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RESEARCH ARTICLE

SOLUBLE EXPRESSION AND PURIFICATION OF HUMAN MONOCYTE CHEMOATTRACTANT PROTEIN-1 IN ESCHERICHIA COLI VIA FUSION WITH A SMALL UBIQUITIN-LIKE MODIFIER

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ABSTRACT

Human monocyte chemoattractant protein-1 (hMCP-1) is one of the main chemokines that controls the migration and infiltration of monocytes and lymphocytes. Both hMCP-1 and its receptor are involved in various diseases, including atherosclerosis, cancer, and rheumatoid arthritis. Therefore, recombinant hMCP-1 (rhMCP-1) can be used to help develop therapeutic modulators of these diseases. However, rhMCP-1 often exists as an inclusion body in *Escherichia coli* expression systems and commercially available rhMCP-1 is expensive. In our current study, a 228-bp gene that encodes hMCP-1 was synthesized. Codon usage was optimized to include the codons used in highly expressed *E. coli* genes. The synthetic hMCP-1 gene was cloned into ap ET SUMO (small ubiquitin-like modifier) vector and overexpressed in *E. coli* as anintact, soluble protein. Because rhMCP-1 was easily solubilized, no furthersolubilization steps were needed for purification and no additional tagging residues were introduced into the rhMCP-1 gene product. Our purified rhMCP-1 demonstrated similar chemotactic properties and molecular weight as commercially available rhMCP-1. Hence, our SUMO fusion system is a promising and efficient approach for scaling-up the soluble expression of rhMCP-1.

INTRODUCTION

Monocyte chemoattractant protein-1 (MCP-1) is a relatively basic chemokine that regulates the recruitment of leukocytes, including lymphocytes andmonocytes, to inflammatory sites (Carr *et al*., 1994; Gunn *et al*., 1997; Loetscher *et al*., 1994).Mature human MCP-1 represents the C-terminal sequence of a 99-amino-acid-long precursor proteinand is glycosylated, but glycosylation does not affect its chemotactic activities(Needham *et al*., 1996).Its ability to induce monocyte infiltration influences several inflammatory diseases, suggesting that MCP-1 inhibitors may affect these diseases. Obtaining the large amount of recombinant MCP-1 required to study and understand the MCP-1 structure-function relationship is the first step toward designing therapeutic modulators of inflammatory diseases.

As previously reported, biologically active recombinant human MCP-1 (rhMCP-1) can be produced via the*in vitro* refolding of *Escherichia coli*-produced inclusion bodies (Be all *et al*., 1992; Reid *et al*., 2006). However, the refolding procedure is cumbersome and time-consuming. Recently, small ubiquitin-like modifier (SUMO) fusion technology was described (Marblestone *et al*., 2006; Yan *et al*., 2009).When fused at the N-terminus of other proteins, SUMO can fold and protect these proteins via its chaperoning properties, and can therebybeused as a tag for the heterologous expression of recombinant proteins. The SUMO fusion tag can enhance the expression and solubility of proteins in *E. coli*. In addition, thehexahistidine (6×His) SUMO fusion construct facilitates purification via Ni-NTA chromatography (Butt *et al*., 2005), and target proteins can maintain native N-terminal amino acids after cleavage using SUMO protease. In our current study, our goal was to develop an effective method to improve the production of the soluble form of active MCP-1 without refolding or solubilization. For this purpose, a 228-bp gene that encodes mature hMCP-1 was synthesized, cloned into the pET SUMO plasmid, and over expressed in *E. coli* BL21 (DE3) cells. Afterwards, ahigh-yield method for the soluble expression of active hMCP-1 with biological activity was established.

MATERIALS AND METHODS

Chemical synthesis of the hMCP-1 gene and the construction of expression vectors

The synthetic hMCP-1 gene was designed according the amino-acid sequence of mature hMCP-1 (Zhang *et al*., 1996)

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using the following strategies. A total of 6 oligonucleotides, which varied in length from 78-79 bp, were assembled and ligated into pUC18 via the *Eco* RI and *Hind* III restriction sites. The hMCP-1 gene was optimized to include the codons used in highly expressed *E. coli* genes. The final synthetic hMCP-1 gene, which was designated as pHMCP-1, was used for gene expression. To construct the expression vectors, the gene encoding mature hMCP-1 was amplified from pHMCP-1 using PCR and the following primers: forward, 5'-CAGCCGGACGCTATCAACGCT-3'; reverse, 5'-TTAGGTTTTCGGGGTCTGGGT-3'. The resulting PCR product was separated using 1.5% gel electrophoresis, purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany), and ligated into the linearized pET SUMO expression vector (Invitrogen, Carlsbad, CA). The ligation mixture was introduced into *E. coli* bacterial strain Mach1^{TM}-T1^{R} (Invitrogen) to propagate the recombinant plasmid. The product containing the inserted fragment was confirmed using DNA sequencing.

rhMCP-1 fusion protein expression

The pET SUMO-hMCP-1 construct was introduced into *E. coli* BL21 cells (DE3) (Invitrogen). The recombinant strain was cultured in LB medium supplemented with 50 μg/ml kanamycin at 37°C with shaking (180 rpm) until an OD_{600}value of 0.5 was achieved. Afterwards, IPTG was added to a final concentration of 0.3 mM and the mixture was cultured overnight at 18°C to induce the expression of therhMCP-1 fusion protein. To obtain the recombinant protein, cells were harvested and disrupted by vigorous agitation in SoluLyse buffer (Genlantis, San Diego, CA) with 5 mM β-mercaptoethanol (β-ME), 500 mMNaCl, and EDTA-free protease inhibitor cocktail tablets (Roche, Penzberg, Germany). After disrupting the cells, the insoluble pellets and soluble supernatant fraction were separated by centrifugation at 27,000 ×*g* for 20 min. Both the supernatant and pellets were analyzed using SDS-PAGE.

Purification of the fusion protein

The solubilized SUMO-hMCP-1 fusion protein was incubated with Ni-NTA resin (Invitrogen) at 4°C overnight. The resin-bound proteins were applied to a miniature column, washed in 50 mM sodium phosphate buffer (pH 8) with 20 mM imidazole and 5 mM β-ME, and eluted in wash buffer that contained increasing concentrations of imidazole (50-300 mM). One ml fractions were collected and analyzed using 10% SDS-PAGE. The fractions containing the fusion protein were combined and dialyzed overnight at 4°C against 20 mMTris-Cl (pH 8) containing 150 mMNaCl.

Cleavage and purification of rhMCP-1

The fusion protein (1μg) and SUMO protease (1 U; expressed and purified as described by Huaping *et al*., 2007) were incubated in reaction buffer (20 mMTris-Cl [pH 8.0] with 150 mMNaCl and 1 mM DTT) at RT for 4 h. The sample was cleaved by the SUMO protease and desalted with 50 mM sodium phosphate buffer (pH 8) and 5 mM β-ME, then applied to a Ni-NTA column to remove the 6×His-tagged carrier and undigested fusion proteins. The purified rhMCP-1 was dialyzed against PBS and analyzed using SDS-PAGE and western blot using rabbit anti-human MCP-1 primary antibody (diluted 1:2000; Cell Signaling, Danvers, MA) followed by goat anti-rabbit, HRP-linked secondary antibody (diluted 1:1000; Cell Signaling). Finally, the purified proteins were passed through an endotoxin-removing column (Thermo Scientific, Waltham, MA). The purified protein was filtered through a 0.22-μm filter (Millipore, Billerica, MA) and stored in PBS containing 0.1% BSA at -80°C until use.

Culturing THP-1 cells

The monocytic THP-1 cell line was routinely grown in RPMI-1640 medium supplemented with 2 mM glutamine, 25 mM HEPES, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were incubated at 37°C in a humid incubator containing 5% CO_2.

MCP-1-mediated migration assay

THP-1 chemotaxis was measured in terms of the number of THP-1 cells that migrated through the filter. Twenty-four-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) with 8-μm pore cell culture inserts (Becton Dickinson Labware) were used in this assay. Commercially available hMCP-1 (R&D Systems, Minneapolis, MN) and purified hMCP-1 were diluted in fetal bovine serum-free RPMI-1640 medium, and 700 μl diluted protein was added to each well. THP-1 cells were suspended in fetal bovine serum-free RPMI-1640 medium at a density of 2.5×10^6 cells/ml, and 200 μl cell suspension was placed on top of the inserts. Migration was allowed to proceed at 37°C overnight. After this time, cells which had moved into the lower compartment were evenly suspended, and 10 μl was stained with usingtrypan blue. Cells were then counted using a light microscope.

RESULTS AND DISCUSSION

Chemical synthesis of the hMCP-1 gene and cloning into the pET SUMO vector

In recent years, the results of numerous animal experiments have suggested the future therapeutic benefits of MCP-1, including promoting healing in inflammatory intra-aneurysmal tissue (Hoh *et al*., 2011) and diabetic wounds (Wood *et al*., 2014). Costs will hopefully drop and MCP-1 will eventually become as affordable as other recombinant products, enabling its use in the indicated patient population. To obtain a large amount of highly purified intact hMCP-1, we used the following strategies. First, to avoid the time-consuming steps for solubilization, refolding, and alkaline lysis of the insoluble rhMCP-1 protein found in bacterial debris fractions, we synthesized the hMCP-1 gene using codons that are exploited in highly expressed *E. coli* genes. Second, we expressed SUMO as a fusion protein using the pET SUMO expression vector system in order to increase hMCP-1 solubility.

The synthetic hMCP-1 gene was initially constructed using plasmid pUC18 as the cloning vector. hMCP-1 cDNA encodes a 99-amino-acid-long precursor protein with a 23-amino-acid signal peptide and a 76-amino-acid mature protein (Deshmane *et al*., 2009). A total of 6 synthetic oligonucleotides were used to assemble the gene segment that corresponded to the mature protein. The designed sequence of the hMCP-1 coding region in pHMCP-1 is shown in Fig. 1. The protein expression vector used in this study was the pET SUMO plasmid. This vector contains a 6×His tag followed by SUMO, a yeast-derived protein with a tertiary structure that is specifically recognized by the SUMO protease, which cuts off conjugated SUMO from the target proteins (Mossessova and Lima 2000). The hMCP-1 coding region in pHMCP-1 was amplified by PCR and ligated into the pET SUMO expression vector using the TA cloning method. The recombinant pET SUMO-hMCP-1 plasmid sequence was confirmed using DNA sequencing. This expression system produced the 6×His-SUMO-hMCP-1 protein.

DNA sequence of the synthetic hMCP-1 gene in pHMCP-1

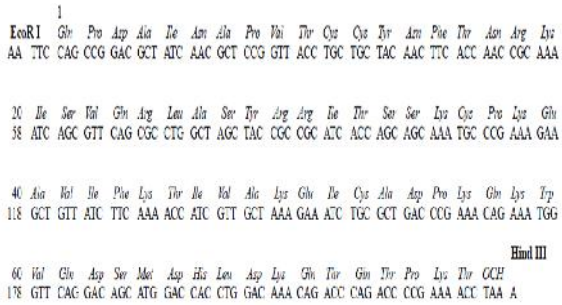

Figure 1 The numbers on the left refer to amino acids (upper) and nucleotides (lower). Position 1 of the amino acid sequence corresponds to the first amino acid (Gln) of the mature hMCP-1.

Expression and purification of the SUMO-hMCP-1 fusion protein

The pET SUMO-hMCP-1 construct was introduced into the *E. coli* bacterial strain BL21 (DE3), and a pilot expression test was performed.

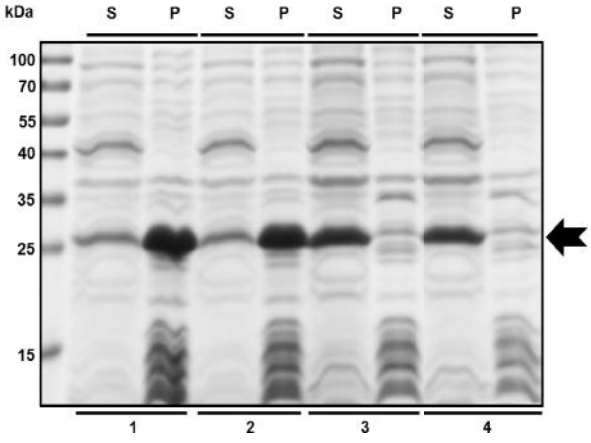

Figure2 Numbers 1-4 indicate the induction conditions: 1) 0.3 mM IPTG at 37°C for 3h; 2) 1 mM IPTG at 37°C for 3h; 3) 0.3 mM IPTG at 18°C overnight; 4) 1 mM IPTG at 18°C overnight. The arrow indicates the locations of the fusion proteins. S, supernatant; P, pellet.

After induction with IPTG at 37°C for 3 h (or overnight at 18°C), the cell pellets were disrupted by vigorous agitation in lysis buffer. The results show that the fusion protein was efficiently overexpressed in its soluble form in the host following induction with 0.3 mM IPTG at 18°C (Fig. 2). Because cold induction enhances the solubility of recombinant proteins (Qing *et al*., 2004), decreasing the temperature from 37°C to 18°C increased the expression of SUMO-hMCP-1 as a soluble protein. In addition, no detergents or further refolding orsolubilization steps were needed throughout the entire protein purification process.

SDS-PAGE analysis of the expressed fusion proteins

The inclusion of the 6×His tag at the N-terminus of the fusion protein allowed for a simplified purification procedure with the lysate supernatant using Ni-NTA chromatography. Most proteins without the 6×His tag were cleared by the wash buffer, and 6×His-tagged SUMO-hMCP-1 was eluted by the elution buffer (50 mM sodium phosphate buffer [pH 8], 5 mM β-ME, and 80-300 mM imidazole gradient) and demonstrated > 95% purity (Fig. 3). The 6×His-SUMO-hMCP-1 complex was the predominant species (approximately 25 kDa).About 144 mg fusion protein was produced per liter of bacterial culture (Table1).

SDS-PAGE analysis of the purified SUMO-hMCP-1 fusion protein

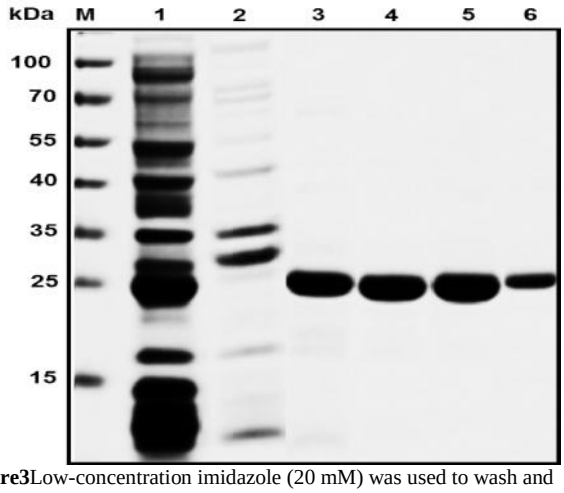

Figure3 Low-concentration imidazole (20 mM) was used to wash and further remove any bacterial contaminants. The eluted protein demonstrated > 95% purity on SDS-PAGE electrophoretic analysis. *Lane M*, protein markers; *lane 1*, low-imidazole wash; *lanes 2-6*, eluates obtained at the respective imidazole concentrations of 50, 80, 100, 150, or 300 mM.

Table 1 Isolation of recombinant hMCP-1 from the pET SUMO-hMCP-1 fusion protein

Purification step	Total protein (mg)	Fusion protein (mg)	Recombinant hMCP-1 (mg)	Purity(%)[d]
Supernatant after cell lysis	1267[a]	228[b]	89[c]	NA
SUMO-hMCP-1 after affinity chromatography	NA	144[a]	56[c]	> 59
Recombinant hMCP-1 after dialysis and cleavage	NA	NA	13[a]	> 79

Estimations are based on 1 l of bacterial culture.
NA not applicable.
[a] Determined using the BCA assay.
[b] The amount of SUMO-hMCP-1 fusion protein was estimated in total proteins using SDS gel scanning.
[c] The amount of recombinant hMCP-1 was calculated as the fraction of SUMO-hMCP-1 fusion protein.
[d] Protein purity was estimated using SDS gel scanning.

Cleaving the SUMO-hMCP-1 fusion protein and purifying rhMCP-1

Cleaving the purified SUMO-hMCP-1 fusion protein was achieved using the SUMO protease and confirmed using SDS-PAGE (Fig. 4). The mixture was subsequently re-applied to a Ni-NTA column to remove 6×His-tagged SUMO and the SUMO protease. Taking advantage of the affinity of 6×His for nickel resin, 6×His-tagged SUMO was separated from the pure protein using a nickel column. Pure, untagged hMCP-1 was collected in the wash step of the separation (lane 3, Fig. 4). Cleaved 6×His-tagged SUMO was retained on the column until elution with 300 mM imidazole (lane 4, Fig. 4). SDS-PAGE indicated that hMCP-1 was successfully purified and demonstrated > 97% purity (lane 3, Fig. 4; lane 1, Fig. 5A). A BSA band was observed because 1 µg of commercially available hMCP-1 contains 50 µg BSA as the carrier protein (lane 2, Fig. 5A**)**. We confirmed MCP-1 using western blot analysis, and rhMCP-1 obtained using our purification protocol demonstrated a similar molecular weight as commercially available hMCP-1 (lanes 1-2, Fig. 5B). rhMCP-1 sequencing yielded the expected N-terminal sequence without the additional tagged residues (data not shown). Finally, the purified recombinant hMCP-1 was produced at a yield of 13 mg/l (Table 1).

SDS-PAGE analysis of the SUMO-hMCP-1 fusion protein that was cleaved using the SUMO protease and rhMCP-1 purification

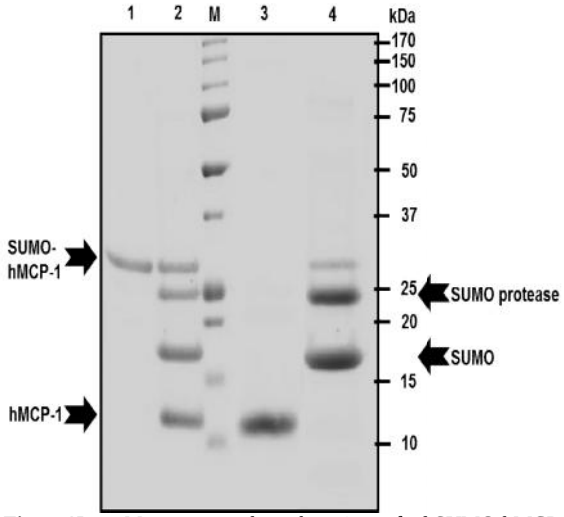

Figure4*Lane M*, protein markers; *lane 1*, purified SUMO-hMCP-1 fusion protein; *lane 2*,mixture of SUMO and hMCP-1 recombinant proteins after cleavage using SUMO protease; *lane 3*, purified rhMCP-1 was collected in the wash step; *lane 4*, SUMO and SUMO protease were eluted using 300 mM imidazole.

Confirmation of hMCP-1 purity and identity

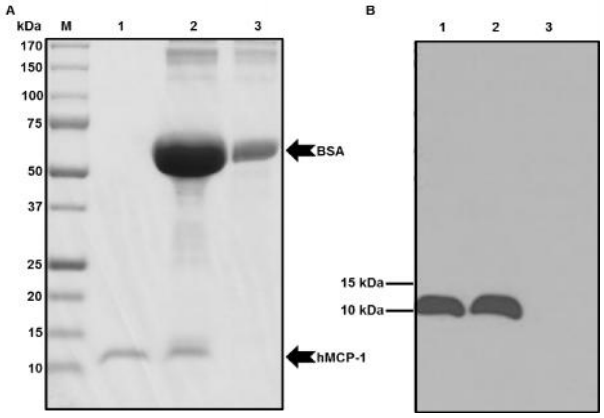

Figure5(A) rhMCP-1 purity was determined using SDS-PAGE. **(B)** The identity of the purified protein was confirmed using western blot analysis with the anti-MCP-1 antibody. *Lane 1*, purified rhMCP-1; *lane 2*, commercially available hMCP-1; *lane 3*, commercially available BSA.

Biological activity of rhMCP-1

A 24-well migration assay was used to determine rhMCP-1 activity. The activity of MCP-1, a chemokine that binds to β-chemokine receptor CCR2b (Paavola *et al*., 1998), was measured. Under the described experimental conditions, rhMCP-1 could induce chemotaxis in monocytic THP-1 cells in a dose-dependent manner. In addition, we were able to demonstrate that our purified rhMCP-1 and commercially available hMCP-1 equally promote chemotaxis in these cells (Fig. 6).

Biological activity of the purified hMCP-1

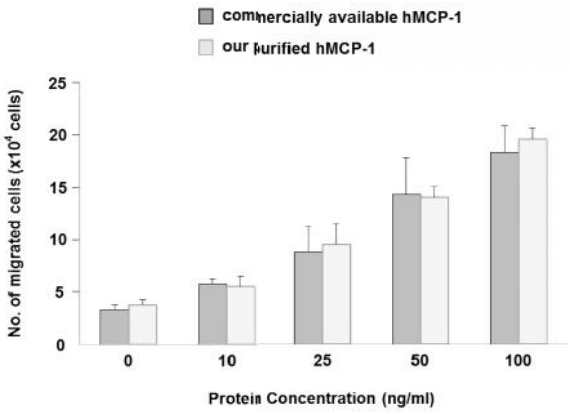

Figure6 The ability of MCP-1 to induce the migration of THP-1 monocytic cells was measured as described in the Materials and methods section.

In our present experiments, SUMO-hMCP-1 was cleaved using SUMO protease, which then released the recombinant protein with a native N-terminus (a prerequisite for maintaining MCP-1 activity) (Butt *et al*., 2005; Zhang *et al*., 1996). The most significant advantage of our SUMO fusion technology over other methods is that no residual amino acids were left on the protein of interest (Yan *et al*., 2009). This is particularly important for producing proteins that depend on a specific N-terminus for activity (e.g., chemokines). We previously used a protein disulfide isomerase fusion expression system to over express soluble rhMCP-1 in *E. coli* (data not published). In

order to cleave the tag, tobacco etch virus protease was used, which generates a non-native N-terminal amino acid after cleaving the fusion proteins. However, the purified rhMCP-1 did not demonstrate any biological activity.

CONCLUSION

The pET SUMO expression system we here describe enabled us to generate a large amount of biologically active hMCP-1 in *E. coli* without refolding or solubilization. This is therefore a new and effective method for improving the soluble expression of biologically active hMCP-1 for use in biomedical and clinical studies.

Acknowledgments

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