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Strategy for enhancing production of recombinant protein in tobacco's suspension culture

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Abstract

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that stimulates the production of granulocytes, macrophages, and white blood cells. The effects of osmotic pressure on secretion of human GM-CSF into the culture medium were investigated in suspension cultures of transgenic tobacco cells. An increase in osmotic pressure caused by the addition of mannitol decreased the cell size index, with the effect being more pronounced when cells were measured wet rather than dry. Increased osmotic pressure enhanced the secretion of hGM-CSF. At 90 g/L mannitol, the maximum concentration tested, hGM-CSF was present in the culture medium at 980 μg/L. As the concentration of mannitol increased, the total amount of protein secreted also increased, but was disproportionately enriched in GM-CSF. NaCl, another osmoticum, had very similar effects on cell growth and hGM-CSF production, but did not cause enrichment for hGM-CSF. Additionally, protein-stabilizing polymer was added to culture broth to enhance stability of secreted recombinant protein. Finally, above two method were applied together to maximize the productivity.

Keywords: GM-CSF; plant cell suspension culture; osmotic pressure; transgenic tobacco; mannitol

1. Introduction

Many proteins of therapeutic or scientific interest are expressed as recombinant proteins in heterologous cell cultures. Although recombinant proteins have typically been produced in microbial or animal cell cultures, plant cell cultures are also used for this purpose. Plant cell culture systems have distinct advantages over microbial and animal cell cultures, including low-cost biomass production and low risk of product contamination by mammalian viruses and bacterial toxins. Plant cells may be cultured as hairy root, immobilized cell, or cell suspension cultures. Among these, suspension cultures are regarded as the most suitable for industrial applications, and have been used in production of a large number of therapeutic proteins (1-4). Rice (5) and tobacco constitute the most popular systems for plant cell suspension culture.

Granulocyte-macrophage colony stimulating factor (GM-CSF) is one of 4 specific glycoproteins that stimulate a population of committed granulocyte-macrophage progenitor cells to generate granulocytes and macrophages, two important types of white blood cells (6). GM-CSF has increasing clinical applications in the treatment of neutropenia and aplastic anemia, and greatly reduces the infection risk associated with bone marrow transplantation by accelerating production of neutrophils (7).

Secretion of recombinant proteins may be accelerated by cell permeabilization. Procedures used for permeabilization usually involve treatment of cells with organic solvents or detergents (8,9). Other methods involve repeated freezing and thawing (10), air-drying (11), freeze-drying (12), or osmotic shock (13) of the cells. Of these methods, the treatment of osmotic shock is simple and less harmful. In this report, we describe the effects of osmotic shock on secretion of human GM-CSF (hGM-CSF) from suspension cultures of tobacco cells.

2. Materials and methods

2.1. Plant cell line and growth medium

Tobacco (*Nicotiana tabacum* L. cv Havana SR) was transformed with *Agrobacterium tumefaciens* LBA4404 harboring the hGM-CSF gene. Suspension cells were obtained from leaf-derived calli of the transgenic plant O64-8. The detailed procedure for generating the transgenic cells was described in (14) including selection marker, plasmid construction, isolation of callus, etc. The cDNA encoding hGM-CSF was placed downstream of a CaMV35S promoter containing a duplicated enhancer region (-417 to -90) including Ω DNA sequence from the coat protein gene of tobacco mosaic virus. A nos terminator was present downstream of the cDNA. One of the hGM-CSF-producing strains was cultivated in 4.3 g/L MS medium (Murashige and Skoog, 1962) supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid, 0.02 mg/l kinetin, 300 mg/l kanamycin, and 3% sucrose. The pH of the medium was adjusted to 5.8 with 0.5 M KOH.

2.2. Suspension culture

Suspension cells were obtained from the leaf-derived calli of transgenic plants and cultured in 300-ml flasks containing 50 ml of MS medium supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid, 0.05 mg/L kinetin, and 3% sucrose. Flasks were shaken at 100 rpm in an incubator at 25°C. The seed culture was propagated by transfer of 20% of the culture into fresh medium every 7 days. For experiments examining secretion of hGM-CSF, filtered fresh cells (2.5 g) were inoculated into 50 ml of medium under various conditions. Osmotic shock was induced by use of medium containing 30 g/L sucrose and various concentrations of mannitol (30, 60, or 90 g/L). Alternatively, NaCl was also used

as an osmotic agent at varying concentrations (50, 100, or 150 mM) equivalent in osmolarity to the mannitol concentrations used above (30, 60, and 90 g/L, respectively). The equivalent osmotic concentrations of NaCl and mannitol were derived from a previous report (15)

2.3. Quantitative analysis of hGM-CSF and total secreted protein

The culture broth was centrifuged at 10,000 rpm for 5 min, and the amount of hGM-CSF in the supernatant was quantitated using an ELISA kit according to the manufacturer's instructions (PharMingen, Inc., USA). Sample concentrations were determined by comparison to a standard curve produced using recombinant hGM-CSF. The total protein concentration in the culture medium was determined by the Bradford assay using a kit supplied by Bio-Rad (USA).

3. Results and discussion

3.1. Effect of mannitol on cell growth and hGM-CSF production

To investigate the influence of initial osmotic pressure on production of recombinant hGM-CSF by transgenic tobacco cells, we used the non-metabolic sugar mannitol to increase the osmotic pressure of the culture medium. The sucrose concentration was kept constant at 30 g/L, and the total sugar concentration was varied by addition of mannitol to 30, 60, or 90 g/L.

The kinetics of cell growth and hGM-CSF production are shown in Fig. 1. The plant cell cultures followed the standard phases of cell growth, with a lag phase of about 2 days, an exponential phase between days 3 and 9, and a final, stationary phase. Cells grew well under the control experimental conditions and reached maximum fresh cell concentrations of 475 g/L at day 12. Addition of mannitol inhibited cell growth. When grown in the presence of 90 g/L mannitol,

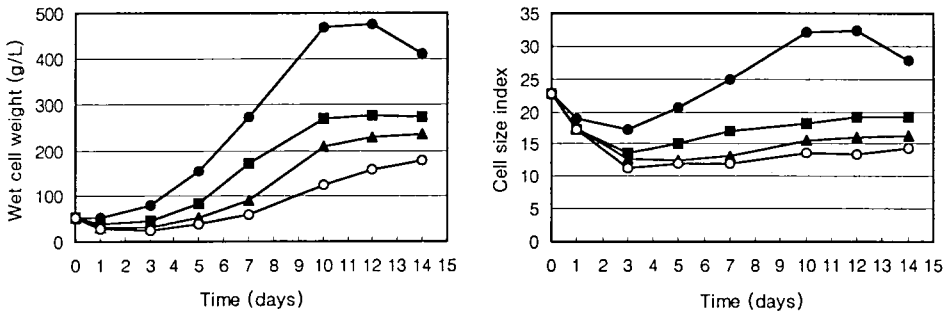

Fig. 1. Effect of mannitol on cell growth and size during batch suspension culture. The symbols represent cells cultured in medium containing 30 g/L sucrose (●), 30 g/L sucrose and 30 g/L mannitol (■), 30 g/L sucrose and 60 g/L mannitol (▲), and 30 g/L sucrose and 90 g/L mannitol (○) respectively. Cell size index was calculated by dividing wet cell weight (WCW) by dry cell weight (DCW).

wet cell weight (WCW) decreased dramatically to about one-fourth that observed under control culture conditions. However, mannitol decreased dry cell weight (DCW) to a lesser extent than it did WCW. As shown in Fig. 1, cell size reduced largely as the initial concentration of mannitol increased. All growth indices, including growth rates and maximum cell masses, decreased as initial osmolarity increased.

We expected that secretion of soluble protein would be enhanced by an increase in initial osmotic pressure of the culture medium. As the mannitol concentration increased from 0 to 90 g/L, we observed that total secreted protein (TSP) decreased from 125 to 87.2 mg/L, but the ratio of TSP:DCW increased for most of the culture period (Fig. 2). At the maximum mannitol concentration (90 g/L), the maximum TSP:DCW ratio (1.36%) was observed. This ratio is 2-fold higher than that observed under normal culture conditions. Thus, mannitol (or osmotic pressure) inhibited cell growth but enhanced protein secretion into the culture medium in our system.

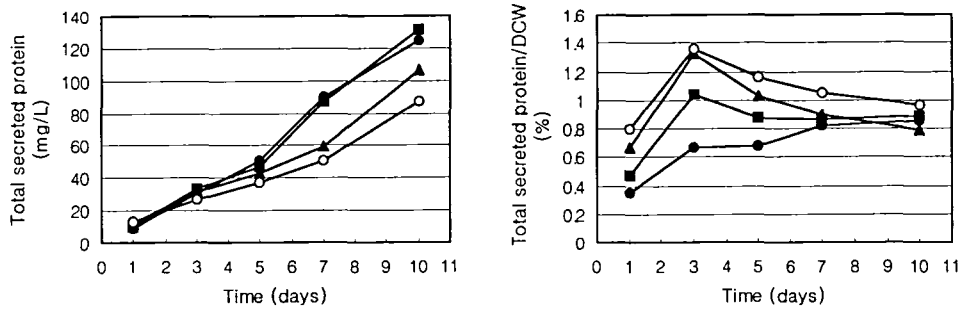

Fig. 2. Effect of mannitol on secretion of total protein (TSP) during batch suspension culture. The symbols represent cells cultured in medium containing 30 g/L sucrose (●), 30 g/L sucrose and 30 g/L mannitol (■), 30 g/L sucrose and 60 g/L mannitol (▲), and 30 g/L sucrose and 90 g/L mannitol (○).

We next examined the effect of initial osmotic pressure on the secretion of recombinant hGM-CSF in particular. As shown in Fig. 3, the extracellular hGM-CSF concentration increased dramatically with the addition of mannitol. On day 5 at the maximum mannitol concentration (90 g/L), hGM-CSF was present at its maximum observed concentration (980.1 μg/L), a 2.9-fold increase over that observed under normal culture conditions. Moreover, the maximum ratio of and

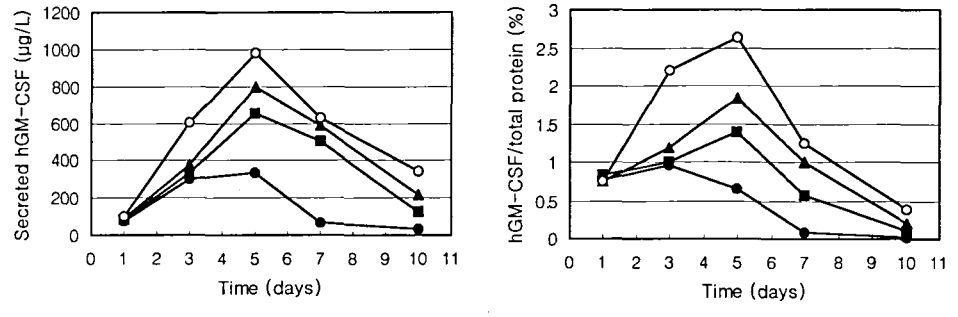

Fig. 3. Effect of mannitol on secretion of hGM-CSF during batch suspension culture. The symbols represent cells cultured in medium containing 30 g/L sucrose (●), 30 g/L sucrose and 30 g/L mannitol (■), 30 g/L sucrose and 60 g/L mannitol (▲), and 30 g/L sucrose and 90 g/L mannitol (○).

hGM-CSF:TSP (2.63%) was also observed on day 5 at 90 g/L mannitol. This value is and 4-fold higher than those observed under normal culture conditions, respectively. Thus, addition of mannitol to the culture medium not only increased TSP per cell weight, but also resulted in a 4-fold enrichment of hGM-CSF in the secreted protein. Obviously, this enrichment obviously would facilitate purification of hGM-CSF from the extracellular medium. We believe that the above effects were caused mainly by the physical force of osmotic pressure (15), with some secondary effects possibly caused by changes in cell physiology (16,17).

The extracellular concentration of hGM-CSF reached a maximum in the mid-exponential phase (day 5), but rapidly decreased after day 5 under all culture conditions. The culture medium environment appeared to become unfavorable for hGM-CSF stabilization after day 5, presumably due to degradation by secreted proteases. Bonner et al. identified protease activity in extracts from suspension-cultured *Nicotiana silvestris* cells (18), and Terashima et al. reported that sucrose depletion in the late phases of cell culture caused the release of sulfhydryl protease (19). Although the recombinant hGM-CSF looked to be degraded specifically in contrast with total protein, this may not be true and could result from that the total protein was measured by Bradford assay which can even detect degraded protein, but hGM-CSF by ELISA which can not detect demolished protein.

3.2 Effect of another osmotic agent (NaCl) on cell growth and secretion of hGM-CSF

To confirm that the effects of mannitol described above were due primarily to osmotic pressure, we analyzed the effect of NaCl on cell growth, TSP, and hGM-CSF. NaCl was added to the culture medium at concentrations (50, 100, or

150 mM) equivalent in osmolarity to the concentrations of mannitol used above (30, 60, and 90 g/L, respectively). Like mannitol, NaCl also inhibited cell growth and reduced cell size significantly (Fig. 4). However, TSP and the ratio TSP:DCW both increased as the concentration of NaCl in the culture medium increased (Fig 5). In the presence of 150 mM NaCl, the maximum ratio of TSP/DCW (3.3%) was obtained at day 5. This ratio is 4.8-fold higher than that obtained under normal culture conditions. Thus, the addition of NaCl had a significant effect on secretion of protein.

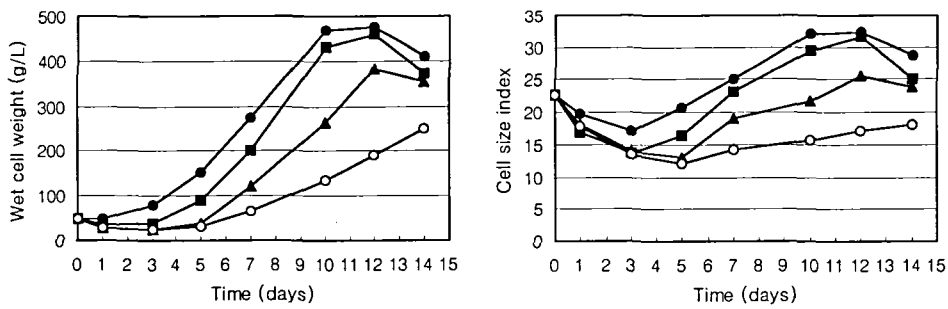

Fig. 4. Effect of NaCl on cell growth and size during batch suspension culture. The symbols represent cells cultured in medium containing 30 g/L sucrose (●), 30 g/L sucrose and 50 mM NaCl (■), 30 g/L sucrose and 100 mM NaCl (▲), and 30 g/L sucrose and 150 mM NaCl (○) respectively. Cell size index was calculated by dividing WCW by DCW.

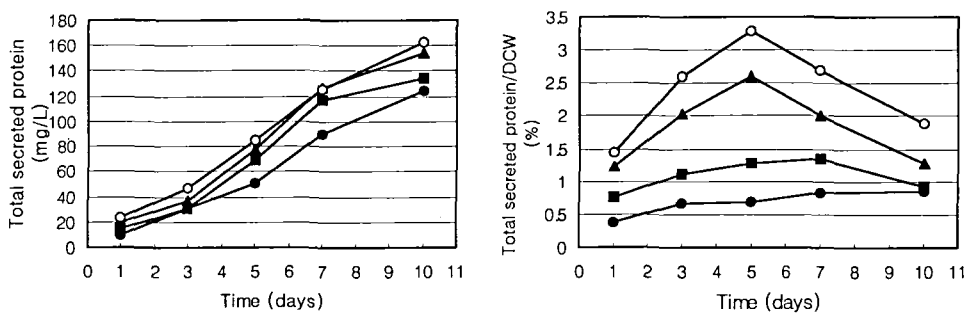

Fig. 5. Effect of NaCl on TSP during batch suspension culture. The symbols represent cells cultured in medium containing 30 g/L sucrose (●), 30 g/L sucrose and 50 mM NaCl (■), 30 g/L sucrose and 100 mM NaCl (▲), and 30 g/L sucrose and 150 mM NaCl (○).

The addition of NaCl also enhanced the secretion of hGM-CSF. As shown in Fig. 6, the maximum hGM-CSF production of 776.2 μg/L was achieved with 150 mM NaCl at day 5, but rapidly decreased after day 5 under all culture conditions. The ratio of hGM-CSF:TSP reached a maximum during the early-exponential phase (day 3), but rapidly decreased after day 3 under all culture conditions. The highest hGM-CSF:TSP ratio (1.3%) was obtained in the presence of 50 mM NaCl. This value is 1.3-fold higher than that obtained under normal culture conditions. James *et al* (20) observed that the addition of 50 or 100 mM NaCl inhibited cell growth and increased the secretion of GM-CSF by 1.5 fold, which coincided with our results.

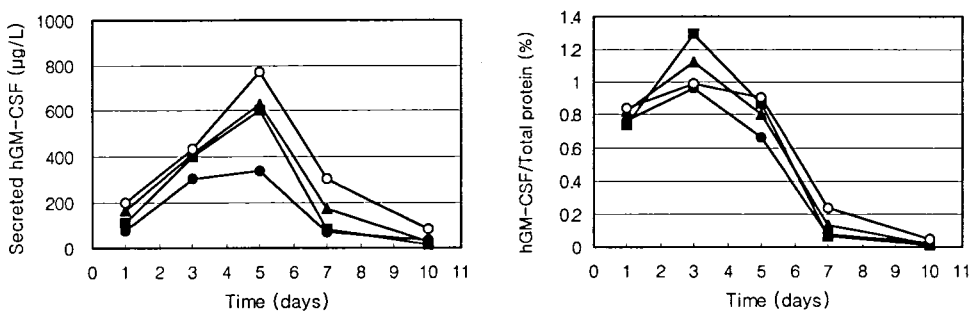

Fig. 6. Effect of mannitol on secretion of hGM-CSF during batch suspension culture. The symbols represent cells cultured in medium containing 30 g/L sucrose (●), 30 g/L sucrose and 50 mM NaCl (■), 30 g/L sucrose and 100 mM NaCl (▲), and 30 g/L sucrose and 150 mM NaCl (○).

3.3. Comparison of effects of mannitol and NaCl on production of recombinant hGM-CSF

As shown in Table 1, mannitol and NaCl had similar inhibitory effects on cell growth as measured by DCW. However, mannitol caused a greater reduction in WCW than did NaCl. NaCl may not have been as effective as mannitol at increasing the osmolarity of the culture medium, since NaCl is more likely to be drawn up by the cells, which would deplete its concentration in the medium.

Table 1. Comparison of effects of mannitol and NaCl on cell growth and hGM-CSF production

Growth conditions	WCW (g/L) at day 12	DCW (g/L) at day 12	Maximum TSP (mg/L)	Maximum secreted hGM-CSF (μg/L)	Maximum hGM-CSF: TSP (%)
Control	475.0	14.7	125.0	334.7	0.96
30 g/L mannitol	279.0	14.5	131.1	655.2	1.39
60 g/L mannitol	227.8	13.4	106.1	793.8	1.84
90 g/L mannitol	158.6	11.8	87.3	980.1	2.63
50 mM NaCl	457.5	14.5	135.0	602.2	1.29
100 mM NaCl	380.2	14.8	154.4	630.2	1.12
150 mM NaCl	192.3	11.2	162.4	776.2	0.10

Both mannitol and NaCl significantly enhanced secretion of hGM-CSF. The enrichment of hGM-CSF in the TSP, which would facilitate subsequent purification, was observed only with mannitol, however. Both agents had positive effects on total secretion of protein and negative effects on cell growth. Both cell growth and osmotic pressure affect the extent of protein secretion. It is not clear why mannitol, and not NaCl, caused enrichment for hGM-CSF. The NaCl treatment may have caused additional physiological effects due to salt stress (21).

Further research in several areas of study is needed to optimize the use of plant cell suspension cultures for production of secreted proteins. The exact mechanism by which osmotic pressure enhances protein secretion remains to be elucidated. Also the use of stabilizing polymers shows promise for enhancing the stability of secreted proteins (1,22). Finally, development of a culture system in which secreted proteins may be harvested before exposure to proteases would be of great utility.

4. Conclusion

We demonstrated that production of recombinant hGM-CSF in tobacco suspension cultures is effectively enhanced by osmotic pressure generated by mannitol and NaCl. Mannitol, but not NaCl, also enriched the total secreted protein for hGM-CSF. Mannitol and NaCl inhibited cell growth. This system provides a useful model for the production of other recombinant proteins from suspension cultures of plant cells.

Acknowledgements

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