Biotechnology and Bioprocess Engineering **2004**, 9: 423-427

A Simple Purification Procedure of Biologically Active Recombinant Human Granulocyte Macrophage Colony Stimulating Factor (hGM-CSF) Secreted in Rice Cell Suspension Culture

Niti Sharma, Seung-Moon Park, Tae-Ho Kwon, Dae-Hyuk Kim, and Moon-Sik Yang*

Institute for Molecular Biology and Genetics, Basic Science Research Institute, Chonbuk National University, Jeonju 561-756, Korea

Abstract A simple purification procedure of bioactive human granulocyte macrophage colony stimulating factor (hGM-CSF) secreted in rice cell suspension culture has previously been described. In this study the protein was purified to apparent homogeneity with an overall yield of 80.1% by ammonium sulfate precipitation and a single chromatographic step involving FPLC-anion exchange chromatography. The purified hGM-CSF revealed at least five glycosylated forms ranging from 21.5~29 kDa, and its biological activity was independent of the glycosylation pattern. This is the first purification report of recombinant hGM-CSF to apparent homogeneity from rice cell suspension cultures.

Keywords: hGM-CSF, rice cell suspension culture, protein purification, glycosylation

INTRODUCTION

Human granulocyte macrophage colony stimulating factor (hGM-CSF) belongs to the family of four glycoproteins that are involved in the proliferation and differentiation of granulocytes, monocyte-macrophages, and related haemopoietic cells [1]. GM-CSF specifically regulates the production and function of white blood cells, thus, it is helpful in fighting infections [2]. In addition, it is an important clinical candidate for the treatment of neutropenia, aplastic anemia, cancer, and AIDS, and is also thought to reduce infection risks associated with bone marrow transplantation [2,3]. As a result of successful clinical trials, recombinant GM-CSF has been approved for clinical use in many countries [2].

Mature hGM-CSF has the form of a single chain polypeptide containing 127 amino acids with four cysteine residues that form two intrachain disulphide bonds [4,5]. It has a molecular mass of 14.7 kDa under non-glycosylated conditions, but it may vary from 18~30 kDa, depending upon the extent of glycosylation and the type of cell from which it is derived [6,7]. In humans, it is produced by multiple cell types like fibroblasts, endothelial cells, stromal cells and lymphocytes, however, the concentration of GM-CSF is quite low in these cells [2]. In order to meet the increasing global clinical demand, various attempts to increase the production of GM-CSF have been made to express it in a variety of foreign hosts such as *E. coli*, yeast, fungi, mammalian cells, and plant cells [8-12]. However, in the literature, GM-CSF production is mostly reported in the inclusion bodies [13-15], which suffers from several limitations like complex processing, poor *in vitro* renaturation, and lower yield and specific activity. Furthermore, upon comparing natural GM-CSF with the product obtained from the inclusion bodies, the latter has an added methionine in the *N*-terminal sequence which stimulates antibody production in the human body, thereby, altering therapeutic properties of the protein [16].

Nowadays, there has been a growing interest in using plant cells for the production of expensive and extremely pure recombinant proteins because they offer several advantages over the other expression systems, such as being conductive to a large scale, low cost production of proteins and having a low risk of protein contamination [17]. Since plant cell culture medium contains no added proteins, the recovery and downstream purification of the secreted protein is relatively inexpensive and easy. Moreover, plant cells can synthesize and process mammalian proteins in a similar way to the natural host. The foreign proteins expressed in plant cell cultures can be harvested from plant biomass, culture supernatant, or from a mixture of the two. Using this latest technique, hGM-CSF has been expressed in tobacco suspension cultures [12, 18,19]. However, this technique encountered secretion and instability problems, and the yield was too low for downstream purification of the recombinant protein.

To overcome this problem, we previously used the rice amylase expression system to produce rhGM-CSF in rice cell suspension culture, and we obtained a significantly

***Corresponding author**
Tel: +82-63-270-3339 Fax: +82-63-270-4334
e-mail: mskyang@chonbuk.ac.kr

higher yield of the protein than the previous experiments [20,21]. This high yield may facilitate downstream purification of the recombinant protein. In continuation of our earlier work, this study attempted to purify hGM-CSF that was secreted in a rice cell suspension culture. To our knowledge, this is the first report of rhGM-CSF, purified to apparent homogeneity from rice cell suspension cultures.

MATERIALS AND METHODS

Materials

A prepacked Mono Q 10/100 column was obtained from Pharmacia and was operated using the FPLC system (Amersham Pharmacia Biotechnology, Uppsala, Sweden). Recombinant hGM-CSF that was derived from *E. coli* was purchased from Endogene (Boston, MA, USA). All other chemicals used in this study were procured from Sigma Chemical Company (St. Louis, MO, USA).

Purification of hGM-CSF

All purification steps were performed at 4°C unless otherwise stated. About 1 liter of the culture supernatant from a thirteen-day-old rice cell suspension culture was lyophilized and dissolved in buffer A (20 mM sodium phosphate buffer containing 1 mM DTT and 0.5 mM EDTA, pH 7.2). After an overnight dialysis in buffer A, the resulting solution was centrifuged at 15,000 × *g* for 30 min. The clarified supernatant was referred to as the crude extract. The proteins were precipitated using 50~90% ammonium sulfate fractionation. The pellet obtained by centrifugation (15,000 × *g* for 30 min) was resuspended in a minimum volume of buffer A and dialyzed against the same buffer for 4 h with repeated changes in the buffer each hour. After filtering a 2 mL sample through a 0.45 μm membrane, it was loaded onto a Mono Q 10/100 FPLC anion exchange column (Pharmacia). The column (10 × 100 mm, bed volume = 8 mL) was pre-equilibrated with buffer A, and the sample was eluted (flow rate = 4 mL/min, P = 4 MPa) with a linear gradient of buffer B (buffer A containing 1 M NaCl), until a 50% salt concentration was reached. The fractions with detectable absorbance (A_{280}) were analyzed by the dot blot and were stored at -20°C until further analyses.

Analytical Methods

Total protein concentration of the samples was estimated by Bradford's method [22], using a protein assay kit (Bio-Rad, USA). Bovine serum albumin was used as a standard. SDS-PAGE was performed according to the procedures described by Laemmli [23], with a 15% acrylamide concentration in the separating gel. The protein bands were visualized by silver staining [24].

Western blot analysis samples were electroblotted onto a Hybond C Extra membrane (Amersham Pharmacia Biotechnology, UK) after SDS-PAGE. Rat-anti human GM-CSF (PharMingen Inc., CA, USA) at 1:500 and anti-rat-IgG biotin conjugate (PharMingen Inc., CA, USA) at 1:10,000 were used as the primary and secondary antibody, respectively.

For the determination of amino acid sequence, the purified proteins were blotted onto the PVDF membrane (Pall corporation, USA) then stained with Coomassie Blue R-250 [25]. The stained protein bands were excised, and their *N*-terminal sequences were determined by automated Edman degradation on a pulse liquid automatic sequencer (Applied Biosystems).

hGM-CSF-specific ELISA Assay

The activity of hGM-CSF in various fractions was measured using the hGM-CSF specific ELISA kit (Endogene, Woburn, MA, USA) according to the procedures provided by the manufacturer.

rhGM-CSF Biological Activity Assay

To determine the biological activity of rhGM-CSF, the amount of thymidine required for the growth of hGM-CSF-dependent TF-1 cells was measured [26]. Briefly, aliquots of growth-factor-starved cells that were suspended in RPMI medium supplemented with 10% FBS (HyClone Laboratories Inc., UT, USA) at 1×10^6 cells/mL, were added to each well of a microtiter plate containing the test samples of hGM-CSF. After incubating the plates for 48 h, 1 μCi of [methyl-^{3}H] thymidine (Amersham Life Science, NJ, USA) was added to each well and incubated again for 16 h. The cells were harvested using an Inotech cell harvester (Switzerland), and the tritium content was measured by liquid scintillation counting.

RESULTS AND DISCUSSION

Literature pertaining to the purification of rhGM-CSF from plant cell suspension cultures is limited. As mentioned earlier, the yield of hGM-CSF expressed in the tobacco suspension culture was too low to purify. So, for the ease of purification, a 6-His tag was added to the 3' end of GM-CSF cDNA. Fortunately, we were able to increase the production of rhGM-CSF greatly in rice cell suspension cultures [20,21]. In this paper, we present a simple procedure for rhGM-CSF purification to apparent homogeneity from rice cell suspension cultures.

Purification of rhGM-CSF

The protein was purified about twenty-eight folds up to homogeneity using only a single chromatographic step after the ammonium sulfate precipitation, which gave an overall yield of 80.1% (Table 1). Ammonium sulfate fractionation (50~90%) proved as a very useful preliminary purification step because α-amylase, the major protein from the rice suspension culture, was effectively removed by this step. After applying the desalted sample on a Mono Q anion exchange column, the sample was eluted

Table 1. Purification of rhGM-CSF from rice cell suspension culture (1 L)

Step	Total protein (mg)	Total activity* ($\times 10^5$ units)	Specific activity ($\times 10^5$ units/mg)	Yield (%)	Purification (Fold)
Crude	312.5	45.8	0.15	100.0	1
$(NH_4)_2SO_4$ ppt.(50~90%)	150.7	40.6	0.27	88.6	1.8
Mono Q column	8.7	36.7	4.22	80.1	28.1

* One unit is defined as the amount of [methyl-^{3}H] thymidine uptake per ng of recombinant hGM-CSF.

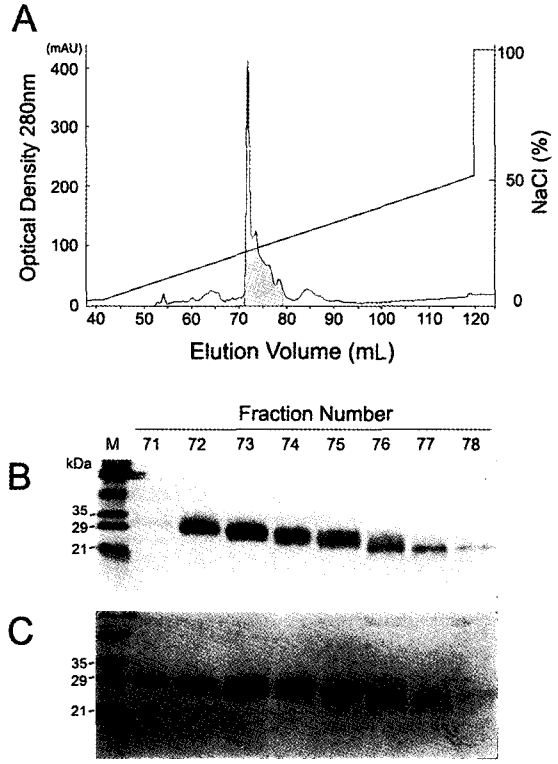

Fig. 1. A, Elution profile of hGM-CSF from Mono Q column. The protein was eluted with buffer B until a 50% salt concentration was reached. The shaded area in the profile indicates active protein fractions. B, Silver stained SDS-PAGE, and C, Western blot analysis of purified protein fractions. M, Molecular weight markers. Numbers on the left refer to the estimated sizes in kDa.

with a linear gradient of 1 M NaCl in buffer A, until a 50% salt concentration was reached. The immunoreactive hGM-CSF was eluted between 72~78 column fractions (Fig. 1A). Upon silver staining of the purified protein fractions, no other band was detected on the gel, which indicated there were no other minor impurities along with the purified protein. Silver staining and Western blot analysis revealed gradual changes in molecular mass that ranged between 21 to 29 kDa, across the elution profile (Fig. 1B, C), which indicated five different species of the purified protein. The relatively high molecular mass species were eluted first at a lower salt concentration when compared to the others. Similar patterns were also observed in the case of GM-CSF purified from human lymphocytes using RP-HPLC [27], where at least nine species, varying in molecular mass from 14.5 to 32 kDa were reported.

Heterogeneity of Purified hGM-CSF Variants

The heterogeneity observed in the molecular mass of the purified protein fractions was due to variable glycosylation reaction, such as hGM-CSF having two potential *N*- and various *O*-glycosylation sites [28], which can cause a big molecular weight shift. A similar heterogeneity in molecular mass has also been observed ranging from 14.5~50 kDa in transformed yeast cells [29], 16~35 kDa in Namalwa cells [30], 30~40 kDa in tobacco suspension culture [12], and 19~21 kDa in transgenic tobacco seeds [35].

It can be predicted that species with a molecular mass between 28~29 kDa have both *N*-glycosylated sites, while those species with a mass of 21~25 kDa have only one. In addition, the size heterogeneity of the low molecular mass variants observed suggests variability in the *O*-linked glycosylation. These results concur with earlier findings [27] where the largest GM-CSF protein observed (28~32 kDa) had both sites glycosylated, the intermediate GM-CSF protein (23~25 kDa) had one site glycosylated, and the smallest one (16~18 kDa) had neither site glycosylated.

N-terminal Sequencing

The purified rhGM-CSF was subjected to *N*-terminal sequencing, and the data for the first five amino acid residues were determined to be Ala-Pro-Ala-Arg-Ser for all purified hGM-CSF variants. This indicated that the precursor of rhGM-CSF is correctly processed at the cleavage site of a rice Amy3D signal peptide [20], thus implying the *N*-terminal of purified rhGM-CSF is identical to its natural counterpart.

Effect of Glycosylation on Biological Activity

The results obtained for the *in vitro* biological activity in various purified fractions and the recombinant standard (Fig. 2) implies that the biological activity is independent of the glycosylation pattern. Although glycosylation does not seem to be essential for the biological activ-

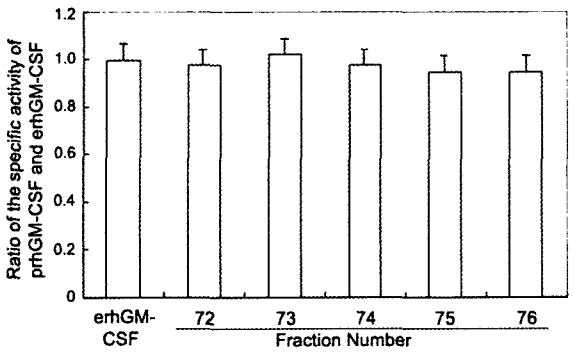

Fig. 2. Biological activity of various purified fractions of hGM-CSF. The activity was compared to commercial rhGM-CSF, which is derived from *E. coli*. Five ng/mL of rhGM-CSF from each fraction were used for the assay. Mean ± S.D., n=3. prhGM-CSF, plant derived recombinant hGM-CSF; erhGM-CSF, *E. coli* derived recombinant hGM-CSF.

ity of GM-CSF, *in vivo* or *in vitro* [31,32], some previous reports described the role of variable glycosylation as the cause of variations in biological activity, immunogenecity, and pharmacokinetics [33,34]. The state and efficiency of glycosylation is affected by various factors like protein structure, molecular interactions, and cellular environment [29]. Furthermore, plant glycosylation differs from the mammalian system in terms of having xylose and fucose in addition to the core glycans that are identical in both systems [35]. Therefore, it is still conceivable that the molecular details of posttranslational modifications, which were not tested for in this study, remain to be elucidated.

This study has clearly shown a simple purification procedure of recombinant protein from rice suspension culture. The high yield and purity of the protein that was obtained, suggest that the purification procedure adopted in this study can be employed for other therapeutic proteins.

Abbreviations hGM-CSF, human granulocyte macrophage colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; rhGM-CSF, recombinant human granulocyte macrophage colony stimulating factor; DTT, dithiothreitol; EDTA, ethylenediaminetetracetic acid; FBS, fetal bovine serum; PVDF, polyvinylidene fluoride; FPLC, fast performance liquid chromatography

Acknowledgments Fellowship granted to Dr. Niti Sharma by Chonbuk National University is gratefully acknowledged. This work was supported by the National Research Laboratory Program 2000-N-NL-C-212. We thank the Research Center for Industrial Development of BioFood Materials at Chonbuk National University for kindly providing the facilities for this research.

REFERENCES

[1] Metcalf, D. (1990) Colony stimulating factors: Discovery, development and clinical applications. *Cancer* 65: 2185-2195.

[2] Metcalf, D. (1991) Control of granulocytes and macrophages: Molecular, cellular, and clinical aspects. *Science* 254: 529-533.

[3] Dale, D. C., W. C. Liles, W. R. Summer, and S. Nelson (1995) Review: Granulocyte colony-stimulating factor: Role and relationships in infectious diseases. *J. Infect. Dis.* 172: 1061-1075.

[4] Kaushansky, K., P. J. O' Hara, K. Berkner, G. M. Segal, F. S. Hagen, and J. W. Adamson (1986) Genomic cloning, characterization, and multilineage growth-promoting activity of human granulocyte-macrophage colony-stimulating factor. *Proc. Natl. Acad. Sci. USA* 83: 3101-3105.

[5] Cantrell, M. A., D. Anderson, D. P. Cerretti, V. Price, K. McKereghan, R. T. Tushinski, D. Y. Mochizuki, A. Larsen, K. Grabstein, S. Gills, and D. Cosman (1985) Cloning, sequence, and expression of a human granulocyte/ macrophage colony stimulating factor. *Proc. Natl. Acad. Sci. USA* 82: 6250-6254.

[6] Price, G. B., J. S. Senn, E. A. McCulloch, and J. E. Till (1975) The isolation and properties of granulocytic colony-stimulating activities from medium conditioned by human peripheral leucocytes. *Biochem J.* 148: 209-217.

[7] Fojo, S. S., M. C. Wu, M. A. Gross, Y. Purcell, and A. A. Yunis (1978) Purification and characterization of a colony-stimulating factor from human lung. *Biochemistry* 17: 3109-3116.

[8] Wang, J., M. Zou, B. Huang, M. Zhang, and N. Mao (1995) Molecular cloning and expression of human granulocyte-macrophage colony stimulating factor in *Escherichia coli* system. *Chin. J. Biotechnol.* 11: 45-81.

[9] Balland, A., D. A. Krasts, K. L. Hoch, M. J. Gerhart, K. E. Stremler, and S. M. Waugh (1998) Characterization of the microheterogeneities of PIXY321, a genetically engineered granulocyte-macrophage colony-stimulating factor/ interleukin-3 fusion protein expressed in yeast. *Eur. J. Biochem.* 251: 812-820.

[10] Kim, M. J., T. H. Kwon, Y. S. Jang, M. S. Yang, and D. H. Kim (2000) Expression of murine GM-CSF from recombinant *Aspergillus niger*. *J. Microbiol. Biotechnol.* 10: 287-292.

[11] Chiou, C. J. and M. C. Wu (1990) Expression of human granulocyte-macrophage colony-stimulating factor gene in insect cells by a baculovirus vector. *FEBS Lett.* 259: 249-253.

[12] James, E. A., C. Wang, Z. Wang, R. Reeves, J. H. Shin, N. S. Magnuson, and J. M. Lee (2000) Production and characterization of biologically active human GM-CSF secreted by genetically modified plant cells. *Protein Expr. Purif.* 19: 131-138.

[13] Burgess, A. W., C. G. Begley, G. R. Johnson, A. F. Lopez, D. J. Williamson, J. J. Mermod, R. J. Simpson, A. Schmitz, and J. F. DeLamarter (1987) Purification and properties of

bacterially synthesized human granulocyte-macrophage colony stimulating factor. *Blood* 69: 43-51.
[14] Belew, M., Y. Zhou, S. Wang, L. E. Nystrom, and J. C. Janson (1994) Purification of recombinant human granulocyte-macrophage colony-stimulating factor from the inclusion bodies produced by *Escherichia coli* cells. *J. Chromatogr. A* 679: 67-83.
[15] Dianhai, L., Y. Guiyun, and Z. Lixin (1997) Purification of secretory recombinant human granulocyte macrophage colony-stimulating factor. *Chin. Biol.* 10: 132-135.
[16] Zhang, X. W., T. Sun, X. Liu, D. X. Gu, and Z. Q. Tang (1999) Production of granulocyte-macrophage colony stimulating factor (GM-CSF) by high cell density fermentation of secretory recombinant *Escherichia coli*. *Prog. Biochem.* 34: 55-58.
[17] Doran, P. (2000) Foreign protein production in plant tissue cultures. *Curr. Opin. Biotechnol.* 11: 199-204.
[18] Lee, J. H., N. S. Kim, T. H. Kwon, Y. S. Jang, and M. S. Yang (2002) Increased production of human granulocyte-macrophage colony stimulating factor (hGM-CSF) by addition of stabilizing polymer in plant suspension cultures. *J. Biotechnol.* 96: 205-211.
[19] Lee, S. Y., J. M. Cho, and D. I. Kim (2003) Stability enhancement of hGM-CSF in transgenic *Nicotiana tabacum* suspension cell cultures. *Biotechnol. Bioprocess Eng.* 8: 187-191.
[20] Shin, Y. J., S. Y. Hong, T. H. Kwon, Y. S. Jang, and M. S. Yang (2003) High level of expression of recombinant human granulocyte-macrophage colony stimulating factor in transgenic rice cell suspension culture. *Biotechnol. Bioeng.* 82: 778-783.
[21] Kwon, T. H., Y. M. Shin, Y. S. Kim, Y. S. Jang, and M. S. Yang (2003) Secretory production of hGM-CSF with a high specific biological activity by transgenic plant cell suspension culture. *Biotechnol. Bioprocess Eng.* 8: 135-141.
[22] Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
[23] Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
[24] Wray, W., T. Boulikas, V. P. Wray, and R. Hancock (1981) Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* 118: 197-203.
[25] Matsudaria, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* 262: 10035-10038.
[26] Kitamura, T., T. Tange, T. Terasawa, S. Chiba, T. Kuwaki, K. Miyagawa, Y. F. Piao, K. Miyazono, A. Urabe, and F. Takaku (1989) Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. *J. Cell Physiol.* 140: 323-334.
[27] Cebon, J., N. Nicola, M. Ward, I. Gardner, P. Dempsey, J. Layton, U. Duhrsen, A. W. Burgess, E. Nice, and G. Morstyn (1990) Granulocyte-macrophage colony stimulating factor from human lymphocytes: The effect of glycosylation on receptor binding and biological activity. *J. Biol. Chem.* 265: 4483-4491.
[28] Okamoto, M., C. Nakayama, M. Nakai, and H. Yanagi (1990) Amplification and high level expression for human granulocyte-macrophage colony stimulating factor from human lymphoblastoid Namalwa cells. *Bio/Technol.* 8: 550-553.
[29] Ernst, J. F., J. J. Mermod, J. F. DeLamarter, R. J. Mattaliano, and P. Moonen (1987) *O*-Glycosylation and novel processing events during secretion of a factor/GM-CSF fusions by *Saccharomyces cerevisiae*. *Bio/Technol.* 5: 831-834.
[30] Okamoto, M., M. Nakai, C. Nakayama, H. Yanagi, H. Matsui, H. Noguchi, M. Namiki, J. Sakai, K. Kadota, and M. Fukui (1991) Purification and characterization of three forms of differently glycosylated recombinant human granulocyte-macrophage colony stimulating factor. *Arch. Biochem. Biophys.* 286: 562-568.
[31] Moonen, P., J. J. Mermod, J. F. Ernst, M. Hirschi, and J. F. DeLamarter (1987) Increased biological activity of deglycosylated recombinant human granulocyte/macrophage colony-stimulating factor produced by yeast or animal cell. *Proc. Natl. Acad. Sci. USA* 84: 4428-4431.
[32] Quesniaux, V. J. F. and T. C. Jones (1998) Granulocyte-macrophage colony stimulating factor. pp. 77-87. In: T. Angus (ed.). *The Cytokine Handbook*. Academic Press, London, UK.
[33] Dorr, R. T. (1993) Clinical properties of yeast-derived versus *Escherichia coli*-derived granulocyte-macrophage colony-stimulating factor. *Clin. Ther.* 15: 19-29.
[34] Robinson, R. L. and L. A. Myers (1993) Preclinical safety assessment of recombinant human GM-CSF in rhesus monkeys. *Int. Rev. Exp. Pathol.* 34A: 149-172.
[35] Sardana, R. K., Z. Alli, A. Dudani, E. Tackaberry, M. Panahi, M. Narayanan, P. Ganz, and I. Altosaar (2002) Biological activity of human granulocyte-macrophage colony stimulating factor is maintained in a fusion with seed glutelin peptide. *Transg. Res.* 11: 521-531.

[Received June 14, 2004; accepted November 18, 2004]