Special Issue

Concentrating *Autographa californica* nuclear polyhedrosis virus and recombinant alkaline phosphatase from insect cells using a temperature-sensitive hydrogel

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Abstract

Recombinant alkaline phosphatase expressed in insect cells was concentrated by a factor of one and half times at a separation efficiency of 54.2% using hydrogel ultrafiltration. Enzyme concentration was confirmed by SDS-PAGE as well as by spectrophotometric measurement. Wild and recombinant *Autographa californica* nuclear polyhedrosis viruses (AcNPV) were concentrated 1.4 and 1.6 times of the feed solution at 48.5 and 60.0% separation efficiency, respectively. Hydrogel ultrafiltration appears to be an attractive alternative for the concentration of AcNPV and recombinant proteins from insect cells.

Abbreviations: AcNPV-*Autographa californica* nuclear polyhedrosis virus; Sf21-*Spodoptera frugiperda* 21 cells; SDS-PAGE-Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCID₅₀-50% tissue-culture infectious dose

Introduction

A temperature-sensitive hydrogel, poly (N-isopropylacrylamide) gel, has been used in separation processes to extract water and low molecular weight solutes from macromolecular solutions (Freitas and Cussler, 1987) and to separate soy protein (Trank *et al.*, 1989), cellulase enzymes (Park and Orozco-Avila, 1992; Park and Orozco-Avila, 1983) and subtilisin (Han *et al.*, 1995). The main characteristic of the hydrogel, making it useful for separation processes, is that it exhibits a discontinuous, reversible and a large volume change upon temperature swing (Freitas, 1986). This hydrogel separation process is especially suited to labile products such as proteins, fermentation products and other biomolecules, because the process does not change the ionic environment or shear conditions of the medium.

To our knowledge, this work is the first report on the concentration of baculovirus and recombinant proteins from baculovirus-infected insect cells using

a temperature-sensitive hydrogel. We evaluated the performance of hydrogel ultrafiltration by concentrating *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant alkaline phosphatase produced by AcNPV-insect cell culture technology.

Materials and methods

Cell culture, recombinant protein and virus production

Spodoptera frugiperda 21 (Sf21) cells were maintained at 28 °C by weekly passage in EX-CELL 400™ medium. The recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV) expressing human secreted placenta alkaline phosphatase was a kind gift of Dr. Al Wood of Boyce Thompson Institute (Ithaca, NY, U.S.A.). To investigate the concentration of recombinant alkaline phosphatase, a culture broth containing

recombinant alkaline phosphatase was harvested at 4 days post-infection of recombinant AcNPV in Sf21 cells using the procedure described elsewhere (Chung and Shuler, 1993). The broth was then centrifuged to remove cells and cell debris for the hydrogel experiments. To investigate AcNPV concentration, the virus solutions were harvested at 2 days post-infection of wild and recombinant AcNPV in Sf21 cells again using the procedure described above. The virus solutions were centrifuged to remove cells and cell debris for the hydrogel experiments.

Preparation of the hydrogel beads

Beads of a temperature-sensitive hydrogel, poly(N-isopropylacrylamide) were prepared using inverse suspension polymerization with paraffin oil as the continuous phase and pluronic L-81 (BASF, Ludwigshafen, Germany) as a nonionic polymeric surfactant. The details of this procedure is described in Han *et al.* (1994).

Gel volume determination

The gel (1.5 g) was pre-swelled in distilled water (30 ml) at 20 °C for 24 h in a 50 ml graduated glass cylinder (2.5 cm i.d.). The change in gel volume was determined in an environmental chamber upon 24 h temperature shifts between 20 °C and 35 °C. For each volume measurement, the void volume between the gel particles was reduced by placing a plunger made with a stainless steel screen on top of the gel for 1 minute.

Hydrogel concentration experiments

A solution of pure human placenta alkaline phosphatase (Sigma, St. Louis, MO, U.S.A.) (10 ml) with a specific activity of 1.8 IU ml⁻¹ (total activity 18 IU) or cell culture broth (10 ml) with a specific activity of 7.8 IU ml⁻¹ (total activity 78 IU) was added to each test tube with 0.5 g of dry gel. The gel was allowed to swell for 3 h in an environmental chamber at 20 °C and then the gel was removed by filtration. Raffinate volume was measured using a graduated cylinder, and the gel was collapsed at 35 °C.

For the experiments of virus (AcNPV) concentration, either wild AcNPV solution (10 ml) of 1 × 10⁵ TCID₅₀ ml⁻¹ or recombinant AcNPV solution (10 ml) of 1 × 10⁵ TCID₅₀ ml⁻¹ was added to each test tube with 0.5 g of dry hydrogel. The same procedure used to concentrate alkaline phosphatase was applied to the

concentration of wild and recombinant AcNPV.

Percent recovery was calculated by dividing total enzyme activity or total virus titer in the raffinate by that in the feed. Separation efficiency (η) was calculated as follows.

$$\eta = \frac{a_r/a_f - 1}{v_f/v_r - 1} \times 100$$

a_r , a_f : enzyme activity or virus titer in the raffinate and in the feed, respectively.

v_f , v_r : volume in the feed and in the raffinate, respectively.

Analytical methods

Viral titers were measured in duplicate by TCID₅₀ as described elsewhere (Reed and Muench, 1938). Alkaline phosphatase activity was determined spectrophotometrically as described by Davis *et al.* (1992). SDS-PAGE was performed by Laemmli's method (1970). A slab gel consisting of 12% acrylamide running gel and 5% stacking gel was used. Gels were stained with Coomassie Brilliant Blue (Sigma, St. Louis, MO, U.S.A.) or silver nitrate.

Results and discussion

Gel volume change with temperature shift and enzyme stability

The change in gel volume during a 24 h period resulting from a temperature shift from 20 °C to 35 °C is shown in Table 1. For a temperature shift from 20 °C to 35 °C, the gel decreased its volume from 27 ml to 10 ml in 1 h and reached an equilibrium volume (4 ml) in about 4 h. For a reverse shift from 35 °C to 20 °C, the gel volume increased from 4 ml to 21 ml in 1 h and reached an equilibrium volume (27 ml) in about 4 h. We swelled the gel for 3 h and then collapsed the gel for another 3 h in all subsequent experiments, since the activity of alkaline phosphatase remained relatively constant for 24 h at 20 °C, but approximately 10% of the initial enzyme activity was lost by thermal deactivation 4 h after exposure to 35 °C (data not shown).

Concentration of alkaline phosphatase

Following hydrogel ultrafiltration of recombinant alka-

Table 1. Gel volume (ml) change over 24 h period with temperature shift

Temperature shift (°C)	Time (h)								
	0	1	2	4	6	9	12	18	24
20 – 35	27	10	6	4	4	4	4	4	4
35 – 20	4	21	23	27	27	27	27	27	27

Figure 1. SDS-PAGE of pure (A) and recombinant (B) alkaline phosphatase. (1, molecular size marker; 2, feed; 3, raffinate; 4, extractant.)

line phosphatase, a feed of 10 ml with 7.8 IU ml⁻¹ was concentrated one and a half times in the raffinate (5.2 ml of raffinate with 11.7 IU ml⁻¹ as determined spectrophotometrically). The separation efficiency (η) and enzyme recovery (%) were 54.2% and 78.0%, respectively. We obtained similar results for pure (Sigma) alkaline phosphatase (1.7 times of concentration, 52.4% of separation efficiency and 73.3% of enzyme recovery), indicating that the virus (AcNPV) contained in the recombinant culture broth does not interfere with hydrogel ultrafiltration of alkaline phosphatase. Raffinate samples of both the recombinant and the pure alkaline phosphatases showed thicker bands than those of the feed in SDS-PAGE analysis, confirming concentration by the hydrogel ultrafiltration process (Figure 1). The separation efficiency of hydrogel ultrafiltration (54.2% for recombinant alkaline phosphatase and 52.4% for pure alkaline phosphatase) was comparable to that reported (Cheryan, 1986) for membrane filtration (21% for β -galactosidase, 69% for trypsin and 77% for penicillinase).

Concentration of virus (AcNPV)

Hydrogel ultrafiltration concentrated wild and recombinant viruses (AcNPV) by 1.4 and 1.6 times of the feed solution at a separation efficiency of 48.5 and 60.0%, respectively. Percent recovery for wild and recombinant AcNPV was 76.0 and 80.0%, respectively. Virus titers were not detectable in the extractants released

from the gel when the swollen gels were collapsed, indicating that no virus infiltrated the gel. The separation efficiency of influenza virus was 25% – 86% using 22% sucrose gradient centrifugation (Arora *et al.*, 1985). Our results indicate that hydrogel ultrafiltration can possibly replace the conventional method used to concentrate mammalian virus as well as baculovirus. Hydrogel ultrafiltration is a simple and economical method to separate viruses since it does not require special apparatus or significant amount of energy.

Conclusions

Hydrogel ultrafiltration using a temperature-sensitive hydrogel (poly (N-isopropylacrylamide)) concentrated pure and recombinant alkaline phosphatases 1.7 and 1.5 fold with a separation efficiency of 52.4 and 54.2%, respectively. The process also concentrated wild and recombinant viruses (AcNPV) 1.4 and 1.6 fold, yielding a separation efficiency of 48.5 and 60.0%, respectively. The results indicated that the hydrogel ultrafiltration process can be used to concentrate baculovirus and recombinant proteins from insect cells.

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Table 2. Hydrogel ultrafiltration of wild and recombinant AcNPV

	Wild AcNPV			Recombinant AcNPV		
	Feed	Raffinate	Extractant	Feed	Raffinate	Extractant
Virus titer (TCID ₅₀ ml ⁻¹)	1 × 10 ⁵	1.43 × 10 ⁵	ND ^a	1 × 10 ⁵	1.6 × 10 ⁵	ND ^a
Volume (ml)	10	5.3	2.5	10	5	2
Total titer (TCID ₅₀)	1 × 10 ⁶	7.6 × 10 ⁵		1 × 10 ⁶	8 × 10 ⁵	
Virus recovery (%)		76.0			80.0	
Separation efficiency		48.5		60.0		

^a ND: not detectable

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