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PERFORMANCE OF A PULSED-CAP MICROCOLUMN FOR PROTEIN EXTRACTION

A.P.B.Rabelo and E.B.Tambourgi*

Campinas State University, UNICAMP, School of Chemical Engineering,
P.O. Box 6066, 13081-970, Campinas (SP), Brazil
E-mail: elias@desq.feq.unicamp.br

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Abstract - This work presents the results obtained using a microcolumn agitated by pulsed caps, using aqueous two-phase systems formed polyethylene glycol and salts (monobasic and dibasic) potassium phosphate for protein extraction. Proteins used were extracted cytochrome b5 and the enzyme ascorbic oxidoreductase. It was observed that operation of this equipment was stable and high efficiency values were achieved.
Keywords: aqueous two-phase system, downstream processing, continuous extraction equipment, protein, purification, solvent extraction.

INTRODUCTION

The viability of biomolecule production and commercialization depends on product costs. About 50% to 90% of the cost of biomolecule production is for the purification steps. Therefore, for large-scale production, it is necessary to use efficient and economic bioseparation techniques, which makes it possible to achieve high levels of recovery and grades of purity, maintaining the biological activity of molecules (Diamond and Hsu, 1992).

Partitioning in aqueous two-phase systems is an alternative to the separation processes commonly used, such as chromatography, for example. It has been studied extensively during the past twenty years. The high water content in aqueous two-phase systems makes them an adequate environment for preservation of biomolecule properties.

To reduce the time expended in processes using liquid-liquid extraction, liquid-liquid extraction equipment that operates continuously can be used. To avoid enzyme denaturation and loss of the main protein properties due to agitation in this kind of equipment, a system formed of pulsed caps, which produces efficient contact between the phases in the column, was used.

The aqueous two-phase system is a kind of extraction system that can preserve protein and enzyme properties; it can be formed of two polymers dispersed in water or a polymer and salt dispersed in water. Aqueous two-phase systems studied were formed of PEG (polyethylene glycol) and salts (monobasic and dibasic potassium phosphate). Proteins used were cytochorome b5 extract at pH 7.3 and extract of the enzyme ascorbic oxidoreductase at pH 6.0. In the extraction a microcolumn was used for continuous operation, with better system conditions obtained in the batch experiments.

METHODS

Equipment

Figure 1 shows a diagram of the pulsed-cap microcolumn. The equipment used is formed of a glass tube 19 cm high with an outside diameter of 2.84 cm and an inside diameter of 2.54 cm. In the

*To whom correspondence should be addressed

centre of this microcolumn, there is a stainless steel shaft onto which three caps have been soldered at a distance of 4 cm from each other. The base of the caps has a 2.49 cm diameter, with a free area for flow of about 38%.

Figure 2 shows the agitation produced by the cap's movement up-and-down. Pulsed caps are proposed to produce an efficient but gentle agitation thereby avoiding enzyme denaturation. The caps increase the contact time between phases inside the column and produce uniform dispersion of drops, which improves mass transfer (Rabelo, 1999).

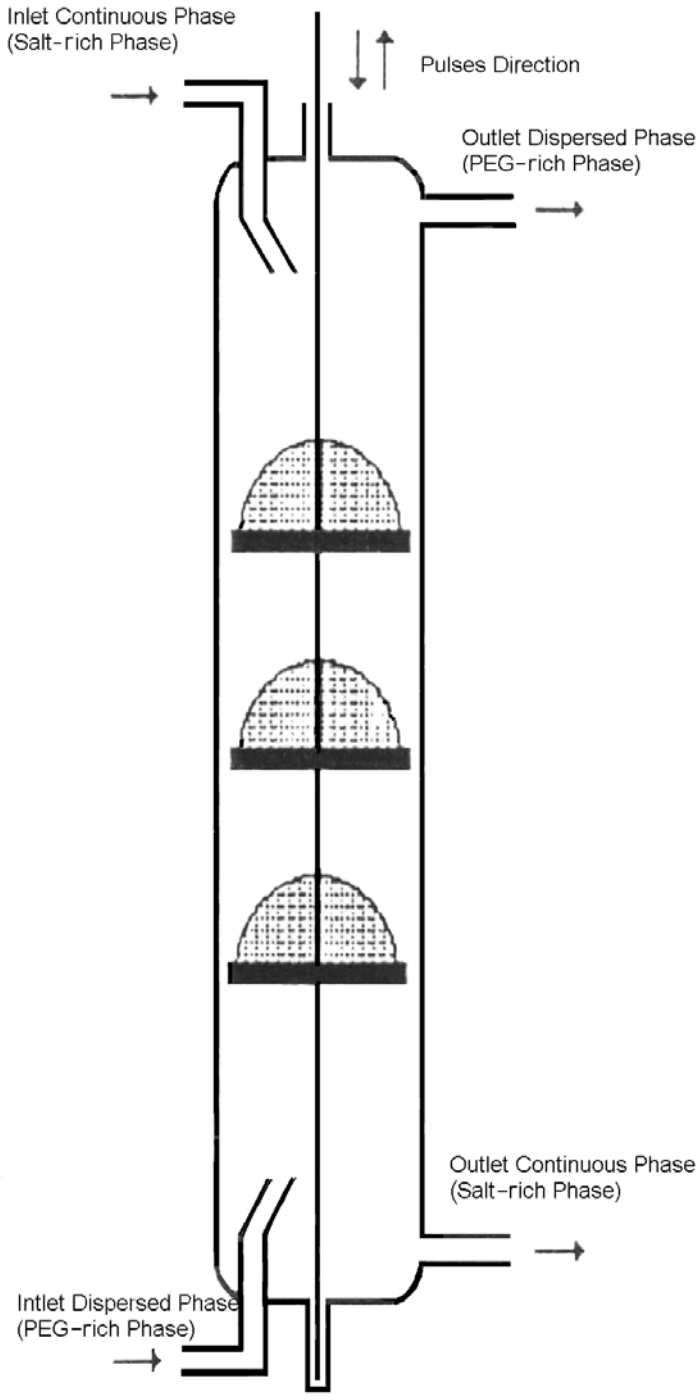

Figure 1: Pulsed-cap microcolumn.

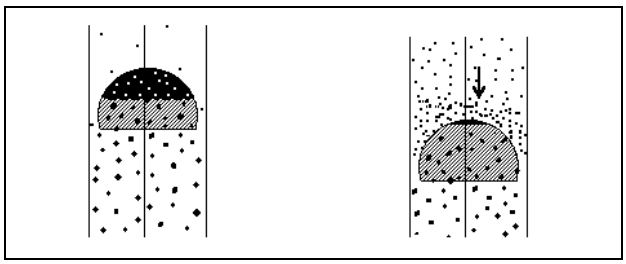

Figure 2: Cap movement and the agitation produced.

Proteins Studied

Two proteins were used: cytochrome b5, which has molecular weight 13.6 kDa and was obtained by fermentation using Escherichia coli and LB - Lurian-Bertani fermentation, and ascorbic oxidoreductase, which has a molecular weight of about 150 kDa and was extracted from curcubita maxima.

Aqueous two-phase extraction systems

The extraction system used in operation of the equipment was determined with batch experiments as the system with the highest extraction and separation efficiencies.

For cytochrome b5 extraction, the system used in continuous extraction was PEG 1000 (polyethylene glycol with a molecular weight of 1000 Da) and potassium phosphate. The system concentration was 17.7% PEG 1000/15.7% potassium phosphate (% w/w), and the ratio of monobasic to dibasic potassium phosphate was chosen to achieve pH 7.3. The system formed of 16.2% PEG 1500/14.3% potassium phosphate (% w/w) was also used, and the ratio of monobasic to dibasic potassium phosphate was chosen to achieve pH 7.3.

The aqueous two-phase system for ascorbic oxidoreductase extraction during continuous operation of the microcolumn was 16.2% PEG 1500/14.3% potassium phosphate (% w/w), and the ratio of monobasic to dibasic potassium phosphate was chosen to achieve pH 6.0.

ANALYTICAL METHODS

Cytochrome b5 Concentration

The concentration of cytochrome b5 was determined by measuring its absorbance at 411 nm (extinction coefficient (ε) =130 $(mmol/L)^{-1}.cm^{-1}$), read in a spectrophotometer (Porto, 1998).

Ascorbic Oxidoreductase Activity

Ascorbic oxidoreductase activity was determined by the method proposed by Carvalho et al. (1981), which uses ascorbic acid in citrate/phosphate solution at pH 6.0 as substract.

Solute Recovery Fraction

The solute recovery fraction is the ratio of the protein or enzymatic activity recovery in the solvent phase to the protein in the feed (extract).

Phases in the column

Protein extract is added to the PEG-rich phase and this solution is the dispersed phase, which is the light phase in the column. The salt-rich phase is the solvent and is the continuous phase in the column.

RESULTS

These proteins were chosen because of their different molecular weights, which allows study of the influence of molecular weight on extraction and equipment operation.

In Figure 3, the behaviour of cytochrome b5 extraction using the aqueous two-phase system described earlier was shown. The time of microcolumn operation was fixed at 70 minutes for every study conducted.

This figure shows that the best operational conditions studied for the system using PEG 1000 are an overall flowrate of 4.7 mL/min and a pulse frequency of 1 pulse/second. The ratio of dispersed to continuous phases was fixed at 1.

According to the results presented, a tendency to attain the stationary state at 30 minutes of column operation is verified in spite of flutuations in operation time. The solute recovery fraction obtained under these conditions was about 72% ± 8%, which was the highest recovery obtained.

Figure 4 shows the influence of overall flowrate and pulse frequency on the solute recovery fraction. It can be observed that the highest protein recovery was achieved at high pulse frequencies and low overall flowrates. The ranges of these parameters were changed as follows:

Overall Flowrate: 4.7 mL/min to 10.9 mL/min;

Pulse Frequency: 0.1 pulse/second to 1 pulse/second.

High pulse frequency increased the mixing of phases and contact between phases is what improved the extraction of protein from the fermentation broth.

Low overall flowrates caused the contact time between phases to increase because the flow speed was lower and phases remained in contact longer inside the column.

The same behaviour was observed using PEG 1500 instead of PEG 1000 and different proteins under the best operational conditions for the pulsed-cap microcolumn, determined earlier.

The solute recovery fraction obtained was about 81% ± 8%. This result is similar to that obtained in the batch experiments, which is about 71.4%.

Ascorbic oxidoreductase was extracted using an aqueous two-phase system formed of PEG 1500/ potassium phosphate, as described earlier.

Figure 5 shows behaviour observed for ascorbic oxidoreductase extraction during column operation. It can be observed that the extraction behaviour is similar in both cases, cytochrome b5 extraction and ascorbic oxidoreductase extraction. Stationary conditions were observed at 30 minutes of microcolumn operation, as observed for cytochrome b5 extraction.

The solute recovery fraction obtained was about 61.7% ± 8%. This result is the same as that obtained in the batch experiments, i.e. 61.7%. Therefore, the molecular weight of the proteins does not influence extraction behaviour for extraction in the pulsed-cap microcolumn.

The lower solute recovery fraction for ascorbic oxidoreductase extraction was also observed in the batch experiments. Therefore, the reduction in the solute recovery fraction is characteristic of protein extraction in continuous or batch experiments and is not related to the continuous extraction equipment used in the experiments. It is known that extraction of proteins with high molecular weights is more difficult, depending on the salt and polymer phase used and their concentrations. This shows that the pulsed-cap microcolumn can be used to extract different proteins using different aqueous two-phase systems.

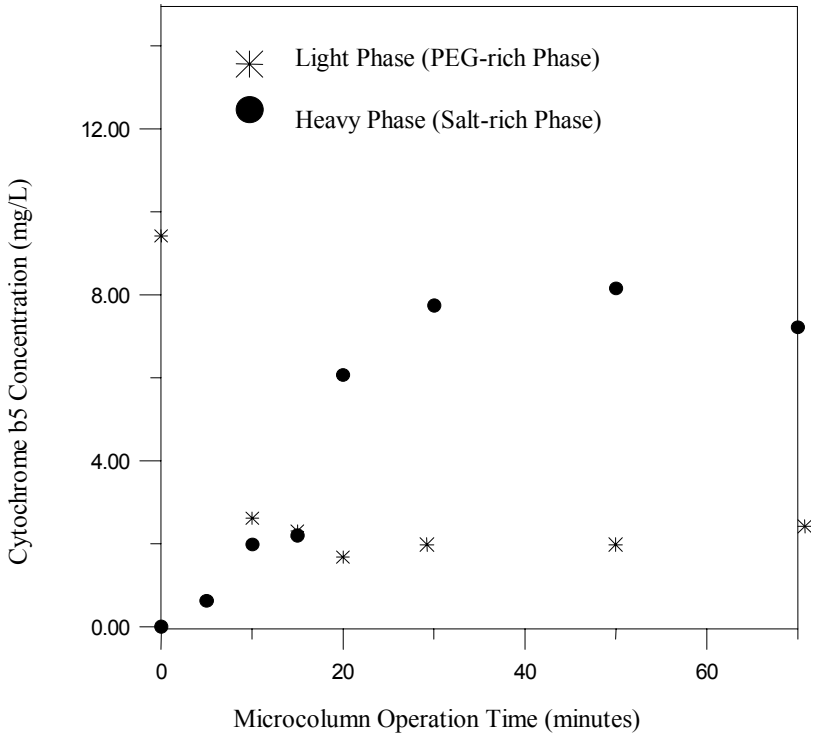

Figure 3: Cytochrome b5 extraction during microcolumn operation.

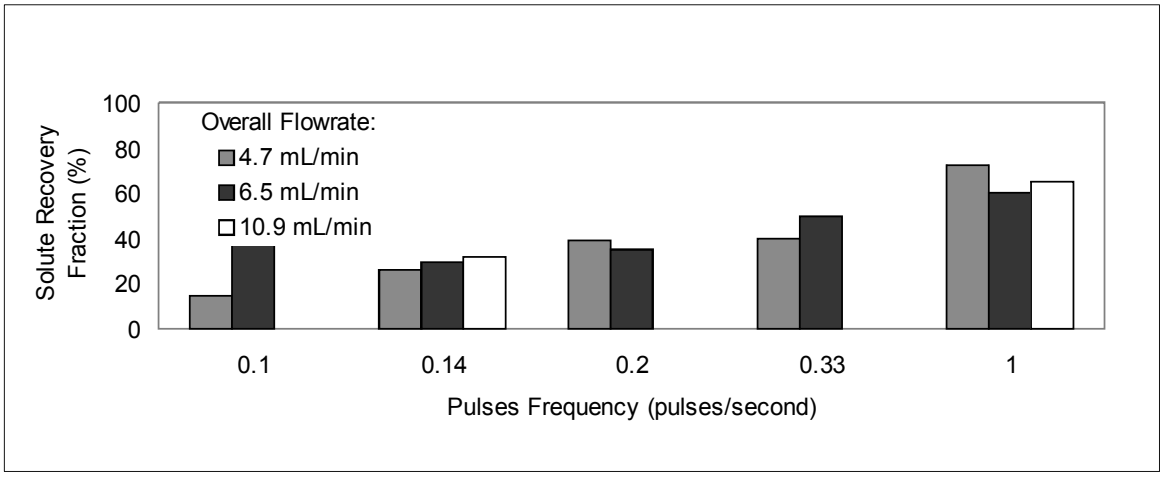

Figure 4: The influence of overall flowrate and pulse frequency on cytochrome b5 extraction.

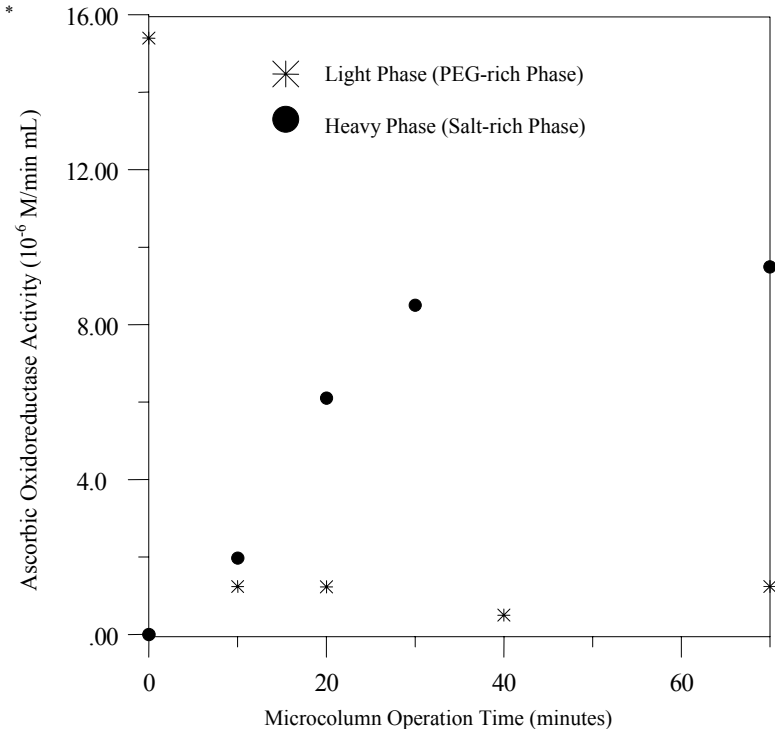

Figure 5: Ascorbic oxidoreductase extraction during microcolumn operation.

CONCLUSIONS

This study shows that the equipment developed, the pulsed-cap microcolumn, is adequate for biomolecule extraction. The pulsed-cap microcolumn had an excellent performance and stable operation under conditions studied. It was proven that agitation imposed on the system by pulsed caps does not cause denaturation of the enzyme studied (ascorbic oxidoreductase) under the operational conditions studied. The best results were obtained at high pulse frequencies and low overall

flowrates. Equipment behaviour was shown to be independent of molecular weight of the proteins. Values for the solute recovery fraction were higher than values obtained in batch experiments, but in less processing time. Results show that it is possible to operate a pulsed-cap microcolumn continuously using aqueous two-phase systems for extracting different proteins and enzymes. Stationary state was attained in a short time and phase separation during operation of the column was fast.

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