High-yield Purification and Characterization of Recombinant Human Leukotactin-1 in *Pichia pastoris*

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Abstract The human chemokine, the short version of leukotactin-1 (shLkn-1; molecular weight = 7.2 kD and 66 amino acids), was expressed and secreted into a culture medium using the methylotrophic yeast, *Pichia pastoris*. The recombinant shLkn-1 was purified from the culture supernatant using a simple two-step procedure consisting of cation exchange and reverse phase chromatography (RPC), in which shLkn-1 was highly purified (99.5%) with a high recovery yield of 82.7%. The C-terminal truncated derivative of shLkn-1 was found in the supernatant and was separated by RPC. The physicochemical properties of the purified shLkn-1 were verified to be the same as expected. The biological activity of the purified recombinant shLkn-1 was also quantified using a chemotaxis assay. It was observed that the recombinant shLkn-1 had the maximum migration activity at a concentration of 10 nM, as potent as MIP-1α.

Keywords: C-terminal truncation, leukotactin, *Pichia pastoris*, secretion

INTRODUCTION

The chemokines are a family of 8~12 kD basic, heparin-binding proteins that are implicated in a wide range of biological activities. It has been reported that these molecules are involved in immunoregulatory functions, a potential role in wound healing, regulation of hematopoietic stem cell and progenitor cell proliferation, and human immunodeficiency virus (HIV)-suppression [1]. Leukotactin-1 (Lkn-1), a recently described CC chemokine, has been known to contain two extra cysteins. It was demonstrated that Lkn-1 is identified on chromosome 17, and the expression analysis showed not only its regular monocistronic transcript but also a bicistronic transcript of the adjacently localized gene for HCC-1 [2]. The recombinant Lkn-1 induces chemotaxis on human monocytes, on T-lymphocytes, and to a lesser degree, in eosinophils [2]. Lkn-1 mediates its effect through the CCR (CC chemokine receptor)-1 and CCR-3 receptors [3,4]. Lkn-1 exhibits the same structural elements as the other chemokines, *i.e.* a triple-stranded antiparallel beta-sheet covered by an alpha-helix [5].

In this paper, we have established efficient cultivation and purification procedures, in which the *Pichia pastoris* (*P. pastoris*) expression system was used to express the short version of Lkn-1 (shLkn-1) [6], and its high-cell-density culture and simple two-step purification procedures were developed. The purified shLkn-1 has been characterized physicochemically, and its biological activity has also been investigated.

MATERIALS AND METHODS

Clone Construction

The vector, pPIC9 was used to express the shLkn-1 gene. The existing multiple cloning site of pPIC9 was modified to include *stu* I restriction site. The 198bp fragment encoding 66 amino acids of the recombinant shLkn-1 was PCR amplified from the Lkn-1 DNA using the following mutagenic primer: 5'-CACTTTGCTGCTG CTGC-3' and 5'-GAATTCTTATATATTGAGTAGGGCTT -3'. The PCR product was cloned in frame with α-factor signal peptide in pPIC9. The resulting expression cassette was sequenced to verify shLkn-1 sequence. Constructed expression plasmid (pPM2-HF) was introduced into *Pichia pastoris* using the spheroplasts transformation procedure essentially as described by *Pichia* expression kit manual (Invitogen catalog No. K1710-01).

Cultivation and Purification

A 2.5-L BioFloIII bioreactor (New Brunswick Scientific Co., NJ, USA) was used to grow *P. pastoris*. A 100 mL of seed culture was cultivated for 18 h in YPD (1% yeast extract, 2% peptone, 2% dextrose) at 30°C, while shaking at 200 rpm. Then, it was inoculated into a BioFloIII bioreactor containing 1.0 L of a basal salt medium (glycerol, 40 g/L; H_3PO_4, 27 mL/L; $CaSO_4$, 0.9 g/L; K_2SO_4,

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18.0 g/L; MgSO$_4$, 10.24 g/L; KOH, 4.13 g/L; NH$_4$OH, 30 mL/L; yeast trace metal solution, 4.4 mL/L). The yeast trace metal solution was composed of CuSO$_4$·5H$_2$O, 6.0 g/L; KI, 0.09 g/L; MnSO$_4$·H$_2$O, 3.0 g/L; NaMo·H$_2$O, 24.0 g/L; H$_3$BO$_4$, 0.02 g/L; CoCl$_2$, 0.5 g/L; ZnCl$_2$, 20.0 g/L; FeSO$_4$·H$_2$O, 65.0 g/L; Biotin, 0.2 g/L; H$_2$SO$_4$, 5.0 mL/L. The temperature, pH, and aeration were controlled at 30°C, 5.0, and 2.0 vvm, respectively. The feed medium was composed of 50% glycerol and 12 mL/L of the yeast trace metal solution. After about 500 mL of the feed medium had been supplied, the feeding was stopped and the culture was starved for 0.5 h. Finally, methanol induction was initiated at a feed rate of 10 mL/h with 100% methanol and 12 mL/L of the yeast trace metal solution [8].

The cells were removed from the culture broth by centrifugation, and the supernatant was diluted two fold with deionized water. The diluted supernatant was loaded onto a cation-exchange column (SP-Sepharose FF 400 mL, Amersham Bioscience) and was subsequently washed with 50 mM pH 7.4 Tris-HCl buffers. The captured shLkn-1 was eluted by applying a stepwise salt gradient (0.3 M NaCl in 50 mM Tris-HCl at pH 7.4) at 20 mL/min. The eluted fractions from the cation exchange chromatography were loaded onto a reverse-phase chromatography column (RPC) (Source 15 RPC 20 mL, Amersham Bioscience). RPC was applied with a step gradient of solvent A (0.1% trifluoroacetic acid/H$_2$O) and solvent B (0.1% trifluoroacetic acid/acetonitrile) at a flow rate of 2 mL/min.

Analytical Methods

Analytical reverse phase HPLC, reverse isoelectric focusing (IEF), and size-exculsion HPLC were performed to check the purity of shLkn-1. Gelatin zymography was performed in 12% SDS-PAGE that had been cast in the presence of 0.1% gelatin. Samples were prepared in non-reducing loading buffer. After electrophoresis, the SDS was removed by 2.5% Triton X-100 to renature the proteases. The gels were then incubated at 37°C for 24 h in an incubation buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 10 mM CaCl$_2$), and then, they were stained with 0.25% Coomassie Blue R 250.

N-terminal sequencing was performed using an automatic peptide sequencer (Model 492, Procise, Perkin-Elmer, USA). For the amino acid composition analysis, shLkn-1 was hydrolyzed and derived with phenyl isothiocyanate (PTC), and then the derivatives were separated as described by Bidlingmer *et al.* [9]. Electrospray mass spectrometry (Platform II, Micromass, UK) was conducted to measure the molecular weight of shLkn-1.

Chemotaxis Assay

The chemotaxis assays were measured using a 24-well micro chemotaxis chamber (Costar, Cambridge, MA, USA). The lower and upper chambers of this plate were separated by a 5 μm pore size polycarbonate membrane. In order to measure the number of migrating cells, we performed the alamar blue staining method. This method allows easy quantitative measurement of the viable THP-1
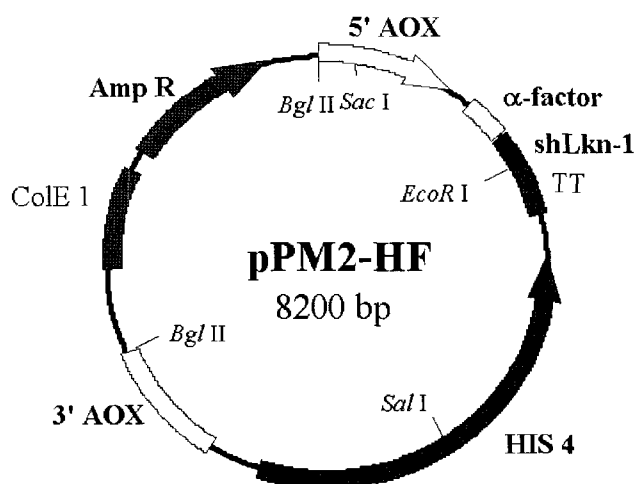

Fig. 1. Expression vector for recombinant shLkn-1.

cell because alamar blue exhibits fluorescence and colorimeteric change in the appropriate oxidation-reduction range created by cellular metabolism. Cultured THP-1 cells were washed twice with serum-free RPMI1640 (Life Technologies Inc., Grand Islands, NY, USA) and were resuspended at 5 × 10^6 cells/mL in serum-free RPMI1640. The lower wells were filled with 600 μL of serum-free RPMI media alone or with the media containing various concentrations of tested shLkn-1, and the upper wells were placed with 100 μL of the prepared cell suspensions. The chamber was then incubated for 3 h at 37°C in a CO$_2$ incubator, after which the lower wells were removed and treated with 10% FBS (fetal bovine serum) and 10% Alamar Blue staining solution (Serotec, Oxford, UK) for 16 h at 37°C. All assays were carried out in triplicate, and the migration of the cells was determined using a microplate fluorescence reader (Molecular Devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 530 and 590 nm, respectively.

RESULTS AND DISCUSSION

Construction of Expression Vector and Transformation into *P. pastoris*

The shLkn-1 cDNA was cloned in frame with the α-factor signal peptide in pPIC9, which targets them to the secretary pathway. To express proteins also contain the KEX2 recognition sequence directly preceding histidine, which is first amino acid of shLkn-1, α-factor signal peptide in pPIC9 was modified by polymerase chain reaction. We made cloning site, to add *Stu* I site at the directly after KEX2 recognition sequence (Lys-Arg) [7]. shLkn-1 gene was amplified from the Lkn-1 DNA and modified by PCR mutagenesis to add an *EcoR* I site to the 3'end PCR products of Lkn-1 DNA were cloned directly into the unique *Stu* I and *EcoR* I site of pPIC9, resulting in the expression plasmid pPM2-HF (Fig. 1).
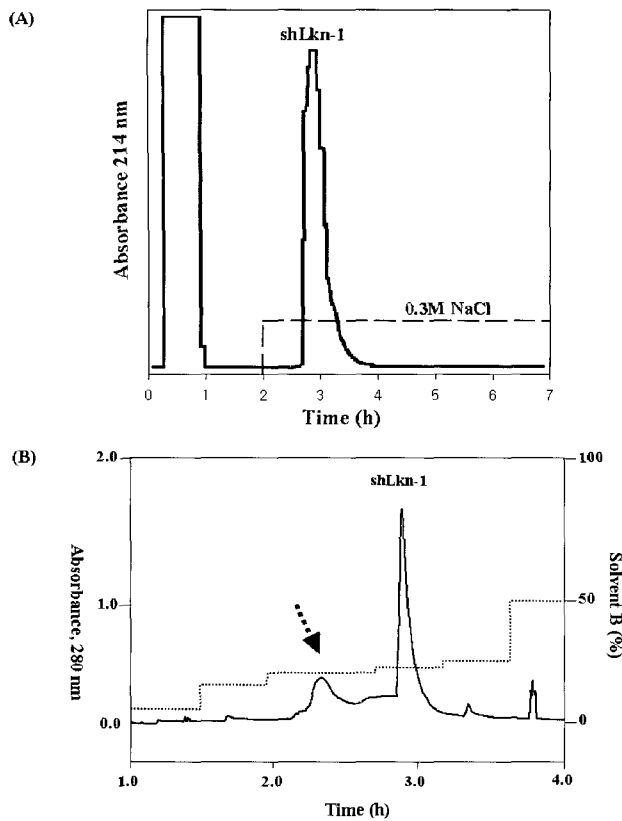
(A)

(B)

Fig. 2. Elution profiles of shLkn-1 from a SP-Sepharose (A) and Source 15 reverse phase chromatography (B). A fraction, marked by the dotted arrow, in reverse phase chromatography was used for the sample for mass spectrometry.

Purification of Recombinant shLkn-1 Produced Using *P. pastoris*

The shLkn-1 was produced using the methylotrophic yeast, *P. pastoris*, as the host in a two-step growth process in order to create a high-cell density, which was followed by a methanol-induced production phase (data not shown). The expression level of the total recombinant shLkn-1 in the culture medium was up to about 300 mg/L of yeast culture supernatant, in which the 2.5-L bioreactor was used, and the culture supernatant was 1.6 L, respectively. The cells were removed from the culture broth by centrifugation, and then the supernatant was diluted two-fold with deionized water. The first chromatography step was prepared with applying a buffer (50 mM Tris-HCl, pH 7.4) to a cation exchange column (SP-Sepharose FF 400 mL) at 20 mL/min. And, then the diluted supernatant was loaded onto a cation-exchange column and subsequently washed from column with 2 L of 50 mM pH 7.4 Tris-HCl. The shLkn-1 was eluted with 50 mM Tris-HCl, 0.3 M NaCl, pH 7.4 (Fig. 2A). Most impurities in the culture supernatant were removed as a result of the cation exchange step, and shLkn-1 was purified using the reverse phase chromatography as a polishing step. At the RPC step, a derivative of shLkn-1 could also be purified, in
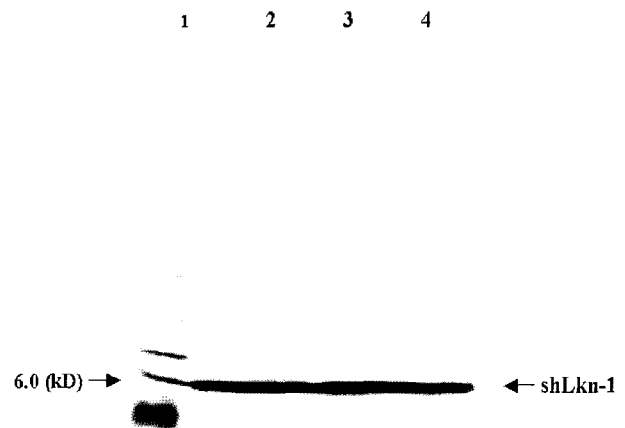

Fig. 3. Coomassie-stained 4~20% SDS-PAGE of the fractions from Fig. 2. The right and left arrows describe shLkn-1 and size marker 6.0 kD, respectively. Lane 1, Molecular weight markers [3.5 (smallest), 6.0, 14.4, 21.5, 31.0, 36.5, 55.4, 66.3 kD]; Lane 2, Culture supernatant; Lane 3, Elution fraction from SP-Sepharose chromatograpy; Lane 4, Elution fraction from reverse phase chromatography.
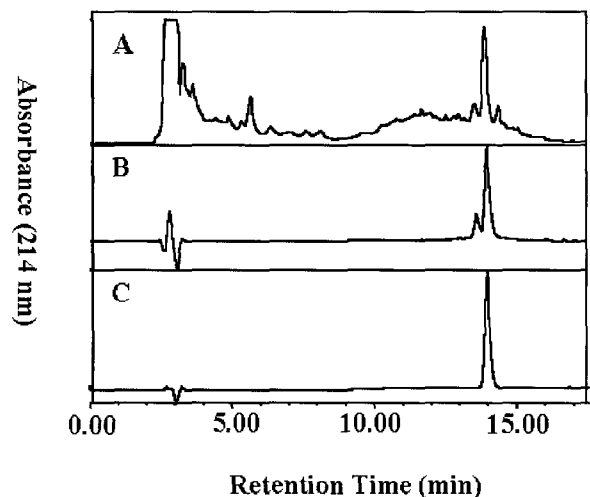

Fig. 4. C18 reverse phase HPLC analysis of the fractions from each purification step. (A) Fermentation supernatant. (B) Eluted fraction from the cation exchange chromatography. (C) Purified fraction after reverse phase chromatography.

which both shLkn-1 and its derivative were eluted between 20% and 22% of solvent B (Fig. 2B). The purity of each step was assessed by SDS-PAGE (Fig. 3) and analytical C18 reverse phase-HPLC respectively (Fig. 4). The single major band and peak were respectively exhibited in these analyses.

After the cation exchange chromatography had been performed, the eluant was shown to contain two portions, a major portion of 71% and a minor portion of 29%, which were analyzed by RP HPLC analysis. The minor
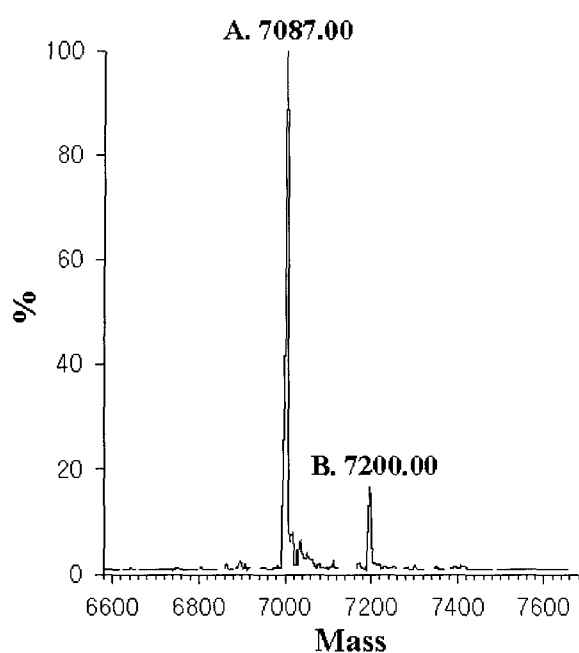
Fig. 5. Mass spectrum of the fraction from the reverse phase chromatography of Fig. 2. Electrospray ionization mass spectrometry was used. The analytical condition were as follows- Tuning parameters: capillary, 3.38 kV: cone, 34 V; ionization mode, ES+; ion energy, 2.7 V, analyzer vacuum pressure, $3.1e^{-5}$ mbar.

Fig. 6. Gelatin zymography of the culture supernatant. The sample was prepared from a culture broth harvested at 142 hrs after induction. Lane 1; Standard marker, Lane 2; Concentrated culture supernatant (20×). Arrows a and b describe the proteolytic activity of the culture supernatant.

portion could be separated by RPC in a second step (Fig. 2B). According to the results from the mass spectrometry, the minor portion contained two molecules where one was 7.200 kD, and the other was 7.087 kD (Fig. 5). However, both 7.200 kD and 7.087 kD were identified as shLkn-1 by N-terminal sequencing. Therefore, we speculated that the 7.087 kD of shLkn-1 was a truncated form which came from the loss of isoleucine residue (113 Da) at the C-terminus of shLkn-1. This cleavage was probably due to the activity of a protease derived from the *P. pastoris*. In the case of secretory expression of human epidermal growth factor (hEGF) (MW =6 kD) in *Hansenula polymorpha* (*H. polymorpha*) [10], it had been demonstarted that the C-terminal-truncated derivative was produced by carboxypeptidase. The C-terminal truncation was also observed in the expression of recombinant hirudin (MW = approx. 7 kD) of *Sacchromycess cerevisiae* [11]. In the culture supernatant of shLkn-1-expressing *P. pastoris*, protease activity was also detected by gelatin zymography, in which two proteases were found with sizes of about 90 kD and 40 kD (Fig. 6). This result led to the deduction that the two proteases were probably KEX2 protease (MW = 90 kD) [12] and carboxypeptidase Y (MW = 44.6 kD) [13], respectively, based on their molecular weight. Therefore, it can be concluded that KEX2 protease worked for the cleavage of the leader peptide during secretion, and carboxypeptidase Y caused the C-terminal truncation in the culture broth.

Finally, the performance of the 1.6-L scale purification

Table 1. Summary of the recombinant shLkn-1 purification procedure. This table summarizes the performance of the 1.6-Lscale shLkn-1 purification processes. The amounts of total protein and shLkn-1 were quantified by BCA protein assay and C18 RP-HPLC analysis, respectively. *The amounts of shLkn-1 in parenthesis describe the C-terminal truncated derivatives. The calculations of yield and purity are based on intact shLkn-1.

	Total protein (mg)	shLkn-1* (mg)	Yield (%)	Purity (%)
Culture supernatant	5100	355 (145)	-	6.9
SP-Sepharose chromatograpy	456	323.8 (132.2)	91.2	71.0
Reverse phase chromatography	295	293.5 (1.5)	82.7	99.5

procedures was summarized (Table 1). The purification yield of 82.7% and the purity of 99.5%, respectively, were accomplished by a simple two-step purification procedure. In particular, the purification yield (82.7%) is of considerable value when compared with the 68% of recomninant guamerin (MW = 6.11 kD) [8] and 63% of recombinant hirudin [14] in *P. pastoris*, and the 25% of hEGF in *H. polymorpha* [10].
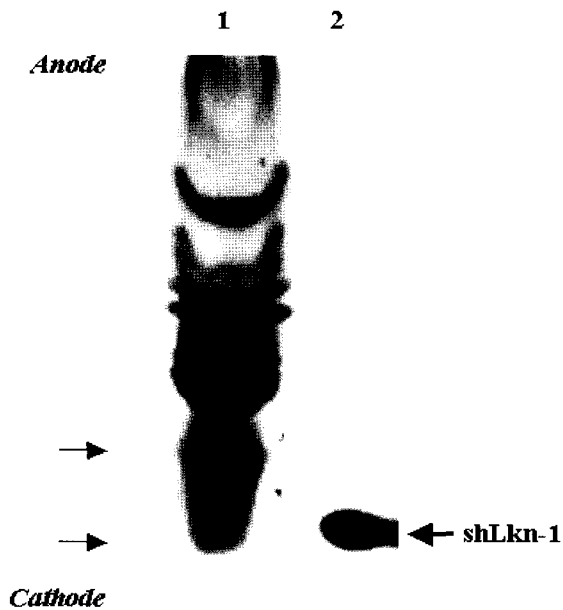
Biotechnol. Bioprocess Eng. 2004, Vol. 9, No. 1

5

Fig. 7. Reverse isoelectric focusing (IEF). Lane 1; IEF standards. The left arrows indicate pH 8.0 (upper) and 9.6 (lower). Lane 2; Purified shLkn-1 (arrow). pI of purified shLkn-1 is approximately 9.0.

Characterization of Purified shLkn-1

The purified recombinant shLkn-1 was further analyzed by mass spectrometry, N-terminal sequencing, and amino acid composition analysis. There was also a peak in the mass spectrum of 7.200 kD, which was exactly identical to the calculated molecular mass for the shLkn-1 (data not shown). It was shown that the N-terminal amino acid sequence (HFAADCCTSY-), which was obtained after 10 cycle-sequencing analysis, was the same as that of the predicted sequence, and the amino acid composition (data not shown) was also identical to the theoretically expected composition. Reverse IEF, which is used to resolve basic proteins with pI's above 8, was performed to check the ionic property of purified shLkn-1and to also check its purity. It was found that one major band was between pH 8.0 and 9.6 markers (Fig. 7).

Biological Activity of Purified shLkn-1

The shLkn-1 elicited a marked, transient rise in $[Ca^{2+}]_i$ in monocytes and eosinophils and acted as a potential chemoattractant for neutrophils, eosinophils, monocytes, and lymphocytes [4]. The biological activity of recombinant shLkn-1, derived from *P. pastoris*, was compared in a chemotaxis assay to that of a commercially available macrophage inflammatory protein-1 alpha, MIP-1α. MIP-1α has an important role in the development of inflammatory responses during infection through the regulation of leukocyte trafficking and function [15]. Both chemokines had chemotactic activity for THP-1 at con-
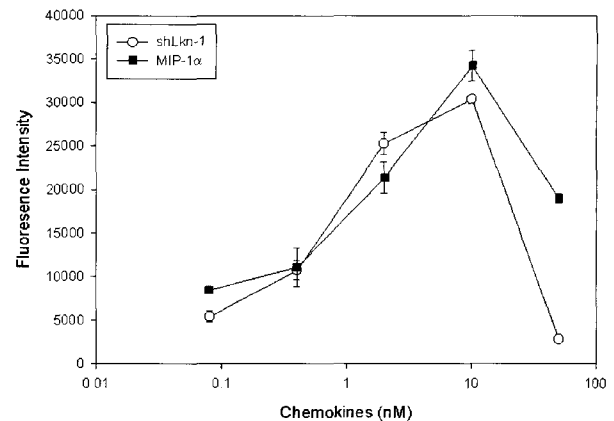

Fig . 8. Chemotactic activity of shLkn-1 (○) derived from *P. pastoris* and MIP-1α (■) from the human myelomonocytic leukemia cell line, THP-1.

centrations from 0.1 to 100 nM. The shLkn-1 was as potent as MIP-1α, as indicated by the observed maximum cell migration at a concentration of 10 nM (Fig. 8). Hence, the Lkn-1 purified from the recombinant *P. pastoris* exerted biological activity.

CONCLUSION

In this study, we demonstrated the high-yield purification procedure of recombinant shLkn-1 using *P. pastoris*, in which the recombinant shLkn-1 was simply purified by cation exchange and reverse phase chromatography. A shLkn-1 derivative, with which one amino acid of C-terminal residue was truncated during the culture process, was effectively separated by reverse phase chromatography. The recombinant shLkn-1 reached the maximum migration activity at 10 nM, with similar to human MIP-1α in a chemotaxis assay. During the development of the biological therapeutics, there are many obstacles to be overcome on the scale-up of the purification process. From this study, we achieved an applicable platform technology for the large-scale production of shLkn-1 for commercial production and/or the chemokine research. This technology might be used widely for the purification of the secreted recombinant protein in *P. pastoris*.

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