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Separation and Purification of Useful Proteins Using Hydrogel Ultrafiltration

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The hydrogel process is a different form of ultrafiltration and has been used to separate biological molecules. In this study, the gel pore size was predicted by pulse NMR technique and neural network using a data base obtained from gel filtration chromatography and diffusion experiment. Recombinant alkaline phosphatase expressed in insect cells was concentrated 1.5 times by hydrogel ultrafiltration by swelling at 20°C and collapsing at 35°C at 53-65% separation efficiency and 78-83% enzyme recovery. Wild and recombinant *Autographa californica* nuclear polyhedrosis viruses (AcNPV) were also 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.

Key words: NMR, neural network, *Autographa californica* nuclear polyhedrosis viruses, insect cells, recombinant alkaline phosphatase

INTRODUCTION

A temperature-sensitive hydrogel, poly (N-isopropylacrylamide) gel, was used in separation processes to extract water and low molecular weight solutes from cellulase enzymes [1] because the process does not change the ionic environment or shear conditions of the medium. To use hydrogel as a filter, its pore size has to be characterized. Gel filtration chromatography (GPC) is a very time-consuming technique. Instead, NMR technique can measure the water mobility in gel pores, which is related to the pore size. NMR relaxation curve is easy to obtain. However, the information contained in the NMR relaxation curve is very complex. It is difficult to correlate the pore size with NMR results using conventional statistical analysis techniques such as regression analysis. A new tool, neural network, combined with NMR technique was used to determine the pore size of gel [2]. 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

Preparation of the Hydrogel Beads, Gel Filtration Chromatography and Pore Size Measurement with Diffusion Experiments

Details are described in [3].

NMR Analysis and Neural Network Prediction

A low field pulse NMR machine was used to determine the gel relaxation curves at five different temperatures (15, 20, 25, 30 and 35°C). A total of 100 NMR relaxation curves (20 for each temperature) were obtained. A three layer neural network system was used to predict the gel pore size at different temperatures. NMR relaxation curves were used as inputs, and pore sizes determined by gel filtration chromatography were used as outputs in the neural network. Seventy percent data sets were used for training the neural network system, 18% for testing and 12% for prediction of pore size.

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 from 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. 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.

Hydrogel Concentration Experiments

A solution of commercial 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

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IU) and 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 1×10^5 TCID50 ml^{-1} or recombinant AcNPV solution (10 ml) of 1×10^5 $TCID_{50}$ 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.

Repeated Hydrogel Concentration Experiment

One hour swelling/1 h collapsing cycles were repeated three times. Ten ml of culture broth with specific activity of 7.8 unit/ml was added to test tubes with 0.5 g dry gel. The gel was allowed to swell for 1 h at 20°C, filtered and rinsed with phosphate buffer before collapsing for 1 h at 35°C. The same gel was used for the second hydrogel ultrafiltration using a mixture of raffinate and rinsed buffer as a feed. The same procedure as in the first cycle was used for the second and third cycles. Three hour swelling (at 20°C)/3 h collapsing (at 35°C) experiments were made in the same way.

Analytical Methods

Viral titers were measured in duplicate by $TCID_{50}$ as described elsewhere [4]. Alkaline phosphatase activity was determined spectrophotometrically as described by Davis *et al.* [5]. SDS-PAGE was performed by Laemmli's method [6]. 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

Pore Size Analysis by NMR and Prediction with Neural Network

Nuclear magnetic resonance (NMR) spectroscopy is based on the measurement of resonant, radio-frequency energy absorbed by nonzero spins of nuclei in the presence of an externally applied homogeneous magnetic field. When a sample containing hydrogen protons is placed in a magnetic field, and is subjected to a radio-frequency pulse with the correct frequency, phase, amplitude and duration, the sample emits a signal, which can be measured. This signal is called the free induction decay (FID). The amplitude of the FID decays with time and is recorded as a relaxation curve. This decaying signal is analyzed to give structural information about the sample. This NMR technique has been used to study the state of water in food systems and the microstructure of food materials. Similarly, the relaxation characteristics of water in hydrogel pores can be used to explore the pore size distribution of the hydrogel.

The relaxation curves of the hydrogel at different temperatures are shown in Figure 1. Both the initial intensity and the relaxation rate were larger at a lower temperature. Initial intensity was larger at a lower temperature. This was because there was more water in the hydrogel pores due to the larger pore sizes. In addition, the net difference in populations of protons is also larger than that at a higher temperature. The relaxation rate increased (or relaxation time decreased) at a lower temperature, especially during initial relaxation period. We expect that the relaxation rate increases at a lower temperature and decreases at a larger pore size. Our observation suggests that among these two opposing effects the temperature effect overwhelmed the pore size effect.

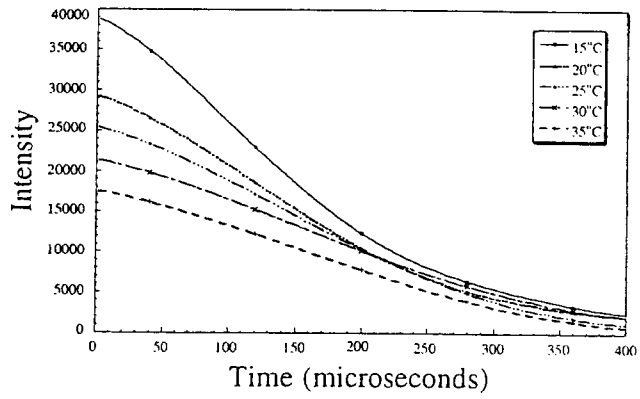

Fig. 1. NMR relaxation curves at different temperatures.

A relaxation curve at each temperature consists of 200 data points (400 microseconds long) and each point was used as an input node (processing element) for neural network processing. The output has only one node, the value of mean pore size. Neural network is a massively parallel system comprised of highly interconnected, interacting processing elements or units that are based on neurobiological models. The neural network was trained starting with random weight setting. This system (backpropagation) would adapt itself to the characteristics of the relaxation curve by changing the weights inside the network. In each training cycle, an input pattern was presented to the network and an output was generated by the network. This output was compared with the actual output. The connection weights were then adjusted to minimize the error between the network-predicted output and actual output. When all were examined, the network started over with the first instance and repeated until the desired error was reached, or the network was trained and ready to predict.

The results of the neural network prediction are shown in Table 1. The predicted values at 15°C and 35°C were the same as those obtained from the gel filtration chromatography and diffusion experiment. Between 20°C and 30°C, the error was larger than that at 15°C and 35°C, since the relaxation curves were close to each other. However, compared with the pore size range, the error was small, indicating that the neural network worked well in predicting the gel pore size from the NMR relaxation curve. By increasing the number of data points used for training, we can possibly increase the prediction accuracy.

Concentration of Alkaline Phosphatase

Hydrogel ultrafiltration concentrated recombinant

Table 1. Comparison of pore sizes from diffusion experiments and neural network prediction

Temperature	Pore size estimated from the diffusion method (DM)	Pore size predicted by neural network (NN)	Fractional error*(%)
15°C	9630	9630	0.0
20°C	5640	5550	1.6
25°C	4550	4480	1.7
30°C	2530	2600	2.7
35°C	678	678	0.0

*Fractional error=(| DM-NN |)/DM × 100%

alkaline phosphatase in the culture broth by 1.5 times. Separation efficiency (%) and enzyme recovery (%) were 54% and 78%, respectively. For commercial alkaline phosphatase (Sigma), hydrogel ultrafiltration concentrated the feed by 1.7 times. Separation efficiency and enzyme recovery were 52% and 73%, respectively. Similar results for recombinant and commercial alkaline phosphatse indicate that the virus (AcNPV) contained in the recombinant culture broth does not interfere hydrogel ultrafiltration of alkaline phosphatase. Low separation efficiencies (54% for recombinant alkaline phosphatase and 52% for commercial alkaline phosphatase) were due to enzyme entrapment between gel particles and attachment on the gel surfaces. When the enzyme activity in the rinsed buffer was added to the total enzyme unit, higher values of enzyme recovery and separation efficiency were obtained (95% and 89%, respectively for recombinant alkaline phosphatase and 95% and 92%, respectively for commercial alkaline phosphatase). The rinsed buffer contains enzymes in the raffinate volume entrained between gel particles and enzymes attached on the gel surface.

Hydrogel ultrafiltration of recombinant culture broth was repeated three times by 3 h swelling (at 20°C)/3 h collapsing (at 35°C) cycles (Table 2). The separation efficiency was 57% in the first cycle, 65% in the second cycle, but decreased in the third cycle. Enzyme recovery was 79% in the first cycle, 83% in the second cycle, but decreased to 65% in the third cycle. Separation efficiency and enzyme recovery % were notably lower in the third cycle. On the other hand, separation efficiency (62%, 54% and 53%, respectively in the first, the second and the third cycle) and enzyme recovery (82%, 81% and 81%, respectively, in the first, the second and the third cycle) were relatively constant for 1 h swelling/1 h collapsing experiment (Table 3). Activity of alkaline phosphatase remained relatively constant for 24 h at 20°C, but decreased to 78% of the initial level 9 h after at 35°C (data not shown). From this we can state that thermal deactivation was responsible for the decrease of enzyme recovery (%) and separation efficiency (%) during three cycles of the 3 h swelling (at 20°C)/3 h collapsing (at 35°C) experiments.

Concentration of Baculovirus (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 [7]. 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.

In conclusion, we showed that the neural network system can relate the hydrogel pore size information

Table 2. Swelling (3 h)/collapsing (3 h) experiment using recombinant alkaline phosphatase

	1st cycle			2nd cycle			3rd cycle		
	F	R	E	F	R	E	F	R	E
Enzyme activity (IU/ml)	7.8	11.9	0.1	7.2	11.4	0.1	6.4	9.2	0.1
Volume (ml)	10.0	5.2	3.2	8.0	4.2	3.2	7.7	3.5	3.0
Total activity (IU)**	78.0	61.9 (73.8)	0.3	57.6	47.9 (53.9)	0.3	49.3	32.2 (41.2)	0.3
Activity recovery (%)**		79 (95)	<1		83 (94)	<1		65 (84)	<1
Separation efficiency (η)		57 (89)			65 (86)			37 (70)	

*F=feed; R=raffinate; E=extractant
**The value in parenthesis was obtained by adding the activity in rinsed buffer to total enzyme activity of the raffinate.

Table 3. Swelling (1 h)/collapsing (1 h) experiment using recombinant alkaline phosphatase

	1st cycle			2nd cycle			3rd cycle		
	F	R	E	F	R	E	F	R	E
Enzyme activity (IU/ml)	7.8	11.9	0.2	7.9	10.9	0.1	7.2	9.9	0.1
Volume (ml)	10.0	5.4	2.7	8.5	5.0	2.5	8.0	4.7	3.0
Total activity (IU)**	78.0	64.3 (73.0)	0.5	67.2	54.5 (64.0)	0.3	57.6	46.5 (54.5)	0.3
Activity recovery (%)**		82 (94)	<1		81 (96)	<1		81 (95)	<1
Separation efficiency (η)		62 (86)			54 (89)			53 (87)	

*See the footnote in Table 2.

Table 4. 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*	1×10^5	1.6×10^5	ND*
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	

* ND: not detectable

obtained from gel permeation chromatography and diffusion experiments to the relaxation curve obtained using NMR. Once the system is trained, neural network can be a powerful and convenient tool to predict the hydrogel pore size under different conditions (temperature, gel composition and preparation method, etc.). The pulse NMR technique and neural networks will be especially useful to predict the nonequilibrium pore size under dynamic volume changing conditions.

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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