Partitioning of Recombinant Human Granulocyte-Macrophage Colony Stimulating Factor (hGM-CSF) from Plant Cell Suspension Culture in PEG/Sodium Phosphate Aqueous Two-phase Systems

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Abstract Partitioning of human granulocyte-macrophage colony stimulating factor (hGM-CSF) was achieved in the aqueous two-phase systems (ATPSs) using a crude extract of transgenic tobacco cell suspension culture. This study examined the effects of polyethylene glycol (PEG) molecular weight and concentration and the effects of sodium phosphate concentration in different PEG/sodium phosphate systems on the partition coefficient, K. The best ATPS system was 5% PEG 8,000/1.6 M sodium phosphate after 2 h of incubation at room temperature. In this system, hGM-CSF was partitioned in the PEG-rich phase with a yield of 57.99% and $K_{hGM\text{-}CSF}$ of 8.12. In another system, 3% PEG 10,000/1.6 M sodium phosphate, hGM-CSF was also partitioned primarily in the top phase with a yield of 45.66% and $K_{hGM\text{-}CSF}$ of 7.64 after 2 h of incubation at room temperature.

Keywords: aqueous two-phase system, hGM-CSF, purification, plant cell culture, recombinant protein

INTRODUCTION

Plant cell cultures have been used for over 20 years to produce a variety of natural products [1] and, more recently, they have been used to produce recombinant proteins [2-4]. Plant cells seem to be a good source for recombinant material because the cells can be grown on relatively simple media with no added protein [5]. If a foreign protein is produced in cell culture and is secreted into the medium, rather than being stored inside the cells, the product recovery and purification can be carried out without large quantities of contaminating protein [6]. There is a particular interest in the use of plants as bioreactors, because they offer production of recombinant proteins in large quantities and at relatively low cost [7]. Plant-derived proteins are also likely to be safer for human use than those derived from mammalian cells, because plant cell contaminants and viruses are usually not pathogenic to humans [2].

Human granulocyte-macrophage colony-stimulating factor (hGM-CSF), a hemopoietic growth factor, is one of four specific glycoproteins that stimulate a population of granulocyte-macrophage progenitor cells to generate granulocytes, macrophages, and two important types of white blood cells [8]. Human GM-CSF has clinical applications in the treatment of neutropenia and aplastic anemia, and has greatly reduced the risk of infection associated with bone marrow transplantation by accelerating neutrophil count recovery [9]. Human GM-CSF has been expressed in various foreign hosts, such as *Escherichia coli* [10], yeast [11], *Aspergillus niger* [12], mammalian cells [13], and plant cells [2-4], and is now produced for clinical use [14].

The aqueous two-phase polymer/polymer or polymer/salt systems have been widely and successfully used to extract and purify biological macromolecules. Separation is achieved by the difference in the distribution of the target compound and the contaminants between the two phases. Due to the high water content in both phases and low interfacial tension, these systems provide mild conditions that are especially suited for biological macromolecule separation [15]. This technology offers the advantages of high capacity, high activity yields, and simplicity when increasing scale [16,17]. The aqueous two-phase systems, especially the polyethylene glycol (PEG)/salt systems, have been widely used for the bioseparation of proteins, because of their low cost and the wide range of hydrophobicity differences among the two-phase systems. Employing these phase systems can enhance the partition selectivity of target proteins, especially for hydrophobic

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products [18].

Here, we have reported the partitioning of hGM-CSF, which was obtained from a crude extract of transgenic tobacco cell suspension culture, in different PEG/sodium phosphate aqueous two-phase systems.

MATERIALS AND METHODS

Plant Cell Suspension Culture

Cell suspension cultures of the hGM-CSF transgenic tobacco plant (*Nicotiana tabacum* L. *cv.* Havana) O64-8 were used for all experiments. Briefly, the hGM-CSF gene was incorporated into the genome of the suspension cells by *Agrobacterium* transformation. The hGM-CSF was produced under the control of a cauliflower mosaic virus (CaMV) 35S promoter and with the kanamycin resistance gene as a selectable marker. Tobacco cells were cultured in 300-mL flasks containing 50 mL of liquid MS [19] medium supplemented with 1 mg/L of 2,4-dichlorophenoxyacetic acid, 0.05 mg/L of kinetin, and 30 g/L of sucrose. Flasks were shaken at 100 rpm in an incubator at 25°C. The 4-day-old culture broth was then centrifuged at 5,000 rpm for 5 min and the supernatant (crude extract) was used for the partition experiments using different PEG/sodium phosphate systems.

Aqueous Two-Phase Systems

Polyethylene glycol (MW = 8,000 and 10,000) and sodium phosphate salts ($Na_2HPO_4 \cdot 7H_2O$, NaH_2PO_4) were obtained from Sigma Chemical Company (St. Louis, MO, USA) and were used to prepare the aqueous two-phase systems. The other chemicals were of the analytical grade. The two-phase systems were created by adding the required amounts of PEG and sodium phosphate to the supernatant to achieve the concentrations listed in Table 1. In formulating the partitioned systems, stock solutions of PEG and sodium phosphate were prepared. An aliquot of these polymer/salt solutions was added to the supernatant to make a final volume of 10 mL. After different incubation times (2, 6, or 24 h) at room temperature, phase separation was accomplished through centrifugation in graduated centrifuge tubes at 5,000 rpm for 5 min. The volumes of the top and bottom phases were measured, and the samples were withdrawn for the hGM-CSF assay and total protein determination.

Table 1. Concentrations of the phase-forming PEG/sodium phosphate in the aqueous two-phase systems

System designation	Concentration of PEG (%)		Concentration of sodium phosphate (M)
	MW 8,000	MW 10,000	
PS1	5	-	1.2
PS2	10	-	1.2
PS3	5	-	1.6
PS4	10	-	1.6
PS5	-	3	1.2
PS6	-	8	1.2
PS7	-	3	1.6
PS8	-	8	1.6

The hGM-CSF partitioning coefficient ($K_{hGM\text{-}CSF}$) and total protein partitioning coefficient ($K_{protein}$) were defined as the ratio of hGM-CSF concentration and total protein concentration in the top and bottom phases, respectively. The volume ratio (V) was the ratio of the volume in the top (V_T) phases to the volume in the bottom (V_B) phases. The partitioning yield of hGM-CSF in the top phase, Y, is given by the following equation [20]:

$$Y = \frac{100}{1 + (V_B/V_T) \cdot (1/K_{hGM\text{-}CSF})}$$

hGM-CSF Assay

The concentrations of hGM-CSF in the top and bottom phases were detected and quantified by the enzyme-linked immunosorbent assay (ELISA). The ELISA was performed following the manufacturer's instructions (PharMingen, Inc., San Diego, CA, USA). The hGM-CSF concentrations were calculated using the standard curve of recombinant hGM-CSF.

Total Protein Determination

The concentrations of total protein in the top and bottom phases were determined through spectrophotometry [21]. The samples of each phase were mixed with distilled water and Bradford reagent, and then the absorbance was measured at 595 nm. The total protein concentrations were calculated using the standard curve of bovine serum albumin.

RESULTS AND DISCUSSION

Plant Cell Suspension Culture

The dynamics of hGM-CSF and total protein production are shown in Fig. 1, with a lag phase of about 2 days, an exponential phase between days 3 and 9, and a final, stationary phase. The extracellular concentration of the total protein increased continuously from days 1 to 10 of the culture, with a final value of 125 mg/L. However, the extracellular concentration of hGM-CSF reached a maximum value of 180 μg/L at day 4 (exponential phase), and then rapidly decreased after day 5. Bonner *et al.* [22] identified protease activity in extracts from a cell suspension culture of *Nicotiana sylvestris*. These results indicate that *in situ* purification is needed to develop an efficient production system of hGM-CSF using plant cell culture.

Table 2. Effects of PEG (molecular weight and concentration) and sodium phosphate (concentration) on V, $K_{hGM\text{-}CSF}$, and $K_{total\ protein}$

System	V			$K_{hGM\text{-}CSF}$			$K_{total\ protein}$		
	Incubation time (h)			Incubation time (h)			Incubation time (h)		
	2	6	24	2	6	24	2	6	24
P1	0.25	0.19	0.18	0.95	0.84	0.74	0.92	0.88	0.75
P2	0.43	0.43	0.37	0.92	0.84	0.67	0.89	0.85	0.87
P3	0.18	0.15	0.15	8.12	7.27	5.73	1.09	1.07	1.02
P4	0.33	0.33	0.30	2.00	1.79	1.67	0.99	0.98	0.98
P5	0.12	0.11	0.10	1.42	1.18	1.00	0.89	0.85	0.85
P6	0.39	0.33	0.30	0.51	0.46	0.40	0.94	0.89	0.79
P7	0.11	0.10	0.10	7.64	6.60	5.30	1.23	1.22	1.16
P8	0.30	0.25	0.25	1.00	0.84	0.76	0.96	0.94	0.92

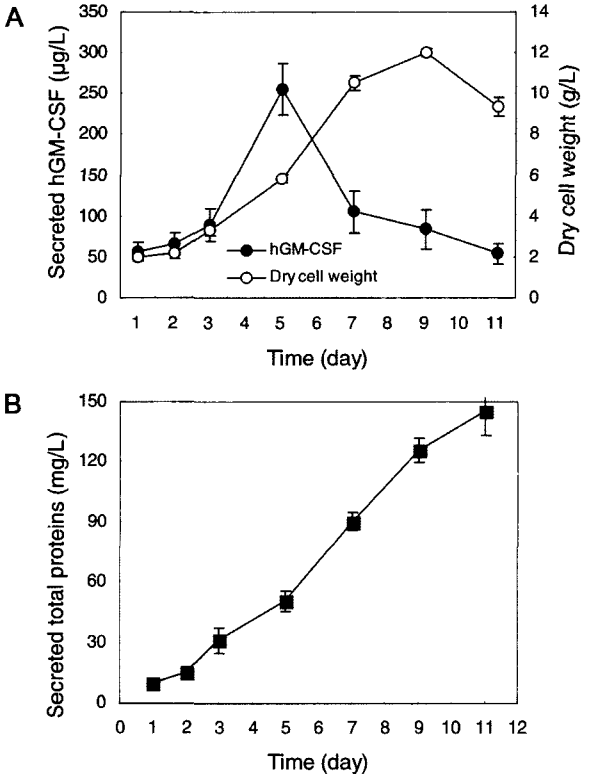

Fig. 1. Kinetics of growth (A), extracellular hGM-CSF (A), and total protein (B) during batch suspension culture. The values represent the means of triplicate results, and the error bars represent standard deviations.

In these cells, protease activity was highest during the stationary phase (data not shown). For the partitioning experiments, we used the culture broth of day 4 (mid-exponential phase), which contained the greatest amount of extracellular hGM-CSF.

Partitioning of hGM-CSF and Total Protein

The partitioning of hGM-CSF and total protein obtained from the supernatant of transgenic tobacco cell suspension culture in the PEG/sodium phosphate systems was investigated. As shown in Table 2, hGM-CSF in the nearly aqueous two-phase systems concentrated in the top phase, the PEG-rich phase, with the $K_{hGM\text{-}CSF}$ ranging from 1.00 to 8.12, and the total protein tended to concentrate in the bottom phase, with the $K_{total\ protein}$ ranging from 0.75 to 0.99. While PEG molecular mass had little influence on the partitioning of hGM-CSF and total protein, the sodium phosphate concentrations affected the hGM-CSF and total protein partition significantly. The $K_{hGM\text{-}CSF}$ remained at a minimum value of 0.40 to 0.51 in an 8% PEG 10,000/1.2 M sodium phosphate system and at of 0.67 to 0.93 in 10% PEG 8,000/1.2 M sodium phosphate system. The $K_{hGM\text{-}CSF}$ increased with an increasing sodium phosphate concentration, and reached its asymptotic value of about 8 in the 5% PEG 8,000/1.6 M sodium phosphate system and 3% PEG 10,000/1.6 M sodium phosphate system. Similarly, sodium phosphate concentrations significantly affected the total protein partition. In the 5% PEG 8,000/1.6 M sodium phosphate or 3~8% PEG 10,000/1.6 M sodium phosphate systems, the total protein was partitioned to the top phase ($K_{total\ protein} > 1$), whereas the partition was reversed in the other aqueous two-phase systems containing 1.2 M sodium phosphate. Thus, the sodium phosphate concentration in the ATPS has a substantial effect on $K_{hGM\text{-}CSF}$ and $K_{total\ protein}$.

The partitioning coefficients of hGM-CSF and total protein were also influenced by several other factors, such as PEG concentration and incubation time. For example, we found that a high partitioning coefficient of 8.12 for hGM-CSF was obtained in a phase system composed of 5% PEG 8,000/1.6 M sodium phosphate after a 2-h incubation. Whereas, the partitioning coefficient for hGM-CSF was only 2.00 in a phase system composed of

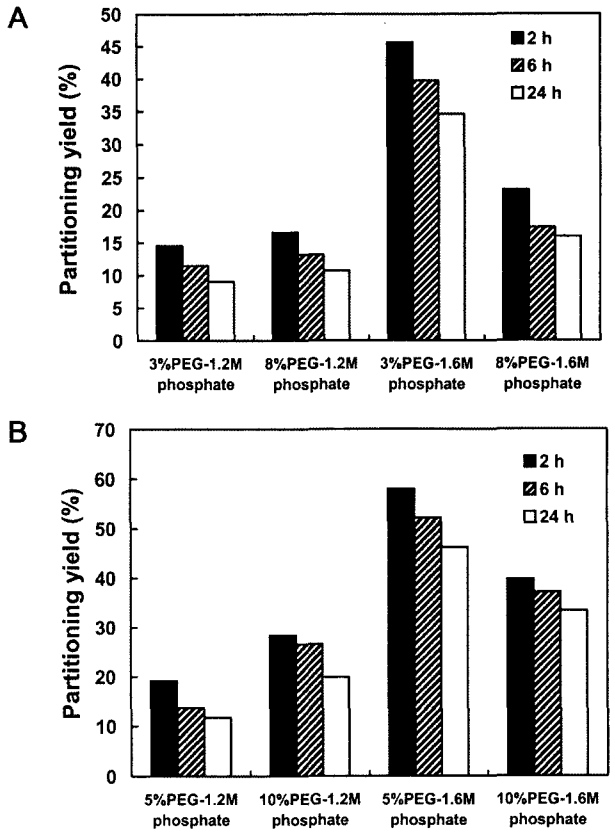

Fig. 2. Human GM-CSF partitioning yield (%) in the top phase of ATPS using various molecular weights of PEG. The molecular weights of PEG were 10,000 (A) and 8,000 (B) respectively. The values are the means of duplicate results.

10% PEG 8,000/1.6 M sodium phosphate after a 2-h incubation, or 1.67 in the same system after a 24-h incubation. Total protein analyses gave similar results, as shown in Table 2.

Partitioning Yield of hGM-CSF in the Top Phase

The partitioning yield of hGM-CSF depends on two parameters: V_B/V_T and $K_{hGM\text{-}CSF}$. In most of the aqueous two-phase systems investigated, hGM-CSF partitioned more towards the top phase, but the volume values, V_T, were usually small. Therefore, the partitioning yielded low values. The highest hGM-CSF yields were 57.99% and 45.66% with partition coefficients of 8.12 and 7.64, and V_B/V_T values of 5.67 and 9.00 (with V_T/V_B respective ratios of 0.18 and 0.11) in the phase compositions of 5% PEG 8,000/1.6 M sodium phosphate and 3% PEG 10,000/1.6 M sodium phosphate, respectively (Fig. 2).

CONCLUSION

In an aqueous two-phase system separation process, the hGM-CSF and total protein from a plant cell suspension were concentrated primarily in the top phase of a 5% PEG 8,000/1.6 M sodium phosphate system, with partition coefficients of up to 8.12 and 1.09, respectively, for a 2-h incubation. Generally, the hGM-CSF concentrations in the top phase were higher in the systems containing PEG 8,000 than in those containing PEG 10,000, while the total protein concentrations were reversed. The concentrations of sodium phosphate strongly influenced the partitioning of hGM-CSF and total protein. The partitioning of hGM-CSF into the top phase was increased by increasing the concentration of sodium phosphate and decreasing the molecular weight and concentration of PEG. The highest yields of hGM-CSF were 57.99% and 45.66% in 5% PEG 8,000/1.6 M sodium phosphate and 3% PEG 10,000/1.6 M sodium phosphate systems, respectively.

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