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Short communication

Variation of penicillin acylase partition coefficient with phase volume ratio in poly(ethylene glycol)–sodium citrate aqueous two-phase systems

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Abstract

The influence of phase volume ratio on partition and purification of penicillin acylase from *Escherichia coli* on poly(ethylene glycol)–sodium citrate aqueous two-phase systems was studied. In PEG 1000 systems both partition coefficients of the enzyme and total protein increased with decreasing phase volume ratio. However, in PEG 3350 containing NaCl, penicillin acylase follows a reverse trend, while total protein behaves in the same way. Implications for protein purification designs are discussed.

Keywords: Aqueous two-phase systems; Partitioning; Penicillin acylase; Enzymes

1. Introduction

Aqueous two-phase systems have been widely used for the purification of proteins. Their technical simplicity, ease of operation and scale-up, makes them very attractive for industrial applications. However, this simplicity is only apparent as there are many factors that influence the partition and purification of proteins in these systems. Currently, there is no model allowing the design of specific conditions for the purification of a known protein. Most studies in this field are empirical and purification is achieved by systematic variation of several factors.

In a previous work [1] we reported the partial purification of penicillin acylase from osmotic shock extracts of *Escherichia coli* on poly(ethylene glycol) (PEG)–sodium citrate systems. In the absence of NaCl the best results were obtained with PEG 1000, long tie-line and pH 6.9. The purification factor was 2.6 and the yield 83%. The addition of 8.8% (w/w) NaCl to PEG 3350–sodium citrate pH 6.9 systems, improved the purification to 5.7 with 85% yield.

Theoretically, the purification factor is proportional to the separation factor, which is the ratio between the partition coefficients of the target protein (K_e) and total protein (K_p). However, in our former study, the systems with the higher separation factors did not yield the best purification. This could be due to the phase volume ratio employed. It is known that this parameter also influences the purification factor and yield in aqueous two-phase systems. In systems with $K_e > K_p$, the purification factor increases with the decrease of the phase volume ratio, whereas the yield decreases.

Previous studies were performed with a phase volume ratio close to one; optimisation of purification could, therefore, be achieved by manipulating this ratio, and trying to find a good compromise

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between purification factor and yield. This is only possible if the partition coefficients both for total protein and target protein remain constant along a given tie-line. Some authors observed increase of total protein partition coefficient with the decrease of phase volume ratio [2,3] and other studies report increase of single protein partition coefficient under the same conditions [4].

In this work we studied the influence of the phase volume ratio on the partition coefficients of total protein and penicillin acylase, and its implication on purification.

2. Experimental

2.1. Materials

All reagents used were of analytical grade. PEG 1000 and PEG 3350 were obtained from Sigma (St. Louis, MO, USA).

2.2. Production and extraction of penicillin acylase

A mutant strain of *Escherichia coli* ATCC 9637 was grown in 1000 cm^3 shake flasks with 250 cm^3 of medium, containing 1% (w/v) yeast extract and 0.3% (w/v) phenylacetic acid, at pH 7.0, 300 rpm and 25°C. Cells were harvested by centrifugation at 12 000 *g*, for 10 min at the end of the exponential phase (40 h), washed with 200 m*M* phosphate buffer pH 7.5, and stored at 4°C until used.

Enzymatic extract was obtained by cold osmotic shock rupture of the cells. Cells were suspended in 250 m*M* Tris–HCl pH 8.0+12.5 m*M* EDTA+20% (w/w) sucrose and stirred for 15 min at 4°C. Cells were separated from the previous solution by centrifugation and resuspended in cold distilled water for 30 min with stirring at 4°C. Intact cells and cell debris were removed by centrifugation and the extract was stored at 4°C.

2.3. Characterisation of aqueous two-phase systems

Binodal curves were determined by titration according to Albertsson [5]. Small amounts of water were added to several biphasic systems of defined composition until turbidity disappeared. Total composition of the system was then calculated and taken as a binodal point. Tie-lines were defined by determining the composition of top and bottom phases for selected system. Citrate was quantified by isocratic elution on a Merck 5 μm LiChrospher 100 RP-18, (250×4 mm I.D.) column, with 200 m*M* phosphoric acid–methanol (90:10, v/v) as eluent, at a flow-rate of 1.0 ml/min. Detection was at 220 nm and the concentration was calculated from the calibration curve. Chloride concentration was determined by the Volhard titration method according to Ref. [6]. In systems containing sodium chloride its concentration was estimated by assuming that it was equal to chloride concentration. PEG concentration was determined by refractometry after correcting for the contribution of the citrate and NaCl, if present.

2.4. Preparation of aqueous two-phase systems

Stock solutions of 50% (w/w) PEG 1000, 50% (w/w) PEG 3350 were prepared and stored at 4°C. Concentrated sodium citrate solutions (35.3%, w/w) at the required pH were prepared by mixing appropriate amounts of equimolar solutions of trisodium citrate dihydrate and citric acid monohydrate. Systems were prepared at 20±1°C by the mixture of enzymatic extract with suitable amounts of PEG and citrate solution in 15-ml graduated tubes with conical tips. Solid NaCl was added when needed. Water was added to a final amount of 8 g. After vortex shaking for 1 min the two phases were separated by centrifugation and assayed for protein concentration and penicillin acylase activity.

Protein concentration was determined by the method of Bradford [7]. To correct for the interferences of PEG and citrate, samples were diluted and read against blanks with the same composition but without enzymatic extract.

Penicillin acylase activity was assayed by the method of Kutzbach and Rauenbusch [8]. The hydrolysis of 6-nitro-3-(phenylacetamido)benzoic acid (NIPAB) was followed spectrophotometrically by the increase in absorbance at 410 nm. The reaction was performed at 37°C in 100 m*M* phosphate buffer pH 7.5 in stirred cells. Under these conditions neither PEG nor citrate interfered with the enzymatic activi-

ty. Enzymatic activity (Act.) was calculated from the following expression:

$$\text{Act. (U/ml)} = \frac{\Delta\text{Abs}}{\Delta t v 4.49} \quad (1)$$

where v is the volume of the sample analysed.

3. Results and discussion

3.1. PEG 1000–sodium citrate systems

In our former study [1] higher separation factors were obtained in systems containing PEG 1000–sodium citrate pH 7.6. These should be, theoretically, the most selective and were thus chosen for phase volume ratio variation studies. The tie-lines for three composition previously studied were defined and designated as short (27.1%, w/w), medium (42.1%, w/w) and long (48.9%, w/w) tie-lines (Fig. 1). To confirm these determinations several systems along the calculated long tie-line were analysed for top and bottom phase composition. Within the experimental error, the composition of top and bottom phase of these systems was equal (Fig. 2).

The effect of phase volume ratio on the extraction of penicillin acylase and total protein was studied in several systems at the long tie-line. Partition coefficients of the enzyme and total protein increased with decreasing phase volume ratio (Fig. 3). A small increase of purification factor and yield (Fig. 4) was due to a greater increase of K_e than K_p (Fig. 3). The maximum values of purification factor (3.4) and yield (92%) were obtained for a phase volume ratio of 0.2. No significant improvement of purification could be achieved by manipulating the phase volume ratio.

Similar experiments at medium and short tie-lines were carried out in order to get a better understanding of the PEG 1000–sodium citrate pH 7.6 system. For systems of the medium tie-line, the behaviour was the same as previously observed (Figs. 3 and 4). However, for the short tie-line, while penicillin acylase partition coefficient remains almost constant, total protein coefficient increased as observed for the medium and long tie-line systems (Fig. 3). As expected in these conditions a decrease in purification factor and a significant decrease of yield were observed with decreasing phase volume ratio (Fig. 4).

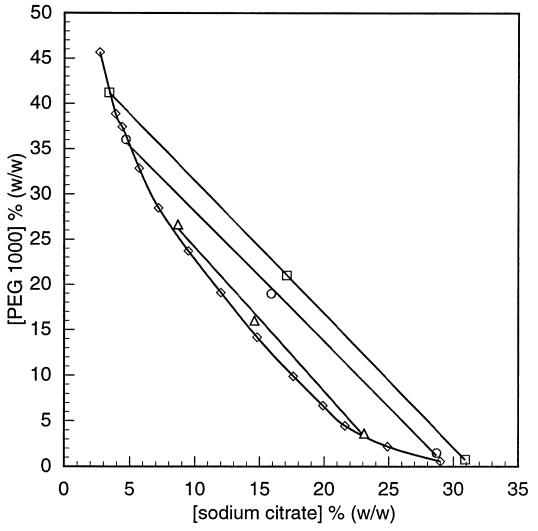

Fig. 1. Phase diagram for PEG 1000–sodium citrate system at pH 7.6. (◇) Binodal line. (△) Short tie-line (27.1%, w/w). (○) Medium tie-line (42.1%, w/w). (□) Long tie-line (48.9%, w/w).

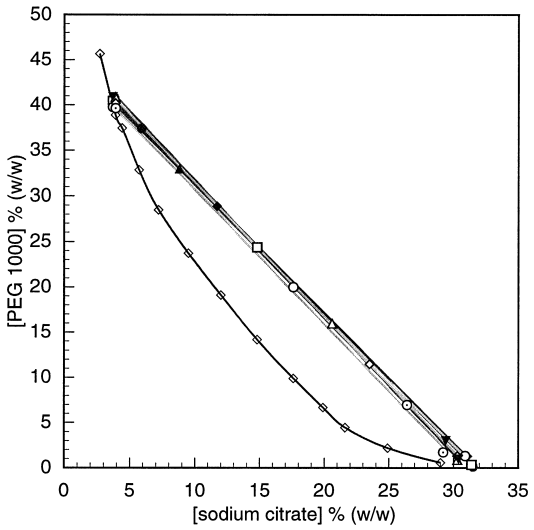

Fig. 2. Confirmation of the long tie-line determination. Systems along the previously determined tie-line were prepared and analysed for bottom and top phase composition. The total composition of citrate and PEG in the systems were, respectively: (●) 5.9%, 37.4%. (▲) 8.8%, 33.0%. (◆) 11.7%, 28.9%. (□) 14.8%, 24.4%. (○) 17.6%, 20.0%. (△) 20.6%, 16.0% (◇) 23.5%, 11.5%. (⊙) 26.6%, 7.0%. (▼) 29.4%, 3.0%.

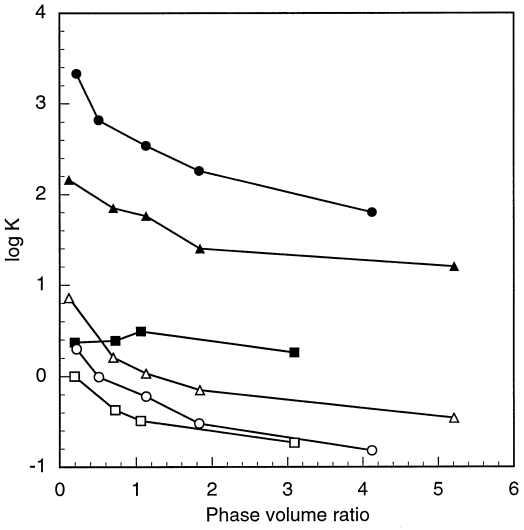

Fig. 3. Influence of phase volume ratio on partition coefficient of total protein (open symbols) and of penicillin acylase (filled symbols) at different tie-line lengths in PEG 1000–sodium citrate pH 7.6 systems. (□, ■) Short tie-line. (△, ▲) Medium tie-line. (○, ●) Long tie-line.

These results agree with those obtained by Huddlestone et al. [4] in PEG–potassium phosphate systems for a single pure protein (bovine serum albumin), who also reported that the protein partition

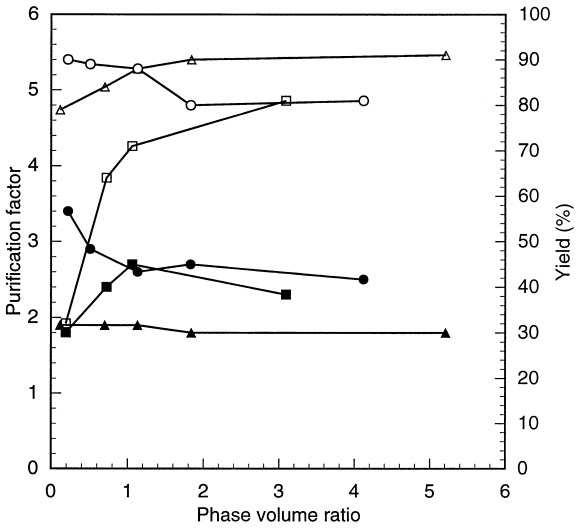

Fig. 4. Influence of phase volume ratio on yield (open symbols) and purification factor (filled symbols) at different tie-line lengths in PEG 1000–sodium citrate pH 7.6 systems. (□, ■) Short tie-line. (△, ▲) Medium tie-line. (○, ●) Long tie-line.

coefficient only remains constant along a given tie-line when it is close to the binodal. This suggests that in PEG–salt systems optimal behaviour is observed only in phase diagram regions where the differences between the two-phase compositions are lower. However, in other studies the phase volume ratio was shown to have no influence on protein partition coefficient [2,3]. Schmidt et al. [2] argued that the differences observed by Huddelston et al. [4] could be due to working with systems that do not have similar top and bottom phase compositions and/or that are saturated. None of these possibilities seem true here. As shown in Fig. 2 the systems employed have the same top and bottom composition. Although the determinations were made in the absence of enzymatic extract, the total concentration of protein is too low to significantly change the composition (<0.8 mg/ml) or to imply saturation.

The different results reported by several authors must be due to differences in physicochemical properties of the proteins studied. Characteristics such as molecular mass, isoelectric point (pI) and surface hydrophobicity/hydrophilicity determine their behaviour and could account for the differences observed. Further information is required in order to establish the most significant factors.

3.2. PEG 3350–sodium citrate, NaCl systems

The system containing 14% (w/w) PEG 3350, 12.4% (w/w) sodium citrate pH 6.9, 8.8% (w/w) NaCl gave the best purification results in the previous study [1]. Phase volume ratio variation was also studied in an attempt to improve purification. The tie-line for this system was determined and confirmed as for PEG 1000 systems. K_p values increased with the decrease of phase volume ratio (Fig. 5). However, unexpectedly, K_e showed a reverse trend in these systems decreasing with the decrease of phase volume ratio (Fig. 5). Nevertheless, the purification factor increases under these conditions (Fig. 6). This was only possible with a yield decrease (Fig. 6).

As far as we know, this is a first report on the decrease in protein partition coefficient with the decrease in phase volume ratio. This is an interesting result, as we observed an inversion of the trend with the change of system composition for a single

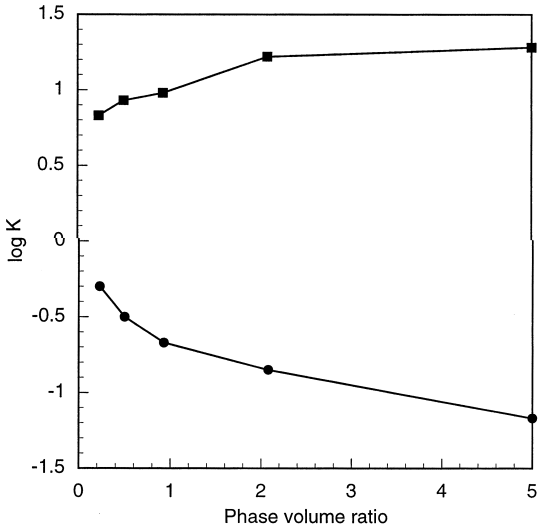

Fig. 5. Influence of phase volume ratio on partition coefficient of total protein (■) and of penicillin acylase (●) in PEG 3350–sodium citrate pH 6.9, 8.8% (w/w) NaCl, medium tie-line (35.6%, w/w).

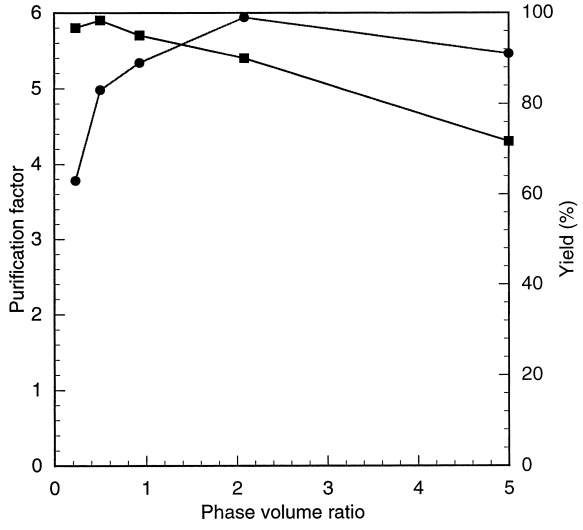

Fig. 6. Influence of phase volume ratio on purification factor (■) and yield (●) in PEG 3350–sodium citrate pH 6.9, 8.8% (w/w) NaCl, medium tie-line (35.6%, w/w).

protein, whereas the behaviour of the total protein remained similar. This result shows that each protein behaves differently depending on its characteristics.

4. Conclusions

The results reported here reveal the importance of the phase volume ratio in the optimisation of protein purification in PEG salt systems. In general, it does not seem possible to significantly increase the purification factor by decreasing phase volume ratio due to the large variation of protein partition coefficient along a given tie-line. However, under particular conditions, it may be possible to reduce the phase volume ratio without loss of yield, which would happen if the protein partition coefficients remained constant. This allows concentration of samples as well as purification.

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