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Large-Scale Purification of Human Granulocyte-Macrophage Colony-Stimulating Factor Expressed in *Bombyx mori* Pupae

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Abstract

Human granulocyte-macrophage colony-stimulating factor (hGM-CSF) acts on many different kinds of cells, including monocytes, macrophages, granulocytes, eosinophils, and multipotential stem cells. To explore further explore pharmaceutical action, we expressed hGM-CSF by the *Bombyx mori* nucleopolyhedrovirus expression system in silkworm pupae. However, purifying recombinant proteins from silkworm pupae on a large scale has been a big challenge. To establish purification methods suitable for mass production, we tried two crude preparation methods: $(NH_4)_2SO_4$ fractional precipitation and isoelectric precipitation with a combination of gel filtration and ion-exchange chromatography. The isoelectric precipitation method was found to be more efficient. With this method, we eventually obtained approx 11.7 mg of 95% pure product from 1000 g of infected silkworm pupae. The recovery of purified protein was greatly increased, by approx 40%, compared with the other method. The biologic activity of this protein was determined up to 9.0×10^6 colony-forming units/mg in the final purified product.

Index Entries: *Bombyx mori* nucleopolyhedrovirus; silkworm pupae; human granulocyte-macrophage colony-stimulating factor; purification; isoelectric precipitation; $(NH_4)_2SO_4$ fractional precipitation; target protein.

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Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that promotes the differentiation and activation of granulocytes, monocytes, macrophage, and dendritic cells and enhances the function of these cells. The various responses (i.e., division, maturation, activation) are induced when GM-CSF binds to specific receptors expressed on the surface of target cells. Mature human GM-CSF (hGM-CSF) is an acidic glycoprotein of 127 amino acid residues, with a variable molecular mass from 14.4 to 35 kDa (on sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]), depending on the degree of glycosylation. Clinical studies have suggested that hGM-CSF may result in a rapid rise in white blood cell count and is beneficial in treating many diseases such as bone marrow disease caused by cytotoxic chemotherapy *(1)*, visceral leishmaniasis *(2)*, and acquired immunodeficiency syndrome *(3)*.

Up to now, hGM-CSF has been expressed by yeast mammalian and bacterial cell expression systems *(4–6)*. At present, recombinant hGM-CSF (rhGM-CSF) for clinical application is mainly expressed in *Escherichia coli*. However, this hGM-CSF is incorrectly folded and lacks posttranslational modifications. In addition, the yeast and mammalian cell expression systems have the disadvantages of overglycosylation and high cost, respectively.

The baculovirus expression system, including the *Autographa californica* nucleopolyhedrovirus (AcNPV) system and the *Bombyx mori* nucleopolyhedrovirus (BmNPV) system, has been proved to be a very effective and versatile tool for the expression of heterologous proteins. To date, thousands of recombinant proteins have been successfully produced by the systems *(7)*, which have the advantages of high yield and proper posttranslational modifications *(8)*.

We have successfully expressed and characterized hGM-CSF in silkworm pupae using BmNPV, which presented as a 29-kDa protein glycosylated with a high level of mannose residues *(9)*. The protein was successfully purified with a poor recovery of approx 10%. Here, we describe an efficient procedure of improved large-scale purification methods for hGM-CSF expressed in silkworm pupae.

Materials and Methods

Cell Line, Silkworm Pupae, and Culture Conditions

The BmN cell line (maintained in our laboratory), derived from the silkworm (*B. mori*) ovary cell line, was cultured in TC-100 medium (Sigma) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco-BRL) at 27°C. Silkworm pupae (*B. mori* hybrid strain Qingsong × Haoyue) were commercially available and were maintained at 2–8°C before infection.

Recombinant Baculovirus and Expression of hGM-CSF in Silkworm Pupae

The hGM-CSF gene was cloned from human embryo fibroblast tissue using reverse transcriptase polymerase chain reaction. The hGM-CSF-cod-

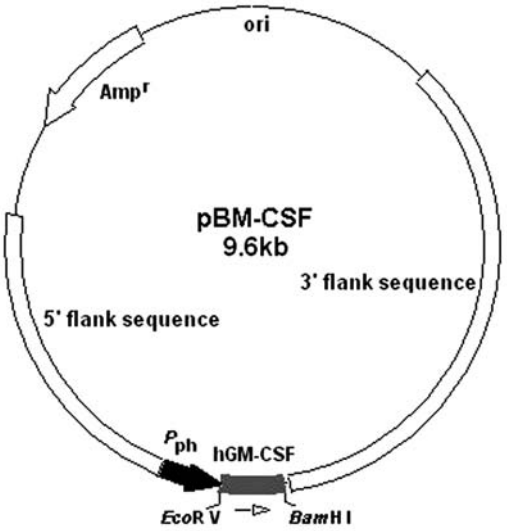

Fig. 1. Construction of recombinant transfer plasmid. This plasmid pBM-CSF contains sequences derived from pUC19 including the origin of replication (ori) and ampicillin resistance gene (Amp^r) and sequences from BmNPV genome. The 3' and 5' flank sequences indicate the 3' and 5' flank fragments of *B. mori* polyhedrin gene, respectively; *P*ph, polyhedrin promoter. The hGM-CSF gene was inserted under the control of polyhedrin promoter.

ing DNA without N-terminal signal peptide was excised and inserted into the baculovirus transfer vector pUBM-4 to produce recombinant transfer vector pBM-CSF (Fig. 1). This recombinant plasmid was cotransfected with linearized BmNPV DNA (Bm-BacPAK6) into BmN cells. Pure recombinant virus was screened according to the method of Summers and Smith *(10)*. Silkworm pupae were inoculated with an amplified virus stock for the expression of hGM-CSF. Five days after infection, the silkworm pupae were harvested and stored at –20°C.

Purification of hGM-CSF Expressed in Silkworm Pupae

Before purification, the infected pupae were defrosted, then homogenized at 20,000 rpm with a homogenizer, followed by filtration through double-layer gauze. The resulting homogenate was centrifuged at 20,000*g* for 30 min at 4°C. From top to bottom, the contents in the centrifuge tube were oil-lipid, solution, and solid residue layers. The solution layer was drawn out with a 100-mL syringe.

The obtained solution was pooled together and mixed with 4 vol of 50 m*M* Tris-HCl (pH 8.0) containing 1 m*M* EDTA and 1 m*M* dithiothreitol. The solution was adjusted to pH 5.0 with 1 *M* HAc and was maintained overnight at 4°C. The acidified samples were centrifuged at 16,000*g* for 15 min at 4°C. The pelleted protein samples were dissolved in buffer A (20 m*M* Na-phosphate, pH 7.4; 1 m*M* EDTA), and the protein solution was applied to a Sephacryl S-200 gel column (5.0 × 90 cm; Amersham Bio-

sciences) equilibrated with the same buffer. Proteins were eluted at a flow rate of 8 mL/min. The pooled active fractions were concentrated by ultrafiltration (NOVA membrane, mol wt cutoff10; Pall). The resulting samples were loaded onto an SP-Sepharose High Performance column (2.6 × 10 cm; Amersham Biosciences) equilibrated with buffer A. After a washing step with 5 column volumes, the bound proteins were eluted at 5 mL/min with 320 mL of a 0–1 *M* NaCl linear gradient. Active fractions from the SP-Sepharose column were dialyzed against buffer B (20 m*M* Na-phosphate, pH 6.8) and concentrated by ultrafiltration with the same filter membrane. The concentrated samples were eventually applied to a Mono Q column (1.0 × 10 cm; Amersham Biosciences) equilibrated with buffer B. After a washing step with 10 column volumes, the bound proteins were fractionated at 2 mL/min with 80 mL of a 0–1 *M* NaCl linear gradient. The eluted active fractions were desalted and concentrated by ultrafiltration with 20 m*M* Na-phosphate buffer, pH 7.4. The final product of purification was freeze-dried for preservation. All chromatographies were performed at 4°C.

Protein Assay

The protein concentration of the obtained samples during purification was measured with a Micro BCA Protein Assay kit (Pierce) with bovine serum albumin as a standard curve. SDS-PAGE and Western blotting were carried out using the buffer system of Laemmli *(11)*, and proteins were visualized by Coomassie brilliant blue R-250 staining. The purity of purified protein was measured by densitometry. Isoelectric focusing (IEF) was performed using an Amersham Biosciences Phasystem according to the method of Righetti and Drysdale *(12)*.

Immunoassay and Biologic Assay

The protein of interest expressed in pupae was determined and quantified using a Quankine hGM-CSF enzyme-linked immunosorbent assay (ELISA) kit (R&D System) according to the manufacturer's instructions. The immunoblotting assay for the expressed recombinant protein was performed with a primary antibody (goat antihuman GM-CSF antibody; R&D System) and a secondary antibody (horseradish peroxidase–labeled rabbit antigoat IgG; DAKOcytomation, Glostrup, Denmark). The biologic assay of rhGM-CSF was carried out with human bone marrow cells in accordance with the method described by Okamoto et al. *(13)*.

To determine the active fractions in real time during the purification, GM-CSF-dependent cell line TF-1 (maintained in our laboratory) was used to measure the biologic activity of fractionated samples. Briefly, after being washed three times with serum-free RPMI-1640 medium (Gibco-BRL), TF-1 cells were seeded into a 96-well plate at a density of 1.0×10^4 cells/well and incubated with RPMI-1640 containing 10% FCS at 37°C in a humidified atmosphere of 5% CO_2. The purified samples were diluted 10,000-fold and

then added to the corresponding wells at 10 µL/well. The cell wells without purified samples were made as a negative control. After 48–72 h of incubation, when 95% of the cells in the negative control were dead, 10 µL of MTT (50 m*M*; Sigma) was added and the incubation continued for 4 h. At the end of incubation, the medium was discarded, and 100 µL of cell lysate solution (10% SDS, 50% [v/v] dimethylsulfoxide) was added to each well. Finally, the absorbance at 450 nm was measured for evaluation of the biologic activity of rhGM-CSF samples.

Results

Preparation of Recombinant Virus and Expression of hGM-CSF in Silkworm Pupae

The recombinant transfer vector pBM-CSF (Fig. 1) was constructed according to the method described by Maeda *(8)*. Recombinant baculovirus was obtained by three rounds of plaque screening according to the method of Summers and Smith *(10)*. One thousand grams of silkworm pupae (approx 700 heads) was inoculated in 10 µL of an amplified recombinant virus stock (1×10^6 plaque-forming units/mL) per pupa between abdominal knobs on the backside. A 29-kDa recombinant protein (named Bm-hGM-CSF) was detected by Western blot. The expression level of recombinant protein was up to 100 µg/pupa, qualified by the hGM-CSF ELISA kit. The biologic activity of Bm-hGM-CSF was measured up to approx 7.0×10^6 colony-forming units (CFU)/mg in the crude extract and 9.0×10^6 CFU/mg in the purified product.

Purification of Bm-hGM-CSF

Bm-hGM-CSF was purified with a combination of crude purification and three-step column chromatography. On the basis of known p*I* of Bm-hGM-CSF *(9)*, we adjusted the pH of pupal homogenate to 5.1 so as to obtain as much of the target protein precipitate as possible. As Fig. 2 shows, the p*I*s of precipitated proteins were near 5.2 (between 4.0 and 6.0).

To remove impurities as much as possible, especially the colored materials, which are easily adsorbed by ion-exchange media and difficult to elute, the precipitated protein samples were applied to a gel filtration column. This purification step is helpful in removing most of the predominant, small molecular portion of colored materials. As shown in Fig. 3A, active fraction (peak B) was almost completely separated from small molecular materials (peak C). The latter largely contained abundant colored materials, which presented a color zone in the gel column, and small molecular polypeptides. However, peak B partially overlapped with peak A. As a result, a few colored materials of large molecule remained in the active fraction. The obtained samples were then loaded onto a cation-exchange SP-Sepharose column, from which these colored materials could be eluted relatively easily. Active fractions were eluted from the SP column

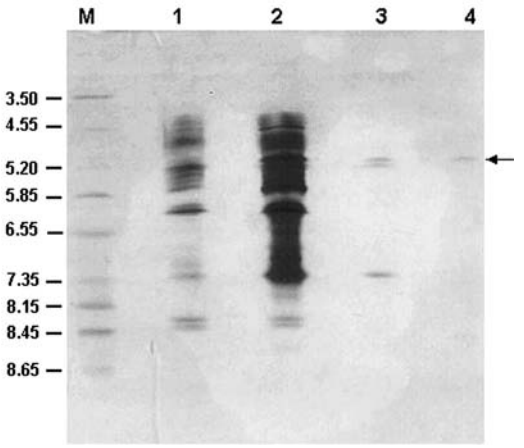

Fig. 2. IEF analysis for p*I* of purified rhGM-CSF on a Coomassie blue–stained gel. M, broad p*I* calibration markers; lane 1, sample of isoelectric precipitation; lane 2, active fraction of gel filtration; lane 3, active fraction of SP-Sepharose; lane 4, active fraction of Mono Q4. The arrow indicates the purified protein.

at 0.4 *M* NaCl (Fig. 3B). In this purification step, a significant increase in the purification factor up to 1047 was observed with maximal loss of 31%. The final purification was performed on an anion Mono Q column. The target protein was eluted with 0.45–0.6 *M* NaCl (Fig. 3c). In this last step, a purified protein was yielded with a very high purification factor of 7400 and a final purity of approx 95% (Table 1).

Characterization of Purified Bm-hGM-CSF

To identify the purified protein, the active fraction obtained in each purification step was analyzed by SDS-PAGE and Western blot. A protein band of approx 29 kDa reacting against the anti-hGM-CSF IgG was revealed (Fig. 4). IEF analysis suggested that the p*I* of purified protein was about 5.1 (Fig. 2).

To check activity loss of the target protein during purification, the active fractions were also bioassayed with human bone marrow cells. The biologic activity of Bm-hGM-CSF increased slightly at the beginning of purification, arrived at the peak in the gel filtration step, and decreased slowly at the end of purification (Fig. 5). Because many sophisticated components exist in the pupal body, in our opinion, some unknown factors in the crude extract may affect the biologic activity of Bm-hGM-CSF.

Discussion

Over the last 20 yr, it has been proved that recombinant baculovirus is a very useful means of expressing heterologous proteins. Since its first introduction *(14)*, the BmNPV expression system has been used to express many proteins such as α-interferon *(14)*, hepatitis B virus surface antigen

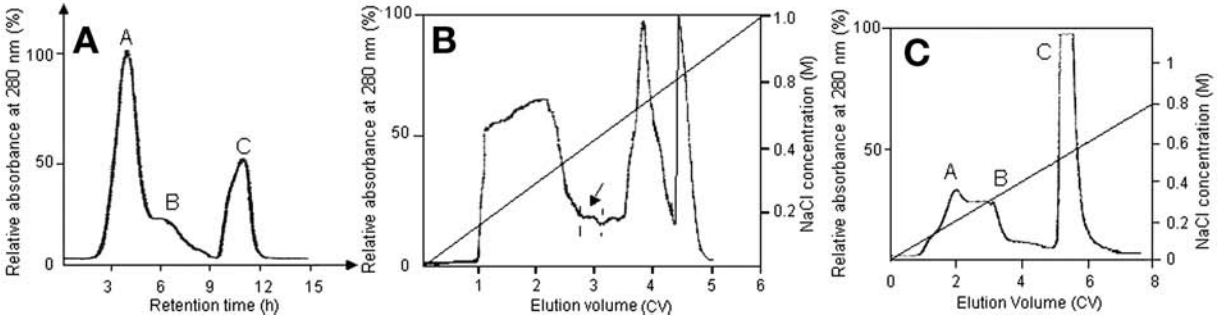

Fig. 3. Purification procedure of Bm-hGM-CSF: profile of **(A)** gel filtration; **(B)** SP-Sepharose; **(C)** Mono Q ion-exchange chromatography. The active fractions were located at peak B (A), the arrow-indicated peak (B), and peak C (C). CV, column volume.

Table 1
Purification Profile of Bm-hGM-CSF

Purification step	Volume (mL)	Protein (mg)	Specific activity (μg/mL)	Recovery (%)	Purification factor (fold)
Diluted homogenate	3500	628,500	20	100	1
Isoelectric precipitation	—	106,000	—	73	4.33
Sephacryl S-200	420	4452	96.7	58	81.8
SP Sepharose	30	162	630	27	1047
Mono Q	9.5	11.7	1015	13.8	7400

(15), human growth factor *(16)*, angiostatin (k1-3) *(17)*, lactoferrin *(18,19)* and hPON 1 Q *(20)*. All of these BmNPV-based recombinant proteins are highly similar to their native forms. Usually, silkworm cells or larvae are used as a host for recombinant virus in these systems. The latter are preferred because of their high yield and low cost. However, the feeding of silkworm larvae is severely time limited because their food is mainly mulberry leaves, which are produced only between April and November. In addition, the harvesting of larvae hemolymph, which has to be completed in a short time, is a time-consuming and laborious task. These limitations make the larvae unsuitable for mass production of recombinant proteins.

Compared with silkworm cells or larvae, silkworm pupae, which need no feeding, could be commercially available in large quantities and directly homogenized for purification. If stored at an appropriate low temperature such as 2–8°C, silkworm pupae can survive for a long time and can be taken out of storage to infect with recombinant virus at any time. Thus, silkworm pupae may be more suitable for mass production of the target protein as a bioreactor.

Some proteins have been successfully expressed in silkworm pupae *(21–24)*. In particular, approx 205 μg of functionally active rPLF was obtained from a single pupa *(18)*. This case suggests that heterologous proteins also can be highly expressed in silkworm pupae. In our work, we chose silkworm pupae as a bioreactor for expression of hGM-CSF. The expression level of hGM-CSF in pupae was up to 100 μg/pupa (approx 100 μg/mL), at least 20-fold higher than in insect cell BmN or Sf-9 culture (data not shown). The disadvantage of BmNPV expression systems using *B. mori* pupae as a bioreactor is the difficulty of achieving product purification, as shown in Table 1 (only 13.8% of final recovery). Therefore, the key is to establish a purification method for mass production of recombinant proteins with BmNPV expression systems.

Our laboratory has expressed hGM-CSF in Sf-9 insect cells using AcNPV *(25)*, and then the protein was also expressed in the silkworm lar-

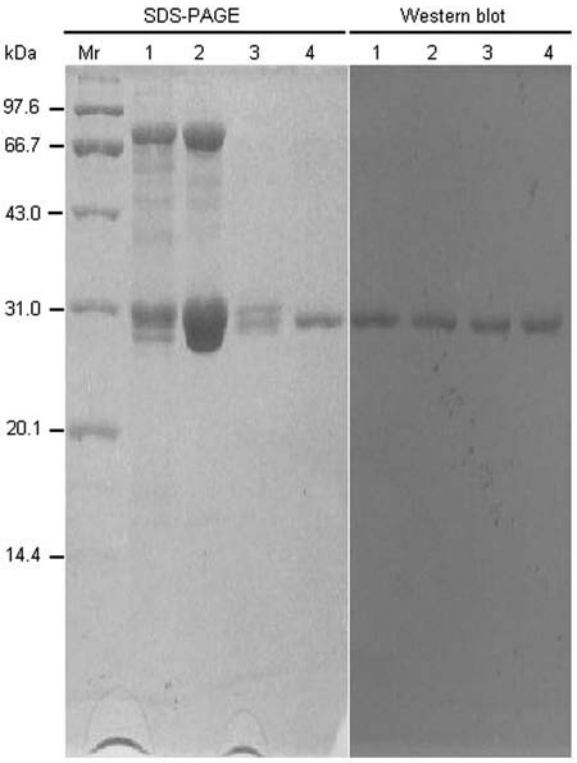

Fig. 4. SDS-PAGE and Western blotting analysis of rhGM-CSF samples purified from pupae. Proteins were visualized on a Coomassie blue–stained gel. Mr, protein size markers; lane 1, sample of isoelectric precipitation; lane 2, active fraction of gel filtration; lane 3, active fraction of SP-Sepharose; lane 4, active fraction of Mono Q.

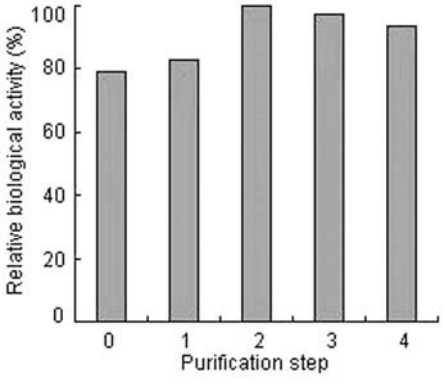

Fig. 5. Biologic activity of Bm-hGM-CSF in each purification step. Step 0, pupal homogenate; step 1, sample of isoelectric precipitation; step 2, active fraction of gel filtration; step 3, active fraction of SP-Sepharose; step 4, active fraction of Mono Q.

vae using BmNPV and successfully purified using antibody affinity chromatography *(26)*. However, neither of these systems is suitable for mass production of recombinant proteins. Some methods were established for purification of recombinant proteins from the hemolymph *(23,26–29)*. We have purified >90% pure rhGM-CSF from the hemolymph with a combined method of salt precipitation, gel filtration, and Ni-chelating Sepharose chromatography (unpublished data). These methods, however, are only applicable for relatively clean materials, not for pupae. The latter contain many colored materials of various molecules and many protein components pertaining to physiologic and biochemical reactions. To date, only a few pupae-expressed recombinant proteins have been successfully purified.

To establish large-scale purification methods for mass production of Bm-hGM-CSF, we tried two crude preparation methods of salt precipitation and isoelectric precipitation. At the beginning, we used $(NH_4)_2SO_4$ fractional precipitation for purification of the target protein. This method had the advantage of obtaining as much target protein as possible. However, at the same time, many unwanted proteins were also precipitated together with the target protein, which made the purification more difficult. By contrast, isoelectric precipitation was able to greatly decrease unwanted proteins, obtain a high yield of target protein, and simplify the process of purification. Compared with the findings of a previous report *(9)*, the biologic activity of purified product was higher, perhaps owing to the improvement in the processing method for purified active fractions. In addition, the removal of most of the small molecular colored materials in gel filtration greatly increased the recovery in the ion-exchange chromatography step. Finally, the protein of interest was purified with an improved recovery of about 40% more than the former. The in vivo biologic activity of purified rhGM-CSF was confirmed without impairment (unpublished data).

Conclusion

We successfully established an efficient procedure of purification methods for mass production of Bm-hGM-CSF. This work may offer a feasible means of industrial production of recombinant biomedical proteins on a large scale in silkworm pupae. Further studies are needed to improve overall recovery of the target protein.

Acknowledgments

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