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Stability Enhancement of hGM-CSF in Transgenic *Nicotiana tabacum* Suspension Cell Cultures

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Abstract Proteolytic enzymes existing in plant cell cultured media are the major reason for the loss of secreted human granulocyte-macrophage colony-stimulating factor (hGM-CSF). The addition of pepstatin, aprotinin and PMSF relatively decreased the proteolytic degradation of hGM-CSF in a conditioned medium, but sufficient prevention against the proteolytic activity could not be obtained with chemical protease inhibitors. Gelatin, as a competitive substrate for protease, showed a stabilizing effect in a conditioned medium. Compared to the initial hGM-CSF concentration in a conditioned medium, with 10 g/L of gelatin, 68% of the hGM-CSF remained after 5 days. In a cell culture experiment, 5 g/L of gelatin significantly stimulated the hGM-CSF production and accumulation in culture media, with no growth inhibition. Compared to the controls (4.72 μg/L), the extracellular hGM-CSF level could be increased to 39.78 μg/L with the addition of 5 g/L of gelatin.

Keywords: transgenic plant cell culture, hGM-CSF, proteolytic degradation, stabilizing agent, gelatin

INTRODUCTION

The secretion of newly synthesized foreign protein can simplify the downstream processing. In contrast to animal cell cultures, media for plant cell growth contain few proteins, therefore, the purification of secreted foreign proteins in plant cell cultured media have greater economical advantages over other cell culture systems. However, the secretion of foreign proteins into culture media often encounters problems in their stability, which deteriorate the biological function and production yield of the proteins. The formation of unfavorable inter- or intra-chemical bonds and aggregation can explain the instability of synthesized proteins. In addition, proteolytic degradation can also be a reason for the loss of foreign proteins in conditioned media. Actually, the presence of proteolytic activity in plant cell cultures has been reported, and the degradation by proteases has been suggested as a reason for the rapid decrease of extracellular foreign proteins. In order to increase the stability of secreted proteins, and prevent proteolytic degradation, extensive efforts have been applied toward cell cultures. For example, the developments of protease-deficient cell lines, use of protease inhibitors and the addition of stabilizing compounds, have all been examined. Chemical protease inhibitors can decrease the proteolytic degradation through the suppression of protease activity, but the high cost, relatively short half-life and cytotoxicity, significantly restrict their use in cell cultures [1]. Polymeric compounds, such as polyethylene glycol (PEG), polyvinylpyrrolidone (PVP) and gelatin, have been reported as stabilizing compounds; and dimethyl sulfoxide (DMSO) has been reported to also increase foreign protein production from plant cell cultures by enhancing the stability of the extracellular foreign proteins [2-6]. Bacitracin, a cyclic polypeptide mixture, has also been known to act as an inhibitor of all four classes of proteolytic enzymes [7,8] In addition, the properties of plant cell culture media, and their components, are known as factors that also influences the protein stability [9]. Nevertheless, despite extensive efforts to improve production yields, through increasing the protein stability and reducing the protease activity, the exact mechanism and the reason for the rapid loss of foreign proteins in conditioned media still remain to be elucidated.

In this study, the stability of hGM-CSF, in cell-free conditioned media, was examined. In order to clarify the major protease responsible for the proteolytic degradation in plant cell cultured media and to examine the feasibility of protease inhibitor as a medium additive, several chemical protease inhibitors were added to the conditioned media and their stabilizing effects were investigated. Also, the addition of gelatin to *Nicotiana tabacum* suspension cell cultures, as a stabilizing agent to increase the hGM-CSF production, was studied.

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MATERIALS AND METHODS

Plant Cells and Culture Conditions

Transgenic *Nicotiana tabacum* L. cv Havana SR cells were subcultured every 7 days, using a 20% inoculum in a modified MS medium, containing 30 g/L, 0.2 mg/L 2,4-D (dichlorophenoxyacetic acid) and 0.02 mg/L kinetin. The medium pH was adjusted to 5.9 prior to sterilization at 121°C. The cell suspension was incubated at 25°C, with continuous shaking on an orbital shaker at 120 rpm. For fresh cell weight measurement, the cell suspension was filtered through a Whatman No. 1 filter paper, under vacuum, and washed three times with distilled water to remove the residual sugar from the cell surfaces. The cells were then transferred to a preweighed dish and their mass was measured. The dry cell weight was estimated after drying at 60°C for 2 days.

Preparation of Conditioned Medium

To test the stability of the hGM-CSF in the conditioned medium, the cell suspensions cultured for 7 days were filtered using a Whatman No.1 filter paper, with the cell-free conditioned medium filtered again, under sterile conditions, using Steritop (Millipore Inc., USA). The experiments for the test of stability were conducted in buffered (0.1 M Tris/HCl, pH 7.0) or autoclaved conditioned medium and the hGM-CSF exogenously added at appropriate concentrations.

Preparation of Protease Inhibitor

To examine the effects of various protease inhibitors on hGM-CSF degradation, several chemical protease inhibitors were added into the conditioned media containing synthesized hGM-CSF. The types and doses of the chemical protease inhibitors are summarized in Table 1. All the chemical protease inhibitors were purchased from Sigma Chemical Co. (St. Louis, USA).

Determination of hGM-CSF Concentration

The extracellular hGM-CSF was quantified using an ELISA kit, according to the manufacturer's instructions (PharMingen Inc., USA).

RESULTS AND DISCUSSION

Stability of hGM-CSF in Conditioned Medium

The rapid loss of foreign proteins in media, following spontaneous secretion, has been reported in transgenic plant cell cultures. In a preliminary experiment, it was found that the extracellular hGM-CSF reached a maximum concentration on day 4 or 6, and then suddenly decreased. Therefore, it would be useful to understand whether the reason for the hGM-CSF loss in the culture media was due to the instability of the protein or because

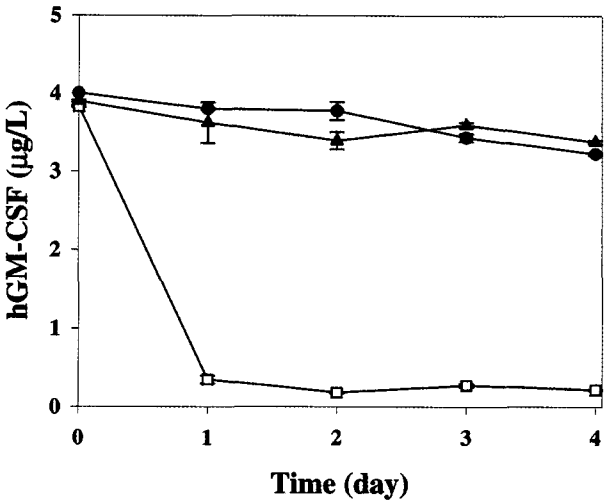

Fig. 1. The stability of the hGM-CSF in 0.1 M Tris/HCl buffer (pH 7.0) and conditioned media, without any cells: ●, buffer; □, conditioned medium; ▲, autoclaved conditioned medium.

of proteolytic degradation. As an initial step, the stability of the hGM-CSF, in a buffer solution and a conditioned medium, was examined under cell-free conditions. Additionally, the conditioned medium was autoclaved to inactivate the proteases. The stability results are shown in Fig. 1. The initial concentration of the hGM-CSF was 3.8~3.9 μg/L, which was similar in all cases. In the buffer solution and an autoclaved conditioned medium, the hGM-CSF concentrations were maintained at the same initial level, without severe reduction over a 4 day period, while the hGM-CSF in the conditioned medium immediately decreased. The results show that the presence of proteases in the conditioned medium plays an important role in the degradation of the secreted hGM-CSF during the culture period. It was also thought that the hGM-CSF had no significant problem in its intrinsic stability when its stability in the autoclaved conditioned media and buffer solution were considered. Consequently, it could be concluded that the protease activity was a major cause for the loss of the secreted hGM-CSF in plant cell cultures. Therefore, the reduction of the protease activity during the culture period, or a decrease in the exposure time, of the extracellular hGM-CSF to proteolytic enzymes, would be necessary to improve the productivity of foreign protein production.

Effect of Protease Inhibitors on hGM-CSF Stability

Proteases can be classified into four groups; *i.e.* aspartyl protease, serine protease, cysteine protease and metalloprotease. Several chemical protease inhibitors against each type of protease were tested, as summarized in Table 1. In Fig. 2, the effects of the chemical protease inhibitors, on the hGM-CSF stability, in the conditioned media under cell-free conditions are shown. In the absence of a protease inhibitor, the initial hGM-CSF concentration (4.14 μg/L) decreased to 0.97 μg/L within 24 h. The

Table 1. The chemical protease inhibitors and their effects on hGM-CSF degradation in the conditioned media

Protease type	Inhibitor	Action	Spectrum	Dose
Aspartyl protease	Pepstatin	reversible	Pepsin, cathepsins, renin	10 μM
Serine protease	PMSF	irreversible	General	1 mM
	Aprotinin	reversible	Trypsin, chymotrypsin, kallilreins	1% (v/v)
Cysteine protease	E64	irreversible		10 μM
Metalloprotease	EDTA	reversible	General	5 mM
–	Protease cocktail	–	–	1% (v/v)

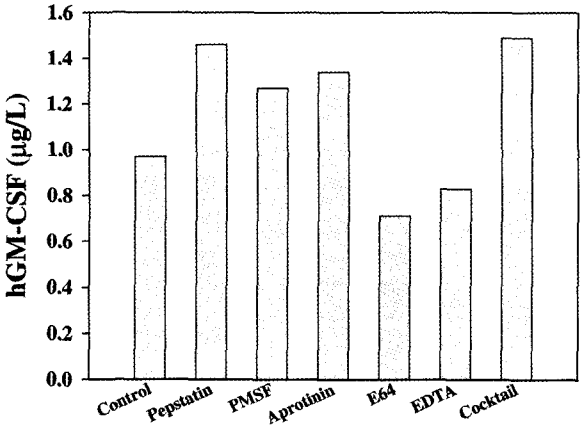

Fig. 2. The effects of the chemical protease inhibitors on the stability of the hGM-CSF in conditioned media. The initial hGM-CSF concentration in the conditioned media was approximately 4.14 μg/L. The bars indicate the residual hGM-CSF concentration, both with and without the chemical protease inhibitors, after 24 h.

addition of pepstatin (10 μM, aspartyl protease inhibitor) reduced the degradation of the extracellular hGM-CSF in the conditioned medium. The residual hGM-CSF concentration after 24 hours-incubation was 1.46 μg/L. It was observed that the serine protease inhibitors, PMSF and aprotinin, were also effective in the prevention of the proteolytic degradation, and increased the remaining hGM-CSF concentrations to 1.27 and 1.34 μg/L, respectively. However, a cysteine protease inhibitor, E64, and a metalloprotease inhibitor, EDTA, showed no protective effects compared to the controls. Although inhibitors for the aspartyl and serine proteases showed somewhat positive effects, the effects did not reach the expected levels. These less beneficial effects might originate from the limited half-life of the protease inhibitors.

Several facts can be deduced from this stability study. Firstly, of the proteolytic enzyme mixtures in the plant cell cultured media, the aspartyl and serine proteases were more responsible for the proteolytic degradation of the hGM-CSF than the cysteine protease and metalloprotease. Secondly, the expected use of chemical protease inhibitors, as media additives is difficult due to their short half-life, as mentioned above. Thirdly, alternative media additives, or approaches, are necessary to prevent the extracellular proteins from proteolytic degradation. For example, it was found that DMSO and gelatin imparted strong stabilization effects to this system.

Effect of Gelatin on hGM-CSF Production

As reported by James and Lee [10], granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) were only slightly stabilized on the addition of bovine serum albumin (BSA), and the use of PVP and gelatin, widely known as a stabilizing agents, were ineffective. Nevertheless, the stabilizing effect of gelatin has been shown to be in dispute. According to Lee *et al.* [3], the addition of gelatin to transgenic *N. tabacum* suspension cultures significantly increased the extracellular hGM-CSF production. In order to confirm the effects of gelatin, 1, 5 and 10 g/L of gelatin were added to conditioned medium, under cell-free conditions, and the stability of the hGM-CSF investigated, aseptically. Beside gelatin, the effects of DMSO, PVP and PEG, on the hGM-CSF stability were also investigated. The PVP and PEG did not enhance the hGM-CSF stability in conditioned medium, with only the DMSO giving a stabilizing effect. However, further investigation into the use of DMSO was not carried out, as the cell growth was severely inhibited on its addition, even though it could increase the hGM-CSF secretion and improved the stability in culture media.

Fig. 3 shows the effects of the gelatin concentration on the hGM-CSF stability in the conditioned medium. In the absence of gelatin (the control), the initial hGM-CSF concentration (3.14 μg/L) rapidly decreased to 0.30 μg/L within a day. Whereas, the addition of gelatin, to the conditioned medium, apparently improved the stability of the hGM-CSF, and the improvement was proportional to the increase in the gelatin concentration. As a result, 2.12 μg/L of hGM-CSF remained, with 10 g/L of gelatin, after 5 days. In addition, a similar result was obtained when casein was tested in the conditioned medium. Therefore, it can be concluded that gelatin acts as a competitive substrate for protease, and therefore much more hGM-CSF could remain with the use of gelatin.

In Fig. 4, the cell growth and extracellular hGM-CSF production, with different concentrations of gelatin, are shown. Because an excess amount of gelatin could induce

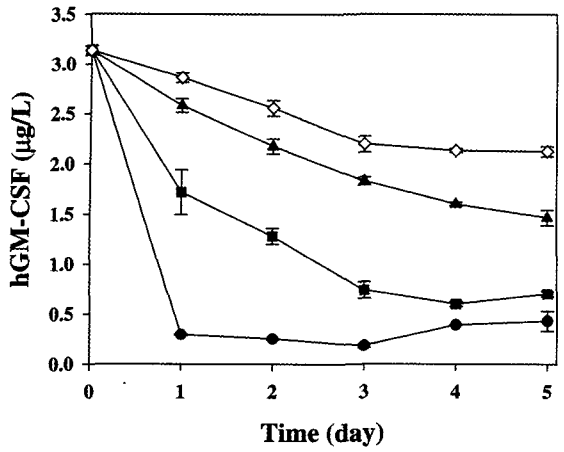

Fig. 3. The effect of the gelatin concentration on the hGM-CSF degradation in the conditioned media: ●, control; ■, 1 g/L; ▲, 5 g/L; ◇, 10 g/L.

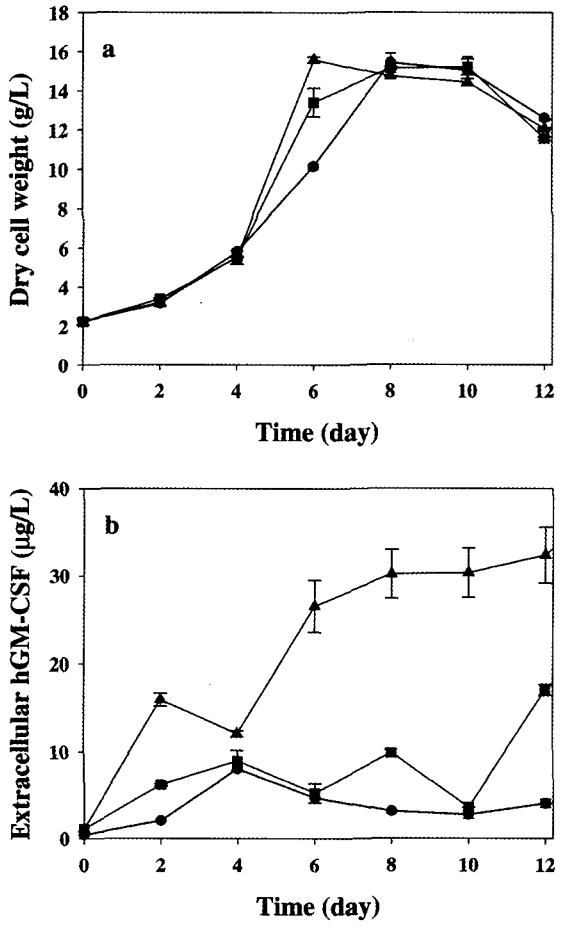

Fig. 4. The time course changes of (a) the transgenic *N. tabacum* cell growth and (b) extracellular hGM-CSF production with gelatin: ●, control; ■, 1 g/L; ▲, 5 g/L.

some problems in the downstream process step, and increase the culture viscosity, up to 5 g/L of gelatin was initially supplemented. The transgenic *N. tabacum* cell growth was slightly affected by the addition of gelatin. It is not clear why the cell growth increased on the addition of gelatin on the 6th day. One possible explanation is that the attachment gelatin to the cell surfaces led to the overestimation in the cell mass. Nevertheless, the maximum cell densities, both with and without gelatin, showed similar levels (14.4 ~ 15.0 g/L). Nonetheless, it was apparent that up to 5 g/L of gelatin did not inhibit the cell growth.

In view of the hGM-CSF production, the addition of gelatin as a stabilizing agent gave a prominent improvement in the extracellular hGM-CSF accumulation, although 1 g/L of gelatin did not significantly increase the hGM-CSF production. In the control culture, the level of the hGM-CSF reached a maximum of 4.72 μg/L on day 6, and the level of the extracellular hGM-CSF level decreased due to proteolytic degradation. In contrast, 5 g/L of gelatin drastically increased the hGM-CSF production. With 5 g/L of gelatin, the extracellular hGM-CSF concentration continuously increased, until day 12, and 39.78 μg/L of extracellular hGM-CSF was obtained, which was a 8.4-fold increase compared to that of the control.

CONCLUSION

The aspartyl- and serine-proteases were found to be responsible for the proteolytic degradation of hGM-CSF in plant cell cultures. Proteolytic degradation can be important factor in secretory foreign protein production system. In order to lower the production cost, and increase production yield, in cell culture for foreign protein production, the facilitated secretion, and improved stability, of synthesized foreign proteins were indispensable steps. However, the use of protease inhibitors, as stabilizing agents, has to be restricted due to their cost, short half-life and cytotoxic effects. It was found that the addition of gelatin was effective in the stabilization of the hGM-CSF, with no growth inhibition in transgenic plant cell cultures. Furthermore, it is expected that the synergistic increase in the hGM-CSF production can be achieved by the application of Pluronic F-68, for the enhanced secretion, and gelatin, as a stabilizing agent.

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