Journal of Chromatography, 319 (1985) 43–50
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,262

SEPHAROSE 6B SUBSTITUTED WITH POLYETHYLENE OXIDE-LIKE DERIVATIVES FOR CHROMATOGRAPHIC FRACTIONATION OF PROTEIN MIXTURES

R. MATHIS, P. HUBERT* and E. DELLACHERIE
Laboratoire de Chimie-Physique Macromoléculaire, C.N.R.S.—E.R.A. No. 23, 1, rue Grandville, 54042 Nancy Cédex (France)
(First received September 11th, 1984; revised manuscript received October 2nd, 1984)

SUMMARY

The principle of batch multistep partition between aqueous polymer two-phase systems can be applied in a chromatographic procedure, simply by immobilizing one of the phase-forming polymers on a stationary support and using the other one as the mobile phase. Polyethylene oxide (PEO) was covalently bound on Sepharose 6B by means of an epoxy-activated spacer arm. This support was used to study the purification of $\Delta_{5\rightarrow4}$ 3-oxosteroid isomerase by elution with buffers containing solutes known to be immiscible with PEO (dextran, potassium phosphate, etc.). Experiments carried out on a "blank gel" devoid of PEO molecules gave results comparable to those obtained on the PEO-bound support, indicating that this spacer arm, acting as a PEO-like derivative, plays a primordial role in the fractionation process.

INTRODUCTION

Partition between aqueous two-phase systems has been used for many years to study binding modes between ligands and biological materials (nucleic acids, enzymes, receptors, etc.) and it also constitutes a fractionation technique[1]. In this latter field, the need for high purification ratios generally requires the use of multi-step extraction procedures, such as countercurrent distribution. Although many successful purification experiments have been reported[1–3], it is clear that the high viscosity of the solutions generally used makes this technique slow and tedious and affords a likely explanation for its rather limited practical use.

It seems logical that the multi-step partition operation could be carried out according to a column chromatographic procedure if one of the phase-forming polymers were immobilized on a gel (stationary phase) and the other one used as the mobile phase. This idea is not totally new, since Morris[4], and more recently Anker[5], exploited this concept. However, this improvement did not induce any spectacular development of the technique, probably due to the difficulties caused by the fact that the immobilized aqueous phase was merely soaked into the support beads.

We bound polyethylene oxide (PEO) covalently onto Sepharose 6B in order

0021-9673/85/$03.30

to study the fractionation of a complex protein mixture by elution with buffers containing solutes known to form immiscible two-phase systems with PEO (dextran, potassium phosphate, etc.). To test the efficiency of this technique, we applied it to the purification of $\Delta_{5\rightarrow4}$ 3-oxosteroid isomerase from *Pseudomonas testosteroni* (isomerase) for which we had already gained some experience during purification studies carried out by batch affinity partition in PEO–dextran two-phase systems[6,7].

MATERIALS AND METHODS

Polyethylene oxide (PEO 6000, MW = 6000) was obtained from Roth (F.R.G.) and 1,4-butanediol diglycidyl ether from Janssen Chimica (Belgium). Dextran T 70 (MW 70,000) and Sepharose 6B were supplied by Pharmacia (Uppsala, Sweden). The isomerase-containing acetonic powder and Δ_5-androstene-3,17-dione were kindly supplied by Roussel-Uclaf (Romainville, France). All other reagents were the purest available.

$\Delta_{5\rightarrow4}$ 3-Oxosteroid isomerase (E.C. 5.3.3.1) (referred to as isomerase in the following text) was extracted by adding 100 ml of 0.02 *M* Tris–HCl buffer pH 7.2 at 20°C to 10 g of the acetonic powder. After stirring for 3 h in the cold and centrifugation at 20,000 *g* for 1 h, the supernatant was collected. The activity of the isomerase, measured with Δ_5-androstene-3,17-dione as substrate, was 525 U/ml. The total protein concentration, measured according to the Amido Black technique[8], was 5.5 mg/ml.

The modified Sepharose 6B (see later, about 13 ml) was packed in a column (20 × 1 cm) and equilibrated with the desired mobile phase at a flow-rate of 4 ml/h. After the sample to be fractionated (0.3–0.5 ml) had been applied, the elution profile was obtained by continuous measurement of the absorbance of the effluent at 280 nm. Fractions of 1 ml were collected and those containing the isomerase were pooled. The enzymatic activity recovered in the pool was measured, as well as the total protein concentration.

RESULTS AND DISCUSSION

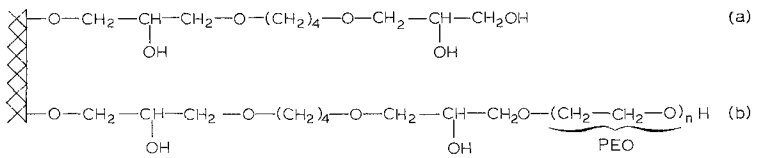
Immobilization of polyethylene oxide on Sepharose 6B

The stationary phase was prepared by covalent immobilization of PEO 6000 on Sepharose 6B. This carbohydrate gel was selected because of its large pore size, to rule out as far as possible the interference of gel-permeation phenomena in the fractionation process.

The immobilization procedure first consists in treatment of Sepharose 6B by 1,4-butanediol diglycidyl ether according to Sundberg and Porath[9], which results in the introduction of reactive oxirane groups and the simultaneous stabilization of the gel by cross-linking. The amount of epoxy groups bound, determined according to Sundberg and Porath[9], was around 850 μmol per g freeze-dried gel in all experiments performed under standard conditions.

The epoxy-activated Sepharose 6B thus obtained is subsequently treated with high concentrations of PEO 6000 in a basic medium (pH 12), using the procedure described by Matsumoto and Shibusawa[10]. The residual unreacted epoxy groups are inactivated by treatment with sodium hydroxide (pH 14) overnight at room temper-

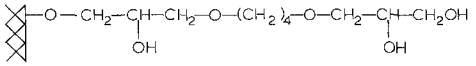
ature, or alternatively with 0.1 *M* perchloric acid for 1 h rather than with 1 *M* aminoethanol, which, although frequently used, would introduce large amounts of undesirable ionizable groups on the gel. The schematic structure of the stationary phase obtained (gel A)



Gel A

reflects the only partial substitution of available oxirane groups by PEO and the resulting presence of both moieties a and b along the chain of the carbohydrate support.

In order to rule out the eventual interference of unsubstituted spacer arm molecules in the fractionation process, we prepared a "blank gel" according to the method described above, except for the coupling step with PEO 6000 which was omitted (gel B). The stationary phase thus obtained can be schematically represented as follows:



Gel B

Chromatographic experiments

Fractionation of the isomerase-containing mixture on gel B as stationary phase was attempted with mobile phases containing various concentrations of dextran T 70.

Table I shows that an increase in the concentration of dextran in the mobile phase results in a more marked separation of the peaks containing isomerase and the

TABLE I

EFFECT OF THE DEXTRAN CONCENTRATION IN THE MOBILE PHASE ON THE SEPARATION OF ISOMERASE FROM CONTAMINANTS

Stationary phase: gel B (see text). Mobile phase: dextran T 70 at various concentrations (%, w/v) in 0.03 *M* sodium phosphate buffer pH 7.5. Other experimental conditions as described in Materials and Methods.

$$\alpha = R_{\text{isomerase}}/R_{\text{contaminants}} = \left(\frac{V_{\text{isomerase}} - V_0}{V_0}\right)\Big/\left(\frac{V_{\text{contaminants}} - V_0}{V_0}\right)$$

where $V_{\text{isomerase}}$ and $V_{\text{contaminants}}$ = elution volumes of the corresponding species and V_0 = void volume of the column.

Dextran concentration (%)	α
4	1.29
6	1.40
8	1.75
10	1.90
12	2.45

bulk of contaminating proteins. It is even possible to obtain complete separation when the concentration of dextran in the elution buffer reaches 12% (Fig. 1). Control experiments carried out as described in Table II did not result in any separation of isomerase from the contaminants. This indicates that the fractionation is not due to a gel-permeation phenomenon (a) and depends neither on the dextran (b) nor on the immobilized spacer arm (c) alone, but requires the combined effect of both species.

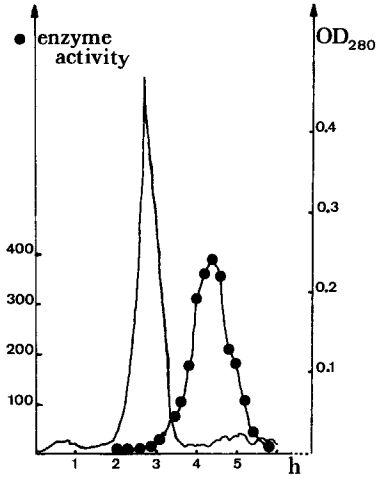


Fig. 1. Fractionation of the isomerase-containing solution. Stationary phase: gel B (see text). Mobile phase: 12% (w/v) dextran T 70 in 0.03 *M* sodium phosphate buffer pH 7.5. Experimental conditions (column size, flow-rate, etc.) as described in Materials and Methods. ●—●, Isomerase enzymatic activity in arbitrary units.

TABLE II

STATIONARY PHASE AND MOBILE PHASE CONDITIONS EMPLOYED DURING CONTROL EXPERIMENTS

	Stationary phase	*Mobile phase*
a	Sepharose 6B	0.03 *M* Phosphate buffer
b	Sepharose 6B	10% Dextran T 70
c	Gel B	0.03 *M* Phosphate buffer

This first series of experiments demonstrates the ability of the butanediol diglycerol 1-ether spacer arm to play a significant rôle in the fractionation process. This result, although unexpected, appears logical *a posteriori*, considering the similarity in structure between butanediol diglycerol 1-ether and PEO, and the resulting possibility of considering this spacer arm as a low-molecular-weight PEO-like derivative.

Experiments carried out earlier by batch partition between aqueous PEO–dextran systems[6,7] had shown that contaminating proteins favour the dextran-rich phase ($\sigma \approx 0.4$)* while isomerase establishes preferential interactions with the PEO-

* Partition coefficient:

$$\sigma_i = \frac{C_i \text{ Top phase (PEO-rich)}}{C_i \text{ Bottom phase (dextran-rich)}}$$

rich top phase ($\sigma \approx 4$). The retardation of isomerase on the PEO-like immobilized Sepharose 6B is therefore in agreement with these results.

Further experiments were carried out with mobile phases containing potassium phosphate*, a solute known to form aqueous two-phase systems with PEO[1]. Elution of the isomerase-containing sample with mobile phases of various potassium phosphate concentrations affords results even more spectacular than with dextran. For potassium phosphate concentrations above 8%, the peak corresponding to contaminating proteins is eluted in the range of $2V_0 < V_t < 4V_0$, while no enzymatic activity is eluted before $V_t = 10\ V_0$. In order to avoid too long elution times and the resulting recovery of the enzymatic activity in very dilute solutions, the potassium phosphate concentration of the mobile phase was decreased to 0.9% immediately after the bulk of the contaminants had been eluted. This stepwise elution results in the recovery of isomerase enzymatic activity in a narrow peak containing 55–60% of the initial activity injected. The total protein amount in the pool of isomerase-containing fractions was 7 μg, which corresponds to the removal of over 99.5% of the contaminants. A subsequent decrease in the potassium phosphate concentration (elution with water) reveals the presence in the protein mixture of species interacting with the gel even more strongly than isomerase (Fig. 2).

A recent article by Ling and Mattiasson[11], on the fractionation of an artificial mixture of bovine hemoglobin and serum albumin on polyvinylalcohol-immobilized Sepharose with sodium chloride as eluent, suggests that the phenomenon should not be restricted to species forming aqueous two-phase systems in batch. Our own results

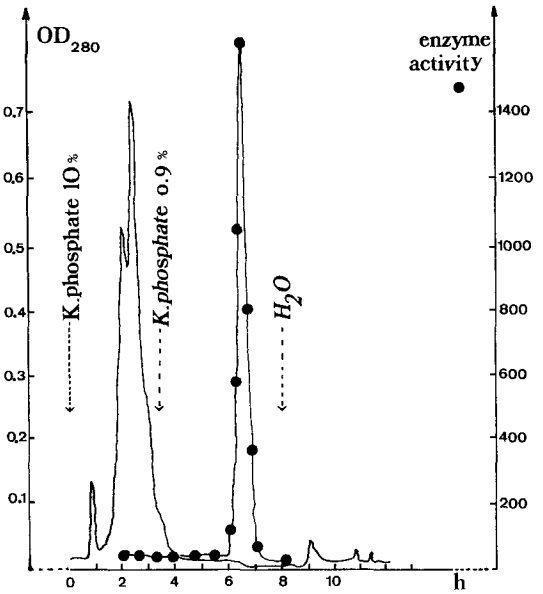


Fig. 2. Stepwise elution of the isomerase-containing sample (0.4 ml) successively with 10 and 0.9% potassium phosphate (pH 7.0) and finally with distilled water. Other experimental conditions as described in Materials and Methods. ●—●, Isomerase enzymatic activity in arbitrary units.

* The term "potassium phosphate" implies a mixture of K_2HPO_4 and KH_2PO_4 with the ratio 306.9 g K_2HPO_4 to 168.6 g KH_2PO_4, as suggested by Albertsson[1].

with isomerase confirm this finding. The elution with mobile phases containing salts known to be compatible with PEO (sodium chloride, ammonium chloride, etc.) makes it possible to fractionate the protein mixture as efficiently as with incompatible salts (potassium phosphate), provided that their concentration is high enough (≈ 3 *M*). The need for high ionic strengths in the mobile phase suggests that the fractionation observed may be interpreted in terms of differential hydrophobic interactions with the PEO-like immobilized derivative. Experiments carried out in ammonium chloride and ammonium sulphate at the same ionic strength (1.8 *M*) (Fig. 3) indicate clearly that the nature of the ions, as well as the ionic strength itself, plays a significant rôle in the fractionation process. The importance of this fact has been illustrated in a procedure called "salting-out chromatography", where high-salt mobile phases can afford efficient fractionations of complex protein mixtures even on underivatized Sepharose[12–14]. Hydrophobic interactions with the stationary phase, together with "salting-out" effects in the mobile phase, therefore appear as the cumulative driving forces of the phenomenon observed.

In the second part of this study we wanted to investigate the eventual additional effect of PEO on the fractionation quality. For this purpose, a PEO-immobi-

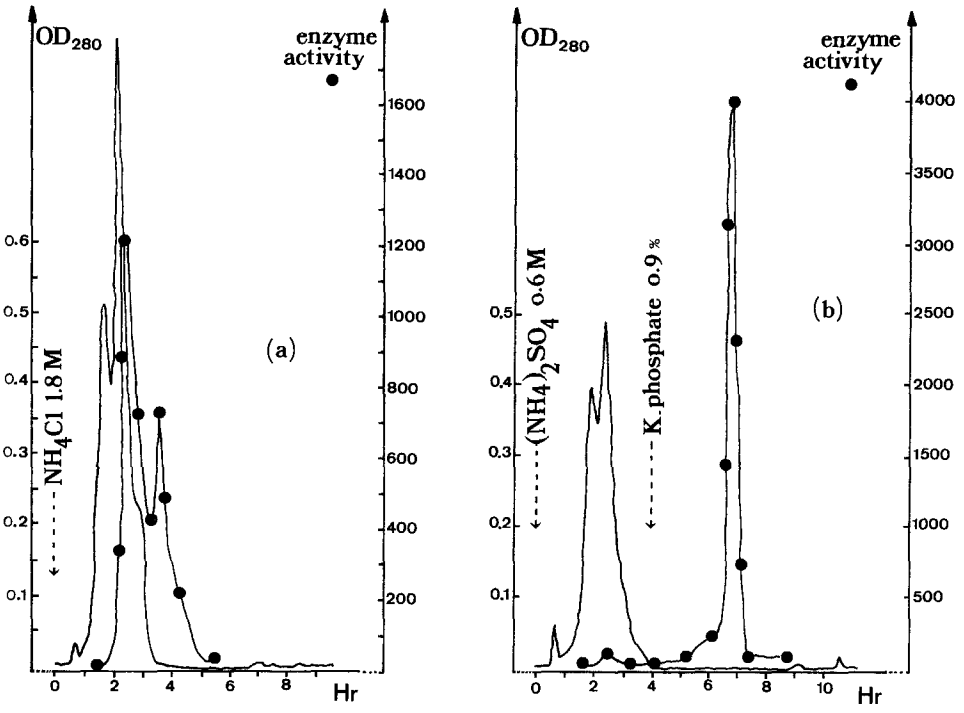


Fig. 3. Effect of the ion composition of the mobile phase on the fractionation profile, at the same ionic strength. The stationary phase (gel B, see text) was eluted with 1.8 *M* NH_4Cl (a) or 0.6 *M* $(NH_4)_2SO_4$ (b) in both cases under the same experimental conditions (column size, flow-rate, buffer pH, etc.). If isocratic elution is possible with NH_4Cl (a), the use of $(NH_4)_2SO_4$ results in a much stronger retention of isomerase and requires a subsequent decrease of the ionic strength (elution with 0.9% potassium phosphate) to recover the enzymatic activity within reasonable elution times (b).

lized stationary phase (gel A) was prepared by reaction of PEO 6000 with epoxy-activated Sepharose 6B under the conditions described by Matsumoto and Shibusawa[10].

We found that when dextran or potassium phosphate solutions are used as the mobile phase, experiments carried out with gel A as stationary phase give very similar, if not identical, fractionation profiles to those obtained with the blank gel we have previously described. This result prompted us to investigate the fractionation of bovine serum albumin and hemoglobin on our blank gel (gel B) devoid of PEO molecules, under the conditions described by Ling and Mattiasson[11]. Fig. 4 shows that our fractionation profile is quite comparable to that obtained with the so-called PVA-substituted Sepharose 6B and that the phenomenon observed is mainly, if not totally, due to the butanediol diglycerol 1-ether spacer arm itself. One should therefore question either the role played by PEO (or PVA), or the coupling reaction of the polymer on the stationary phase under the conditions used.

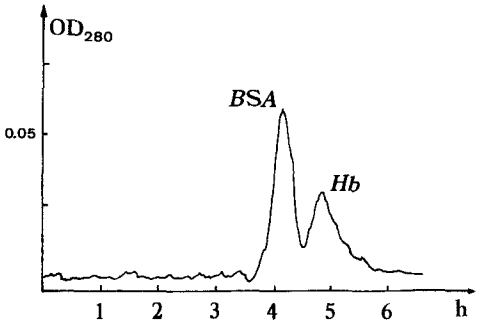


Fig. 4. Separation of bovine serum albumin (BSA) and hemoglobin (Hb) on a column (25 × 1 cm) packed with gel B (see text) under the same conditions as described by Ling and Mattiasson[11]: elution at room temperature with a flow-rate of 4.8 ml/h. The mobile phase consisted of 3 *M* NaCl and 10 m*M* Tris–HCl pH 7.5. A 30-μl sample was applied, containing 17 mg/ml BSA and 6 mg/ml Hb.

In our opinion, there is no doubt as to the potential value of PEO in this chromatographic procedure, since the similarity in structure between butanediol diglycerol 1-ether and PEO, along with the convincing results obtained with our blank gel, make very unlikely a total inefficiency of PEO. On the contrary, the coupling reaction seems very questionable. The absence of any titrable atom or chromophore on PEO prevents the direct determination of immobilized molecules. Besides, the indirect measurements (≈ 15 μmol per g freeze-dried gel according to Matsumoto and Shibusawa[10]) very likely affords unreliable results, due to the error range inherent to the techniques employed (spectrophotometric titration of the washings or elementary analysis of the gel). On the other hand, the weak nucleophilic character of PEO together with the rate of hydrolysis of epoxide groups at different pH values[9,15] argue in favour of low substitution ratios of the stationary phase by PEO, not sufficient to produce a significant effect in the fractionation process.

The synthesis of a new stationary phase, by immobilization of PEO with a titrable feature in its structure, allowing the direct determination of the amount bound, and in the absence of any spacer arm liable to interfere with the fractionation

phenomena, is currently under investigation. A stationary phase of this kind should make it possible to investigate without ambiguity the role of PEO or other polymers during the fractionation of protein mixtures by this particular type of chromatography.

REFERENCES

1 P. Å. Albertsson, *Partition of Cell Particles and Macromolecules,* Almqvist and Wiksell, Stockholm, and Wiley-Interscience, New York, 2nd ed., 1971.
2 K. H. Kroner, H. Hustedt and M. R. Kula, *Biotechnol. Bioeng.,* 24 (1982) 1015–1045.
3 A. Veide, A. L. Smeds and S. O. Enfors, *Biotechnol. Bioeng.,* 25 (1983) 1789–1800.
4 C. J. O. R. Morris, *Protides Biol. Fluids,* 10 (1963) 325.
5 H. S. Anker, *Biochim. Biophys. Acta,* 229 (1971) 290–291.
6 P. Hubert, E. Dellacherie, J. Néel and E. E. Baulieu, *FEBS Lett.,* 65 (1976) 169–174.
7 P. Hubert, *Thèse de doctorat,* Nancy, 1979.
8 W. Schaffner and C. Weissmann, *Anal. Biochem.,* 56 (1973) 502–514.
9 L. Sundberg and J. Porath, *J. Chromatogr.,* 90 (1974) 87–98.
10 U. Matsumoto and Y. Shibusawa, *J. Chromatogr.,* 187 (1980) 351–362.
11 T. G. I. Ling and B. Mattiasson, *J. Chromatogr.,* 254 (1983) 83–89.
12 J. Raymond, J.-L. Azanza and M. Fotso, *J. Chromatogr.,* 212 (1981) 199–209.
13 G. Sawatzki, V. Anselstetter and B. Kubanek, *Biochim. Biophys. Acta,* 667 (1981) 132–138.
14 A. R. Ashton and L. E. Anderson, *Biochim. Biophys. Acta,* 667 (1981) 452–456.
15 *Affinity Chromatography. Principles and Methods,* Pharmacia, Uppsala, 1979.