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Production and Purification of Soluble Recombinant Human Lymphotoxin in *Escherichia coli*

CHOI, YOON-HO[1], DOE-SUN NA[2], JAE-GU PAN, SEUNG-KIEL PARK, HYANG-SOOK YOO, KOOK-HEE KANG[1], AND MOON-HEE SUNG*

Genetic Engineering Research Institute, Korea Institute of Science and Technology, P.O. Box 17, Taedok Science Town, Taejon 305-606, Korea
[1]Department of Dairy Science, Sung Kyun Kwan University, Suwon 440-746, Korea
[2]College of Medicine, University of Ulsan, Seoul 138-040, Korea

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Human lymphotoxin (LT) was produced in *E. coli* as a soluble protein. The level of recombinant human LT production was about 4% of the total souble proteins of *E. coli* extracts. Recominant human LT was purified to apparant homogeneity by a simple procedure utilizing FPLC on Mono Q and Mono S columns. The specific activity of the purified LT was 1.0×10^7 units/mg.

Lympotoxin (LT), now often refered to as tumor necrosis factor-β (TNF-β), is exclusively produced by T-lymphocytes, both $CD4^+$ and $CD8^+$ cells, following the appropriate stimulation in the context of class II and class I restriction, respectively (15). The gene for LT is closely linked to the gene for TNF-α (about 1200 bp distance) within the MHC region (13). TNF-α and LT show pleiotropic activites *in vitro* and *in vivo* including the followings; cytotoxic effects against tumors and transformed cells (7, 26-28), stimulation of interleukin-1 and colony stimulating factor secretion (8), stimulation of prostaglandin E2 and collagen production (5), and stimulation of various immune effector cells (9). TNF-α and LT share 30% amino acid homology and bind to the same receptor (2, 16, 18, 23). Although TNF and LT are structurally and functionally related, various differences in their three-dimensional structure have been inferred. Thus, biological activity of TNF-α is fairly stable to detergents, organic solvents and acids whereas that of LT is fairly unstable to these conditions. Conversely, TNF-α is sensitive and LT is highly resistant to various proteases (6). Other studies indicated disparate effects of TNF-α and LT (3, 17).

To produce and purify a large amount of TNF-α and LT for clinical trials, recombinant technology for these proteins would be desirable. Recombinant TNF-α was easily produced in *E. coli* at a high level (16). On the other hand, the high level of expression of natural LT was unsuccessful by using the cDNA. (7, 22). Attempts to increase the expression level by using the synthetic gene designed according to the preferred codons of *E. coli* had been unsuccessful (7, 22). The high level expression has been obtained by using the combination of the synthetic and natural genes (25). In the previous study, we have cloned and expressed the LT cDNA in *E. coli* and partially purified the protein (14). Although the expression level was high , LT was mainly produced as the inclusion body which was not active biologically. Denaturation and renaturation processes were required to obtain the biologically active protein. These processes may decrease the yields of purification and/or the specific activity of the protein. In this contribution, we describe the production of recombinant human LT as a soluble protein in *E. coli*. We also establish the methods for purification of the soluble LT from *E. coli*.

*Corresponding author
Key words: Recombinant human lymphotoxin (TNF-β, LT), soluble production, purification

MATERIALS AND METHODS

E. coli Strains

E. coli JM109: *relA1, supE44, hsdR17, gyrA96, relA1, thi,* Δ*(lac-proAB)/F'[traD36, proAB*$^+$*, lacI*^{q}*, lacZ*Δ*M15]*,

mcrA$^-$, *mcrB*$^+$ was used for cloning. *E.coli* MM294: *supE 44, hsdR, endA1, pro, thi* was used as a host strain to produce recombinant human LT (25).

Enzymes and Chemicals

Enzymes were purchased from KOSCO Biotech (Seoul, Korea), Promega Biotech (U.S.A) and other suppliers. Enzymatic reactions were carried out as recommended by the suppliers. All chemicals were reagent grade from either Sigma (U.S.A.) or Merck (West Germany).

Oligonuleotides for Polymerase Chain Reaction (PCR) as the Primers

Oligonucleotides were chemically synthesized with an automatic DNA synthesizer at the Service Department of Genetic Engineering Research Institute, Korea Institute of Science and Technology.

Assay for LT Cytotoxicity

The cytotoxic activity of LT was determined as the lysis of mouse L-929 fibroblast cells as described (1). Briefly, mouse L-929 fibroblast cells were trypsinized and inoculated in micotiter plates at the concentrations of 1.25×10^4 cells/100 μl medium per well. After 12~18 h incubation, these cells were exposed to 100 μl of serially diluted test samples of LT. The test samples removed after 48 h, the plates were washed, and cell lysis was detected by staining the plates with a 0.5% crystal violet solution in methanol: water (1 : 4) (v/v). The end points of the cell lysis were determined by measuring the absorbance at 540 nm wavelength using Titertek Multiskan^R(Labsystems). The cells exposed to culture medium alone were set at 0% lysis and those exposed to a 3 M guanidine hydrochloride solution provided the control for 100% lysis. One unit of LT was defined as the amount required for 50% lysis of 12,500 cells inoculated in each well.

Purification of Recombinant Human LT

All operations were performed at room temperature unless otherwise stated.

Step 1. preparation of crude extract: The washed cells (about 5.7 g, wet weight) were suspended in 20 ml of 50 mM Tris-HCl buffer (pH 8.8) containing 1 mM phenylmethylsulfonyl fluoride and 2 mM EDTA. Those cells were disrupted at 4℃ with 20-kHZ Sonifier 450 (Branson). The cell extract was obtained by centrifugation (20,000×g, 30 min) and was dialyzed at 4℃ against 5,000 volumes of the same buffer.

Step 2. Mono Q chromatography: The dialyzed protein solution was applied to a Mono Q HR 10/10 column (1 by 10 cm; Pharmacia, Uppsala, Sweden) on a fast-protein liquid chromatography (FPLC) system in 2 ml portion. The recombinant LT did not bind Mono Q column equilibrated with 50 mM Tris-Hcl buffer (pH 8.8) containing 0.01 mM phenylmethylsulfonyl fluoride and 0.2 mM EDTA. Every fraction was applied to the assay for LT cytotoxicity. Fractions containing LT activity were pooled and concentrated by ultrafiltration with an Amicon PM-10 membrane.

Step 3. Mono S chromatogaphy: The concentrated protein solution was dialyzed against 50 mM sodium phosphate buffer (pH 7.2) and applied to a Mono S HR 5/5 column (0.5 by 5 cm; Pharmacia) equipped on the FPLC system . The LT was eluted at a flow rate of 2.0 ml/min with a 20-min linear gradient of 200 mM NaCl in the same buffer. The purified LT was concentrated to above 1 mg/ml by ultrafiltration, dialyzed against 100 volumes of the same buffer, and stored at −20℃.

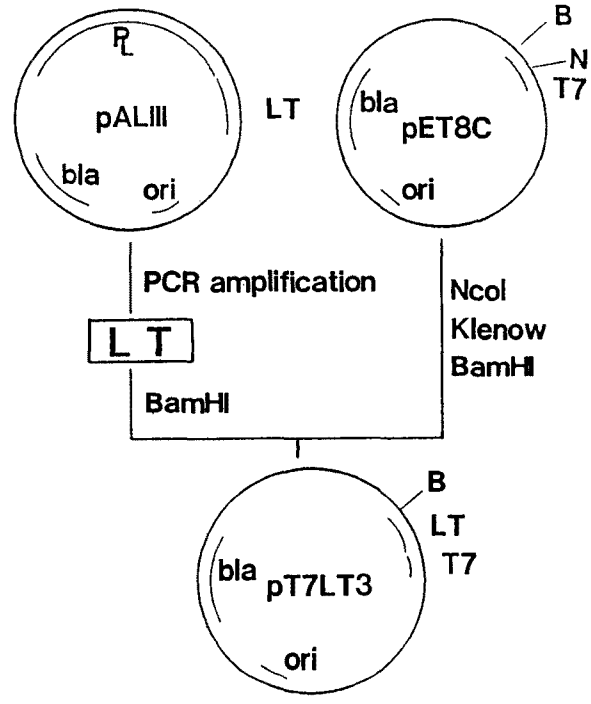

Fig. 1. Construction of plasmid pT7LT3.

Plasmid pT7LT3 was constructed by inserting the LT gene into the *Bam*HI and *Nco*I site of the vector DNA (pET-8C). LT gene fragment was obtained by polymerase chain reaction (PCR) and digestion with *Bam*HI. The sequences of primers used for PCR were 5′-CTCCCAGGCGTTGGTCTTACCCCATCAGCTGCCCAGACTGCCCGT-3′ and 5′-GGAGACTAAGTCTTATTACAGAGCGAAGGCTCCAAAGAA-3′. This was ligated to pET-8C serially treated with *Nco*I, Klenow fragement of DNA polymerase I, and *Bam*HI. Abbreviation: B; *Bam*HI, N: *Nco*I, T7; T7 promoter, bla; β-lactamase producing gene.

RESULTS

Recombinant Human LT Production

Plasmid pT7LT3 contains the coding sequence under the control of phage T7 promoter. The mother plasmid pET-8C has been described (24). The construction scheme for pT7LT3 is shown in Fig. 1. *E. coli* strain MM294 (DE3) was used as a host for this plasmid. *E. coli* cells were grown at 21℃ in M9 medium supplemented with tryptone (10 g/liter), yeast extract (5 g/liter), NaCl (5 g/liter), and ampicillin (50 mg/liter). The grown culture was inoculated to 25 m*l* of the same medium in a 250 m*l* flask to adjust an A600=0.1 and incubated at 21℃ with shaking (200 rpm) continuously. IPTG was added when the culture has grown to an A600=0.6, and the cells were incubated for an additional 36 h. Souble recombinant human LT was prepared by removing cell debris after sonication, using centrifugation at 20,000×g for 20 min. Comparing the sonicated and clarified extracts of *E. coli* cells analyzed by SDS-PAGE before and after the induction of expression, there was a detectable production of a new protein of 18.5 kD, the expected size of recombinant human LT (Fig. 2). Densitometric scanning of the SDS-PAGE gel revealed that recombinant human LT produced in *E. coli* accounted for 4% of the total soluble proteins at 36 h after induction of T7 promoter. Sonicated and clarified extract of *E. coli* MM294 harboring plasmid pT7LT3 contained cytotoxic against mouse L-929 fibroblast cells while that from *E. coli* MM294 transformed with pET-8C was not cytotoxic for L-929 cells. These results indicate that biologically acitive recombinant human LT was produced in *E. coli* as a soluble protein with the expected size (18.5 kD).

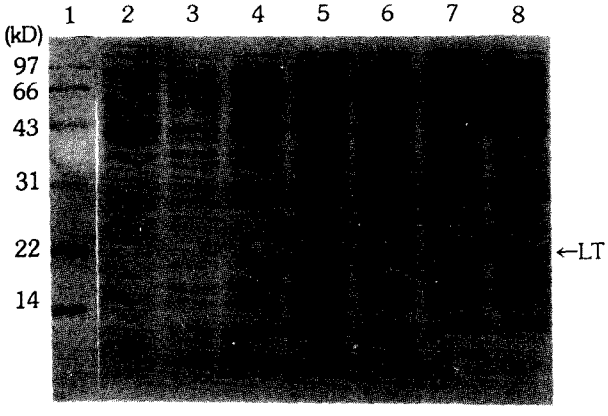

Fig. 2. Production of soluble recombinant human LT in *E. coli* MM294 harboring plasmid pT7LT3.
E. coli cells were grown at 21℃ for 36 h after induction of the T7 promoter. Soluble factions were separated by 15% SDS-PAGE and stained with Coomassie blue. Lane 1; molecular weight standards, lane 2; 0 h, *E. coli*/pT7LT3 was induced to produce recombinant human LT by adding IPTG to 0.1 mM, lane 3; 4 h, lane 4; 8 h, lane 5; 12 h, lane 6; 16 h, lane 7; 24 h, lane 8; 36 h after producing recombinant human LT. The arrow indicates recombinant human LT.

Purification of Recombinant Human LT from *E. coli* MM294 Harboring Plasmid pT7LT3

From the crude extract of *E. coli* MM294 harboring plasmid pT7LT3, the amount of recombinant human LT occupied 4% of the total proteins. The recombinant human LT was purified to homgeneity in two column chromatograpy steps, with a final yield of 45%. A summary of the purification is given in Table 1 and Fig. 3. The specific activity of recombinant human LT from *E. coli* MM294/pT7LT3 was 1.0×10^7 units/mg. The recombinant LT was purified efficiently and highly from the *E. coli* cells: it took only three to five days to purify the recombinant LT to homogeneity from cells of a 1.5 liter culture.

Table 1. Purification of recombinant human lymphotoxin

Purification step	Total protein (mg)	Lymphotoxin[a] (mg)
Crude extract	288	11.5
Mono Q	11.0	8.6
Mono S	5.2	5.2

[a]The amount determined by the 18.5 kD band from the gel.

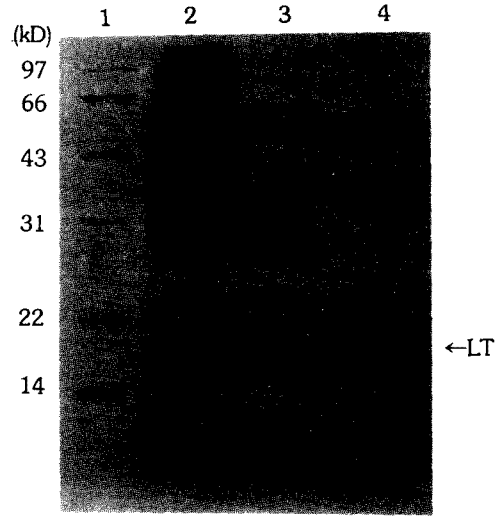

Fig. 3. Analysis of samples of the purification steps by SDS-PAGE.
Lane 1; molecular weight standards, lane 2; cell extracts, lane 3; after Mono Q chromatography, lane 4; after Mono S chromatography.

DISCUSSION

We have cloned human LT cDNA and expressed the gene in *E. coli* at a high level (9% of the total *E. coil* proteins) previously (14). In this case, no biological activity was observed in the soluble fraction of *E. coli*. Sonicated and clarified extract of transformed *E. coli* cells did not contain cytotoxic activity. Highly refractive inclusion bodies were visible under the phase contrast microscope. These indicated that recombinant LT was produced in *E. coli* as biologically inactive insoluble aggregates. In general, eukaryotic proteins expressed at high levels in *E. coli* are insoluble products (10-12, 21). We have tried to purification and renaturation of insoluble recombinant LT from *E. coli*. It was unsuccessful to purify the large amount of biologically active recombinant human LT due to the difficulties of renaturation step of recombinant human LT. Many researchers have investigated the effects of temperature on *E. coli* to get more soluble recombinant proteines (4, 19, 20). Therefore, production of soluble recombinant LT in *E. coli* at low growth temperature (30, 28, and 21℃) was applied (4, 19, 20). When *E. coli* MM294 (DE3) harboring plasmid pT7LT3 was grown at 21℃, there was a detectable soluble production of LT. Sonicated and clarified extracts of *E. coli* MM294 harboring plasmid pT7LT3 showed the cytotoxic activity against mouse L-929 fibroblast cells, and were confirmed to contain the recombinant human LT with Western blot analysis using rabbit anti-human LT polyclonal antibody (Genzyme, USA) (data not shown). These results indicated that the soluble human LT was produced.

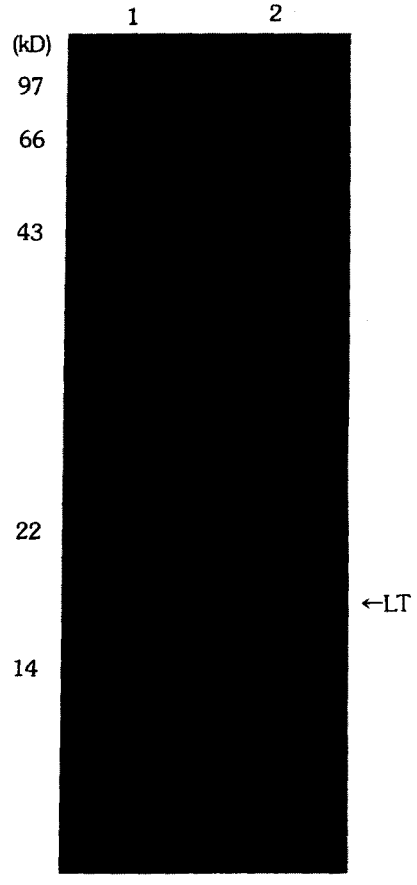

Fig. 4. Analysis of purified LT by SDS-PAGE with silver staining.
Lane 1; molecular weight standards. The size are 97, 66, 43, 31, 22 and 14 kilodaltons (kD) from the top. Lane 2; collection of active fractions from the Mono S chromatography. The arrow indicates recombinant human LT.

For the clinical trials of recombinant LT, a large amount of highly purified recombinant LT is necessary. We have developed a fast and simple purification method with high recovery yield. The purification scheme consisted of only Mono Q and Mono S chromatography on FPLC. The purity of recombinant human LT was above 99% confirmed with silver staining of 15% SDS-polyacrylamide gel (Fig. 4). The result of the specific activity of recombinant human LT (1.0×10^7 units/mg) was similar to that of natural human LT purified from a human lymphoblastoid cell line RPMI1788 culture(1). We are in the process of pursuing the clinical application of the recombinant human LT purified in this study.

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