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PMT1 gene plays a major role in *O*-mannosylation of insulin precursor in *Pichia pastoris*

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ABSTRACT

Protein mannosyltransferases (PMTs) catalyze the *O*-mannosylation of serine and threonine residues of proteins in the endoplasmic reticulum. The five *PMT* genes coding for protein mannosyltransferases, designated as *PMT1, 2, 4, 5* and *6*, were identified from *Pichia pastoris* genome based on the homology to *PMT* genes in *Saccharomyces cerevisiae* genome, which has seven *PMT* genes. The homologues of *S. cerevisiae PMT 3* &*7* genes are absent in *P. pastoris* genome. Approximately 5% of the recombinant insulin precursor expressed in *P. pastoris* is *O*-mannosylated. In this study, we attempted to prevent *O*-mannosylation of insulin precursor *in vivo*, through inactivation of the Pichia *PMT* genes. Since multiple PMTs are found to be expressed, it was important to understand which of these are involved in *O*-mannosylation of the insulin precursor. The genes encoding *PMT1*, *4*, *5* and *6* were knocked out by insertional inactivation method. Inactivation of *PMT* genes *4, 5 and 6* showed ~16–28% reductions in the *O*-mannosylation of insulin precursor. The *PMT1* gene disrupted Pichia clone showed ~60% decrease in *O*-mannosylated insulin precursor, establishing its role as an important enzyme for insulin precursor *O*-mannosylation.

Introduction

Protein *O*-mannosylation is an essential post-translational modification which is evolutionally conserved from bacteria to higher eukaryotes [1,2]. *O*-mannosylation is abundant in fungi, while it is less common in metazoa. Protein *O*-mannosylation differs from other forms of *O*-glycosylation in that it begins in the endoplasmic reticulum [3]. In the first step, mannose from dolichol monophosphate-activated mannose is transferred to Serine or Threonine residues of secretory proteins [4]. The number of mannose residues added further can vary from 5 in *Candida albicans* and 2–3 in *Pichia pastoris* (*P. pastoris*) to 13 in *Saccharomyces cerevisiae* (*S. cerevisiae*) [5–7]. In mammals, other sugars such as galactose, fucose, *N*-acetylglucosamine, glucose and sialic acid are also added to the *O*-linked carbohydrate chain. Protein *O*-mannosylation also occurs on cell wall proteins in *S. cerevisiae* and inactivation of some *PMT* genes retards the growth while simultaneous inactivation of multiple *PMT* genes is lethal [8].

During evolution in fungi, *PMT* genes have undergone gene duplication. In *Aspergillus fumigatus*, there are only three *PMT* genes [9] while *P. pastoris* has 5 *PMT* genes [10]. *S. cerevisiae* has 7 putative *PMT* genes [8]. It is not clear at present whether there are any mechanisms by which a particular protein substrate is recognized and *O*-mannosylated by a specific PMT. Also whether *O*-mannosylation of a protein is to facilitate its secretion or identify it as a misfolded protein to target its retention within endoplasmic reticulum and whether there is a segregation of such functions by different members of PMT family is an interesting question to explore.

Many heterologus proteins secreted from *S. cerevisiae* have *O*-mannosylation. The PMT responsible for this post-translational modification and the reason for this modification are not understood in the majority of the cases. In one example, *PMT1* and *PMT2* genes were found to be absolutely essential to *O*-mannosylation. Knocking out either *PMT1* or *PMT2* gene led to 100% reduction in *O*-mannosylation of the recombinant protein. Knocking out other PMTs led to a decrease from 45% to 59% reduction in *O*-mannosylation of the secreted recombinant protein. It was suggested that *O*-mannosylation led to a loss of biological activity and was not an essential modification [11]. Thus by knocking out the *PMT* genes responsible for *O*-mannosylation, the quantity and quality of the recombinant product was significantly improved. In this study we have identified the role of *PMT* genes in *O*-mannosylation of insulin precursor in *P. pastoris*. Knocking out various PMTs in Pichia and examining their impact on the level of *O*-mannosylation of insulin precursor led to identification of PMT1 as a critical gene.

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Materials and methods

Strains, plasmids and media

The *P. pastoris* strain GS115 (*his*) host was used for expression of insulin precursor (IP).[1] The expression vector used was pPIC9K (Invitrogen, USA) that provides the promoter and terminator sequences of the *P. pastoris* AOX1 gene and the *S. cerevisiae* Matα signal sequence for secretion. The *Escherichia coli* strain DH5α was used for routine cloning and propagation of plasmids. Yeast extract–peptone–dextrose (YPD) medium containing 10 g/l yeast extract, 20 g/l peptone and 20 g/l dextrose was used for routine growth and subculturing of Pichia strains. YNBD medium used for selection contained 13.4 g/l yeast nitrogen base (YNB) without amino acids and 20 g/l dextrose (D). Luria broth/agar was used for culturing *E. coli*. Media components used were either from Himedia (Mumbai, India) or Difco (USA).

Construction of the insulin precursor-secreting P. pastoris

The IP gene sequence was codon optimized for the expression in *P. pastoris* and the synthetic gene (GenBank accession No. KC168055) was obtained from Geneart Inc., Germany. The synthetic gene was subcloned into pPIC9K vector in *XhoI* and *EcoRI* restriction sites in frame with Matα signal sequence for secretion. The resulting plasmid is pPIC9K/IP. The design of the IP producing single chain amino acid sequence was based on the literature [12,13].

Transformation of P. pastoris

The plasmid pPIC9K/IP was digested with *BglII* and the large fragment was transformed into *P. pastoris* strain GS115. Transformation was carried out by electroporation of freshly prepared competent cells in 0.2 cm cuvettes. The pulse was delivered by Gene Pulser (BioRad) at 1500 V, 25 μF, and 200 Ω. The electroporated cells were allowed to recover for 1 h in 1 M Sorbitol at 30 °C and then spread onto YNBD agar plates. The resulting transformants were selected on YPD plates, containing progressively increasing concentrations of G418 (0.5–2.0 mg/ml) to identify multicopy clones. Several clones that were viable at 2 mg/ml concentration of G418 were tested in small scale induction experiments. Extracellular IP concentrations were analyzed by RP-HPLC to identify the best expressing clone. IP clone #1 was selected as the lead clone.

LC–ESI-MS data acquisition and analysis

The HCT-ultra PTM discovery system (Bruker Daltonics, Germany) equipped with an electron-spray ionizer was used to acquire the LC–ESI-MS data. The ESI-MS was in tandem with the Agilent HPLC (1100 series). Positive ionization mode was used for this acquisition which was done with nebulizer gas pressure at 65 psi, dry gas flow of 11.0 l min^{-1} and drying temperature of 350 °C. The mass scan range was 700–2200 m/z. MS-MS data was acquired in the Electron Transfer Dissociation (ETD) mode. The Data Analysis 4.0 software (Bruker Daltonik, GmbH) was used to analyze the mass data.

[1] *Abbreviations used:* PMT, protein mannosyltransferase; IP, insulin precursor; RP-HPLC, reverse phase high performance liquid chromatography; YPD, yeast extract–peptone–dextrose; YNB, yeast nitrogen base; ETD, electron transfer dissociation; MGHI, mono-glycosylated human insulin; DGHI, di-glycosylated human insulin.

Enzymatic digestion of insulin for peptide mass fingerprinting

Insulin was digested using the enzyme Endoproteinase Glu-C to generate the peptide fragments. Purified insulin was solubilized in 0.01 N HCl at a concentration of 1 mg/ml, followed by adjustment of the pH to 8.0 using Tris Buffer. Finally the enzyme Endoproteinase Glu-C (1 mg/ml) was added to the protein solution in the enzyme to protein ratio of 1:50, and the reaction mixture was incubated at 37 °C for 4 h [6]. The peptide mass fingerprint was generated using analytical RP-HPLC in tandem with ESI-MS Agilent 1100 LCMS) to identify each chromatographic peak.

PMT1 gene disruption

The 477 bps *PMT1* coding gene fragment was amplified by PCR using primers PMT1FP and PMT1RP (Table 1) using Taq polymerase (Bangalore Genei, India). The amplicon obtained was cloned into pTZ57R vector (MBI Fermentas) and sequence verified. *PMT1* gene fragment was subcloned into pPICZA vector in *BamHI*/*BglII* sites to obtain PMT1/pPICZA vector. After linearization of PMT1/pPICZA by *BstEII*, the construct was transformed into the IP clone #1 strain producing insulin precursor. Transformation was done by electroporation as described above, except that after allowing cells to recover in 1 M Sorbitol, one volume of YPD medium was added, and the cells were incubated for 1 h at 30 °C. Cells were then plated on YPD agar medium supplemented with 1 M Sorbitol and 100 μg/ml Zeocin (Invitrogen Inc.). The insertion of the disruption vector into Pichia genome *PMT1* locus was verified by PCR screening using the primer combinations, P1 and P2 to confirm from the upstream of *PMT1* genomic locus and with primers P3 and P5 to confirm from the downstream region of *PMT1* genomic locus.

Disruption of PMT2, 4, 5 and 6

The same strategy as described above for *PMT1* gene was used for the disruption of the remaining *PMT* genes but with different gene specific set of primers. Briefly, the *PMT2* gene was amplified using PMT2FP and PMT2RP and used restriction enzyme *KpnI* for linearization. The specific primer SPMT2DCFP was used for screening and confirmation along with P1. Similarly, the *PMT4, 5* and *6*

Table 1
Primers used for vector construction and gene disruption verification. The P1, P2, P3, P4 and P5 primers location shown in the Fig. 2C.

Primer name	Nucleotide sequence (5′–3′ direction)
PMT1FP	GGA TCC TAA TAG CCC ACT CTG ATC TAC CTC ACT
PMT1RP	GGA TCC AAA GCC CTC ATG TCC ATA AGC AGA
PMT2FP	GGA TCC TAA TAG GTG GGT TTA TTT GTC ACA GTA
PMT2RP	GGA TCC GAA ACA CCC AAT CAT TGT TGG CA
PMT4FP	GGA TCC TAA TAG GTT CAT TTC GCT ATT CTA AGC A
PMT4RP	GGA TCC TTT CGA CTT CAA AGG ACG GGT T
PMT5FP	AGA TCT TAA TAG ATC CTA CCA GTG ATC ATT TAC CT
PMT5RP	AGA TCT TCA CTA ATT GGA AGG TCT AGA ATC
PMT6FP	GGA TCC TAA TAG CTT GCC GTT AAG AGA TAC GAT GA
PMT6RP	GGA TCC TGA GAA TGC AAG TTT GCA CCA GTA
P1	GGA CTT ATG GTT CAT CAT TGG TGA
P2	TAG CAG AGC GAG GTA TGT AGG CGG TGC
P3	GAG TCC GAG AAA ATC TGG AAG AGT
P4	GCT ACA CTA GAA GGA CAG TAT TTG GTA
P5	GCC CTA ACG TTT TCC TTA GCT
SPMT2DCFP	GGC TTC TGC TTA CTG GTA TTT CCA
SPMT4DCFP	GTT GGT AAA CAC TTT GCT GCA CGA
PMT4DSCKRP	GTC CTC TGC AAT ATC GAC GA
SPMT5DCFP	CGA TCG TGA TGT GGT AAA GCA TGT CT
PMT5DSCHKRP	GTC TCC TAT AAT ACT GA
SPMT6DCFP	GGA TGT GTA TGC AGT GTC AAA CTT GT
PMT6DSCHKRP	CCA CTC ATC GTT TCC GTC TGA