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Expression and Purification of Bioactive High-Purity Recombinant Mouse CXCL14 in *Escherichia coli*

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Abstract Mouse CXCL14/BRAK is a monocyte-selective chemokine which is expressed in almost all normal tissues. A flood of reports on its new functions of tumor suppression and fat metabolism modulation has left CXCL14 a potential therapeutic candidate for these diseases. Therefore, a simple accessible method is on demand for large-scale production of recombinant mouse CXCL14 protein for in vivo animal studies. Here, we introduce an efficient method for large-scale production of recombinant mouse CXCL14, by which an 18-mg protein is produced from 2-L *Escherichia coli* culture with good bioactivity and low level of endotoxin.

Keywords Mouse CXCL14 · Nickel affinity · Large-scale preparation · Chemokines

Introduction

CXCL14 is a younger member of chemokine family. It was first discovered in human breast and kidney, and finally proved to be constitutively expressed in many tissues [1].

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Originally, its function was mainly focused on inflammation [2] because of its selectivity on monocytes and monocyte-derived dendritic cell and nature killer cell [3–5]. However, more and more investigators have paid their attentions to the associations between *cxcl14* and oncogenesis and obesity recently [4, 6, 7]. The accumulated proofs suggest that CXCL14 plays a key role in suppressing tumorigenesis and motility [8–10]. The mouse cxcl14 mRNA encodes 99 amino acids (AAs), in which N-terminal 22 AAs serve as a signal peptide which is cleaved as the protein is secreted. The remaining C-terminal 77 AAs construct the mature chemokine with a calculated molecular weight of 9,440 Da and pI 9.9. Four conserved cysteines in the mature protein form two intrachain disulfide bonds.

A molecular evolution study has shown that the *cxcl14* gene emerged at the Devonian period (359.2~416 Ma ago) and considerably diverged from other chemokines [11]. Besides, the study also revealed a high AA sequence conservation of CXCL14 between species (75/77 AAs identities between human and mouse and 76/77 between human and rat) that enlighten the fact that CXCL14 might perform important functions.

As more and more functions were reported in the areas mentioned above, a simple accessible method is urgently needed for large-scale production of recombinant mouse CXCL14 protein for in vivo animal studies. In this work, the rmCXCL14 expressed in *Escherichia coli* was purified by nickel affinity column followed by varied ion exchange chromatography after refolding. Final purity of the produced rmCXCL14 was higher than 95%. The endotoxin level was less than 0.058 EU/μg, and the bioactivity was verified using monocyte chemotaxis and cell proliferation assays.

Materials and Methods

Construction of rmCXCL14 Expression Vector

The plasmid pET28a(+) and strain *E. coli* BL21 (DE3; Novagen, USA) were used as expression vector and recombinant host strain, respectively. Reverse-transcription kit was purchased from TaKaRa Company (Dalian, China) and *pfu* polymerase from Transgene Company (Beijing, China). The mouse CXCL14 gene was cloned from the cDNA, which was reversely transcribed from total mRNA of mouse liver tissue. The mature CXCL14 protein was encoded by the full-length sequence from 445 to 678 bp (GeneBank accession no. NM_019568.2) without the signal peptide coding sequences. The coding sequence was amplified by PCR with primers 5′-GTGGCTAGCTCCAAGTGTAAGTGTTC and 5′-TCGCTCGAGCTATTCTTCGTAGACCCTGC. The NheI and XhoI sites (underlined) were incorporated into primers to facilitate directional cloning of the PCR product into polyclonal sites of the expression vector pET28a(+). Through genetic manipulations described previously [12], a rmCXCL14 expression plasmid, pET28a-rmCXCL14, was constructed and analyzed by restrictive enzyme digestion and finally confirmed by DNA sequencing (Fig. 1). The rmCXCL14 protein was expressed as a fused protein with a polyhistidine tag (His-tag) and thrombin cleavage site at N-terminal.

Expression Condition Optimization of rmCXCL14

The recombinant strain *E. coli* BL21 (DE3) containing the plasmid pET28a-rmCXCL14 was cultured in the LB medium containing 100 μg/ml kanamycin, with continuous shaking at 300 rpm, and at 37 °C for 12 h. One-hundred-microliter culture was inoculated into 5-ml LB medium and grown in same condition until the optical density at 600 nm (OD_{600}) of the

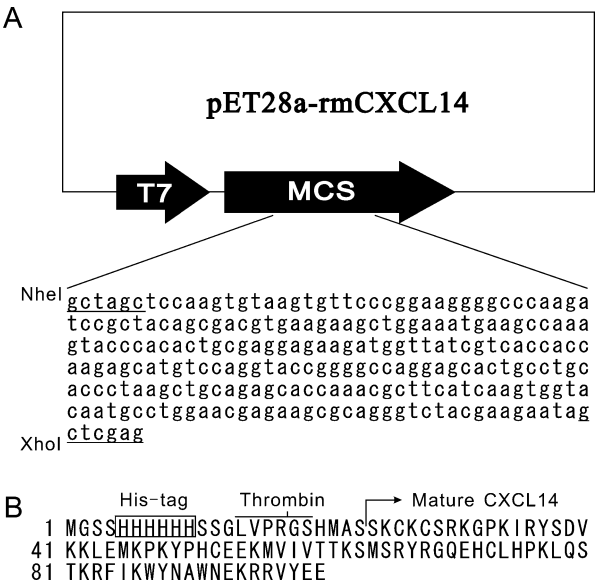

Fig. 1 **a** Construction of rmCXCL14 expression plasmid pET28a-rmCXCL14. The coding sequence of mature CXCL14 protein was inserted into pET28a (+) between NheI and XhoI restrictive sites. **b** The rmCXCL14 protein was expressed as a fusion protein with a His-tag and a thrombin cleavage site at N-terminal

culture suspension reached 0.8. The expression of rmCXCL14 protein was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) at different concentrations and incubating at 37 °C for 2 or 6 h. Total protein prepared from the culture was analyzed by 15% (*w*/*v*) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and stained with Coomassie brilliant blue R250 (Shenggong, Shanghai, China).

Preparation of Cell Lysate and Affinity Purification of rmCXCL14

Two liters of the culture was harvested by centrifugation at 8,000×*g* for 5 min at room temperature. After washing once with 40-ml ice cold PBS buffer, cells were centrifuged at the same condition. Cell pellet was resuspended in denatured His-tag binding buffer (50 mM imidazole, 0.5 M NaCl, 0.02 M Tris-Cl pH 7.9, 0.1% Tween-20, and 6 M Guanidine Hydrochloride) with 1 mM phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 100 mg/ml and sonicated on ice-bath (400 W power, sonicate repeatedly for 3 s on and 10 s off). Then, the cell lysate was centrifuged at 20,000×*g* for 30 min at 4 °C, and the supernatant was collected and filtered using 0.45-μm microfiltration membrane. Three milliliters Ni-Sepharose (Pharmacia, USA) was loaded onto column and recharged by 5 column volumes (CV) of 50 mM $NiSO_4$ and equilibrated by 3 CV of denatured binding buffer. Cell lysate was loaded onto the purification column at rate of 0.5 ml/min at 4 °C. Then, the column was washed with 10 CV of denatured wash buffer (60 mM imidazole, 0.5 M NaCl, 0.02 M Tris-Cl pH 7.9, 0.1% Tween 20, and 6 M Guanidine hydrochloride) before elution by denatured elute buffer (1 M imidazole, 0.5 M NaCl, 0.02 M Tris-Cl pH 7.9, 0.1% Tween-20, and 6 M Guanidine hydrochloride).

Reduction and Refolding of the Protein

The intermolecular disulfide bonds were reduced by adding dithiothreitol (DTT) into protein solution to final concentration of 0.2 M. After overnight incubation at 4 °C, the

solution was gradually diluted with refolding buffer (20 mM Tris-Cl pH 7.9, 0.1 M NaCl, 1 mM reducing glutathione, 1 mM EDTA, 10% sucrose, 0.1% Triton-X114) to 20-fold of the initial volume over 1 h with gentle stirring at room temperature and then incubated at room temperature for 1 h. Precipitate was removed by centrifugation at 20,000×*g* for 30 min.

Ion Exchange Purification

The supernatant was loaded onto an SP Sepharose column (Amersham Biosciences) with a volume of 20 ml, which had been pre-equilibrated with Buffer A (20 mM Tris-Cl, pH 8.0), with flow rate maintained at 5.0 ml/min. After washing with 5 CV of Buffer A, protein was eluted using programmed gradient Buffer B (20 mM Tris-Cl and 1.0 M NaCl, pH 8.0), which was linearly increased from 20% to 80% with a flow rate of 1.0 ml/ml.

The eluted fractions containing the target protein were desalinated using a Sephadex G25 column (height 60 cm and diameter 1.5 cm), which was pre-equilibrated with 2 CV of Buffer A. The elution fraction containing target protein was collected.

The endotoxin of the protein was removed by anion exchange chromatography as described [13]. The desalinated protein solution was loaded onto a Q Sepharose column (Amersham Biosciences) of about 20 ml volume, pre-equilibrated with 5 CV of Buffer A, with flow rate maintained at 5.0 ml/min. The flow through fraction containing endotoxin-free target protein was collected.

For reducing the final protein volume and increasing the protein concentration, the protein solution was loaded onto dialysis bags with molecular weight cutoff 2 kDa and dehydrated by PEG10000 until the solution volume decreased to about 10 ml. Samples from each purification steps were quantified by the Bradford method.

SDS–PAGE and Western Blotting

SDS–PAGE was performed using 15% (*w*/*v*) gel on the PowerPac Basic (Bio-Rad, USA). Protein samples were separated by electrophoresis at 120 V for 1.5 h, and then stained using Coomassie brilliant blue R250. In Western blotting experiment, proteins were transferred to polyvinylidene fluoride (PVDF) membranes by wet transfer with 200 mA for 1.5 h. After blocking in 10% nonfat milk PBS, the membrane was incubated with 0.5 μg/ml anti-mCXCL14 rat IgG (R&D, USA) in PBST (PBS buffer +0.05% Tween 20) plus 5% nonfat milk. After washing four times by PBST, the membrane was incubated with peroxidase-conjugated anti-rat IgG goat IgG (PTG, USA) at 1:5,000 dilution ratio in PBST plus 5% nonfat milk at room temperature for 2 h. The membrane was washed four times with PBST for 5 min each, and then, electrogenerated chemiluminescence (ECL) reaction was performed using Pierce ECL Western Blotting Substrate reagent (Thermo) for 1 min and visualized in X-ray film.

Endotoxin Assay

The endotoxin level of the protein was analyzed semiquantitatively using a *Tachypleus amebocyte* Lysate (TAL) kit from Xiamen Houshiji, Ltd. (Xiamen, China) according to the protocol given by reagent supplier. Briefly, a 1-EU/ml endotoxin standard induces TAL reagent to catalyze gel-clot reaction, while the gel-clot reaction cannot be induced by protein samples with endotoxin level less than 1 EU/ml. Protein sample was diluted a tenth in series using endotoxin-free water and assayed as above.

Reverse Phase High-Performance Liquid Chromatography Assay

The purity of the protein was analyzed by a Shimadzu LC2010AHT system. Purified protein (8 μg) in PBS was loaded onto an Inertsil C4 column (250×4.6 mm, 5 μm, Japan), and eluted with linear gradient of acetonitrile from 0% to 100% (*v*/*v*) in 0.1% (*v*/*v*) trifluoroacetic acid in 65 min. The flow rate was maintained at 0.8 ml/min. Absorbance was read at 280 nm, and profile was compared with the standard.

Bioactivity Assay

The bioactivity of the protein was verified both through measuring its chemotaxis activity on monocytes and proliferation promoting activity on fibroblast NIH3T3, individually. Briefly, the fresh monocytes were isolated from peripheral blood of a male Sprague–Dawley (SD) rat by Ficoll density gradient centrifugation and adherent culture, and then, chemotaxis was performed using 24-well transwell devices for 3 h in duplicate for each concentration. For each well, about 50,000 monocytes were used. Migrated cell number was counted in five random selected ×200 field. In the cell proliferation promoting activity assay, 5,000 NIH3T3 fibroblasts were seeded in 24-well plates (Corning, USA). Fibroblasts were grown 2 days in DMEM supplemented with 2% calf serum and different concentration of purified rmCXCL14, and cell numbers were determined using MTT method. Two independent experiments were performed in triplicate. Student *t* test was used to assess the difference between groups.

Results

Expression of rmCXCL14

The *E. coli* BL21 strain harboring plasmid pET28a-rmCXCL14 was induced by 0.1 and 1.0 mM IPTG at 37 °C after the OD_{600} reached 0.8. After induction, cells were harvested by centrifugation and lysed by resuspending in SDS–PAGE loading buffer, and then, SDS–PAGE was performed with the equal amount of cell lysates of different treatments. Coomassie brilliant blue staining showed that a protein with molecular weight of about 16 kDa was induced (Fig. 2a). Western blot analysis proved this band to be the target protein (Fig. 2b). As far as the expression amount was concerned, there was no clear difference between amounts of protein expressed under 0.1- and 1.0-mM IPTG induction (Fig. 2a). However, for both IPTG concentrations, the target protein was significantly more produced at 6 h than at 2 h. Therefore, 0.1-mM IPTG concentration and 6-h induction time were used as optimized induction condition in further protein production. Under this condition, 2-L medium yielded 165-mg target protein, which was approximately 10% of the total cell protein.

Nickel Affinity Purification of rmCXCL14

The cells from 2-L culture, wet weight about 9 g, were harvested for the following preparation. The cell lysate was prepared, and affinity chromatography was performed as described above. The eluted solution was collected in 2-ml fractions. Eluted protein samples were assayed by SDS–PAGE, and the fractions 2–4 containing the target protein were combined for the following steps (Fig. 3). Bradford and SDS–PAGE assays indicated that 76-mg protein with 90% CXCL14 was recovered.

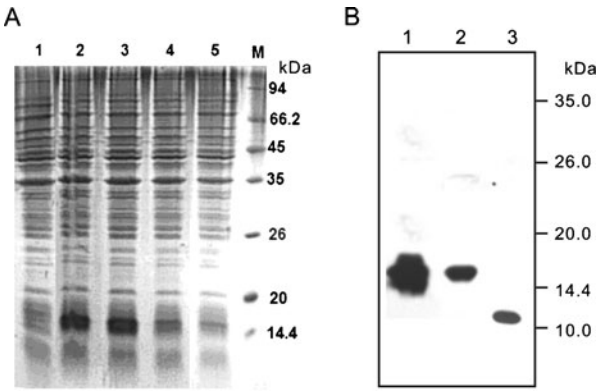

Fig. 2 Optimization of induction condition of rmCXCL14 and western blot identification. **a** *E. coli* harboring plasmid pET28a-rmCXCL14 was induced by 0.1 mM of IPTG (*lane 2* and *4*) and 1.0 mM IPTG (*lane 3* and *5*) for 6 h (*lane 2* and *3*) or 2 h (*lane 4* and *5*). *Lane 1* contained lysate of noninduced cell. **b** Induced (*lane 1*) or noninduced (*lane 2*) cell lysate and commercial rmCXCL14 from R&D company (*lane 3*) were detected by western blot

Ion Exchange Purification

The eluted protein was reduced and refolded as described above, and the precipitate was removed by centrifugation before ion exchange purification. Refolded protein solution was loaded onto S sepharose column and eluted using PBS with gradient NaCl concentration from 0.2~0.8 M. According to the elution profile (Fig. 4a), the rmCXCL14 was eluted until the eluent conduction reached to 35 mS/cm. Twenty-milliliter peak (peak 2, Fig. 4a) was collected and loaded onto a G25 column to remove high concentrated saline (peak 4, Fig. 4). The protein peak in flow through (peak 3, Fig. 4) was collected and loaded onto Q sepharose column to remove the endotoxin. The protein peak (peak 5, Fig. 4) was collected and concentrated by PEG10000, and dialyzed overnight in PBS (pH 7.4). In total, 18.2-mg protein in 14-ml PBS was obtained.

Characterization of Purified rmCXCL14

Purity of the purified rmCXCL14 was analyzed by reverse phase high-performance liquid chromatography (RP-HPLC). The result indicated that the purity of rmCXCL14 was >95% (Fig. 5). The endotoxin level of prepared protein was assayed using the TAL kit and found to be lower than 0.058 EU/μg. The protein was filtered through 0.22-μm membrane and stored at −80 °C. In chemotactic activity assay, freshly isolated monocytes showed significant chemotaxis at 1-μM rmCXCL14 which agreed with the result obtained by Kurth et al. [3]. In fibroblast proliferation promoting assay, the rmCXCL14 significantly promoted the proliferation of NIH-3T3 cell lines at 2.4 nM (Fig. 6).

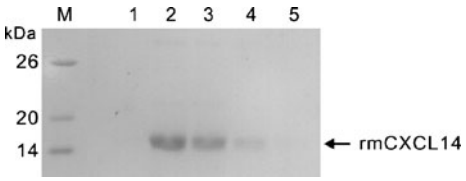

Fig. 3 SDS–PAGE of nickel affinity purification of rmCXCL14 protein. Protein bound to the resin was eluted by elute buffer and collected in 2-ml fractions (*lane 1–5*) and discriminated by reducing SDS–PAGE

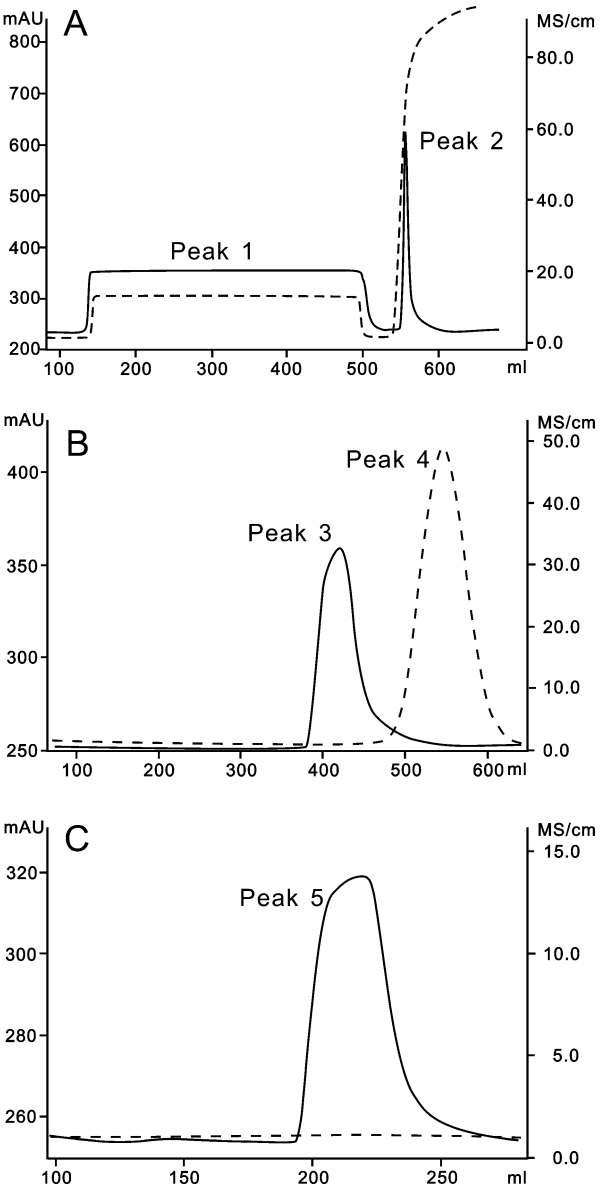

Fig. 4 Chromatography spectrum of ion exchange. The elution profiles were given by UV absorption at 280 nm (*solid curve*) and solution conduction (*dash curve*). **a** The refolded protein was loaded onto the S column and adsorbed by resin. The fraction containing a high concentration of imidazole flowed through the column and formed a constant absorption at 280 nm (*Peak 1*). The rmCXCL14 was eluted (*Peak 2*) when the conduction came to 35 mS/cm. **b** The high salt protein solution was loaded onto the G25 column, and the protein (*Peak 3*) and saline (*Peak 4*) were separated. **c** Peak 3 fraction was loaded onto the Q column and protein rmCXCL14 flowed through without binding to resin

Discussion

In the present study, we reported a method of expression and purification of recombinant mouse CXCL14. Briefly, the fusion CXCL14 protein was highly expressed in *E. coli*, and denatured and primarily purified by nickel affinity, and further purified using ion exchange after refolding.

The denaturation and refolding were the key operations and the outstanding features of the method. Firstly, the denaturation of target protein was necessary in this craft. Although about 50% of the induced CXCL14 protein existed in cytosol and was soluble in PBS

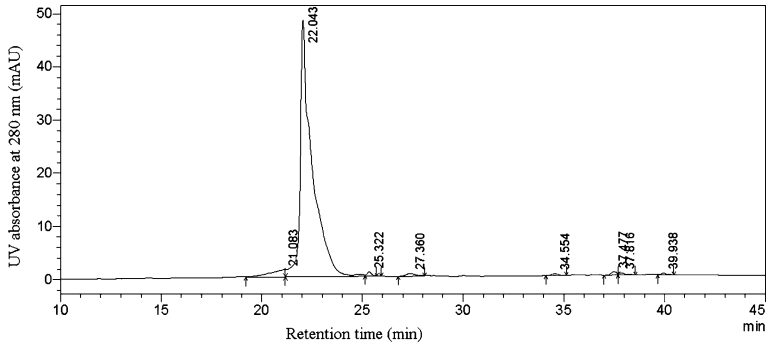

Fig. 5 The RP-HPLC spectrum of purified rmCXCL14. The purity of prepared rmCXCL14 was analyzed by RP-HPLC, and the result showed a single peak with a retention time of 22.04 min which contributed >95% UV absorbance of the sample

buffer after released from cell by ultrasonication, the protein showed unexpected features. (1) Only a part of the soluble protein (10%) could not be adsorbed by nickel resin efficiently, indicating that the protein molecules were polymerized so that obstructed the affinity adsorption. (2) The eluted protein from nickel affinity chromatography was unstable in variant dialysis solutions, such as PBS and Tris buffer, and precipitated after dialysis. A possible reason might be the mismatched intramolecular disulfide bonds established or even intermolecular bonds, since nonreducing SDS–PAGE revealed a series of rmCXCL14 polymers with varied degree of polymerizations (not shown), while after denaturation and refolding, the protein mostly existed in a monomer form [14, 15]. Secondly, we employed uncommon renaturation craft, in which the renaturation buffer was added into protein solution gradually rather than reversely as usual. In most purification method, denatured protein was added into a large volume of renaturation buffer to prevent the polymerization between molecules. However, the usual method did not work in our experiment. When the denatured CXCL14 protein dropwisely added into renaturation buffer, almost all protein precipitated soon. On the contrary, when the renaturation buffer was added into denatured protein, 40% CXCL14 protein was recovered as soluble protein. This craft is also suitable for purification of the homologous gene, CXCL4 (not published). The success of the renaturation craft is possibly due to that the high concentration of denaturation reagents (guanidine hydrochloride) that prevented the polymerization between monomers [16]. The

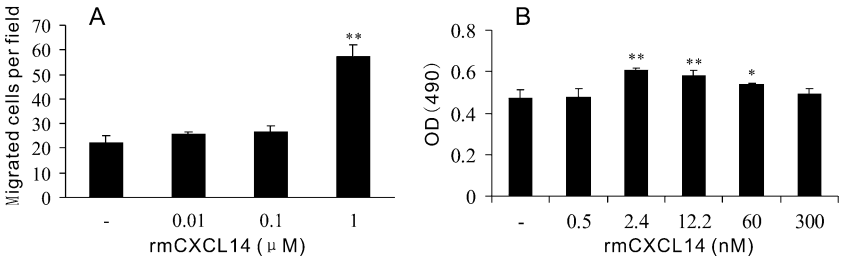

Fig. 6 Result of bioactivity assay of rmCXCL14. **a** Freshly isolated peripheral blood monocytes showed chemotaxis responses to the prepared rmCXCL14 at 1 μM concentration ($p<0.01$). **b** MTT cell proliferation assay revealed that the prepared rmCXCL14 at concentration 2.4~60 nM could significantly promote the proliferation of NIH-3T3 ($p<0.01$). The p value of significant difference was indicated by *<0.05 and **<0.01

two crafts ensured a high recovery rate (21.6%) and refolding efficiency. After the following routine procedures, total 18-mg target protein could be obtained from 2-L culture (Table 1).

No paper about large-scale production of CXCL14 protein was found while searching PubMed database. The 25-μg pack commercial product of rmCXCL14 is available in R&D company which is not sufficient for even a normal in vivo experiment. Compared to the rmCXCL14 of R&D company, the protein produced according to our method has the equal biological activity and much less endotoxin level (<0.058 EU/μg in our protein vs. <1.0 EU/μg in R&D's). The shortcoming of the protein produced using our method is that it is fused with a 20 AA peptide (Fig. 1), which could be cleaved by thrombin if necessary [17], though it does not affect the bioactivity.

The biological activity is the most important character for protein preparation; therefore, we measured the bioactivity of the purified rmCXCL14 by two different methods. Firstly, the monocyte chemotaxis was the earliest discovered bioactivity of CXCL14. Kurth et al. examined the monocyte chemotaxis activity of chemical synthetic CXCL14, and the result showed that CXCL14 exhibited significant chemotaxis to freshly isolated monocyte when the concentration reached 1 μM [3]. We repeat the chemotactic assay using our prepared protein and got a consistent result (Fig. 6). Secondly, the proliferation-promoting effect of CXCL14 on NIH3T3 cell was discovered on a NIH3T3 cell line which was stably transfected with a CXCL14 expression plasmid [18]. The proliferation promoting effect of CXCL14 was also discovered on an immortalized human fibroblast (BJhTERT cells) by incubating with recombinant human CXCL14. The effect showed a bell-shaped curve against the protein concentration, and was highest at 25 ng/ml (~2 nM). Inspired by the study, we assayed the bioactivity of our rmCXCL14 by incubating it with a mouse fibroblast, NIH3T3 cell, and obtained a consistent result. The effect also showed a bell-shaped curve against protein concentration, with the highest point at 2.4 nM (Fig. 6). The comparisons indicate that the purified rmCXCL14 has equal bioactivity with the chemical synthetic and the commercial recombinant protein.

In summary, we reported a purification method for mouse recombinant CXCL14 protein, by which milligrams of high purity (>95%) protein with bioactivity, and low level endotoxin could be produced in four workdays. The ability to produce milligram quantities of bioactive rmCXCL14 with simple steps will certainly facilitate the in vivo study of its functions. The purification strategy should also have reference values for production of human protein at a large scale.

Table 1 Purity and yield in each purification step

Purification step	Total protein (mg)	rmCXCL14 (mg)	rmCXCL14 (%)	Protein yield (%)
Cell lysate	1,600	165	10.3	100
Ni+ affinity	97	87.3	90.0	52.9
Reduction and renaturation	38.2	35.6	93.2	21.6
Cation ion exchange	23.3	22.6	97.0	13.7
G25 desalination	19.9	19.3	97.0	11.7
Anion–ion exchange	18.8	18.2	97.0	11.0

The protein concentration after each step was determined by Bradford, and purity of protein was evaluated by SDS–PAGE or RP-HPLC

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