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Enhancement of Protein Secretion in *Pichia pastoris* by Overexpression of Protein Disulfide Isomerase

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Abstract: A potential vaccine candidate, *Necator americanus* secretory protein (Na-ASP1), against hookworm infections, has been expressed in *Pichia pastoris.* Na-ASP1, a 45 kDa protein containing 20 cysteines, was directed outside the cell by fusing the protein to the preprosequence of the α-mating factor of *Saccharomyces cerevisiae*. Most of the protein produced by single copy clones was secreted outside the cell. However, increasing gene copy number of Na-ASP1 protein in *P. pastoris* saturated secretory capacity and therefore, decreased the amount of secreted protein in clones harboring multiple copies of Na-ASP1 gene. Overexpression of the endoplasmic reticulum (ER) resident, homologous chaperone protein, protein disulfide isomerase (PDI) was able to increase the secretion of (Na-ASP1) protein in high copy clones. The effect of PDI levels on secretion of Na-ASP1 protein was examined in clones with varying copy number of PDI gene. Increase in secreted Na-ASP1 secretion is correlated well with the PDI copy number. Increasing levels of PDI also increased overall Na-ASP1 protein production in all the clones. Nevertheless, there was still accumulation of intracellular Na-ASP1 protein in *P. pastoris* clones over-expressing Na-ASP1 and PDI proteins. © 2005 Wiley Periodicals, Inc.

Keywords: *Pichia pastoris*; protein secretion; protein disulfide isomerase; *Necator americanus* secretory protein; Na-ASP1; protein overexpression; secretion bottleneck

INTRODUCTION

The methylotrophic yeast, *Pichia pastoris*, is a highly successful system for expression of heterologous proteins (Daly and Hearn, 2005; Macauley-Patrick et al., 2005). Several factors have contributed to its rapid acceptance over other expression systems, including the tightly regulated alcohol oxidase I promotor (*AOX1*) (Cregg and Higgins, 1995) and a strong respiratory growth system that facilitates high cell densities (Brierley et al., 1990; Cereghino et al., 2002; Zhang et al., 2000). It is also well suited for production of therapeutic proteins, which require post-transcriptional modifications such as disulfide bond formation (Macauley-Patrick et al., 2005).

The first choice of expression mode for many proteins is to secrete the protein of interest outside of the cell for ease of purification and required post-translational modifications as the protein moves through the secretory pathway. Standard steps to improve recombinant protein expression include optimizing codon usage and increasing the gene dosage. However, overexpression of a protein in a nonoptimal environment may cause deleterious problems, such as saturation of the secretory pathway resulting in by misfolded proteins (Inan et al., 2005; Parekh et al., 1995; Smith and Robinson, 2002). The folding of proteins destined for the secretory pathway occurs in the endoplasmic reticulum (ER) where a quality control system insures that secreted proteins are correctly folded, including the correct formation of disulfide bonds (Helenius et al., 1992). Disulfides that form early in the folding process are often incorrect; cysteines can be mispaired or disulfides can be formed in the wrong temporal order, making it difficult to oxidize buried cysteines (Creighton, 1984). To rectify these errors, the incorrect disulfides must be broken and new ones formed in a different configuration. The ER contains folding assistants that help proteins achieve their correct disulfide arrangement (Helenius et al., 1992). Protein disulfide isomerase (PDI) is a 57 kDa protein of the ER that helps in rearrangement of incorrect disulfide pairings by isomerase activity because of CGHC domains and substrate binding site, accelerating both processes without drastically altering the refolding pathway (Gilbert, 1998). Improvements of secretion of recombinant proteins expressed in yeast, Chinese Hamster ovary (CHO) cell and *Escherichia coli* by over-expressing PDI have been documented in literature. (Davis et al., 2000; Humphreys et al., 1996; Robinson et al., 1994). However in some instances overexpression of chaperone proteins in ER did not improve the secretion of recombinant proteins (Butz et al., 2003).

In our previous study, we described expression of *Necator americanus* secretory protein (Na-ASP1) in *P. pastoris*

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(Inan et al., 2005). We observed that increasing copy number of Na-ASP1 gene resulted in reduced secretion of the product with a most of the protein accumulated inside the cell. Here we evaluate the effect of introducing multiple copies of the PDI gene into *P. pastoris* expressing multicopies of Na-ASP1 gene.

MATERIALS AND METHODS

Materials

The construction of *P. pastoris* X-33 strains expressing multiple copies of Na-ASP1 gene was described elsewhere (Inan et al., 2005). pPICZαNa-ASP1 plasmid was a gift from Dr. Peter Hotez of The George Washington University. Codon optimization of Na-ASP1 gene for *P. pastoris* was done by Aptagen LLC (York, PA). *E. coli* TOP10 competent cells, *P. pastoris* host strain X-33, expression plasmid pPIC3.5K, and plasmid pCR-Blunt II-TOPO cloning vector was purchased as a kit from Invitrogen (Carlsbad, CA). Restriction enzymes were from New England Biolabs (Beverly, MA). Plasmid propagations were done in *E. coli* TOP10 cells using either LB Miller (ampicillin 100 μg/mL) or LB Lennox Zeocin (25 μg/mL) medium depending on plasmid used.

Construction of PDI Expression Plasmid pPIC3.5K-PDI

The PDI gene was amplified with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) from chromosomal DNA of wild type *P. pastoris* X33 using forward primer 5′-ATGCAATT-CAA-CTGGGATATTAAAACTG-3′ and reverse primer 5′-TTAAAGCTGTCGTGAGCGTC-3′ based on published sequence of *P. pastoris* PDI gene (EMBL AJ302014) (Warsame et al., 2001).

After amplification, PCR product was purified and ligated into pCR-Blunt II-TOPO cloning vector by following the instruction manual. The positive clones from the ligation reactions were analyzed by restriction digestion and sequencing the insert fragments at UNL DNA sequencing facility. After confirmation of the correct sequence and orientation, the vector (pPCRII-PDI) was digested with *Eco*R I restriction enzyme. A 1.5 kb PDI gene was purified from agarose gel and ligated into *Eco*R I site of pPIC3.5K using Quick Ligation Kit (New England Biolabs). The resulting plasmid was designated as pPIC3.5K-PDI (Fig. 1). After confirmation of correct orientation of the insert, plasmid midiprep kit (Qiagen, Valencia, CA) was used to isolate plasmid DNA for transformation experiments.

Transformation of *P. pastoris*

Plasmid pPIC3.5K-PDI was linearized with *Hpa I* at the *HIS4* locus and transformed into *P. pastoris* X-33 competent cells harboring 2, 3, or 4 copies of the Na-ASP1 by electroporation. The cells were pulsed in 2 mm electroporation cuvette at

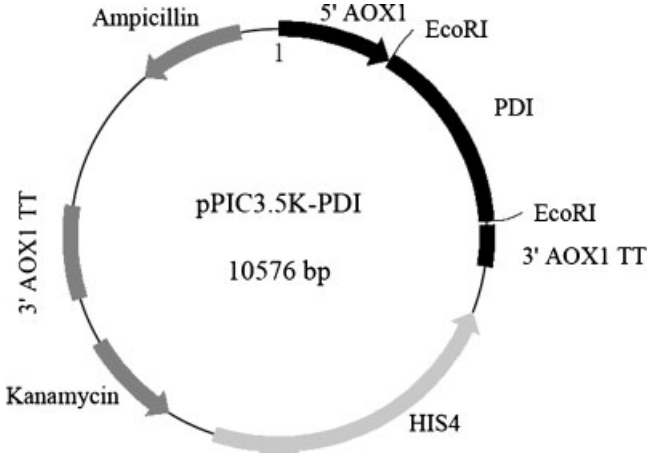

Figure 1. PDI over-expressing vector pPIC3.5K-PDI.

1.5 kV, 200 Ω, and 25 μF in a Gene Pulser electroporator (Bio-Rad, Hercules, CA) and 1 mL of ice-cold 1 M sorbitol was added immediately. Direct selection of geneticin (G418) resistance in yeast does not work well. The procedure to generate geneticin resistant clones requires an initial selection of His+ transformants followed by screening for varying levels of geneticin. Thus, transformed cells were spread on minimal dextrose (MD) plates allowing sufficient time to express sufficient amounts of resistance factor. Increasing amounts of the transformation mixture was spread on YPD agar plates containing (0.25, 1, 2, 4 mg/mL) Geneticin and incubated at 30°C for selection of putative multi-copy recombinants. After about 2 days, the colonies were picked and streaked on YPD plates for single colony isolation.

Southern Blot Analysis

Genomic DNA of the selected clones was extracted from the freshly grown YPD cultures of *P. pastoris* strains expressing Na-ASP1 and coexpressing Na-ASP1 and PDI proteins using the MasterPure™ Yeast DNA Purification Kit (Epicenter, Madison, WI). Chromosomal DNA concentrations were quantified using Picogreen DNA Quantification kit, (Invitrogen). One microgram of chromosomal DNA of *P. pastoris* (pPICZα/Na-ASP1) and *P. pastoris* (pPIC3.5K-PDI and pPICZα/Na-ASP1) were digested with restriction enzymes *Nde* I and *Xho* I, respectively, separated on 0.7% TAE agarose gel. Through capillary action, the DNA was transferred to a positively charged nylon membrane (Bio-Rad), using the method described by Southern (1975). DIG labeled *AOX1* promoter was used as a probe in hybridization solution to estimate Na-ASP1 gene copies. DIG labeled PDI gene was used to estimate the PDI gene copy number. DIG DNA Labeling and Detection kit (Roche Applied Science, Indianapolis, IN) was employed for capturing chemiluminescence signal on X-ray films. The copy number was determined by band intensity on the Southern blots by densitometric analysis using Alpha Innotech 8800 gel

imaging system and Alpha EaseFC software (San Leandro, CA).

Northern Blot Analysis

Total RNA was prepared using TRI Reagent® from Molecular Research Centre, Inc (Cincinnati, OH) from methanol induced cells as described in induction experiments. One hundred milligrams of induced cells were distrupted with Mini-Beadbeater-48; using zirconia/silica beads (Biospecs, Bartlesville, OK) with five cycles of 1 min beating and 5 min resting. Total RNA was quantified using Ribogreen Quantitation kit, Invitrogen. Ten microgram of total RNA was separated on a 0.9% agarose, 5% formaldehyde gel. RNA was then transferred to positively charged nylon membrane Zeta-Probe membrane, (Bio-Rad), probed with nonradioactive DIG labeled Na-ASP1 and PDI gene to estimate m-RNA of Na-ASP1 and PDI, respectively. The signals were detected and quantified as described for Southern blot analysis.

Small Scale Induction Experiments

Single colonies were grown in 10 mL YPD broth (1% yeast extract; 2% peptone; 2% dextrose) overnight, which was used to inoculate 15 mL of BMGY (1% yeast extract, 2% peptone, 1% glycerol, 1.34% YNB (w/o amino acids), 4×10^{-5}% biotin, and 100 mM potassium phosphate, pH 6.0). The starting optical density (at 600 nm) for the shake flasks was 0.05 in 50 mL baffled shake flask. The culture was grown in a rotary shaker at 30°C and 250 rpm (Innova 4000, New Brunswick Scientific, NJ) up to 10 OD (600 nm) for about 24 h. The cells were centrifuged at 3,000*g* for 12 min at room temperature and resuspended in 15 mL BMMY (1% yeast extract, 2% peptone, 1% methanol, 1.34% YNB (w/o amino acids), 4×10^{-5}% biotin, and 100 mM potassium phosphate, pH 6.0, and 2 mM phenylmethylsulfonylfluoride (PMSF). Induction time was 12 h at the same conditions. The cells were harvested at 3,000*g* for 12 min at 4°C. Supernatant were stored at 4°C and cells kept frozen in (−80°C) until further analysis.

Western Blot Analysis

For evaluation of intracellular accumulation of proteins, 250 mg of the cells were disrupted with Mini-Beadbeaster-48; using zirconia/silica beads (Biospecs). One minute vibrating and 5 min resting on ice were done for total of five cycles. The cell lysates were centrifuged at 10,000*g* at 4°C, the supernatants containing the soluble cytosolic proteins were removed and the pellets containing insoluble proteins were further treated with NuPAGE LDS sample buffer and NuPAGE Sample reducing agent form Invitrogen, at 70°C for 10 min and centrifuged at 10,000*g* and 4°C for 10 min. Ten micrograms of soluble protein and 10 μL fermentation broth and insoluble fraction were separated in 10% Bis-Tris, NuPAGE gels (Novex Pre-cast gel, Invitrogen) at reducing and denaturing conditions. The proteins were transferred to PVDF membrane and treated with a 1:8,000 dilution of Rabbit anti-ASP-1 (2 h) and a 1:5,000 dilution of goat anti-rabbit antibody (1 h). The signals were visualized with ECL plus detection reagent and hyperfilm ECL from Amersham Biosciences (Piscataway, NJ). Images were quantitated by densitometric analysis using Alpha Innotech 8800 gel imaging system using Alpha EaseFC software.

N-Terminal Sequencing

N-terminal sequence of PDI protein was carried out from an expected protein band blotted on Sequi-blot polyvinylidene fluoride (Sequi-Blot PVDF, Bio-Rad) membrane. Ten microgram of total protein was separated on 10% Bis-Tris NuPAGE gels (Novex Pre-cast gel, Invitrogen). After wet transfer protocol, the membrane was stained with amido black stain for 5 min. The visible 57 kDa band was cut out, and N-terminal sequence was obtained by Edman degradation at University of Nebraska Medical Center (Omaha, NE).

RESULTS

Previously, we have described the expression of Na-ASP1 in *P. pastoris* (Inan et al., 2005). We originally screened 90 clones expressing Na-ASP1 extracellularly. The putative copy number of the clones ranged from one to four copies. A negative correlation was observed between copy number and extracellar protein production although increasing copy number resulted in increased Na-ASP1 transcript level. We postulated that bottleneck for secretion of Na-ASP1 in *P. pastoris* could be protein folding in ER since the Na-ASP1 protein contains 20 cysteine molecules.

Copy Number Confirmation on Selected Na-ASP1 Expressing Clone

Copy number of the Na-ASP1 gene in selected clones, 7, 28, 46, and 80 was confirmed. The chromosomal DNA of the clones were digested with *Nde* I restriction enzyme, which does not cut the expression vector pPICZα/ASP1, and the *AOX1* promoter was employed as a probe for Southern blot experiment. Results of Southern blot analysis of selected clones are shown in Figure 2. The host strain (X-33) shows a single band around 9 kb, which corresponds to the *AOX* loci. Theoretically, every additional insertion of the expression vector at the *AOX1* loci should result in 4.7 kb increment in band size. The clone 46 showed a single band size of 13.7 kb, which is the expected band for a single copy clone. Similarly, clones 80, 28, and 7 had 18.4 kb, 23.1 and 27.8 kb band, respectively. Therefore, the clones 46, 80, 28, and 7 had one, two, three and four copies of the expression vector, respectively (Fig. 2).

Small Scale Induction Experiments

The selected clones were tested for Na-ASP1 production in shake flask by inducing with methanol in BMMY media.

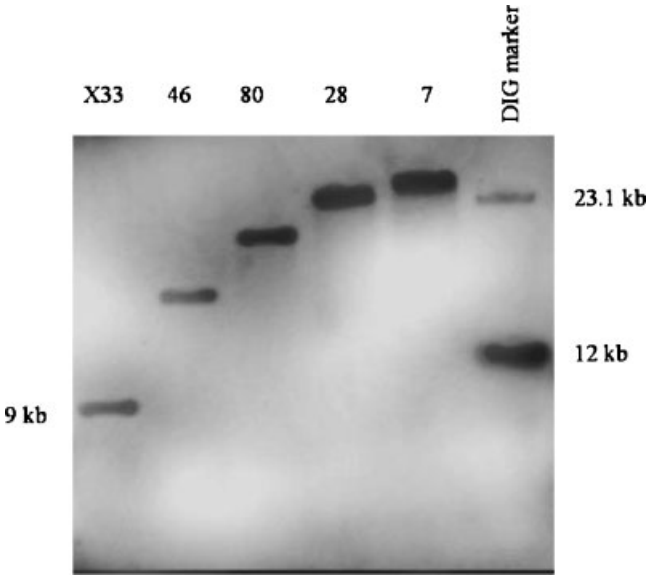

Figure 2. Southern blot analysis of selected Na-ASP1 clones. The lane numbers correspond to the clone ID numbers and copy number is determined by increase in plasmid size of 4.7 kb to the host X33.

Expression levels were determined for each clone through Western blots analysis (intracellular protein and supernatants) (Fig. 3 and Table I). Since an analytical quantitative method was not available at the time of experiments, a semi-quantitative method was used to compare Na-ASP1 production rates. *E. coli* produced Na-ASP1 standard was found to be not stable; therefore, it could not be used for quantification purposes (Fig. 3). The band intensities on developed films were quantified with Alpha Innotech 8800 gel imaging system using Alpha EaseFC Software (Alpha Innotech). All the clones showed Na-ASP1 production, although the amount of protein secreted per cell decreased significantly as the gene copy number increased. The single copy clone (no. 46) showed the highest secretion of Na-ASP1 protein. The four-copy clone had fourfold less Na-ASP1 in the supernatant compared to the single copy clone (Fig. 3A).

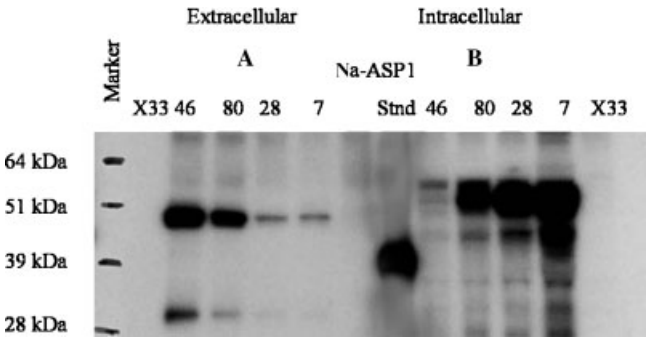

Figure 3. Western blot analysis for expression levels of selected Na-ASP1 clones. All the numbers shown above the picture are clone ID. A: A fixed amount of supernatant for all samples from shake flasks was loaded. B: Ten micrograms of intracellular protein from cell lysate was loaded for analysis. *E. coli* Na-ASP1 standard was degraded and was not used for Na-ASP1 quantification.

Western analysis of soluble cell extract revealed that, in the high copy number clones, Na-ASP1 protein was retained inside the cells (Fig. 3B). The size of Na-ASP1 protein band (~52 kDa) was higher than the expected molecular weight of 45 kDa in soluble cell extracts. This is indication of inefficient cleavage of α-mating factor by KEX2 protease. Increase in 7 kDa molecular weight accounts for the size of pro-region of α-mating factor. This is also an indication of the entrapment of the Na-ASP1 protein in the ER after removal of signal sequence (pre-region of α-mating factor), before the golgi where the pro-region of α-mating factor is cleaved by KEX2.

In contrast to secreted Na-ASP1, the amount of Na-ASP1 produced intracellularly increased with copy number (Fig. 3) indicating an increase in total Na-ASP1 production (Table I).

Overexpression of Protein Disulfide Isomerase in Na-ASP1 Multicopy Clones

It has been shown that PDI, a chaperone protein, plays a key role in folding and secretion of heterologous proteins in yeast (Robinson et al., 1994) and CHO cells (Davis et al., 2000). Therefore, we have over-expressed homologous PDI protein in multiple (2-4) Na-ASP1 copy clones. The endogenous PDI gene was amplified from *P. pastoris* X-33 chromosome based on a published gene sequence (Warsame et al., 2001). The amplified PDI gene was subcloned into pPIC3.5K vector under the control of *AOX1* promoter. The pPIC3.5K vector carries kanamycin marker gene for selection, which confers resistance to geneticin in *P. pastoris* and kanamycin resistance in *E. coli*. Geneticin hyper-resistant clones arise from multiple copies of the G418 resistance gene, which in turn corresponds to multiple plasmid copies, and thus multiple copies of the heterologous gene being expressed. By selecting against the G418 resistance, the Na-ASP1 copy is not affected since Na-ASP1 expressing plasmid carries the zeocin resistance gene.

After transformation, 26 colonies were selected from YPD plates with varying concentration of G418. The copy number of the selected clones was estimated by Southern blot employing DIG-labeled PDI gene as a probe. Estimated PDI copy number of the clones calculated densitometrically from band intensities as shown in Table II. The highest PDI copy number was obtained with the clone (80-18) with eight copies, one copy being endogenous PDI plus seven copies from the expression plasmid. The clone (80-19) did not contain the integrated PDI plasmid and was used as control for expression experiments.

To estimate levels of PDI transcript, Northern blot analyses was performed. Ten selected transformants were examined for PDI transcript covering high and low PDI copy transformants together with host clones with different copy numbers of pPICZα-NASP1. A 1.9 kb band was detected on Northern blot assays (Fig. 4), which showed the mRNA levels of clones, where clone (no. 80-18) produced highest level of PDI mRNA, followed by clones (no. 80-17) and (no. 80-20) correlating well to their copy numbers. A positive correlation

Table I. Estimated protein expression levels of selected Na-ASP1 clones.

Clone ID	Copy number	Extracellular Na-ASP1 levels[a]	Intracellular Na-ASP1 levels[b]	Total Na-ASP1 protein	Total Na-ASP1 produced relative to single copy clone
46	1	1	0.3	1.3	1.0
80	2	0.82	1.8	2.6	2.0
28	3	0.5	2.7	3.2	2.5
7	4	0.25	3.8	4.1	3.1

[a]Extracellular protein levels were determined relative to clone no. 46.
[b]Intracellular protein levels measured relative to extracellular product of one copy clone no. 46.

was obtained between the PDI gene copy numbers and corresponding mRNA levels.

Our attempts failed to show PDI protein was overexpressed intracellularly in selected clones using commercially available antibodies against *Saccharomyces cerevisiae* PDI, and *P. pastoris* PDI specific antibody was not available. Separation of soluble proteins in SDS–PAGE gel indicated an increased amount of a 57-kDa protein, the expected size of PDI protein (Fig. 5). Intensity of band correlated well with copy number of the clones (Table II). Therefore, the cell extract of the clone no. 80-18 was separated in SDS–PAGE gel, transferred to PVDF membrane, and N-terminus of the protein was sequenced via Edman degradation. The sequence confirmed that 57-kDa band indeed was PDI protein, and processed as expected. The first five amino acid of the N-terminus of the 57-kDa protein was SDQEA, the expected sequence for PDI as found in the literature (Warsame et al., 2001).

Effect of PDI Overexpression on Na-ASP1 Secretion

The effect of PDI overexpression on secretion of multicopy Na-ASP1 clones (2-4) was evaluated in selected clones (Table II). Na-ASP1 expression levels were determined for each transformant by Western blots analysis of the cell extract and fermentation supernatant. Increase in relative Na-ASP-1 levels was determined by comparing to a single copy Na-ASP1 clone (no. 46) since purified Na-ASP1 produced in *E. coli* was not stable and found to degrade over time (Fig. 3). The band intensities of developed films were quantified by densitometric analysis as described above. The expected size of Na-ASP1, 45 kDa, was present for all PDI transformants (Fig. 6A). There was approximately a threefold increase in protein secretion for clones no. 80-18 compared to the single copy Na-ASP1 clone no. 46 (Table II). There was an increase in the amount of Na-ASP1 secreted for all the clones over-expressing PDI protein as compared to the host strain. However, the highest secretion was observed in clone no. 80-18, which has two copies of Na-ASP1 gene and eight copies of the PDI gene (Fig. 6 and Table II). Clones 7-4 and 7-5, which have four copies of Na-ASP1 and four copies of PDI gene, did not secrete as much as the clone 80-17 and 80-18. However, the highest relative improvement was observed with PDI overexpresing the four copy clone Na-ASP1 clones (7-4, 7-5, and 7-14) with respect to their host.

A 54 kDa band reacting to anti Na-APS1 antibodies, corresponding to the pre- and pro-region of the α-mating factor attached to Na-ASP1, was still present inside the cells coexpressing PDI and Na-ASP1 (Fig. 6B).

Intracellular insoluble Na-ASP1 was also monitored in the clones expressing both Na-ASP1 protein and Na-ASP1/PDI over-expressing clones (Fig. 7). A single copy Na-ASP1 clones had a minute amount of insoluble Na-ASP1, however, amount of insoluble Na-ASP1 increased with increasing Na-ASP1 copy numbers. Overexpression of PDI reduced insoluble Na-ASP1 especially in two copy Na-ASP1 (80-18) clone (Fig. 7). Although a reduction was observed in amount of insoluble Na-ASP1 in three (28-9) and four (7-14) Na-ASP1 copy clones, insoluble Na-ASP1 accumulation was still observed in corresponding clones.

Table II. Effect of PDI overexpression on secretion of Na-ASP1 secretion.

Clone ID	Na-ASP1 copy number	PDI copies	Relative extracellular Na-ASP1 amount*	Relative Na-ASP1 fold increase with respect to host**
X33	0	1	0	0
46	1	1	1	1
80	2	1	0.82	1
28	3	1	0.50	1
7	4	1	0.25	1
80-17	2	7	3.55	4.3
80-18	2	8	3.64	4.4
80-19	2	1	0.75	1
80-20	2	7	3.23	3.9
28-9	3	6	2.21	4.42
28-11	3	5	1.63	3.26
7-4	4	4	1.73	6.9
7-5	4	4	1.77	7.1
7-13	4	3	1.57	6.1
7-14	4	5	1.98	7.9

*Extracellular Na-ASP1 amount was calculated from densitometric analysis of Western results. The results are reported relative to extracellular amount of Na-ASP1 produced from single Na-ASP1 (46) clone.

**Individual clones with extra PDI gene are compared to their host. The results are reported relative to multi-copy host stains.

DISCUSSION

Potential bottlenecks for optimum expression of recombinant proteins are promoter strength, the codon usage of the expressed gene, the gene copy number, translocation of nascent protein to ER, folding and processing in ER and golgi, and finally secretion out of the cell. The feasibility of improving an expression system for a recombinant protein by

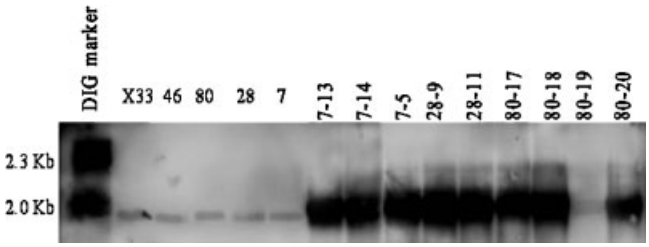

Figure 4. Northern blot analysis of PDI over-expressed Na-ASP1 clones. Northern blot analysis was performed to assess the levels of PDI transcript. Ten micrograms of total RNA was run and subjected to Northern hybridization using DIG labeled PDI as a probe. The numbers above the picture indicate the clone ID numbers. Clone no. 80-19 is an untransformed clone and is used as control.

a certain measure depends on whether one or several steps of protein processing are close to their limits. Codon optimization and gene copy numbers are the first critical parameters of an expression system to evaluate. Numerous examples have been described in literature that shows an increase of gene copy number can significantly increase productivity. (Clare et al., 1991; Vassileva et al., 2001).

In our previous study, we observed adverse effect of copy number for secretion of NA-ASP1 (Inan et al., 2005). We have demonstrated that one part of the bottlenecks for secretion of Na-ASP1 in *P. pastoris* was the secretion process itself and not the codon usage, transcription, or translation process. Increasing copy number resulted in an increased Na-ASP1 transcript and total Na-ASP1 produced, but not an increase in secreted Na-ASP1, in fact a decrease (Fig. 3).

Protein folding in the ER is a critical step both in mammals and yeast and a prerequisite for secretion (Gething and Sambrook, 1992; Helenius et al., 1992). The newly synthesized heterologous protein enters the lumen of the ER as part of a translocated polypeptide chain; it encounters a change in the redox environment that ultimately promotes

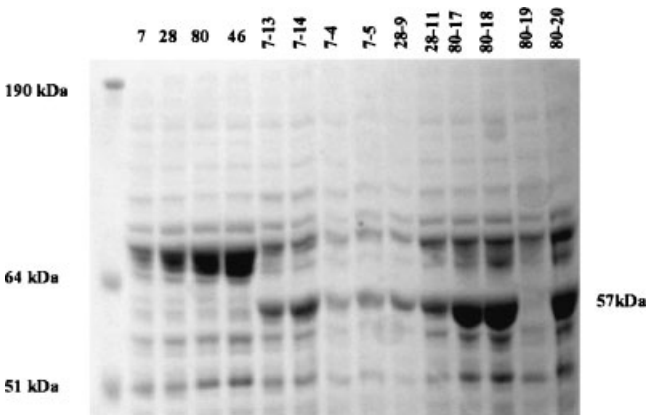

Figure 5. SDS–PAGE for intracellular samples of PDI over-expressed Na-ASP1 clones. SDS–PAGE was performed for confirmation of PDI protein. Ten micrograms of samples were loaded for N-sequencing from a blot. The protein sequence was determined by Edman degradation. All numbers above the figure show the clone ID.

formation of intrachain and/or interchain disulfide bonds. Chaperons assist in the folding of the nascent polypeptide chain by slowing folding, preventing aggregation, and ensuring the correct disulfide bonds are formed (van Vliet et al., 2003). Thus, it is not surprising that many over-expressed proteins in yeast accumulate in the ER (Ruohonen et al., 1997). Components of the folding machinery may become rate limiting and heterologous protein folding is inefficient. Overexpression of the components of the folding machinery, such as the PDI may lead to enhanced secretion (Robinson et al., 1994). Proteins secreted from eukaryotes must be correctly folded, or aggregates may be formed and unfolded proteins can be targeted for degradation through the translocon and the proteosome (Lord et al., 2000). Disulfide bond formation plays a critical role in the folding of proteins in the ER and efficient processing is necessary in obtaining high yields (Ngiam et al., 2000).

Overexpression of PDI has been successful in increasing the yields of some heterologous proteins from *S. cerevisiae* and *E.coli* but not others (Humphreys et al., 1996; Robinson et al., 1994). Coexpression of human PDI in *E. coli* strains producing antibody Fab fragments only increased secreted yields of one Fab variant, which was more susceptible to incorrect disulfide formation but not the other (Humphreys et al., 1996), suggesting that proteins which are susceptible to the formation of kinetically trapped intermediates may require higher concentrations of PDI, and overexpression of PDI could then ease this bottleneck.

The limiting step for efficient secretion of Na-ASP1 in multicopy clones may be the nature of the Na-ASP1 protein itself. Na-ASP1 contains 20 cysteine amino acids and preliminary structural studies of Na-ASP indicate 10 disulfide bonds (unpublished data). A large number of disulfide bonds increases the potential for aggregation due to misfolding in the ER, and thus our justification for over-expressing homologous PDI. Clones having higher copies of the PDI gene had higher Na-ASP1 levels secreted into the supernatant, regardless of Na-ASP1 copy (Table II). Interestingly, there was also an increase in the soluble intracellular Na-ASP1 levels and decrease insoluble intracellular Na-ASP1 levels with PDI over-expressing clones (Figs. 6B and 7). The mechanism by which increased PDI levels leads to increased overall Na-ASP1 production is also an intriguing question. One possible explanation is that nascent polypeptide proteins are synthesized and translocated into the ER; the sequence is presented vectorially to the ER lumen so that folding may begin as protein emerges from the ribosome. When PDI is limiting in the ER, the proteins tend to aggregate forming nonnative disulfide bonds. The aggregated proteins are degraded through ER-associated protein degradation pathway (ERAD) (Lord et al., 2000) and unfolded protein response (Ellgaard and Helenius, 2003). These two quality control mechanism are shown to be linked (Friedlander et al., 2000). Hence, over-expressing PDI may facilitate proper folding of Na-ASP1 and rescuing it from ERAD or UPR pathways and resulting in increased level of soluble Na-ASP1. Limitations in other secretory steps may contribute to

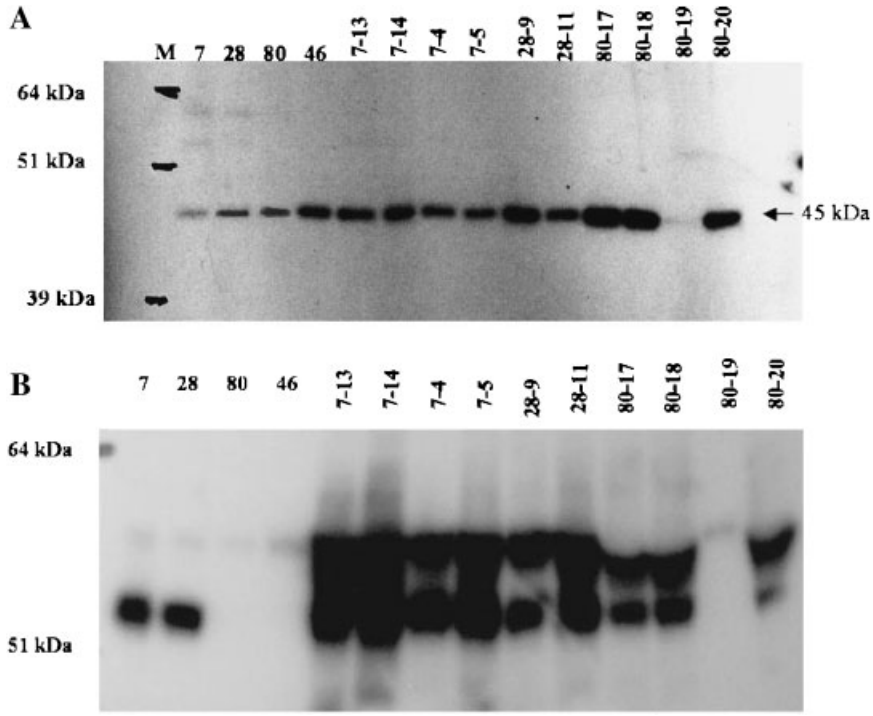

Figure 6. A: Extracellular Western blot analysis of PDI over-expressed Na-ASP1 clones. All numbers shown above the picture are clone ID. The intensity of the bands was compared using semi-quantitative method for quantification. Clone no. 80-19 was used as negative control. B: Intracellular Western blot analysis of PDI over-expressed Na-ASP1 clones. Ten micrograms of samples were loaded and numbers shown above are clone ID. Clone no. 80-19 was used as negative control.

the accumulation of Na-ASP1 in secretory organelles. Western blot analysis of intracellular soluble proteins showed two distinct molecular weight Na-ASP1 products. The nature of these bands could be a result of unprocessed and incomplete processing of the α-factor leader (Laboissiere et al., 1995), 52 and 54 kDa, respectively. The 52 kDa accounts for cleavage of the pre-region from the α-mating factor signal sequence with the pro-region still attached to Na-ASP1 protein. The 54 kDa band was observed in hosts not over-expressing PDI, that is, clones 7 and 28, and accounts for the unprocessed form of the α-mating factor-NaASP1 fusion. The extracellular product from all the clones had expected size of 45 kDa indicating that pre- and pro-region of alpha mating factor was efficiently removed. There was no fully processed Na-ASP1, that is, 45 kDa, found intracellularly, indicating that if the pre- and pro-region of α-mating factor were cleaved, it was transported outside the cell.

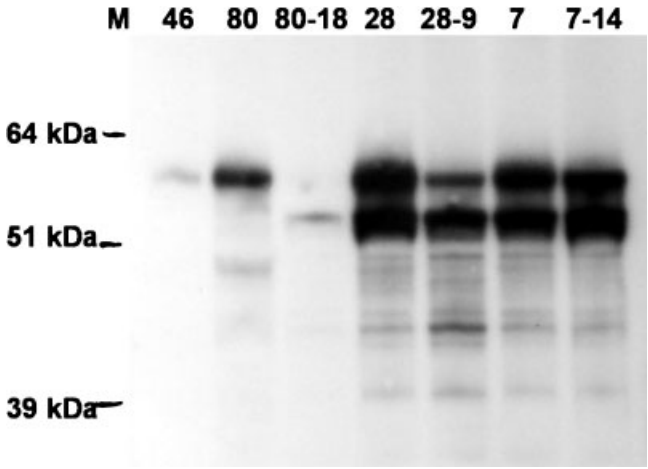

Figure 7. Western blot analysis of insoluble fractions of selected clones. The numbers shown above the picture are clone ID numbers.

Highest impact of PDI on Na-ASP1 secretion was observed in two copy-Na-ASP1 clone (clone no. 80-18). Two copies of Na-ASP1 (clones no. 80) reduced the amount of secreted Na-ASP1 amount compared to a single Na-ASP1 (clone no. 46) (Fig. 1). This result was due to accumulation of Na-ASP1 intracellularly both soluble and insoluble form (Figs. 1 and 7). When PDI (eight copies) was overexpressed in this clone no. 80-18, amount of insoluble Na-ASP1 was almost diminished (Fig. 7) and extracellular Na-ASP1 was increased (Fig. 6B), however, it also resulted in increased soluble intracellular Na-ASP1 (Fig. 6B). Since both soluble and insoluble intracellular form Na-ASP1 size was higher than 45 kDa, it is likely that other limitations, for example, KEX2 cleavage, translocation from ER to Golgi, play a role in extracellular production of Na-ASP1.

In conclusion, this study demonstrates overexpression of a chaperone protein, PDI, in *P. pastoris* and shows the importance of refolding activity of PDI in ER for secretion of a recombinant protein containing disulfide bonds. Overexpression of PDI may improve secretion of recombinant proteins, which normally misfolded in ER, and the process development technique described here could be applicable for other proteins and aid understanding secretory pathways of *P. pastoris*.

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