted onto Qiabrane membrane (Qiagen). The blot was probed with the digoxigenin-labeled human ATHIII cDNA fragment. Colorimetric detection of the membrane was performed with the reaction of anti-digoxigenin alkaline phosphatase (Boehringer Mannheim).

Purification of His-tagged GOD

The yeast culture supernatant containing the recombinant GOD-(6xHis) was concentrated by ultrafiltration (YM30 membrane, Amicon). The 6xHis-tagged GOD was purified from the 40-fold concentrated culture supernatant using Ni-NTA spin column according to the manufacturer's instruction (Qiagen).

RESULTS

Secretion and Glycosylation of Human α_1-AT in the *pmr1Δ* Strain

Human α_1-antitrypsin (α_1-AT), a 394 amino acid glycoprotein, is a major serum protein with the protease inhibitory activity. The protein is primarily synthesized in the liver and secreted into the plasma as a glycosylated form with the complex type of carbohydrate at three asparagine residues 46, 83, and 247 [24]. The human α_1-AT was expressed in the wild type and the *pmr1Δ* strains to test if the protein could be supersecreted in the *pmr1* mutant strain. However, unexpectedly, the *pmr1* null strain secreted a substantially reduced amount of α_1-AT compared to the wild type strain, indicating that the disruption of *PMR1* apparently inhibited the expression of α_1-AT (Fig. 2 and Table 1).

Our previous study on the glycosylation state of the human α_1-AT produced by *S. cerevisiae* revealed that the recombinant α_1-AT displayed a considerable heterogeneity with the two major core-glycosylated forms around 52 kDa and many additional hypermannosylated forms of higher apparent molecular weights [14].

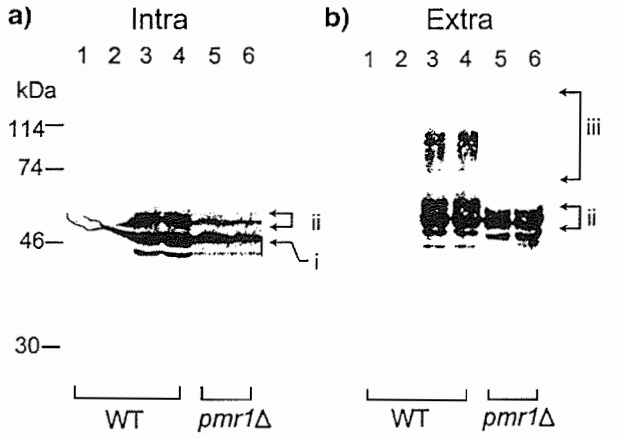

Fig. 2. Immunoblot analysis of α_1-AT expressed in the *pmr1Δ* strain. The transformants of the wild-type (lanes 3 and 4) and the *pmr1* deletion (lanes 5 and 6) strains, harboring the α_1-AT expression vector pYInuAT, were cultured in the presence of galactose for 2 days. The total cell extract (a) corresponding to 0.5 OD_{600} and the culture supernatant (b) corresponding to 40 μL (wild type) or 160 μL of cell culture (*pmr1*), respectively, were fractionated on 10% SDS-polyacrylamide gel and analyzed with the polyclonal antibody raised against human α_1-AT. Lanes 1 and 2 contain the samples from the untransformed wild-type strain. (i) Unglycosylated form of α_1-AT, (ii) core-glycosylated form of α_1-AT, (iii) hyperglycosylated form of α_1-AT.

Table 1. Effect of *pmr1* null mutation on the secretion of heterologous glycoproteins in *S. cerevisiae*

Protein	Strain	Expression level (μg/OD_{600})		Secretion level (μg/mL)
		Int[1]	Ext[2]	
α_1AT	WT	0.4	0.3	24
	pmr1Δ	0.15	0.1	2
ATHIII	WT	0.05	0.04	3
	pmr1Δ	0.4	0.25	5
GOD	WT	5	7.3	275
	pmr1Δ	2.8	14.4	163

[1] Intracellular level of each recombinant protein was estimated by densitometry scanning of the Western blots in Fig. 2(a), Fig. 4(a), and Fig. 6(a), respectively.

[2] Extracellular level of each recombinant protein was estimated by densitometry scanning of the Western blots in Fig. 2(b), Fig. 4(b), and Fig. 6(b), respectively.

Compared to the α_1-AT expressed in the wild-type strain, the α_1-AT expressed in the *pmr1Δ* strain did not show any high molecular weight species above 52 kDa (Fig. 2(b)). The present result is consistent with the previous observation that the human α_1-AT secreted from an *ssc1-1* strain appeared to be core-glycosylated

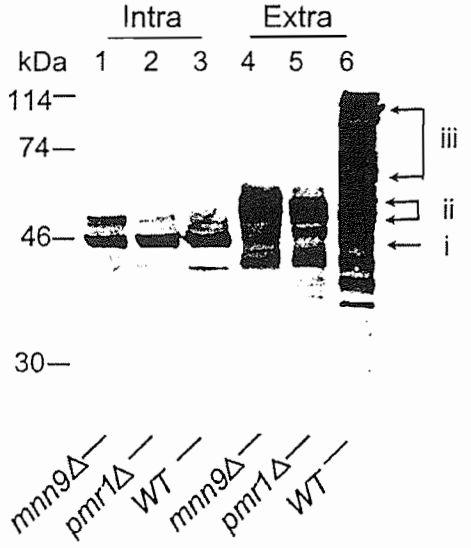

Fig. 3. Comparison of the recombinant α_1-AT expressed in the *mnn9*Δ (lanes 1 and 4), the *pmr1*Δ (lanes 2 and 5), and the wild-type (lanes 3 and 6) strains. Total cell lysates (Intra) and culture supernatants (Extra) from the recombinant strains were analyzed by Western blotting with the polyclonal antibody raised against human α_1-AT. (i) Unglycosylated form of α_1-AT, (ii) core-glycosylated form of α_1-AT, (iii) hyperglycosylated form of α_1-AT.

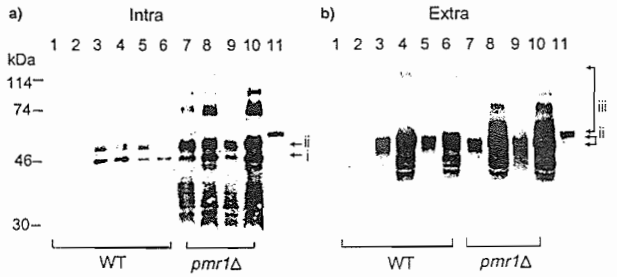

Fig. 4. Expression and secretion of human ATHIII in *S. cerevisiae*. The transformants of the wild-type (lanes 1-6) and the *pmr1*Δ (lanes 7-10) strains, which harbor the ATHIII expression vector pYInuATHIII, were cultured in the presence of galactose for 2 days. The total cell extracts (a) corresponding to 0.4 OD_{600} and the culture supernatants (b) corresponding to 80 μL of the cell culture were prepared, fractionated on 8% polyacrylamide gel, and analyzed with the polyclonal antibody raised against human ATHIII. Lanes 1, 3, 5, 7, and 9 show the samples prepared from the culture grown for 1 day, and lanes 2, 4, 6, 8, and 10 show the samples from the culture grown for 2 days. Lane 11 contains 20 ng of authentic ATHIII (Sigma). (i) Unglycosylated form of ATHIII, (ii) core-glycosylated form of ATHIII, (iii) hyperglycosylated form of ATHIII.

as found in the α_1-AT secreted from an *mnn9* strain [5]. However, it should be noted that the core-glycosylated forms of α_1-AT in the extracellular fraction of the *pmr1*Δ strain migrated slightly faster than those secreted in the wild-type strain on SDS-polyacrylamide gels (Fig. 2(b)). In contrast, the core-glycosylated forms of α_1-AT in the intracellular fraction, mainly corresponding to the ER form, migrated with the same mobility irrespective of strains (Fig. 2(a)). This result suggested that certain modification processes, occurring after transit through the ER, were affected by the loss of Pmr1p. To further analyze the difference, we compared the mobility of the core-glycosylated forms of the α_1-AT secreted in the wild type strain, the *pmr1Δ* mutant strain, and the *mnn9*Δ strain having a defect in the addition of a long mannose outer chain [16]. The core-glycosylation forms of the α_1-AT secreted in the *pmr1*Δ strain showed a slightly faster migration than those secreted in the *mnn9*Δ strain on SDS-PAGE (Fig. 3(Extra)). By contrast, the core-glycosylated forms of the α_1-AT secreted from the *mnn9* strain migrated with the same mobility as those secreted from the wild type strain. These results strongly suggested that some modification processes other than the elongation of α1,6-linked outer chain, mediated by Mnn9p, might be also affected in the *pmr1* mutant strain.

Secretion and Glycosylation of ATHIII in the *pmr1*Δ Strain

Human antithrombin III (ATHIII) is an important regulatory protein in hemostasis and thrombosis. Human ATHIII, a single-chain glycoprotein with a molecular weight of about 60 kDa, is synthesized in the liver and secreted into the plasma as a glycosylated form containing four N-linked carbohydrate chains of complex structure [25]. The inulinase signal sequence, which led to efficient secretion of α_1-AT, was also used to direct secretion of human ATHIII in *S. cerevisiae* (Fig. 1). The wild type and the *pmr1*Δ strains transformed with the ATHIII expression vector pYInuATHIII were analyzed for the secretion and glycosylation of ATHIII in *S. cerevisiae*. As generally observed in glycoproteins expressed in yeasts, the recombinant ATHIII was secreted in the wild-type strain as heterogeneous forms with the major species around 58 kDa and many additional species with higher and lower molecular weights (Fig. 4(b)). Treatment of the culture supernatant with Endo H converted the ATHIII species larger than 58 kDa into a single species of ~ 50 kDa (data not shown), suggesting that the higher forms were hypermannosylated species of ATHIII. Many smaller forms of ATHIII in the culture supernatant appeared to come from the proteolytic degradation of ATHIII in the yeast secretion pathway. In the intracellular fraction of the wild type cell (Fig. 4(a)), the unglycosylated form of 50 kDa was detected along with the core-glycosylated forms.

Unlike α_1-AT (Fig. 2), which was secreted mostly as core-glycosylated forms in the *pmr1* null mutant, ATHIII were secreted as hypermannosylated forms as well as core-glycosylated forms in the *pmr1* null mutant. However, the hypermannosylated species of ATHIII secreted in the *pmr1*Δ strain migrated much faster on SDS-PAGE compared to those secreted in the

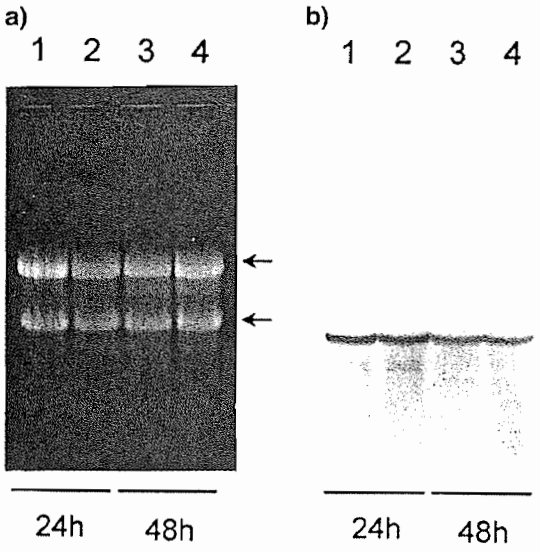

Fig. 5. Northern blot analysis of the ATHIII transcript expressed in *S. cerevisiae* transformants. Total RNAs were prepared from two individual transformants of the wild-type (lanes 1 and 3) and *pmr1*Δ (lanes 2 and 4) strains, respectively, which were cultured in the presence of galactose. 10 μg of RNA samples from 24 h culture (lanes 1 and 2) and 48 h culture (lanes 3 and 4) were fractionated on a 1.2% formaldehyde-agarose gel, blotted to membrane, and hybridized with the 1.35 kb digoxigenin-labeled *BglII*/*SalI* human ATIII cDNA fragment. (a) Photograph of the ethidium bromide-stained gel before transfer to the membrane. Arrows indicate the positions of 18S and 25S ribosomal RNAs. (b) Colorimetric detection of the membrane with the reaction of anti-digoxigenin alkaline phosphatase. The arrow indicates the ATHIII transcript expressed from the *GAL10* promoter.

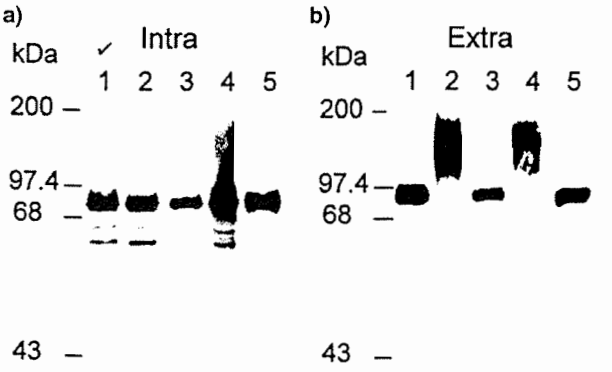

Fig. 6. Expression and secretion of *A. niger* GOD in *S. cerevisiae*. The transformants of the *pmr1*Δ (lane 1), *mnn1*Δ (lane 2), *mnn9*Δ (lane 3), and wild-type (lane 4) strains, which harbor the GOD expression vector pYGOD-His, were cultured in the presence of galactose for 2 days. Lane 5 corresponds to 0.8 μg of authentic *A. niger* GOD. The total cell extracts (a) corresponding to 0.5 OD_{600} and the culture supernatants (b) corresponding to 10 μL of the cell culture were fractionated on 8% polyacrylamide gel, and then analyzed with the polyclonal antibody raised against *A. niger* GOD. Note that the culture supernatant corresponding to 100 μL of cell culture was analyzed in the case of the *mnn9* strain.

wild-type strain, reflecting the reduced hypermannosylation activity in the absence of Pmr1p (Fig. 4(b)). As observed in the expression of human α_1-AT, the core-glycosylated form of the ATHIII secreted in the *pmr1* disruptant also appeared to be smaller in size than that secreted in the wild type strain, although the core-glycosylated forms retained within the cell showed the same molecular weight. Interestingly, some distinct ATHIII species of high molecular weight were shown in the intracellular fraction of the *pmr1*Δ cell (Fig. 4(a)), but not of the wild type cell. The high molecular weight species were resistant to Endo H treatment (data not shown). We will discuss this observation in detail in the Discussion section.

The *pmr1*Δ strain showed about a two-fold increase in the total secretion level (mg of protein per mL of culture broth) of ATHIII compared to the wild-type strain (Fig. 4, Table 1). Considering that the final cell mass of the *pmr1* deletion mutant was just about 30% of that of the wild type cell, the extracellular level of ATHIII per a unit cell mass was actually increased up to six-fold. However, the intracellular level of ATHIII per cell mass was also increased about eight-fold in the *pmr1* mutant. To investigate a possibility that the increased intracellular level of ATHIII in the *pmr1* mutant might result from the enhanced expression level of ATHIII, we measured the steady state levels of ATHIII transcript in the wild type and *pmr1* mutant strains. The result of Northern blot analysis showed the comparable levels of ATHIII transcript in both strains (Fig. 5), ruling out the possibility that the enhanced export of ATHIII was attributed to more efficient expression of ATHIII in the *pmr1*Δ strain than in the wild type strain.

Secretion and Glycosylation of GOD in the *pmr1*Δ Strain

To study the secretion and glycosylation pattern of recombinant proteins in the *S. cerevisiae pmr1*Δ strain in more detail, we expressed another heterologous glycoprotein, *A. niger* glucose oxidase (GOD), which is known to contain 8 potential sites for N-linked glycosylation [26]. The recombinant GOD was expressed as a fusion form tagged with 6 additional His-residues to facilitate purification of the protein (Fig. 1). The GOD expression vector pYInuGOD-His was introduced into several mutant strains of *S. cerevisiae* defective in N-linked glycosylation. The GOD expressed in the wild-type strain was secreted as heavily mannosylated forms (Fig. 6, lane 4) as previously reported [26]. The *mnn1* deletion mutant strain, which is defective in the addition of terminal α1,3-linked mannose, secreted GOD as heavily mannosylated forms although the overall length of outer chain seemed to be slightly reduced (Fig.

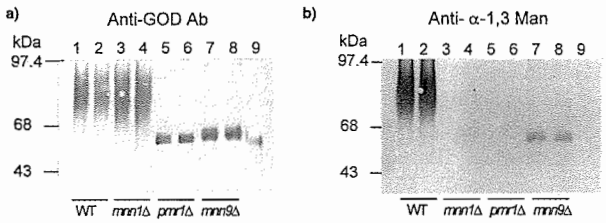

Fig. 7. Western blot analysis of the purified GOD secreted *S. ceevisiae*. The histidine-tagged GOD in the culture supernatants of the wild-type (lanes 1and 2), *mnn1*Δ (lanes 3 and 4), *pmr1*Δ (lanes 5 and 6), and *mnn9*Δ (lanes 7 and 8) strains were purified using Ni-chelate resin. The purified GOD was fractionated on 8 % polyacrylamide gel and then analyzed with the polyclonal antibody raised against *A. niger* GOD (a) or α1,3-linked mannose (b). Lane 9 contains the authentic GOD from *A. niger*.

6, lane 2). However, the GOD proteins expressed in the *pmr1* and *mnn9* null strains were secreted as a homogenous form with the migration rate similar to the authentic GOD on SDS-PAGE (Fig. 6, lanes 1 and 3 vs. lane 5). In the aspect of secretion, the disruption of *PMR1* appeared to increase the amount of secreted GOD per a unit cell mass, but did not improve the ultimate level of GOD secreted into the culture supernatant due to the decreased cell mass of the *pmr1* mutant (Table 1).

As observed in the expression of human α_1-AT and ATHIII, the core-glycosylated form of the GOD secreted from the *pmr1*Δ strain appeared to be slightly smaller than that from the *mnn9*Δ strains. Based on the previous report that the secreted invertase in the *S. cerevisiae pmr1* mutant did not contain α1,3-linked mannose [13], we investigated whether the smaller size of core-glycosylated forms of the heterologous glycoproteins secreted in the *pmr1*Δ strain was, at least in part, due to the lack of α1,3-mannose linkage. The GOD tagged with 6 residues of histidine was purified from the culture supernatant and analyzed by the antibody raised against α1,3-linked mannose. As shown in Fig. 7(b), the GOD secreted in the *pmr1*Δ and *mnn1*Δ strains failed to bind anti-α1,3 mannose antibody unlike the GOD secreted in the wild-type and *mnn9*Δ strains. The present Western blot result strongly supports that the *pmr1*Δ strain is unable to add terminal α1,3-linked mannoses to secretory glycoproteins. Considering that the α1,3 glycan linkages are primarily responsible for the hyper-antigenic nature of glycoyslated proteins from *S. cerevisiae*, the *pmr1*Δ strain is expected to be a suitable host system for the production of glycoproteins which may resemble more closely to the glycoprotein structure of higher eukaryotes.

DISCUSSION

The *pmr1* mutant strain of *S. cerevisiae*, isolated initially as a 'super-secretor' strain (*ssc1*), has shown enhanced secretion of several heterologous proteins compared to the wild type strain. However, the secretion of the plant protein thaumatin could not be improved to any significant extent by disruption of the *PMR1* gene [10], suggesting that improvements in heterologous protein secretion by the *PMR1* disruption are not applicable to all proteins. In this paper we have also observed that the *PMR1* disruption significantly enhanced the secretion level of human ATHIII but not that of human α_1-AT. A notable observation was a significant increase in the intracellular level of ATHIII in the *pmr1* mutant strain over the wild-type strain, despite the similar levels of ATHIII transcript in both strains. This was suggestive of the decreased degradation of ATHIII retained inside the *pmr1* mutant cell. Durr *et al.* [8] has recently proposed that reduced Pmr1p activity could induce the secretion of nonnative proteins that are otherwise retained for ER-associated degradation or salvaged by the vacuole, thus leading to the supersecretion phenotype of a *pmr1* mutant. Therefore, it can be speculated that substantial proportion of yeast-produced ATHIII might be malfolded, possibly because one or more of three disulfide bonds present in the protein [27] do not form or have formed incorrectly. The possibility of misfolded conformations of the recombinant ATHIII is suggested by the appearance of some distinct high-molecular weight species of ATHIII in the *pmr1* deletion strain when analyzed on SDS-PAGE. It is unlikely that the high molecular weight forms of ATHIII consist of oligomers with incorrect intermolecular disulfide bonds because the samples of recombinant ATHIII were analyzed under reducing state containing 1% β-mercaptoethanol. Rather we assume that the high molecular weight forms might be generated by the loop-sheet polymerization of ATHIII, which is known to take place spontaneously under the conditions causing conformational change of ATHIII [28]. Our results are also in agreement with the previous report that the export of intracellularly accumulated prochymosin, which was malfolded due to inefficiency of disulfide bond formation in yeast, was remarkably stimulated in a *pmr1* mutant strain [10].

A number of secretory proteins with industrial importance are glycoproteins, which undergo glycosylation during the secretion process. Glycosylation, the most extensive of all the post-translational modifications of proteins, has a profound effect on properties critical to glycoprotein products, such as antigenicity, specific activity, solubility, and resistance to protease attack [29]. The different processing of core-oligosaccharides attached to proteins, such as hypermannosylation in the Golgi, has thus been presented as a major drawback of the *S. cerevisiae* system for the production of recombinant glycoproteins [30]. A partial solution to the problem of hyperglycosylation of recombinant proteins expressed in *S. cerevisiae* would be the expression of the proteins in glycosylation mutant strains such as the *mnn9 mnn1* [16] and *och1 mnn1* [31] double mutant strains. In these mutant strains, N-

glycosylation would be confined to the core oligosaccharide residues without the terminal α1,3-glycan linkages that are highly antigenic. However, the severe growth defect of the *och1* and the *mnn9* mutants is a major limitation in using these mutants to produce large quantities of recombinant proteins. Considering the faster growth phenotype of *pmr1Δ* strain compared to these mutant strains, the present results suggest that the *pmr1Δ* strain would be a practical host for a high-level production of core-glycosylated recombinant proteins that are devoid of terminal α1,3-mannoses. Taken together, the present results corroborate the superiority of the *pmr1* strain as a host system over the wild type strain especially for the production of some recombinant glycoproteins that are poorly secreted or heavily hypermannosylated at the wild-type background.

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