Special Issue

Preparation of recombinant six-histidine-tagged human LECT2, a chemotactic protein to neutrophils, in *Escherichia coli*

Mie Ito^{1,2}, Satoshi Yamagoe², Kazuo Tomizawa², Satoshi Mizuno², Masaru Tanokura¹ & Kazuo Suzuki²

¹ Biotechnology Research Center, University of Tokyo, Yayoi 1–1–1, Bunkyo-ku, Tokyo 113, Japan; ² Department of Bioactive Molecules, National Institute of Health, Toyama 1–23–1, Shinjuku-ku, Tokyo 162, Japan

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Abstract

LECT2 is a chemotactic protein to neutrophils. A recombinant six-histidine-tagged human LECT2, (His)₆-LECT2, was expressed in *E. coli* using a pET21a(+) vector. The (His)₆-LECT2 was purified from the soluble fraction in *E. coli* as a single band in sodium dodecyl sulfate/polyacrylamide gel electrophoresis using three steps of column chromatography with Ni²⁺-charged nitrilo-triacetic acid (Ni-NTA) agarose, DEAE-Sepharose, and CM-Sepharose. The purified (His)₆-LECT2 was yielded with 96 µg from the soluble fraction of 1,500 ml culture of *E. coli*. The circular dichroism spectrum of (His)₆-LECT2 showed the folded structure, which is rich in β-sheet structure and rare in α-helix.

Introduction

Activated neutrophils kill invaded bacteria and viruses *in vivo* and *in vitro* (Yamamoto *et al.*, 1991). Neutrophils are activated by complement component C5a, interleukin-8 (Suzuki *et al.*, 1989), and by bacterial chemotactic peptide fMet-Leu-Phe (Koshio *et al.*, 1990). After screening other chemotactic proteins produced of leukemia cells, LECT2 has been purified as a chemotactic protein to neutrophils from culture fluid of SKW-3 cells (Yamagoe *et al.*, 1996). Human LECT2 gene (hLECT2 or LECT2) and bovine LECT2 (bLECT2) were cloned and recombinant LECT2 were expressed in CHO cells (Yamagoe *et al.*, 1997).

Bacterial production of LECT2 is essential to obtain a large amount of recombinant LECT2, which is necessary to study the three dimensional structure and physicochemical properties. To prepare a recombinant protein, the protein is often expressed in a form fused with a tag protein such as glutathione-S-transferase (GST) or maltose binding protein (MBP) followed by factor Xa site (Gray *et al.*, 1982; Maina *et al.*, 1988) or tobacco etch virus (TEV) protease site (Parks *et al.*,

1994). On the other hand, six-histidine-tagged recombinant protein has recently been developed (Foutoulakis *et al.*, 1989; Crowe *et al.*, 1994; Kaslow and Shiloach, 1994).

In the present study, we expressed and purified recombinant human LECT2 from *E. coli* as six-histidine-tagged LECT2, (His)₆-LECT2. The secondary structure of the product was confirmed with circular dichroism (CD).

Materials and methods

Chemicals

Ni²⁺-charged nitrilo-triacetic acid (Ni-NTA) agarose was purchased from Qiagen GmbH (Hilden, Germany). Bacto-tryptone and Bacto-yeast extract were obtained from Difco (Detroit, Michigan, U.S.A). Ampicillin, tris[hydroxymethyl]aminomethane (Tris)-HCl, MgCl₂, and EDTA were the products of Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Imidazole was obtained from Nacalai Tesque Inc.

(Kyoto, Japan). DEAE-Sepharose Fast Flow and CM-Sepharose Fast Flow were the products of Pharmacia Biotech AB (Uppsala, Sweden). *Nde*I and *Bam*HI, and pET21a(+) were obtained from Takara Shuzo Co., Ltd (Otsu, Japan).

Synthesis of primer oligonucleotide

An oligonucleotide primer derived from the cDNA sequence of LECT2 was synthesized with an OLIGO 1000 DNA synthesizer (Beckman, CA, U.S.A.). The sequence of the synthesized oligonucleotide was: GCGCATATGCACCACCACCACCAC-CACGGGCCATGGGCTAATAT.

Amplification of DNA fragments by polymerase chain reaction (PCR)

PCR amplification of LECT2 cDNA was proceeded. Reaction mixture (100 μ l) contained 100 ng of the cDNA clone as a template, 8 μ l of equimolar mixtures of the four deoxyribonucleotide triphosphates (2.5 mM each), 10 μ l of 10 times the concentration of reaction buffer containing 500 mM KCl, 200 mM Tris-HCl (pH 8.4), 3 μ l of 50 mM MgCl₂ and 0.5 μ l (2.5 U) of *Taq* DNA polymerase (Gibco BRL), and the synthesized oligonucleotide primer above and the T7 primer (final concentration 1 μ M each). The amplification was performed in a DNA thermal cycler (Perkin Elmer/Cetus) by programmed incubation repeated 25 times of the following turn: for 1 min at 94 °C, for 1 min at 55 °C, and for 1 min at 72 °C.

Construction of (His)₆-LECT2 expression vector

Expression vector of (His)₆-LECT2 was constructed as follows. To make cDNA of (His)₆-LECT2 fragment, *Nde*I-*Bam*HI fragment amplified by the PCR technique was inserted into *Nde*I-*Bam*HI site of pET21a(+). The sequence was confirmed of the amplified PCR fragment. pET21a(+) containing (His)₆-LECT2 was harbored into *E. coli* BL21(DE3).

Production of (His)₆-LECT2

E. coli harboring (His)₆-LECT2 expression vector were inoculated into 30–40 ml of LB medium containing 10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, 5 g of NaCl in 100 ml supplemented with ampicillin (100 μ g/ml) in shaker flasks. The cells were grown with a reciprocal shaking at 350 rpm for 16 h at 37 °C.

Figure 1. Expression of (His)₆-LECT2. *E. coli* BL21(DE3) harboring (His)₆-LECT2 vector was cultivated for 8 h. The lysate from culture was centrifuged to separate the soluble and insoluble fractions. Samples prepared were analyzed by 15% SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining. M, molecular size marker; 1, soluble fraction; 2, concentrated insoluble fraction; and 3, insoluble fraction. The samples for lanes 1 and 3 were derived from the same volumes of cell lysate.

The bacterial suspension (30–40 ml) was diluted with fresh media to 1/50. The cell suspension was further incubated at 37 °C with shaking at 250 rpm. When its absorbance at 600 nm was approximately 0.7–0.9, 1 mM IPTG was added to activate the induction of the protein for further 16 h. The cells were harvested by centrifugation at 4,000 $\times$ g for 10 min and frozen at –80 °C.

Determination of purity of recombinant proteins

Recombinant proteins were applied to sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Laemmli, 1970). Gels were stained with 0.1% Coomassie Brilliant Blue R-250.

Measurements of CD spectra

For CD measurements, a Jasco J-720 CD spectropolarimeter was used with temperature controlled at 20 °C. The protein concentration was 0.1 mg/ml. A blank solution was 40 mM Na-phosphate (pH 7.4). To estimate the contents of the secondary structures, the CONTIN program (Provencher *et al.*, 1981) was used.

Figure 2. CM-Sepharose column chromatography of (His)₆-LECT2. (His)₆-LECT2 was purified as described in Materials and Methods. Numbers, fraction numbers of the chromatography; M, molecular size marker.

Figure 3. Far UV CD spectrum of (His)₆-LECT2 at 20 °C. The concentration of (His)₆-LECT2 was 0.1 mg/ml. The secondary structure was estimated with the CONTIN program to be 0% α -helix and 61% β -sheet.

Results

Production of (His)₆-LECT2 from 1.5 L culture

E. coli harboring (His)₆-LECT2 expression plasmid were inoculated into 30–40 ml of LB medium containing 10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, 5 g of NaCl in 100 ml supplemented with ampicillin (100 μ g/ml) in shaker flasks. The cells were grown with a reciprocal shaking at 350 rpm for 16 h at 37 °C. The bacterial suspension (30–40 ml) was diluted with fresh media to 1/50. The cell suspension was further incubated at 37 °C with shaking at 250 rpm. When its absorbance at 600 nm was approximately 0.7–0.9, 1 mM IPTG was added to activate the induction of the protein for further 16 h. The cells were harvested by centrifugation at $4,000 \times g$ for 10 min and frozen

at –80 °C. Figure 1 shows the expression of (His)₆-LECT2. As shown in Figure 1, (His)₆-LECT2 was mainly produced in the insoluble fraction of the cell lysate. The histidine-tagged LECT2 was purified from the supernatant by three steps of chromatography.

(His)₆-LECT2 was purified at first using an Ni-NTA column (Foutoulakis *et al.*, 1989; Hochuli *et al.*, 1987). Bacterial pellet was resuspended with 100 mM Tris-HCl buffer (pH 7.5) containing 5 mM MgCl₂, 0.5 mM EDTA and 100 mM NaCl. The suspension was sonicated, and then centrifuged at $8,000 \times g$ for 4 min at 4 °C. Ni-NTA column (2 ml pack) was equilibrated with 50 mM Na-phosphate buffer (pH 7.4) containing 300 mM NaCl and 40 mM imidazole. Soluble fraction of cell lysate containing (His)₆-LECT2 was applied to the column. The column was washed with 100 ml of the same buffer and (His)₆-LECT2 was eluted with 15 ml of the buffer containing 500 mM imidazole. Combined fractions containing (His)₆-LECT2 from Ni-NTA column were applied to a DEAE-Sepharose column (2 ml pack) equilibrated with 50 mM Na-phosphate buffer (pH 7.4). The column was washed with 100 ml of the same buffer. The passing-through and washing fractions were combined and applied onto a CM-Sepharose column (2 ml pack) equilibrated with 50 mM Na-phosphate buffer (pH 7.4). The column was washed with 100 ml of the same buffer, and proteins were eluted in 0.7 ml fraction with 9 ml of 50 mM Na-phosphate buffer (pH 7.4) containing 1 M NaCl at a flow rate of 0.3 ml/min, monitoring the absorbance at 280 nm.

(His)₆-LECT2 was purified as a single band in fraction numbers 5 to 7 of a CM-Sepharose column. Production of (His)₆-LECT2 was not enhanced by the treatment with IPTG as was judged by Ni-NTA column chromatography (data not shown). In this production of (His)₆-LECT2, 96 μ g was yielded from 1.5 L culture. To increase the production of LECT2, culture conditions were examined in the combination of LB medium at pH 8.5, the addition of 5 mM glucose, and the cultivating temperature of 26 °C. However, the yield of the protein was not increased under these conditions.

Discussion

A recombinant (His)₆-LECT2 was expressed in *E. coli* using a pET21a(+) vector and was purified from the soluble fraction of the lysate of *E. coli* cells to a single band in SDS-PAGE using three steps of column chromatography with Ni-NTA agarose, DEAE-Sepharose, and CM-Sepharose. In addition to the present expres-

sion systems, the fusion proteins of MBP-factor Xa-digestion site (Xa)-LECT2, MBP-Xa-LECT2, and GST-Xa-LECT2 were examined to produce recombinant LECT2. Since the LECT2 protein has many Arg and Lys residues, it may possibly have the sites recognized by factor Xa. LECT2 in these fusion proteins was indeed digested into fragments with the treatment of factor Xa (data not shown).

On the other hand, instead of Tac promoter in the expression vectors for MBP- and GST-fusion proteins, pET21 vector containing T7 promoter was used for the production of (His)₆-LECT in the present study. The protein was produced, but mainly in the insoluble fraction. (His)₆-LECT2 was purified with yield of 96 µg from the soluble fraction of 1.5 L culture of *E. coli*. To increase the yield, the cultivating temperature was lowered to 26 °C, in which the production of (His)₆-LECT2 was decreased in either of the single production or the production with (His)₆-thioredoxin ((His)₆-Trx) as compared with that in the culture at 37 °C (Ito *et al.*, unpublished). Amount of (His)₆-LECT2 in the soluble fraction was slightly increased when (His)₆-LECT2 was coproduced with (His)₆-Trx.

In conclusion, pET21a(+) vector gave a single protein with the best yield of the soluble recombinant histidine-tagged LECT among the present expression systems.

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Address for correspondence: Masaru Tanokura, Biotechnology Research Center, University of Tokyo, Yayoi 1–1–1, Bunkyo-ku, Tokyo 113, Japan