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High-level production of biologically active chemokines in *Escherichia coli*

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ABSTRACT

The chemokines eotaxin-1 (CCL11) and eotaxin-2 (CCL24), belonging to the CC chemokines family, play key roles in the inflammatory response, allergic asthma and other diseases. When expressed in *Escherichia coli*, chemokines are prone to form inclusion bodies devoid of biological activity, and it is hard to refold them properly. Here an expression and purification protocol for high-level production of soluble and biologically active CCL11 and CCL24 in *E. coli* has been established. A final yield of 8.7 mg/l for CCL11 and 3.9 mg/l for CCL24 has been obtained and the purified proteins were characterized with SDS-PAGE, mass spectrometry and circular dichroism. High binding affinity of purified chemokines with C—C chemokine receptor type 3 (CCR3) has been confirmed with surface plasmon resonance (SPR) and the K_D values are 3.7×10^{-7} M and 3.0×10^{-7} M, respectively, for CCL11 and CCL24. This report provides a straightforward strategy for the efficient production of soluble and biologically active chemokines in *E. coli*.

1. Introduction

Chemokines are a large family of polypeptide signaling molecules that induce cell migration by activating G protein coupled receptors [1]. They can bind matrix glycosamino-glycans (GAG) in order to direct chemotaxis along the chemokine gradient [2]. They are secreted proteins with about 100 amino acids and molecular weight ranging from 8 to 14 kDa. Chemokines contain a typical Greek key shape structure, with 3 β-strands and 1 α-helix and stabilized by disulfide bonds between conserved cysteine residues. More than 50 chemokines have been identified in human [3], which are divided into four sub-families (CC, CXC, CX3C, XC) based on the position of cysteines in the N-terminal domain [4]. Chemokines together with their cognate receptors are involved in many diseases, including tumor progression and metastasis, arthritis, asthma, neurodegenerative diseases and HIV-1/AIDS [5,6]. Therefore, chemokines are extensively studied in pharmaceutical development [7,8].

Eotaxin-1/CCL11 and eotaxin-2/CCL24 belong to the CC chemokine sub-family, with the characteristics of two adjacent cysteines near the N-terminus [9]. Recent work shows that CCL11 and CCL24 together with their receptor CCR3 play a pivotal role in the control of leukocyte chemotaxis and represent attractive targets for the blockade of the progression of inflammatory diseases [7,10]. Antagonists derived from CCL11 and CCL24 can efficiently block the CCR3 signaling pathway and cure related diseases [11,12]. Many efforts have therefore been taken to investigate the properties and functions of CCL11 and CCL24.

There is an increasing demand to obtain milligrams of biologically active chemokines for biological studies and drug screening. Conventionally, chemokines were purified from natural sources. However, a complex purification procedure is often required to isolate various isoforms [13,14]. Alternatively, CCL11 and CCL24 can be obtained through chemical or recombinant synthesis [15–19]. But, it is also very costly to obtain chemokines by chemical synthesis. To increase the efficiency and decrease the cost of production, *Escherichia coli* has been widely used as a powerful and versatile host for high-level protein expression [20]. However, chemokines, such as CXCL12, CCL5, XCL1, CX3CL1 and chemerin, are typically over-expressed with poor solubility and in the form of inclusion bodies devoid of biological activity when expressed in *E. coli* [21,22]. To recover biological activity, chemokines in inclusion bodies normally need to be solubilized using guanidinium hydrochloride or urea and then refolded to form proper disulfide bonds [23], which is time-consuming and costly. It is therefore highly desirable to develop a new procedure to express chemokines in soluble and biologically active form and purify them in an efficient way. To achieve soluble and biologically active expression, chemokines are often expressed as fusion proteins, such as fused with MBP (maltose

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binding protein), Nus, GST and thioredoxin *etc*., to facilitate correct folding and formation of disulfide bonds [24–27]. Chemokines without fusion protein can be obtained after digestion with specific enzymes.

In this study, we describe a protocol for a highly efficient preparation of biologically active CCL11 and CCL24. The genes were synthesized and inserted into different expression systems to express human CCL11 and CCL24 in *E. coli*. After optimization of expression conditions, soluble chemokines with high expression level were obtained as fusion proteins, and the target proteins were then purified with a relatively simple procedure. Their biological activity was further verified with surface plasmon resonance (SPR) experiments. This work has provided an important example for purifying chemokines and will facilitate further studies on protein interaction and pharmaceutical development based on these two proteins.

2. Materials and methods

2.1. Materials

The pET28a, pET32a (Novagen) and pMAL-p4x (New England Biolabs, USA) plasmids were used for sub-cloning and expression of chemokines as fusion proteins. Corresponding *E. coli* strains, including *E. coli* BL21 (DE3), *E. coli* Origams (Novagen) and *E. coli* K12 TB1 (New England Biolabs, Ipswich, MA, USA) were used as the host strains for protein expression. The enzymes, including T4 DNA ligase, *Hin*d III, *Eco*R I and Taq polymerase, were obtained from TaKaRa (TaKaRa, China). Plasmid DNA was purified using a Tiangen Mini Purification Kit (Tiangen, China).

2.2. Construction of CCL11 and CCL24 expression plasmids

The genes of CCL11 and CCL24 were commercially synthesized with optimized codon usage for over-expression in *E. coli* (Supplementary File 1). Then the genes were amplified by PCR using primers as shown in Supplementary Table 1. The PCR-amplified products were purified and digested with *Eco*R I and *Hin*d III, and then subcloned into pET28a, pET32a and pMAL-p4x plasmids, resulting in pET28a-CCL11, pET28a-CCL24, pET32a-CCL11, pET32a-CCL24, pMAL-p4x-CCL11 and pMAL-p4x-CCL24 recombinant plasmids. The recombinant plasmids were further verified by DNA sequencing.

2.3. Optimization of CCL11 and CCL24 expressed in E. coli

Several factors may influence the expression of the target protein in soluble form, such as expression plasmid, host strain, induction phase and expression temperature. To achieve a high-level expression of soluble and active chemokines, these factors were then systematically scrutinized [28].

The expression yield of different expression plasmids was compared. Briefly, recombinant plasmids were transferred into their corresponding *E. coli* strains, *i.e.* pET28a-CCL11 and pET28a-CCL24 were transferred into BL21 (DE3), pET32a-CCL11 and pET32a-CCL24 transferred into Origams, pMAL-p4x-CCL11 and pMAL-p4x-CCL24 transferred into TB1 strain. Single colony was picked up and cultured overnight in 10 ml Luria–Bertani (LB) broth (10 g/l tryptone, 5 g/l yeast extract, 10 g/l sodium chloride) [29] supplemented with appropriate antibiotics (50 μg/ml kanamycin for BL21 and Origams strain, 100 μg/ml ampicillin for TB1 strain) at 37 °C with shaking (170 rpm). After overnight growth, 1 ml of culture was then inoculated into 100 ml of fresh LB supplemented with appropriate antibiotics and cultured at 37 °C with continuous shaking [30,31]. When the $OD_{600\,nm}$ grew up to 0.6, the culture temperature was lowered down to 25 °C, and then 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) was added into the medium. After induction, 2 ml of sample was periodically collected, centrifuged and kept in the refrigerator. At last, the samples were lyzed and centrifuged at 12,000 × *g* for 10 min at 4 °C, and the supernatant was applied for dot blot analysis, where mouse anti-His monoclonal antibody (Tiangen, China) was used as first antibody, followed by HRP-labeled goat anti-mouse (Tiangen, China) as second antibody. The blot was finally stained using the Amersham ECL plus, and then detected on a FLA-5100 imaging system (Fuji, Japan) and analyzed with MultiGauge Ver.3.X software. According to our experiments, the error in dot blot quantification is about 10%.

The induction phase and induction temperature were optimized on a similar procedure by inducing at $OD_{600\,nm}$ of 0.2–0.4, 0.4–0.8, 1.0–1.2 and over 1.3 and under culture temperature of 25 °C, 30 °C and 37 °C, respectively.

2.4. Expression and purification of CCL11 and CCL24 in E. coli

Six liters of *E. coli* harboring pET28a-CCL11 or pET28a-CCL24 were cultured at the optimized conditions, and *E. coli* cells were then harvested by centrifugation at 8000 × *g* for 10 min at 4 °C. The pellets were re-suspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) supplemented with 0.05% (w/v) lysozyme, 1 mM DNase I and 1 mM phenylmethanesulfonyl fluoride (PMSF) and incubated for 30 min at 4 °C. After incubation, *E. coli* cells were cracked on an ultra-high pressure homogenizer (JN3000, JNBio, China). Cell debris was then removed by centrifugation at 12,000 × *g* for 20 min at 4 °C; the supernatant was collected and filtered through a 0.45 μm membrane filter. The filtrate was loaded onto a HiTrap™ Chelating HP column (GE Healthcare) pre-equilibrated with Buffer A (PBS buffer plus 500 mM NaCl) for purification *via* Ni affinity chromatography. The impurities were washed off with PBS buffer containing 500 mM NaCl, 250 mM imidazole (pH 7.4) and the target protein was eluted with PBS buffer containing 500 mM NaCl, 500 mM imidazole (pH 7.4). The protein was concentrated and then loaded onto HiPrep 26/10 desalting column prepacked with Sephadex G-25 for buffer exchanging to PBS buffer. The purified fusion proteins were then digested using His_6-TEV enzyme (courtesy of Fersht's Lab at LMB, UK) [32]. After digestion, the sample was loaded onto a HiTrap™ Chelating HP column pre-equilibrated with Buffer A. CCL11 or CCL24 was recovered in the flow-through, while the cleaved fusion part with His_{12}-tag and the His_6-TEV enzyme were captured in the column.

2.5. SDS-PAGE and western blot analysis

The purified CCL11 or CCL24 was mixed with NuPage LDS sample buffer (4×) and NuPage Reducing agent (10×) (Invitrogen), and then the mixed sample was incubated at 95 °C for 5 min. The purified CCL11 and CCL24 were characterized by SDS-PAGE using a 4–12% NuPAGE bis-tris gel and MES SDS running buffer (Invitrogen). The western blot experiments were carried out according to the procedure as previously described using mouse anti-His monoclonal antibody (Tiangen, China) as the primary antibody and the HRP-labeled goat anti-mouse antibody (Tiangen, China) as the secondary antibody [33]. The blot was finally stained using the Amersham ECL plus, detected on a FLA-5100 imaging system (Fuji, Japan) and analyzed with MultiGauge Ver.3.X software. The protein standards marker used for SDS-PAGE does not contain His_6-tag and cannot be stained in western blot experiments. A specific protein standards marker for western blot, the Easy Western Protein Standard (Genescript), was therefore employed to further confirm the molecular weight of the target proteins in western blot experiments. On the other hand, the protein standards marker for western blotting is too diluted for SDS-PAGE. Different protein standards markers were therefore applied for SDS-PAGE and western blotting.

2.6. Mass spectrum analysis

The mass spectrum measurements were carried out on a Bruker Biflex III matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer as described previously [34]. Briefly, the samples were dissolved with the matrix in the mixture of acetonitrile and water (1:1, v/v) which contained 1% trifluoroacetic acid (TFA). About 0.5 μl of the sample solution was placed on a metal sample plate and then air-dried at ambient temperature. Mass spectra were acquired in positive linear mode and using an acceleration voltage of 19 kV. Spectra were obtained by setting the laser power close to the threshold of ionization and generally 100 pulses were acquired and averaged.

2.7. Circular dichroism spectroscopy

Circular dichroism (CD) experiments were performed on a MOS-450 circular dichroism spectrometer (BioLogic Science Instruments, France). Far-UV CD spectrum was acquired over the wavelength range of 190–250 nm with a 5 mm path length cell at 25 °C in phosphate buffer (20 mM phosphate, pH 7.4), where the step size and acquisition time were set as 1 nm and 2 s, respectively. The final spectrum was background subtracted using corresponding buffer as reference.

2.8. Surface plasmon resonance measurement

The interaction between CCL11/CCL24 and CCR3 was studied by surface plasmon resonance (SPR) on a Biacore T100 (GE Healthcare) instrument using NTA sensor chip at 25 °C. CCR3 was produced with stably transfected HEK293 cells, which has been verified to be biologically active [33]. Two flow-cells were used for the SPR experiments, one of which worked as a reference to subtract possible non-specific signal. The running buffer was Hepes buffer with 10 mM Hepes, 0.15 M NaCl and 0.1% Triton X-100 (pH 7.4). The sensor chips were activated with 0.5 mM $NiCl_2$, and then purified CCR3 with His tag was immobilized on the chips. The binding of CCL11 or CCL24 to the immobilized CCR3 was monitored in real time with a flow rate of 30 μl/min. The data from the reference cell, where CCR3 was not loaded but all the other conditions were identical, was used for background subtraction. The sensorgrams were fitted globally with a 1:1 binding model using Eq. (1) for the association phase, Eq. (2) for the equilibrium phase and Eq. (3) for the dissociation phase [35,36].

$$y = \frac{R_m * C}{k_d/k_a + C}\left(1 - \frac{1}{\exp(k_a * C + k_d)x}\right) \quad (1)$$

$$y = \frac{C * R_m}{C + k_d/k_a} \quad (2)$$

$$y = R_o * \exp(-k_d * x) \quad (3)$$