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Coexpression of Protein Disulfide Isomerase (PDI) Enhances Production of Kringle Fragment of Human Apolipoprotein(a) in Recombinant *Saccharomyces cerevisiae*

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Abstract In an attempt to increase production of LK8, an 86-amino-acid kringle fragment of human apolipoprotein(a) with three disulfide linkages, protein disulfide isomerase (PDI) was coexpressed in recombinant *Saccharomyces cerevisiae* harboring the LK8 gene in the chromosome. Whereas overexpression of the LK8 gene without coexpressing PDI was detrimental to both host cell growth and LK8 production, coexpression of PDI increased the LK8 production level by 2.5-fold in batch cultivation and 5.0-fold in fed-batch cultivation compared with the control strain carrying only the genomic PDI gene.

Key words: Apolipoprotein(a), kringle, *Saccharomyces cerevisiae*, protein disulfide isomerase

Numerous proteins, such as angiostatin and endostatin showing angiogenic activity, have been studied as possible candidates for anticancer therapeutics [1, 11]. Recent studies showed that the kringle fragment of human apolipoprotein(a) KV, termed as LK8, has strong anti-angiogenesis activity [6, 11]: The LK8 protein, a three disulfide-linked 86-amino-acid polypeptide, strongly inhibits new capillary formation *in vitro*.

Saccharomyces cerevisiae is a GRAS (Generally-Regarded-As-Safe) organism that grows readily to high density on simple media [5]. Moreover, its genome has been completely sequenced (www.yeastgenome.org); therefore, a number of genetic manipulation tools are available to construct an optimum "genetic context" for foreign protein production [10, 15].

The rate-limiting step in protein secretion is folding in the lumen of endoplasmic reticulum (ER), where efficiencies of disulfide bond formation and folding are interrelated [18, 20]. Therefore, manipulation of the ER folding environment may allow further improvements in *S. cerevisiae*'s secretory capacity. Several lines of evidences indicate that ER-resident molecular chaperones, including protein disulfide isomerase (PDI), are responsible for the formation of disulfide bonds [18, 19]. PDI, which acts as both disulfide isomerase and oxidoreductase, also rearranges preexisting disulfide linkages. Xiao *et al.* [19] suggested that the catalytic function of PDI, either its oxidase or isomerase activity, is essential for the growth and viability of yeast.

In our previous study, a recombinant *S. cerevisiae* expression system to produce LK8 was constructed, and the dependence of LK8 production level on the gene dosage was verified [2]. Elevation of the LK8 gene copy number through the multiple integration method yielded more than 2-fold increase of LK8 production level, compared with the episomal expression system. However, there was no further increase of LK8 production level as the LK8 gene dosage increased from 11 to 16 copies, strongly indicating that the protein secretory machinery might be saturated in the recombinant *S. cerevisiae* strain, as reported elsewhere [7, 14].

In this work, the effect of PDI coexpression on increasing the LK8 protein production level was explored in batch- and fed-batch cultivations of recombinant *S. cerevisiae*.

Escherichia coli DH5α {F^-, *lacZ*ΔM15, *hsd*R17, (r^-m^-), *gyr*A36} was used for plasmid manipulation. The recombinant *S. cerevisiae* 2805/MδLK8 (*MATα pep4::HIS3 prb1 can1 his3 ura3-52*) strain harboring 16 copies of the *GAL1* promoter-driven LK8 expression cassette in the chromosome was used for LK8 production [2]. To construct plasmid pMK102, two PCR primers, PDI1F (5'-ATAAGAATGCGGCCGCC-ATACATCTATCCCGTTATG-3') and PDI1R (5'-GGACT-AGTTTACAATTCATCGTGAATGGCATC-3'), were designed

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to amplify a DNA fragment containing the PDI ORF of *S. cerevisiae*. The PCR fragment was inserted into the NotI/SpeI sites of the 2-micron *URA3*-marked vector pMK101 that contains the *GAL1-10* promoter and *CYC1* terminator (lab stock). Expression of PDI in pMK102 is under the control of the galactose-inducible *GAL10* promoter.

LB medium (1% NaCl, 1% tryptone, and 0.5% yeast extract) was used for bacterial cultivation. Selective synthetic complete medium (SC) lacked uracil to select for plasmids pMK101 and 102. Batch cultivations were carried out in a bench-top bioreactor (Bioengineering AG, Wald, Switzerland) with 1.5-l YEPD medium (1% yeast extract, 2% peptone, and 2% glucose) supplemented with 3% galactose. Fed-batch cultivations were carried out with 1.5-l working volume of an enriched medium (4% yeast extract, 0.5% casein hydrolysate, and 3% galactose). For fed-batch fermentation, galactose (50%, w/v) was intermittently fed into the reactor as the sole carbon source after depletion of the galactose initially added. Medium acidity was controlled at pH 5.5 by adding either 2 N HCl or 2 N NaOH. All yeast cultures were carried out at 30°C. An agitation speed of 500 rpm and an aeration of 1 vvm were maintained throughout the cultivation.

Dry cell mass concentration was measured spectrophotometerically at 600 nm. Concentrations of ethanol, glucose, and galactose were measured by HPLC (Knauer, Berlin, Germany) equipped with the HPX-87H column (Bio-Rad, Hercules, CA, U.S.A.). In order to measure the amount of secreted LK8 protein, extracellular proteins were harvested by brief centrifugation and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% polyacrylamide). Gels were silver-strained and images were analyzed using a densitometer.

To examine the effect of PDI coexpression on LK8 production, batch fermentations were performed with recombinant *S. cerevisiae* 2805/MδLK8 strains harboring pMK101 (empty vector) and pMK102, respectively, in YEPD medium supplemented with 3% galactose. Preliminary studies showed that plasmid pMK101 and pMK102 were both stably maintained within the cell, even in rich medium (data not shown). During the glucose-consuming growth phase, both strains did not show any discernable differences in growth rate and cell mass yield from glucose (data not shown). However, there were significant differences in rates of cell growth and substrate consumption in the galactose-consuming growth phase (Table 1). Accordingly, PDI coexpression increased the LK8 production level by 2.5-fold and resulted in a maximum LK8 concentration of 25 mg/l in the *S. cerevisiae* 2805/MδLK8 strain harboring pMK102. It was noteworthy that dry cell mass concentration was also increased by PDI coexpression, presumably by relieving cell growth arrest, as observed in our previous reports [8].

The fact that coexpression of PDI increased the amount of multiple disulfide-linked LK8 in recombinant *S. cerevisiae* was more evident in fed-batch fermentations, when galactose was used as the sole carbon source. The *S. cerevisiae* 2805/MδLK8 strain harboring pMK101, an empty vector, showed retarded growth rate on galactose, as observed in batch cultivation. Moreover, it was almost impossible to start the fed-batch mode until 73 h of cultivation, because the cells did not completely utilize galactose (Fig. 1). Specific galactose consumption rate of the control strain was about 2.7-fold lower than that of the *S. cerevisiae* 2805/MδLK8 strain coexpressing PDI. After 96 h of cultivation, the maximum LK8 concentration of 250 mg/l was obtained by coexpression of PDI in the *S. cerevisiae* 2805/MδLK8 strain, which corresponded to an approximately 5-fold increase in LK8 production level, compared with the control strain carrying the genomic PDI only (Fig. 2).

Elevating gene copy number is a typical strategy used to increase heterologous gene expression in recombinant microbial systems [2, 7, 9, 14]. For secreted proteins, however, this strategy can cause saturation or overloading of the secretory pathway, typically in the endoplasmic reticulum that acts as quality-control machinery in eukaryotic cells [14]. Furthermore, overexpression of a foreign protein in recombinant cells often leads to misfolding of the desired protein and/or arrest of host cell growth owing to heavy traffic of the cellular machinery [7, 14].

Manipulation of chaperone/foldase levels to improve foreign protein secretion in *S. cerevisiae* has been reported for a number of proteins [8, 16, 17, 20]. Overproduction of ER-resident BiP has been shown to stimulate the secretion of bovine prochymosin 26-fold, but to exert no effect on the secretion of plant thaumatin [4].

Table 1. Batch and fed-batch fermentation results of recombinant *S. cerevisiae* 2805/MδLK8 strains.

Strain	Plasmid	Fermentation mode	Maximum dry cell mass (g/l)	Specific galactose consumption rate[b] (g galactose/g cell · h)	Maximum LK8 concentration (mg/l)
S. cerevisiae 2805/MδLK8	pMK101	Batch	15	0.6	10
		Fed-batch[a]	22	0.6	50
	pMK102	Batch	28	1.6	25
		Fed-batch[a]	60	1.7	250

[a]For fed-batch fermentation, galactose (50%) was intermittently fed into the reactor as the sole carbon source after depletion of the galactose added initially.
[b]Specific galactose consumption rates were measured in exponential growth phases on galactose, and values were averaged from two separate experiments.

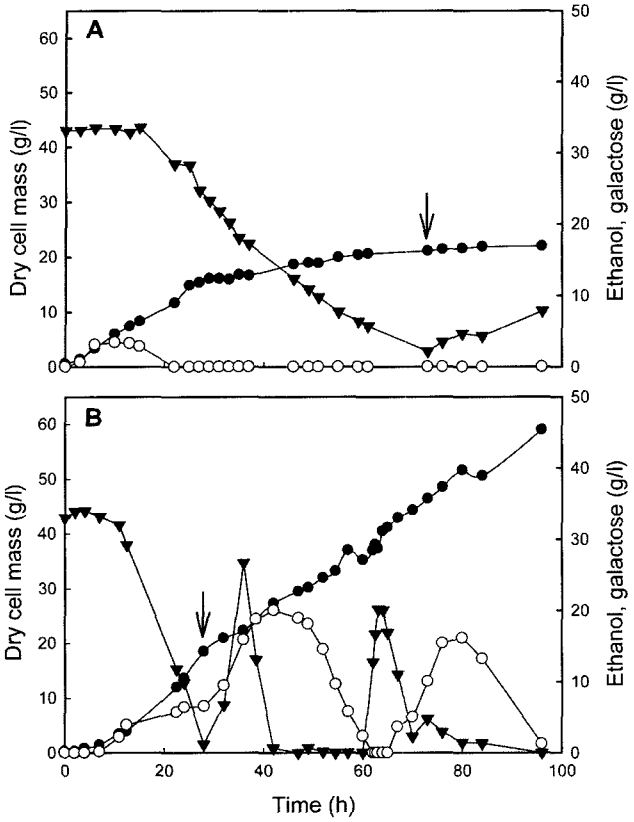

Fig. 1. Fed-batch fermentation profiles of *S. cerevisiae* 2805/MδLK8 strains harboring pMK101 (**A**) and pMK102 (**B**) at pH 5.5 and 30°C.

Each symbol indicates cell growth (●), ethanol (○), and galactose (▼). Galactose (50%, w/v) was intermittently fed into the reactor as the sole carbon source after depletion of the galactose initially added. The arrow indicates initiation of the fed-batch mode.

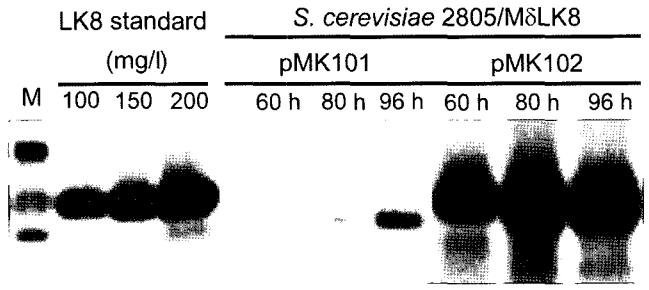

Fig. 2. Comparison of LK8 secretion levels in fed-batch cultivation of recombinant *S. cerevisiae* 2805/MδLK8 strains.

Extracellular proteins were harvested at the indicated cultivation time, separated by SDS-PAGE (12% polyacrylamide), and silver stained. M denotes the lane for the size marker.

In our previous work, a significant enhancement in production level of hirudin, a three disulfide-linked antithrombotic protein from leech, was accomplished in *S. cerevisiae* by coexpression of BiP [8]. Coexpression of BiP not only increased host cell growth, but also prevented an abnormal enlargement of the host cell caused by entrapment of hirudin in the ER.

PDI, an abundant ER foldase, is an essential protein in yeast that catalyzes the oxidation, reduction, and isomerization of disulfide bonds [12, 19]. Smith and Robinson [17] reported a 60% increase in secretion yield of β-glucosidase by overexpressing PDI in *S. cerevisiae*. Approximately 2-fold increase in secretion of a single-chain antibody fragment in yeast was achieved by overexpression of either BiP or PDI [17]. Secretion of thaumatin, a highly disulfide-linked protein, was increased about 2-fold by overexpression of protein disulfide isomerase in fungus *Aspergillus awamori* [13]. It was also reported that coexpression of BiP and PDI acted synergistically to increase secretion of single-chain antibody fragments [16]. However, an increased amount of BiP and PDI in the ER was not always beneficial for foreign protein production, since overexpression of PDI has been found to decrease the production of foreign proteins and to concomitantly cause accumulations of the proteins [3].

Accordingly, it appeared that the protein of interest should be examined experimentally for whether secretion can be improved by overexpression of chaperones such as PDI and BiP in order to overcome secretion block in the ER. In addition, consideration of physiological aspects of host cells and adjustment of genetic context might be crucial in designing an optimal expression system for maximum production of foreign proteins in recombinant microorganisms.

Taken together, it was suggested that the growth of a host cell and the context of gene expression are important for maximizing LK8 protein production in recombinant *S. cerevisiae*, strongly indicating that the gene dosage effect could be complemented by coexpression of secretion-assisting proteins such as protein disulfide isomerase.

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References

1. Carmeliet, P. and R. K. Jain. 2000. Angiogenesis in cancer and other disease. *Nature* **407:** 249–257.
2. Cha, K. H., M. D. Kim, T. H. Lee, H. K. Lim, K. H. Jung, and J. H. Seo. 2004. Selection of optimum expression system for production of kringle fragment of human apolipoprotein(a) in *Saccharomyces cerevisiae*. *Biotechnol. Bioprocess Eng.* **9:** 523–527.
3. Davis, R., K. Schooley, B. Rasmussen, J. Thomas, and P. Reddy. 2000. Effect of PDI overexpression on recombinant

protein secretion in CHO cells. *Biotechnol. Prog.* **16:** 736–743.
4. Harmsen, M. M., M. I. Bruyne, H. A. Raue, and J. Maat. 1996. Overexpresson of binding protein and disruption of the *PMR1* gene synergistically stimulate secretion of bovine prochymosin but not plant thaumatin in yeast. *Appl. Microbiol. Biotechnol.* **46:** 365–370.
5. Kim, H.-E., R. Qin, and K.-S. Chae. 2005. Increased production of exoinulinase in *Saccharomyces cerevisiae* by expressing the *Kluyveromyces marxianus INU1* gene under the control of the *INU1* promoter. *J. Microbiol. Biotechnol.* **15:** 447–450.
6. Kim, J. S., H. K. Yu, J. H. Ahn, H. J. Lee, S. W. Hong, K. H. Jung, S. I. Chang, Y. K. Hong, Y. A. Joe, S. M. Byun, S. K. Lee, S. I. Chung, and Y. Yoon. 2004. Human apolipoprotein(a) kringle V inhibits angiogenesis *in vitro* and *in vivo* by interfering with the activation of focal adhesion kinases. *Biochem. Biophys. Res. Commun.* **313:** 534–540.
7. Kim, M. D., S. K. Rhee, and J. H. Seo. 2001. Enhanced production of anticoagulant hirudin in recombinant *Saccharomyces cerevisiae* by chromosomal δ-integration. *J. Biotechnol.* **85:** 41–48.
8. Kim, M. D., K. C. Han, H. A. Kang, S. K. Rhee, and J. H. Seo. 2003. Coexpression of BiP increased antithrombotic hirudin production in recombinant *Saccharomyces cerevisiae*. *J. Biotechnol.* **101:** 81–87.
9. Lee, F. W. F. and N. A. Da Silva. 1997. Improved efficiency and stability of multiple cloned gene insertions at the δ-sequences of *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **48:** 339–345.
10. Lee, J. S., J. Yu, H. J. Shin, Y. S. Kim, J. K. Ahn, C. K. Lee, H. Poo, and C. J. Kim. 2005. Expression of Hepatitis C virus structural proteins in *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* **15:** 767–771.
11. Lim, H. K., S. G. Kim, K. H. Jung, and J. H. Seo. 2004. Statistical selection of amino acids fortifying a minimal defined medium for a high-level production of the kringle fragments of human apolipoprotein(a). *J. Microbiol. Biotechnol.* **14:** 90–96.
12. Lumb, R. A. and N. J. Bulleid. 2002. Is protein disulfide isomerase a redox-dependent molecular chaperone? *EMBO J.* **21:** 6763–6770.
13. Moralejo, F. J., A. J. Watson, D. J. Jeenes, D. B. Archer, and J. F. Martin. 2001. A defined level of protein disulfide isomerase expression is required for optimal secretion of thaumatin by *Aspergillus awamori*. *Mol. Genet. Genome* **266:** 246–253.
14. Parekh, R., K. Forrester, and D. Wittrup. 1995. Multicopy overexpression of bovine pancreatic trypsin inhibitor saturates the protein folding and secretory capacity of *Saccharomyces cerevisiae*. *Prot. Exp. Purif.* **6:** 537–545.
15. Ro, H. S., M. S. Lee, M. S. Hahm, H. S. Bae, and B. H. Chung. 2005. Production of active carboxypeptidase Y of *Saccharomyces cerevisiae* secreted from methylotropic yeast *Pichia pastoris*. *J. Microbiol. Biotechnol.* **15:** 202–205.
16. Shusta, E. V., R. T. Raines, A. Plückthun, and K. D. Wittrup. 1998. Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments. *Nat. Biotechnol.* **16:** 773–777.
17. Smith, J. D. and A. S. Robinson. 2004. Overexpression of an archaeal protein in yeast: Secretion bottleneck at the ER. *Biotechnol. Bioeng.* **79:** 713–723.
18. Tu, B. P., S. C. Ho-Schleyer, K. J. Travers, and J. S. Weissman. 2000. Biochemical basis of oxidative protein folding in the endoplasmic reticulum. *Science* **290:** 1571–1574.
19. Xiao, R., B. Wilkinson, A. Solovyov, J. R. Winther, A. Holmgrent, J. Lundström-Ljung, and H. F. Gilbert. 2004. The contributions of protein disulfide isomerase and its homologues to oxidative protein folding in the yeast endoplasmic reticulum. *J. Biol. Chem.* **279:** 49780–49786.
20. Zhao, Y., W. Ge, Y. Kong, and C. Zhang. 2003. Cloning, expression, and renaturation studies of reteplase. *J. Microbiol. Biotechnol.* **13:** 989–992.