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EXPRESSION AND SECRETION OF HUMAN LIPOCORTIN-1 BY PROMOTER AND SIGNAL SEQUENCE OF *STA1* FROM *SACCHAROMYCES DIASTATICUS*

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SUMMARY

For the secretion of human lipocortin-1 (LC-1) in yeast, a expression and secretion vector was constructed by using the promoter and signal sequence of glucoamylase gene (*STA1*) of *Saccharomyces diastaticus*. After the cDNA of human LC-1 was ligated with the secretion vector, the resulting hybrid plasmid was transformed into *S. diastaticus*. When the recombinant *S. diastaticus* was cultivated in YPD medium, LC-1 was expressed and secreted into the extracellular medium, yielding LC-1 protein at a concentration of 2.5 μg/mL.

INTRODUCTION

Saccharomyces spp offers several advantages as a host for the production of recombinant proteins. One of the main advantages is that it is capable of secreting proteins of interest into the extracellular medium, which greatly simplifies the purification of proteins and consequently reduces the overall production cost. In addition, secretion of proteins results in the correct formation of disulfide bonds which is essential for recombinant proteins to retain their original biological activities.

The yeast *Saccharomyces* is known to secrete a few proteins into the extracellular medium. Among them, the signal sequences from mating factor-α and invertase of *S. cerevisiae* have been widely used for the secretion of heterologous proteins (De Nobel and Barnett, 1991). However, a large fraction of heterologous proteins expressed by *S. cerevisiae* was found to remain intracellularly, particularly in secretion of proteins with a molecular weight over 20 kDa (Smith *et al.*, 1985; Zsebo *et al.*, 1986; Jigami *et al.*, 1986; De Nobel and Barnett, 1991).

The glucoamylase enzyme, *STA1* gene product, is a heavily glycosylated 250 kDa protein and is known to be secreted into the extracellular medium by *S. diastaticus*. The *STA1* gene was cloned and found to contain a signal sequence (Yamashita et al., 1985). Although the *STA1* gene might have some potential to be used for the expression and secretion of various heterologous proteins from yeast cells, very few studies have been carried out so far. The production of human 5-lipoxygenase in *S. cerevisiae* has been attempted by using the *STA1* promoter and its own signal sequence. However, this enzyme was not successfully secreted into the extracellular culture medium (Nakamura *et al.*, 1990).

Lipocortin-1 (LC-1) is a glucocorticoid-induced protein which mediates the anti-inflammatory function derived from the inhibition of phospholipase A_2 (PLA_2) (Flower, 1988). The cDNA of human LC-1 has been isolated from the cDNA library of human lymphoma cell line or human placental mRNA, and *Escherichia coli* has been genetically engineered to produce recombinant LC-1 in large quantities (Wallner *et al.*, 1986; Huh. *et al.*, 1990). The resulting recombinant LC-1 exhibited an inhibitory activity against PLA_2 and showed anti-inflammatory actions *in vivo*. It consists of 346 amino acids and has a molecular weight of 37 kDa.

In this study the expression vector system with *STA1* promoter and its own signal sequence was constructed to show the secretory production of human LC-1 in *Saccharomyces diastaticus*. The results of this study offered the potential for the general application of this expression system to produce a variety of heterologous proteins in yeast.

MATERIALS AND METHODS

Strains and Culture Conditions. *Saccharomyces diastaticus* YIY345 (*MATa, ura3, leu2-3, -112, his4, sta^o, inh^o*) was used as a host strain. Yeast transformation was carried out as described by Ito *et al.* (1983), and Ura$^+$ transformants were selected on YNBCAD medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acid and 2% glucose). Transformed yeast cells were grown on YPD medium (1% yeast extract, 2% Bacto-peptone and 2% glucose) at 30 °C for 72 hr.

DNA Manipulation and Plasmid Construction. *Escherichia coli* MC1061 was used for the propagation of the plasmids as well as for the cloning procedures. Standard DNA techniques were used (Sambrook *et al.*, 1989). Restriction endonucleases, T4 DNA ligase, mung bean nuclease purchased from Boehringer Mannheim were used as described in the manufacturer's instructions. *Bam*HI linker was from NEB (New England Biolabs Inc. Beverly, MA). The plasmid pYEG27 was constructed by inserting the promoter, signal sequence, and TS regions of *STA1* into the *E. coli*-yeast shuttle vector, pYEp24 (NEB) containing the transcription terminator of *GAL7* gene. The full-length cDNA of human LC-1 was subcloned into the pBluescript SK vector, and the resulting plasmid, pBLP, was used to construct the LC-1 expression cassette in yeast.

SDS-PAGE and Immunoblotting. Proteins in the extracellular medium were precipitated by the addition of trichloroacetic acid (TCA). 100 μl of 1mg/ml deoxycholate solution was added to 1 ml of yeast culture medium, and then the mixture was kept on ice for 15 min. 100 μl of 100 % TCA was added, and the mixture was kept on ice for another 30 min. The tube was spun down for 5 min, and the resulting pellet was dissolved in 50 μl lysis buffer (Laemmli, 1970). The sample was boiled for 5 min, and then 10 μl was loaded on a 12 %

polyacrylamide gel containing SDS (SDS-PAGE). For the analysis of proteins in the cellular fraction, yeast cells in 1 ml culture broth were suspended in 1 ml lysis buffer and broken by vortexing in the presence of glass beads (0.4-0.5 mm) for 5 min. After 5 min centrifugation, the supernatant of 10 μl was used for the SDS-PAGE and immunoblotting. After electrophoresis, proteins were transferred to nitrocellulose membranes (Promega) by electroblotting. LC-1 was detected with the Promega ProtoBlot System (goat anti-rabbit IgG (H+L or Fc)-alkaline phosphatase conjugate [GAR-AP]) using a rabbit anti-lipocortin-1 polyclonal antibody prepared by the standard method (Huh *et al.*, 1990). The amount of LC-1 was quantified by measuring the staining intensity of LC-1 on nitrocellulose filter with a desitometer (Biomed Instrument SCR 2D/1D, USA). The authentic recombinant LC-1 (>95% purity) has been previously obtained after purification of LC-1 from recombinant *E. coli* (Huh *et al.*, 1990), and was used as a standard for analysis.

RESULTS AND DISCUSSION

Construction of LC-1 Expression Plasmid.

Since the correct processing site of signal sequence in glucoamylase was not clearly elucidated (Yamashita, 1989), the cleavage site of endoprotease yscF (*KEX2* gene product)

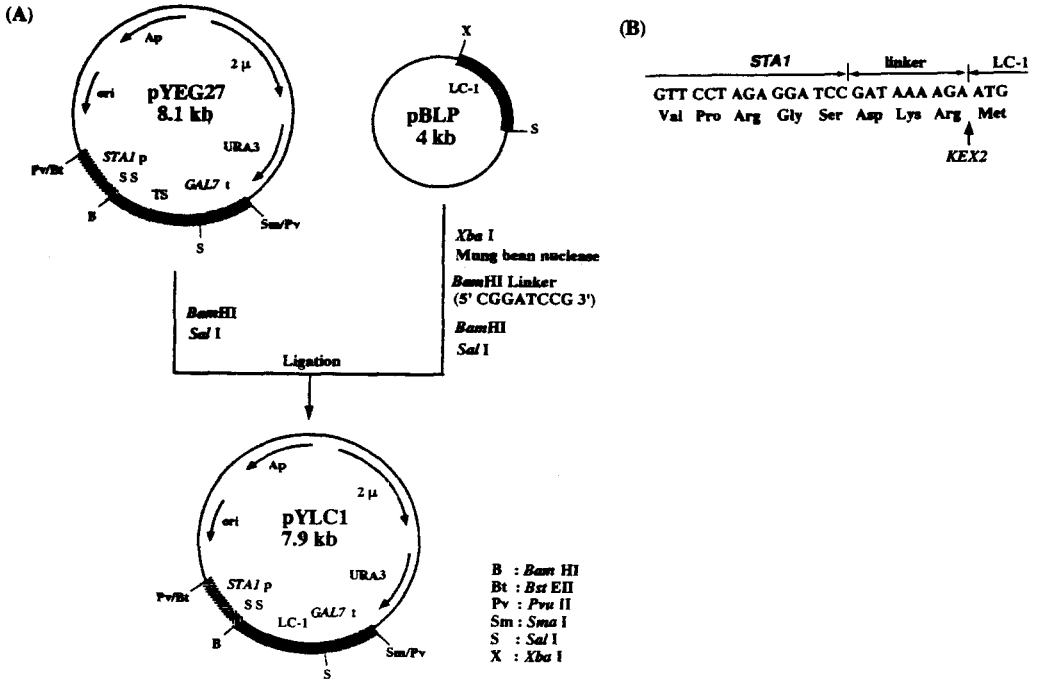

Fig. 1. Construction of human LC-1 expression vector, pYLC1, containing the promoter and signal sequence of *STA1*.
(A) Construction diagram of pYLC1.
A DNA fragment encoding human LC-1 was prepared by excision from pBLP by *Xba*I digestion, mung bean nuclease treatment, *Bam*HI linker attachment, and subsequent *Bam*HI-*Sal*I digestion. The resulting 1.0 kb DNA fragment was inserted into pYEG27, which was digested with *Bam*HI-*Sal*I. *STA1* p, *STA1* promoter; ss, *STA1* signal sequence; *GAL7* t, *GAL7* terminator; TS, Thr-Ser rich region of *STA1*.
(B) Nucleotide sequence of the *STA1* signal and human LC-1 junction in pYLC1.

(Julius *et al.*, 1984), Lys-Arg, was artificially inserted into the upstream of LC-1 cDNA. The synthetic oligonucleotide, 5'- T CTA GAT AAA AGA - 3', was attached to the 5'-ATG of LC-1 cDNA, and then digested with *Xba* I and *Sal* I. The 1.0 kb fragment obtained was subcloned into the pBluescript SK vector, and the resulting plasmid, pBLP, was used to construct the yeast expression vector.

The plasmid pBLP was digested with *Xba*I, and then treated with mung bean nuclease. After the attachment of *Bam* HI linker (5'-CGGATCCG-3') to the 5' end, it was treated with *Bam* HI and *Sal* I. The resulting 1.0 kb fragment was ligated with the pYEG27 plasmid which was previously digested with *Bam* HI and *Sal* I to delete the TS region of *STA1* gene. The final plasmid constructed, pYLC1, was 7.9 kb in size (Fig.1A). The LC-1 expression cassette, pYLC1, contained the *STA1* promoter, *STA1* putative signal sequence (from ^{1}Met to ^{31}Lys), LC-1 cDNA and *GAL7* terminator. Tripeptide, Asp-Lys-Arg, was artificially made in the junction between *STA1* signal sequence and LC-1 cDNA (Fig.1B).

Expression and Secretion of LC-1.

S. diastaticus YIY345 was transformed with the pYLC1 plasmid, and both the transformant and host cells were cultivated in YPD medium. After 3 days cultivation, the culture supernatant and cellular fraction were analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 2A, the LC-1 bands did not appear clearly on a SDS-PAGE gel. As expected, the immunoblotting analysis resulted in clear LC-1 bands so that LC1 in the culture supernatant and the cellular fraction could be analyzed and compared. Recombinant LC-1 expressed in the transformed yeast cell showed two separate bands of about 37 kDa and higher (lane 2 and 4 in Fig. 2B), while no immunoreactive bands were detected in the yeast host cell (lane 1 and 3 in Fig. 2B). The upper one might result from unprocessed and/or glycosylated LC-1 protein. N-linked glycosylation was not observed by digesting the purified yeast LC-1 with Endo H (data not shown), although there existed Asn at position 43 which is a possible site for N-linked glycosylation (Asn-X-Ser/Thr) (Huh *et al.*, 1990). Therefore, the upper band might be an unprocessed LC-1. The concentrations of immunoreactive proteins in the culture medium and the cellular fraction were 2.5 μg and 48 μg per ml culture broth, respectively. The majority of LC-1 was retained in the intracellular or periplasmic space, indicating low secretion efficiency (about 5 % secretion). As observed in the secretion of human 5-lipoxygenase (78 kDa) in yeast (Nakamura *et al.*, 1990), the *STA1* signal sequence directed LC-1 into the yeast secretory pathway, but the product was not secreted so efficiently into the culture medium.

Numerous atttempts have been made to secrete proteins of interest from yeast, particularly with the α-factor or invertase signal sequence. Calf prochymosin (Smith *et al.*, 1985), interleukin-2 (Miyajima *et al.*, 1986) and human lysozyme (Castanon *et al.*, 1988), have been expressed and secreted into the extracellular medium by using these signal

sequences. In cases of human α-1-antitrypsin (Moir and Dumais, 1987) and IFN-Con1 (Zsebo *et al.*, 1986) , a little amount of proteins expressed was secreted, even though these signal sequences were used. According to Zsebo *et al.* (1986), small proteins such as β-endorphin and calcitonin were efficiently secreted into the culture medium, while substantial fractions of large proteins such as IFN-Con1 remained as a cell-associated form. Besides the size, parameters such as net charge and degree of glycosylation can affect secretion efficiency. Since the LC-1 protein does not have a secretory signal sequence (Wallner *et al.*, 1986) and is found intracellularly in many human cell types (Nakata *et al.*, 1990), the passage of LC-1 through the yeast cellular membranes such as ER and plasma membrane might be hampered.

The TS region, consisting of 200 amino acid residues, is located in the downstream near the signal peptide in the *STA1* gene (Yamashita *et al.*, 1985). The *STA1* gene product is not efficiently secreted into the culture medium when the TS region is eliminated (Yamashita, 1989). If the TS region plays a important role in protein secretion, the secretion of proteins may be facilitated by the introduction of this region into the secretion vector systems.

The partially purified LC-1 (about 95% purity) exhibited an inhibitory activity against phospholipase A_2 (data not shown). Further attempts will be carried out to increase the expression level and secretion efficiency of LC-1 with an expression cassette containing *STA1* promoter and its own signal sequence.

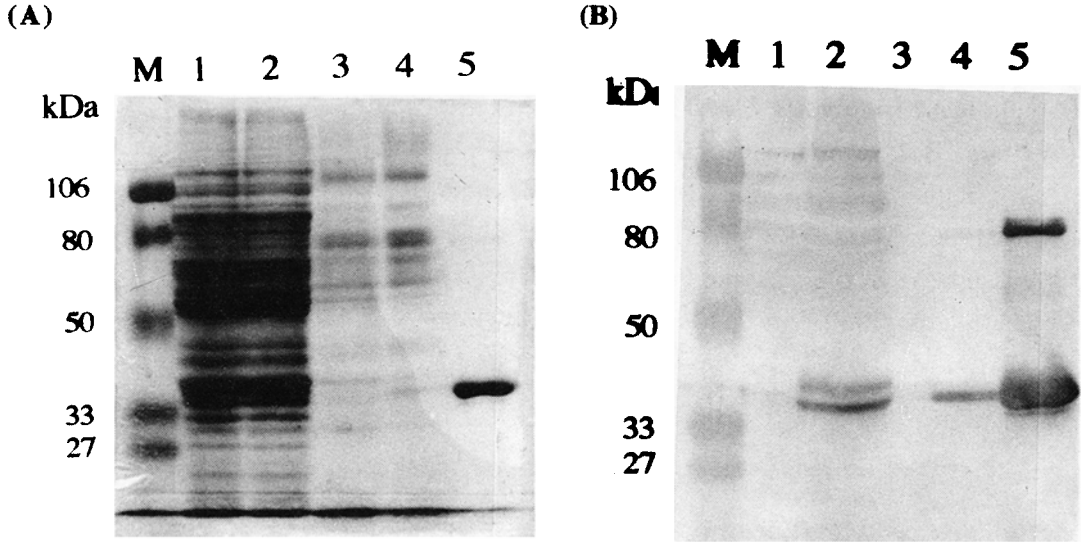

Fig.2. Analysis of expression and secretion levels of LC-1 in transformed yeast cell. Extracellular and cellular fractions were resolved by SDS-PAGE and stained with Coomassie blue (A) or Western blotting (B). Cellular fraction (lane1) and medium (lane3) of untransformed yeast cells. Cellular fraction (lane2) and medium (lane4) of transformed yeast cells. Partially purified LC-1 (lane5, 5 μg). The migration of molecular weight standards (kDa) is indicated as M.

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