Biotechnology and Bioprocess Engineering **2004**, 9: 523-527 

# Selection of Optimum Expression System for Production of Kringle Fragment of Human Apolipoprotein(a) in *Saccharomyces cerevisiae*

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**Abstract** Recombinant *Saccharomyces cerevisiae* expression systems were developed to produce a novel human anti-angiogenic protein called LK8, an 86 amino-acid kringle fragment protein with three disulfide linkages. Galactose-inducible LK8 expression plasmid was constructed, and LK8 production levels by four *S. cerevisiae* strains were compared in order to select an optimal host strain. *S. cerevisiae* 2805 was the most efficient among the strains tested. Elevating the LK8 gene copy number through multiple integration using δ-sequences as target sites resulted in more than a two-fold increase in the LK8 production level compared with the plasmid-based expression system. The maximum LK8 protein concentration of 25 mg/L was obtained from batch cultivation of the yeast transformant that harbors 16 copies of the LK8 gene. In conclusion, the strain integrated with the multiple LK8 gene secreted the protein with relatively high yield, although, the increased LK8 gene dosage over 11 copies did not lead to further enhancement in batch cultivations.

*Keywords*: apolipoprotein(a), kringle, *Saccharomyces cerevisiae*, δ-integration

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, involves various kinds of physiological conditions, such as primary tumor growth, metastasis, and diabetic retinopathy [1-3]. Numerous proteins, such as angiostatin and endostatin showing angiogenic activity, have been studied as possible candidates for anti-cancer therapeutics [1,2]. Recently, it was revealed that the kringle fragment of human apolipoprotein(a) KV, termed as LK8, has strong anti-angiogenesis activity [4]. The LK8 protein, which is a three disulfide-linked 86 amino-acid polypeptide, strongly inhibits endothelial cell migration *in vitro* and suppresses new capillary formation *in vitro* [4].

Since eukaryotic cells secrete a protein with properly folded form through their endoplasmic reticulum (ER) quality control machinery, the production of foreign proteins in the yeast *Saccharomyces cerevisiae* is more advantageous than other bacterial expression systems. This yeast is a GRAS (Generally Recognized As Safe) organism with well-documented genetic information and a long history of applications. The secretion of a foreign protein fused with leader peptides [5,6] is preferable to the expression in recombinant *Escherichia coli* in which translated foreign proteins often accumulate as inactive inclusion bodies. Furthermore, secretion into the medium also facilitates protein recovery and purification [7].

In this study, four laboratory *S. cerevisiae* strains were tested to select an optimal host strain to produce the LK8 protein. On the other hand, to verify the dependence of LK8 production level upon the gene dosage, multiple copy integration was done at δ-sequences in the genome known as long terminal repeats of yeast retrotransposon *Ty1* that are dispersed throughout the yeast genome [8].

*Escherichia coli* DH5α [$F^-$, *lac*ZΔM15, *hsd*R17, ($r^-m^-$), *gyr*A36] was used for plasmid amplification and preparation. Laboratory *S. cerevisiae* strains [11,12] shown in Table 1 were used as host cells for the production of the LK8 protein. The BY4742 strain derived from wild-type S288C was used for the worldwide *S. cerevisiae* gene deletion project [15]. The CEN.PK2-1D strain that has high transformation efficiency was used for the German functional analysis project [16]. BJ3501 and 2805 are protease-deficient (*pep4*) strains, which seemed to be useful for foreign protein production due to their low proteolytic activities. Plasmids p426GAL1 [13] and pδ-neo [8,10] were used as carrier vectors for transforming the LK8 gene into the *S. cerevisiae* strains [14]. Plasmid pPIC9-LK8 (lab stock) was used as the template for pol-

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**Table 1.** *S. cerevisiae* host strains used in this study

| Strain | Genotype | Source or Reference |
|---|---|---|
| BY4742 | *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* | EUROSCARF* |
| CEN.PK2-1D | *MATα ura3-52 trp1-289 leu2-3_112 hisΔ3 α1 MAL2-8$^C$ SUC2* | EUROSCARF |
| 2805 | *MATα pep4::HIS3 prb1-Δ1.6R his3Δ200 ura3-52 can1* | [11] |
| BJ3501 | *MATα pep4::HIS3 prb1-Δ1.6R his3Δ200 ura3-52 gal2 can1* | [12] |

*European *Saccharomyces cerevisiae* Archive for Functional Analysis (http://www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/)

ymerase chain reaction (PCR).

LB medium (1% NaCl, 1% tryptone, and 0.5% yeast extract) was used for *E. coli* cultivation. YPD plates (2% glucose, 1% yeast extract, and 2% peptone) supplemented with various concentrations of G418 sulfate were used to select and maintain the yeast transformants harboring the G418 resistance gene. YNB plates (0.67% yeast nitrogen base without amino acid, 2% agar, and 2% glucose) with proper amino acid supplements were used for the selection of yeast transformants harboring pMCLK8 plasmid.

The batch culture was carried out in a bench-top B. Braun bioreactor (Melsungen, Germany) with one liter of YPD medium (2% peptone, 1% yeast extract, and 2% glucose) supplemented with 3% galactose. Medium acidity was controlled at pH 5.5 by adding either 2 N HCl or ammonia water. All cultures were carried out at 30°C, and an agitation speed of 700 rpm with an aeration of 1 vvm was maintained throughout the cultivation.

All DNA manipulation and bacterial transformations were carried out as described by Sambrook and Russell [14].

Dry cell mass concentration was estimated with a spectrophotometer at 600 nm. Concentrations of glucose and galactose were measured by HPLC (Knauer, Berlin, Germany), which was equipped with the HPX-87H column (Bio-Rad). Colony hybridization was done primarily to select *S. cerevisiae* transformants that produce a high amount of LK8 protein. The copy numbers of the LK8 gene in these transformants were estimated using the Southern blot analysis. Western blot using the anti-rabbit LK8 monoclonal antibody (lab stock) was carried out in order to measure the amount of secreted LK8 protein. All blotting images were analyzed using a densitometer (Bio-Rad).

In order to make plasmid pMCLK8 where LK8 expression is under the control of the galactose-inducible *GAL1* promoter, the LK8 gene fragment containing the *MFα1* leader sequence and the LK8 structural gene was amplified by PCR using plasmid pPIC9-LK8 as the template, and then, it was introduced into the *Bam*HI/*Eco*RI site of the p426GAL1 vector (Fig. 1(a)).

Batch fermentations were performed with the four recombinant *S. cerevisiae* strains harboring plasmid pMCLK8. Extracellular proteins were harvested at 30 and 40 h after the cultivation and they were analyzed by Western blot to measure the amount of secreted LK8 protein. As shown in Fig. 1(b), the amount of secreted LK8 protein from

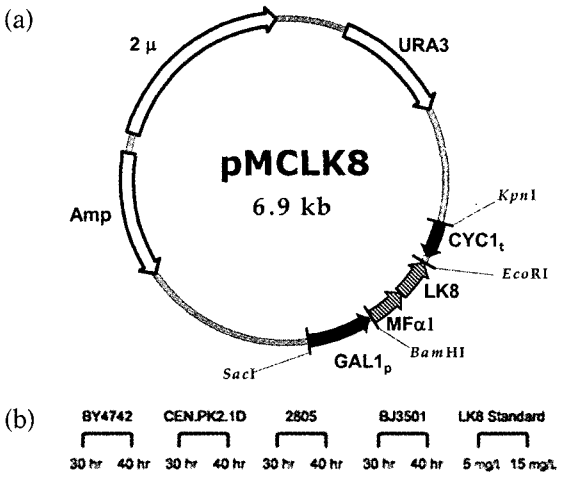


**Fig. 1.** (a) Schematic presentation of pMCLK8 plasmid. (b) Western blot analysis for measurement of the amount of secreted LK8 protein. All host *S. cerevisiae* strains harboring pMCLK8 plasmid were grown in YPD media supplemented with 3% galactose for the *GAL1* promoter induction. Extracellular proteins were harvested after 30 and 40 h of cultivation for analysis. The arrow indicates the LK8 protein.

the BY4742 and CEN.PK2-1D strains was relatively small compared with that from 2805 and BJ3501, which presumably was caused by relatively higher proteolytic activities in the BY4742 and CEN.PK2-1D strains or by different genetic backgrounds. Even though the LK8 production level in the BJ3501 strain was comparable with that in 2805, the BJ3501 strain that had the *gal2* mutation showed significantly retarded growth on galac-

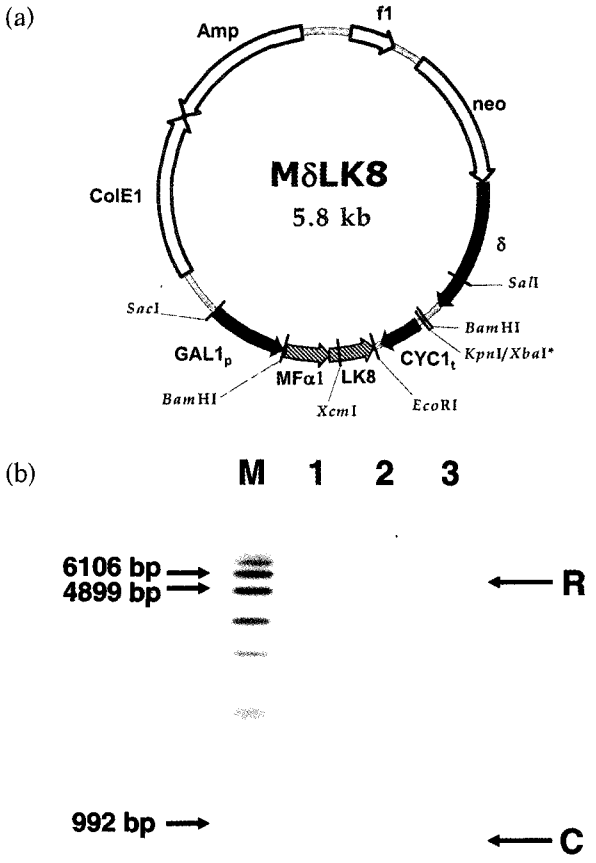


**Fig. 2.** (a) Schematic presentation of MδLK8 plasmid. (b) Southern blot analysis of the recombinant *S. cerevisiae* strains for estimation of the copy number of the LK8 gene in the chromosome. Lane M: size marker; Lane 1: 2805/MδLK8 (clone #102); Lane 2: 2805/MδLK8 (clone #102-17); Lane 3: 2805/MδLK8 (clone #102-17-112). All genomic DNA were double-digested with *Sac*I and *Xba*I and the *GAL1* promoter gene was used as a molecular probe. R denotes the single copy reference band of the *GAL1* promoter gene originally in the host genome. C denotes the band of integrated *GAL1* terminator genes.

tose in comparison with the 2805 strain (data not shown), which might be an undesirable growth phenotype as a host cell for high cell density fed-batch cultivations. Taking all this together, the *S. cerevisiae* 2805 strain was finally chosen as the host cell for LK8 production with an approximate LK8 concentration of 10 mg/L after 40 h of batch cultivation.

A multiple copy integration method was used to elevate the copy number of the LK8 gene in the recombinant yeast strain. To integrate the LK8 expression cassette into the δ-sequences of *S. cerevisiae*, the MδLK8 (5.9 kb, *neo*$^r$) vector was constructed using pδ-neo as the carrier plasmid. Plasmid pMCLK8 was digested with *Sal*I, end-filled with the Klenow fragment, and ligated again to remove the *Sal*I site. The *Xba*I-digested pδ-neo vector was treated with the Klenow fragment to produce blunt ends and was further digested with the *Sac*I enzyme. Then, plasmid pMCLK8 was further digested with *Kpn*I, end-filled with the Klenow fragment, and treated further with *Sac*I in order to obtain the LK8 expression cassette containing the *GAL1* promoter, *MFα1* leader sequence, LK8 structural gene, and *CYC1* terminator. The LK8 expression cassette was then ligated with pδ-neo to construct the chromosome-integrating MδLK8 plasmid (Fig. 2(a)), which was linearized by *Sal*I before transformation into the *S. cerevisiae* 2805 strain.

The number of transformants and the copy number of the LK8 gene in those transformants were greatly dependent on G418 concentration in the selective plate, which was well compatible with the other sources from the literature [8,10,17-19].

The *GAL1* promoter gene was used for Southern blot analysis (Fig. 2(b)) because there was no doubt that the copy number of the integrated *GAL1* promoter gene digested by *Sac*I and *Xcm*I would be the same as the integrated LK8 gene from MδLK8 (Fig. 2(a)). Between 6,106 and 4,899 bp of lane 1, 2, and 3, the *GAL1* promoter gene which was located in the yeast genome was detected and used as a single copy reference band. Finally, recombinant *S. cerevisiae* 2805 strains harboring 11 (clone #102, #102-17) and 16 (clone #102-17-112) copies of the *GAL1* promoter gene were isolated from 1.0% and 1.5% (w/v) concentration of the G418 plate, respectively.

As a consequence, the copy number of the integrated LK8 gene was elevated from 11 to 16 in the genome by a gene dosage-dependent selection method, which is based on the dominant bacterial aminoglycoside 3-phosphotransferase (aph) gene for G418 resistance [8-10]. Afterward, their LK8 production abilities were also investigated in batch cultivation.

The LK8 production levels measured after 40 h of cultivation for the recombinant *S. cerevisiae* strains that harbored 11 (#102-17) and 16 (#102-17-112) copies of the LK8 gene were found to range from 20 to 25 mg/L, respectively, which corresponded to more than a two-fold increase in LK8 protein production when compared with the production from the pMCLK8-driven expression system (Fig. 3). From this, it could be figured out plasmid instability would make a significant difference in the production level between the plasmid-based and integrated systems. Accordingly, the δ-integrated transformants were more efficient in LK8 protein production probably due to the elevated LK8 gene dosages. It was also noteworthy that there was no increase in LK8 production level as the gene dosage increased from 11 to 16 copies, indicating that the protein secretory pathway might be saturated in the recombinant LK8 producing *S. cerevisiae* strain containing above 11 copies of the LK8 gene. Admittedly, there have been some reports concerning the enhanced production of β-glucosidase [9], β-galactosidase [18], bovine pancreatic trypsin inhibitor [20], and an antithrombotic hirudin [10] by increasing the target gene copies in the recombinant *S. cerevisiae* expression system. A linear relationship between the copy number of the target gene and its production level was also observed in our

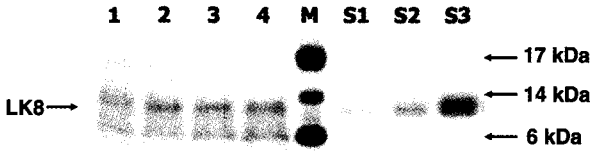


**Fig. 3.** Western blot analysis for determination of the amount of secreted LK8 protein from batch cultivations in recombinant *S. cerevisiae* 2805 strains. After 40 h of cultivation, proteins were harvested and analyzed. Lane 1: 2805/pMCLK8; Lane 2: 2805/MδLK8 #102; Lane 3: 2805/MδLK8 #102-17; Lane 4: 2805/MδLK8 #102-17-112; Lane S1: LK8 standard (10 mg/L); Lane S2: LK8 standard (30 mg/L); Lane S3: LK8 standard (50 mg/L). M denotes the lane for the size marker. The arrow indicates the LK8 protein.

previous report. To summarize, gene dosage often plays an important role on the production of heterologous proteins in recombinant microorganisms [10]. However, elevating the copy number of the target gene often resulted in the entrapment of a translated polypeptide in the secretory pathway along with the the arrest of the host cell growth [21-23]. Overexpression of an antithrombotic hirudin in a recombinant *S. cerevisiae* strain resulted in the enlargement of cell size and arrest of cell growth, which was relieved by the coexpression of the *KAR2* (Bip) gene, which encodes an ER-resident chaperone [24]. In the same line, it can be speculated that the overexpressed LK8 protein might be trapped in the secretion pathway, which was accompanied by retardation in cell growth. More research is now in progress to further increase the LK8 production level engineering the secretion machinery of the yeast *S. cerevisiae*.

**Acknowledgements** This work was supported by the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science and Technology (Grant MG02-0303-002-1-0-0) and Ministry of Education through the BK21 program.

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[Received August 16, 2004; accepted November 30, 2004]