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# Rapid Purification of Recombinant Human Lipocortin-I Secreted from *Saccharomyces cerevisiae*

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**Abstract** Human lipocortin-I was expressed as a secretory product by *Saccharomyces cerevisiae* harboring an expression system consisting of *GAL10* promoter, inulinase signal sequence and lipocortin-I terminator. Fed-batch fermentation was carried out to overproduce recombinant human lipocortin-I. The culture medium was desalted and concentrated by ultrafiltration, and then subjected to hydroxyapatite column chromatography. The lipocortin-I was purified to >98% purity by single-step hydroxyapatite column chromatography. However, it was found that the purified lipocortin-I was a proteolytically-cleaved form which was cleaved immediately after the basic amino acid $Lys^{26}$.

*Keywords*: human lipocortin-I, *Saccharomyces cerevisiae*, secretory product, hydroxyapatite column chromatography

## INTRODUCTION

Human lipocortin-I is implicated as a glucocorticoid-induced, $Ca^{++}$-dependent and membrane-binding protein which mediates the anti-inflammatory function through phospholipase $A_2$ inhibition [1]. Human lipocortin-I has been also shown to exhibit interesting biological activities in the intact central nervous system [2,3], and to be present at increased levels in the brains of cases with multiple sclerosis and in experimental autoimmune encephalomyelitis [4,5]. Because of these biological activities, lipocortin-I has been attracting considerable interest in the pharmaceutical industry.

Recombinant human lipocortin-I has been produced via recombinant DNA technology using a variety of host systems [6-8]. In this study, the yeast *S. cerevisiae* was used as a host to produce human lipocortin-I due to the advantages over other systems such as easy culture, well-known genetics, non-pathogenicity, safety, *etc.* Furthermore, a simple purification process of correctly processed protein can be easily achieved by secreting the heterologous protein of interest out of the cell and harvesting them directly from the culture medium.

Recently, we constructed a novel expression system which efficiently directs the secretion of human lipocortin-I into the yeast culture medium under the control of *GAL10* promoter [9]. The fed-batch fermentation was performed with recombinant *S. cerevisiae* carrying this novel expression cassette, and a simple and rapid purification process was also developed to obtain human lipocortin-I of high purity.

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## MATERIALS AND METHODS

### Strains and Recombinant DNA Techniques

*Escherichia coli* JM109 was used in this study. *S. cerevisiae* 2805 (*MATα pep4::HIS3 pro1-δcan1 GAL2 his3 δ ura3-52*) [10] was used as a host for the expression of lipocortin-I. General DNA manipulation was performed as described by Sambrook *et al.* [11]. Transformation of *E. coli* and *S. cerevisiae* was carried out by the methods of Cohen *et al.* [12] and Ito *et al.* [13], respectively.

### Media and Culture Conditions

YNBCAD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and 0.5% casamino acids) was used for the selection of yeast transformants and for the seed culture. A single yeast transformant on a YNBCAD agar plate was inoculated into 10 mL YNBCAD medium and incubated overnight at 30°C. For further activation, the seed was transferred into a 500-mL Erlenmeyer flask containing 100 mL YNBCAD medium, and cultivated as described above. This culture was used as a seed for the fed-batch fermentation.

### Fed-Batch Fermentation

The start-up medium consists of 40 g/L yeast extract, 5 g/L casamino acids, 20 g/L glucose and 10 g/L $KH_2PO_4$. The seed culture (200 mL) was transferred to a 3.5-L jar fermentor (Bioengineering AG, Switzerland) containing 1.8 L of the start-up medium. After 12 h of culture, glucose was exhausted and the dissolved oxygen concentration began to increase rapidly. At this stage, concen-

trated galactose medium was intermittently fed into the fermentor as described previously [14,15]. The culture temperature and pH were maintained as 30°C and 5.5, respectively, unless otherwise specified. The dissolved oxygen concentration was maintained above 10% air saturation by controlling the aeration rate and agitation speed, and by using oxygen-enriched air.

### Purification of Lipocortin-I

After the cells were separated by centrifugation at 5,000 g, the supernatant was filtered again using a 2-μm filter. The culture supernatant (1 L) was concentrated 10-fold by ultrafiltration using an Amicon membrane (Mol. wt. cut-off = 10,000, Amicon Co., MA, USA), and then dialyzed against 10 mM phosphate buffer (pH 7.0) (Buffer A). The dialyzed solution was loaded on a hydroxyapatite column (3.2 × 25 cm, BioRad, USA) equilibrated with Buffer A. After washing the resin with Buffer A containing 1 mM $CaCl_2$, the lipocortin-I was eluted by step-wise increase of $CaCl_2$ concentration to 50 mM. The eluted fraction containing lipocortin-I was dialized against Buffer A to remove $CaCl_2$, and then lyophilized.

### Analytical Methods

After centrifugation of 1 mL culture broth, 10 μL of the culture supernatant was directly subjected to 10% SDS-PAGE, and the amount of lipocortin-I expressed was quantified by scanning with a densitometer (Biomed Instrument SCR 2D/1D, USA). Two major protein bands, intact lipocortin-I and proteolytically-cleaved lipocortin-I, were found on the gel. Each lipocortin-I concentration was calculated by multiplying the fraction of the scanned peak area of each lipocortin-I band by the total concentration of protein loaded on the gel. The total protein concentration was measured by the modified Lowry method (Sigma protein assay kit #5656, USA). The total lipocortin-I concentration was calculated by the summation of the concentrations of intact lipocortin-I and cleaved lipocortin-I. The cell growth was monitored by measuring the optical density at 600 nm. The concentrations of glucose and galactose were determined by a glucose analyzer (YSI 2700-D, USA), and by the enzymatic method (Boehringer Manheim kit #176303, FRG), respectively.

## RESULTS AND DISCUSSION

### Secretory Production of Human Lipocortin-I

An expression cassette for secretion of heterologous protein in yeast typically consists of "promoter - signal sequence - heterologous gene - terminator". The type of signal sequence used is known to effect the secretion efficiency mainly, while promoter and terminator are known to effect the total expression level more. After extensive optimization studies on the lipocortin-I expression system, we were able to finally construct an optimized expression cassette consisting of "*GAL10* promoter - inulinase signal sequence - lipocortin-I gene - lipocortin-I terminator", and named it the plasmid pYGILPT-5 (Fig. 1).

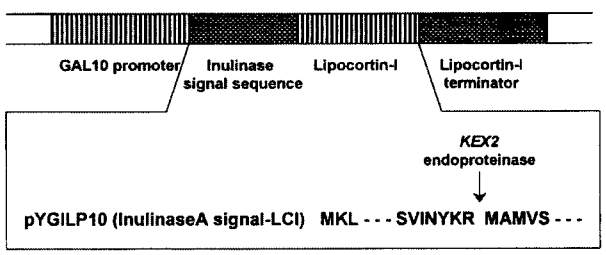


Fig. 1. Basic structure of the plasmid used in this study and amino acid sequence representing the junction region between inulinase leader peptide and human lipocortin-I.

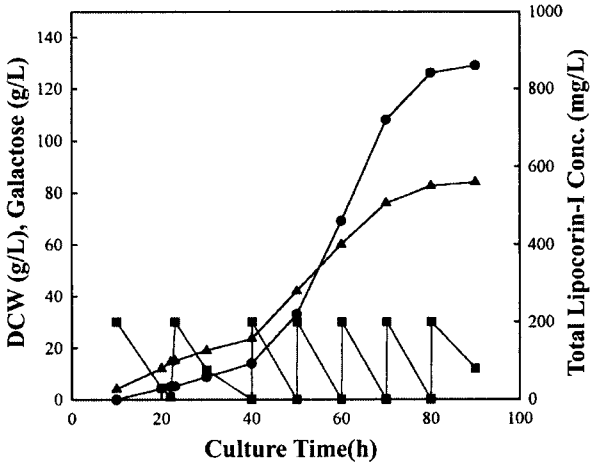


Fig. 2. Time profiles of fed-batch fermentation for lipocortin-I production. ▲, cell concentration; ●, total lipocortin-I concentration; ■, galactose concentration.

A fed-batch culture of the recombinant *S. cerevisiae* 2805 harboring the plasmid pYGILPT-5 was carried out for the overproduction of lipocortin-I using a step-wise feeding of concentrated galactose medium as previously reported [14,15]. This feeding strategy has some advantages over other feeding strategies such as easy control and reproducibility. Fig. 2 shows the results of fed-batch fermentation. As observed in the previous reports [9,16], the aberrant proteolysis occurred at the internal site of lipocortin-I, accumulating intact and proteolytically-cleaved products. The degraded lipocortin-I was previously identified as des1-26-lipocortin-I which was cleaved immediately after the basic amino acid $Lys^{26}$. At the end of the culture, the des1-26-lipocortin-I accounted for about 80% of the total lipocortin-I secreted in the medium (lane 2, Fig. 3), which was higher than that (55%) observed in the fed-batch culture employing a different feeding strategy [9], demonstrating that the extent of proteolysis is greatly affected by the culture methods. It has been reported

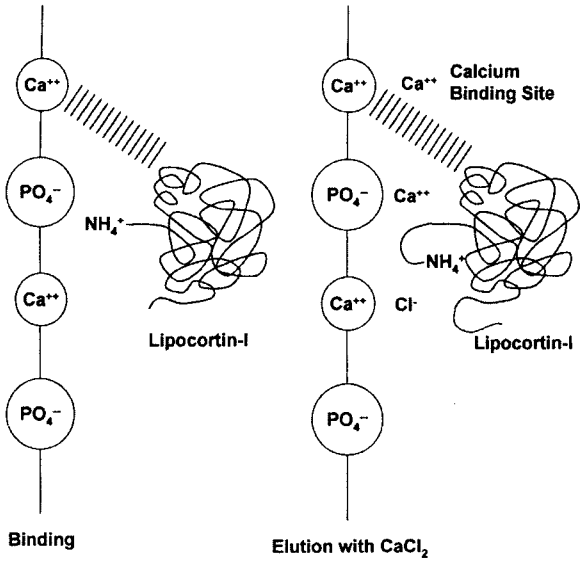


Fig. 3. A schematic diagram representing adsorption and desorption of human lipocortin-I on hydroxyapatite resin consisting of $Ca^{++}$ and $PO_4^{-3}$ ions.

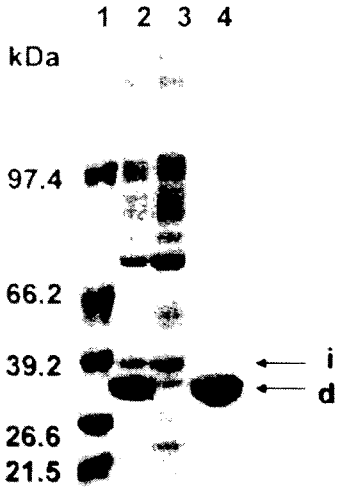


Fig. 4. SDS-PAGE analysis of samples of human lipocortin-I from different steps of purification. Lane 1, Prestained protein molecular weight marker; lane 2, culture medium; lane 3, after washing with 1 mM $CaCl_2$; lane 4, purified sample. Band i, intact lipocortin-I; band d, des1-26-lipocortin-I

that the proteolytic cleavage of human lipocortin-I can be reduced greatly by the addition of high concentrations of L-arginine and L-lysine, with a marked improvement in the yield of intact lipocortin-I [16]. A similar fed-batch fermentation was carried out with the feed medium containing a high concentration of L-arginine or L-lysine. Consequently, the cell growth rate was greatly reduced, resulting in a very low expression level of lipocortin-I.

The maximum level of total lipocortin-I obtained in this study was about 860 mg/L, which was much lower than that (2.1 g/L) obtained in the well-controlled fed-batch fermentation [9]. However, as mentioned above, the feeding strategy employed in this study resulted in very high reproducibility in terms of final total lipocortin-I expression level and final cell density. In contrast, the previous feeding strategy, which yielded 2.1 g/L of lipocortin-I, required continuous monitoring of glucose during the entire period of culture and change in feed rate with respect to the cell growth rate, which is a complicated task to perform in practice.

## Purification of Human Lipocortin-I

The lipocortin-I is a membrane-associated $Ca^{++}$-binding protein. The currently accepted mechanism of phospholipase $A_2$ inhibition of lipocortin-I is that it binds phospholipids in a $Ca^{++}$ dependent manner, thus depleting phospholipid substrates for phospholipase $A_2$ [17]. This $Ca^{++}$-binding property of lipocortin-I leads us to use hydroxyyapatite column chromatography for purification of lipocortin-I, since the functional groups of hydroxyapatite resin consist of $Ca^{++}$ and $PO_4^{-3}$ ions. Fig. 3 shows the schematic adsorption and desorption mechanism of lipocortin-I on a hydroxyapatite resin.

In general, amino groups and carboxyl groups are known to act in the adsorption of proteins to hydroxyapatite as a result primarily of nonspecific electrostatic interactions between their charges and the coun-ter charges on the hydroxypaptite column. Surprisingly, the des1-26-lipocortin-I was purified to homogeneity (Fig. 4) after one-step hydroxyapatite column chromatography, demonstrating that the affinity of des1-26-lipocortin-I for $Ca^{++}$ ion is much higher than that of nonspecific electrostatic interactions. During washing step, most of the contaminant proteins were eluted, indicating that they have a relatively weak binding affinity for $Ca^{++}$. However, the reason why the intact lipocortin-I was eluted during washing step together with contaminant proteins is not clear yet. In contrast, des1-26-lipocortin-I was selectively eluted at 50 mM $CaCl_2$. The purified lipocortin-I was lyophilized and characterized. The flow chart of the purification process is shown in Fig. 5.

## Characterization of Purified Lipocortin-I

The purity of the purified lipocortin-I was > 98% as judged by an analytical rp-HPLC. The purified lipocortin-I was subjected to N-terminal sequencing. The sequence of the first five amino acids was revealed to be Ser-Ser-Lys-Gly-Gly, which is identical to the predicted N-terminal sequence of des1-26-lipocortin-I. The purified des1-26-lipocortin-I was tested for the inhibitory activity of phospholipase $A_2$ by an *in vitro* assay, using autoclaved [$^3$H] oleic acid-labeled *E. coli* as described by Hayashi *et al.* [18]. It inhibited the activity of porcine pancreatic phospholipase $A_2$ in a dose-dependent manner with a half-maximal activity similar to that of human placental lipocortin-I (Fig. 6), demon-

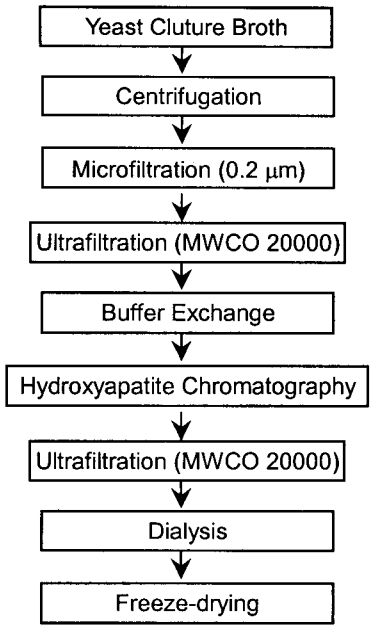


Fig. 5. A process flow diagram for purification of human lipocortin-I.

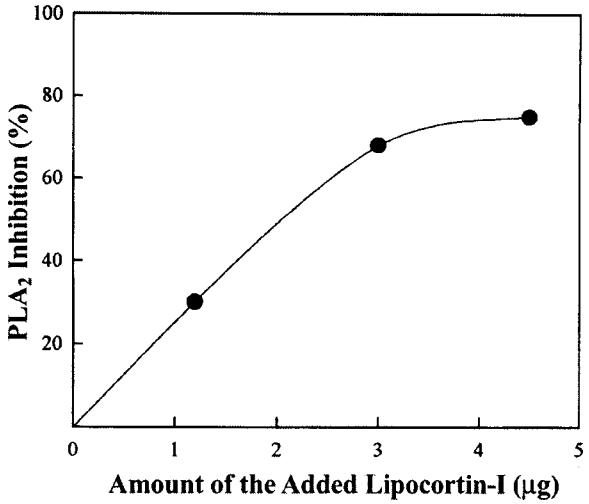


Fig. 6. Dose-dependent phospholipase $A_2$ inhibition of des1-26-lipocortin-I

strating that the N-terminal 25 amino acid truncation of lipocortin-I does not affect the biological activity of lipocortin–I. It has been reported that a similar proteolytic cleavage of lipocortin occurs in human body [19]. A major proteolytic cleavage product secreted by the human prostate gland was not des1-26-lipocortin-I but des1-29-lipocortin-I. These observations imply the possibility that the N-terminal truncated lipocortin-I may have a certain biological function in human body.

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