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Short Communication

Mild hydrophobic interaction chromatography: prediction of chromatographic behaviour by preliminary analysis by "partition between aqueous two-phase systems"

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ABSTRACT

Mildly hydrophobic stationary phases prepared by coupling amino derivatives of various polyoxyalkylene glycols to Sepharose 6B have previously proved successful for the separation of three closely related enzymes present in the same crude extract. A preliminary analysis of the crude mixture of proteins in aqueous biphasic systems consisting of a polyoxyalkylene glycol-rich top phase and a saline bottom phase affords valuable information that can be exploited to predict experimental conditions for optimum resolution by chromatography.

INTRODUCTION

Mild hydrophobic polymers may be covalently immobilized onto inert supports to afford chromatographic stationary phases that are less denaturing towards biomolecules than those generally used in traditional hydrophobic interaction chromatography [1–7]. Such stationary phases were prepared by coupling amino derivatives of various polyoxyalkylene glycols of increasing hydrophobicity [polyoxyethylene (POE) < Pluronic (copolymer of polyoxyethylene and polyoxypropylene) < polyoxypropylene (POP)] to Sepharose 6B previously activated by carbonyldiimidazole. The extraction of three closely related enzymes from *Pseudomonas testosteroni* [$\Delta_{5\rightarrow4}$ 3-oxosteroid isomerase (isomerase), 3α-hydroxysteroid dehydrogenase (α-HSD) and 3β,17β-hydroxysteroid dehydrogenase (β-HSD)] with the double challenge of starting from the same crude extract and performing the purification simply by stepwise elution, was described previously [7]. These chromatographic experiments were carried out on Sepharose–POE and Sepharose–Pluronic, with potassium phosphate as the mobile phase [7].

In this paper, we show that a preliminary analysis by "partition between

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aqueous two-phase systems" [8] affords valuable information that can be exploited to explain our chromatographic results. Such a procedure could be used in mild hydrophobic chromatography as a general approach, prior to chromatographic experiments, in order to predict the most satisfactory conditions for optimum resolution.

EXPERIMENTAL

All chemicals used in this study have been described previously [7], as well as the preparation of *Pseudomonas testosteroni* crude extract [5].

Biphasic systems were prepared as follows. Desired amounts of POE 6000, Pluronic, and potassium phosphate were dissolved in water (3 ml) in haemolysis tubes. After addition of *Pseudomonas testosteroni* crude extract (0.25 ml), the mixtures were adjusted to 5 g with water. The overall final content of polyethers (POE + Pluronic) was 10% (w/w), with the desired POE-to-Pluronic ratios (10:0, 9.5:0.5, 9:1 and 8:2). The final content of potassium phosphate was 10, 15 or 20% (w/w). After 30 inversions, the mixtures were allowed to stand at room temperature for 1 h. Aliquots of both phases were then carefully removed by pipette and assayed for the various enzymatic activities according to procedures already described [9,10].

RESULTS

Mild hydrophobic chromatographic experiments have previously been carried out on stationary phases prepared by covalent immobilization of POE or Pluronic on Sepharose, and with potassium phosphate as the mobile phase [7]. In this work, different aqueous two-phase systems were prepared by mixing potassium phosphate (final concentration 10, 15 or 20%, w/w) with POE + Pluronic mixtures (final overall content 10%, w/w) of various compositions (POE-to-Pluronic ratios from 10:0 to 8:2). The resulting biphasic systems are composed of a phosphate-rich bottom phase and a polyether-rich top phase, the hydrophobicity of which increases with increasing amount of Pluronic.

The partition coefficient of an enzyme, taken as the ratio of its concentration in the top phase to that in the bottom phase, is therefore a direct indication of the ability of the considered enzyme to establish interactions with the polyethers. Consequently, when chromatography with Sepharose–POE or Sepharose–Pluronic stationary phases and potassium phosphate as the mobile phase is applied, high partition coefficients ($K > 1$) should correspond to retention or retardation by the stationary phases. Conversely, biomolecules with low partition coefficients ($K < 1$) favour the phosphate-rich bottom phase and should be eluted without retardation.

Fig. 1. shows the variation of the partition coefficients of the three enzymes with the content of Pluronic in the mixture at three different potassium phosphate concentrations. Irrespective of both the amount of Pluronic in the top phase and the potassium phosphate concentration in the bottom phase, $K_{\text{isomerase}} > K_{\beta\text{-HSD}} \gg K_{\alpha\text{-HSD}}$. Interactions of the polyethers with the enzymes therefore decrease in the order isomerase > β-HSD $\gg$ α-HSD. Chromatography of the three enzymes on Sepharose–POE and on Sepharose–Pluronic, with potassium phosphate as the mobile phase should therefore result in elution in the order α-HSD, β-HSD and finally

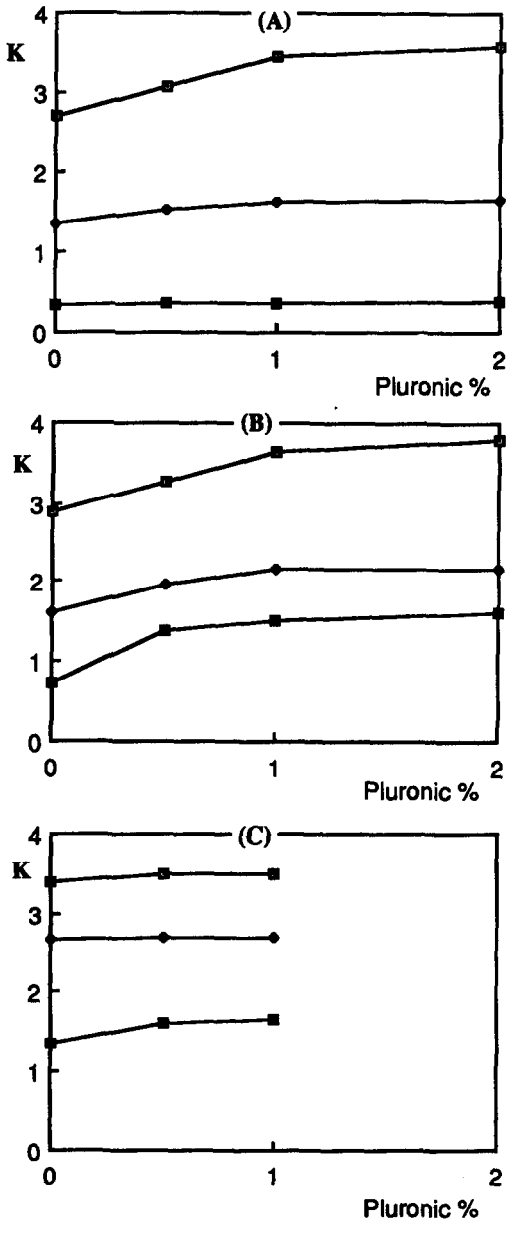

Fig. 1. Partition coefficients (K) of (□) isomerase, (◆) β-HSD and (■) α-HSD *vs.* amount of Pluronic in the biphasic system at different potassium phosphate concentrations: (A) 10, (B) 15, (C) 20% (w/w).

isomerase. This was verified experimentally, as illustrated by elution profiles obtained under various stationary and mobile phases conditions in Figs. 2 and 3.

In addition, the hydrophobic nature of the interactions between the three enzymes and the polyethers is evidenced by two facts. First, all the partition

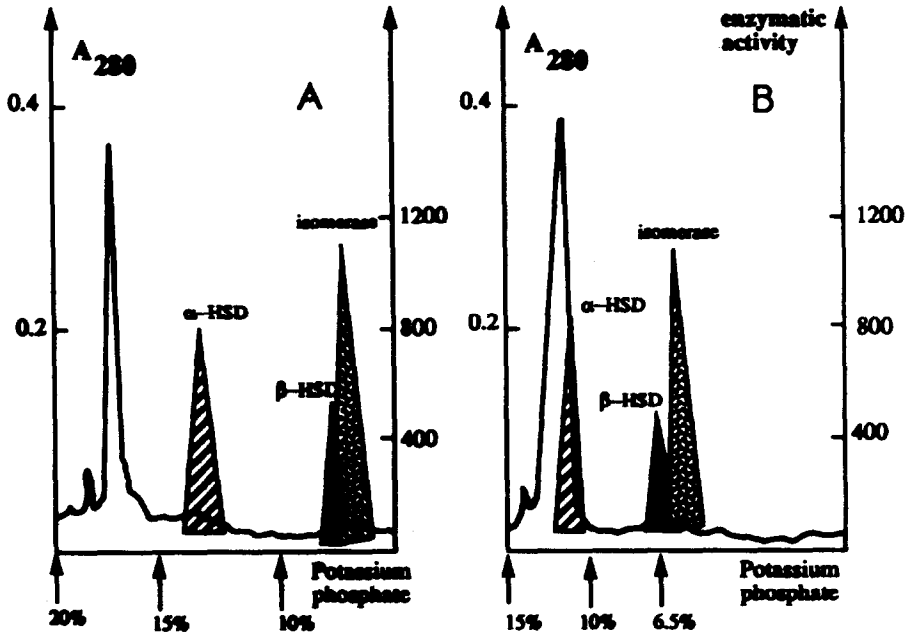

Fig. 2. Results obtained with Sepharose–POE stationary phase. Stepwise elutions of the *Pseudomonas testosteroni* crude extract (0.4 ml) starting with (A) 20% or (B) 15% potassium phosphate (pH 7.0) as the mobile phase (ionic strength changes during the stepwise elutions are indicated by arrows). Column, 38 cm × 1 cm I.D.; flow-rate, 26 ml/h; fractions taken every 5 min; room temperature. The enzymatic activities are indicated in arbitrary units.

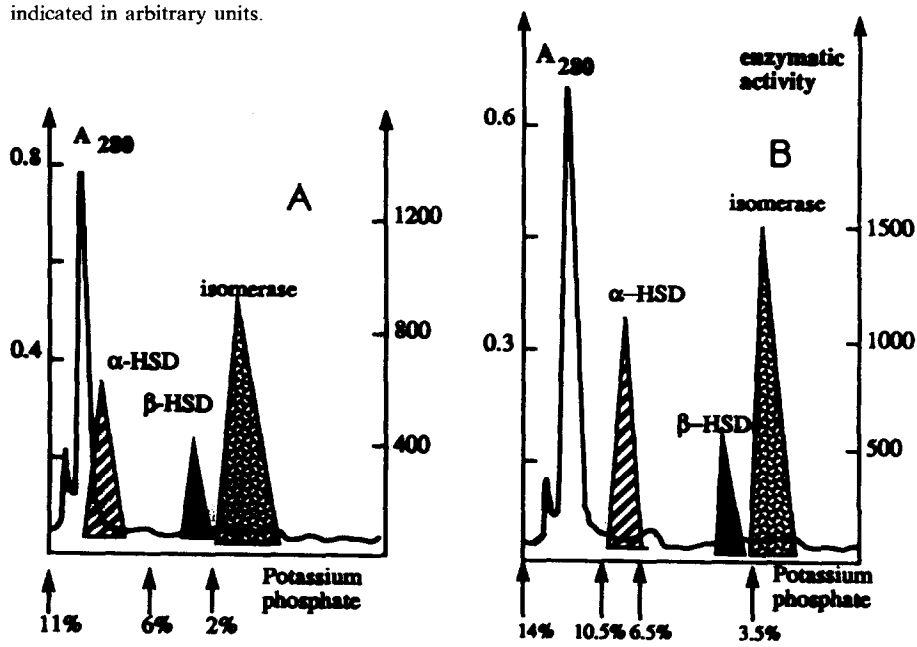

Fig. 3. Results obtained with Sepharose–Pluronic stationary phase. Stepwise elutions of the *Pseudomonas testosteroni* crude extract (0.4 ml) starting with (A) 11% or (B) 14% potassium phosphate as the mobile phase. Column, 29 cm × 1 cm I.D.; other conditions as in Fig. 2.

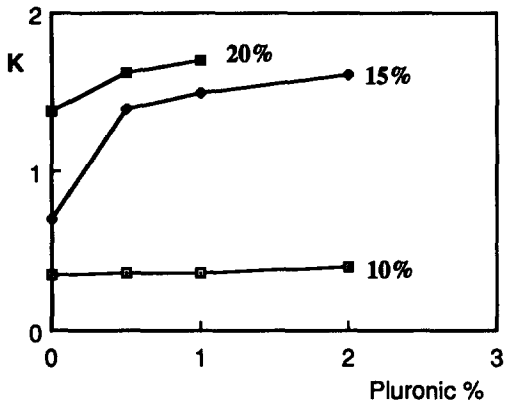

Fig. 4. Partition coefficients of α-HSD *vs.* Pluronic content in the top phase at the three different potassium phosphate concentrations: □ = 10; ◆ = 15 and ■ = 20% (w/w) (rearrangement from values in Fig. 1).

coefficients increase with increasing amount of Pluronic in the top phase, *i.e.*, as the hydrophobic character of this phase becomes stronger. Second, for any given composition of the top phase, an increase in the phosphate concentration in the bottom phase, *i.e.*, an increase in the ionic strength, results in enhanced partition coefficients. This effect is in agreement with general principles governing hydrophobic interactions.

The second observation concerns α-HSD. Obviously, this enzyme exhibits only a very limited tendency to display hydrophobic interactions with the polyethers ($K \ll 1$ at 10% potassium phosphate) (Fig. 1A). Nevertheless, its partition coefficient can be shifted above 1, under the effect of either a higher ionic strength or an increase in the Pluronic content in the top phase, or a combination of both (Fig. 4). Its chromatographic behaviour is in agreement with these results. On Sepharose–POE, retention of α-HSD requires a 20% potassium phosphate mobile phase (compare Fig. 2A and B). Correspondingly, at 0% Pluronic, $K_{\alpha\text{-HSD}} < 1$ for potassium phosphate concentrations of 10% and 15%; $K_{\alpha\text{-HSD}} > 1$ for a potassium phosphate concentration of 20% (Fig. 4). The slightly stronger hydrophobic character of Sepharose–Pluronic allows retention of this enzyme at only a 14% potassium phosphate concentration in the mobile phase (compare Fig. 3A and B), again in agreement with the partition experiment [*e.g.*, with 0.5% Pluronic, $K_{\alpha\text{-HSD}} < 1$ at 10% potassium phosphate and $K_{\alpha\text{-HSD}} > 1$ at 15% potassium phosphate (Fig. 4)].

Finally, β-HSD and isomerase have $K > 1$ for all cases described in Fig. 1. As a result, both should be retarded or retained on Sepharose–POE or Sepharose–Pluronic, provided that the mobile phase phosphate concentration is at least 10%. Further, as ΔK ($K_{\text{isomerase}} - K_{\beta\text{-HSD}}$) increases with increasing amount of Pluronic, a better separation of these two enzymes should be obtained by chromatography on Sepharose–Pluronic than on Sepharose-POE. The chromatographic profiles obtained with these two stationary phases are in agreement with this expectation (compare Figs. 2 and 3).

The results of these preliminary partition experiments allowed us to develop a strategy for optimum chromatograhic resolution, as illustrated in Fig. 3B. The chromatography of the crude mixture was carried out on Sepharose–Pluronic, starting

with a potassium phosphate concentration in the mobile phase (14%) sufficient to afford total retention of the three enzymes. After removal of the unretarded contaminants, the potassium phosphate concentration was decreased to 10.5%, resulting, as expected in the elution of α-HSD. A further decrease in the potassium phosphate concentration to 6.5% allowed the elution of β-HSD and finally isomerase at 3.5% potassium phosphate.

Under these conditions, good recoveries of the enzymatic activities were obtained (α-HSD 60%, β-MHSD 40%, isomerase 65%) together with almost quantitative removal of the contaminants in each fraction (residual contaminants expressed as a percentage of the total proteins in the starting crude extract: α-HSD 1.5%, β-HSD 2%, isomerase not detectable).

The correlation obtained in this study between the analyses by partition and the chromatographic elution profiles suggests that this procedure might be used more generally in the field of mild hydrophobic interaction chromatography.

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