Journal of Chromatography, 474 (1989) 396–399
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 513

Note

Polyoxyalkyleneglycols immobilized on Sepharose 6B for the sequential extraction of three enzymes from a crude extract of *Pseudomonas testosteroni*

R. MATHIS, P. HUBERT* and E. DELLACHERIE
Laboratoire de Chimie-Physique Macromoléculaire, CNRS U.A. 494, 1 rue Grandville, BP 451, 54001 Nancy Cédex (France)
(First received December 21st, 1988; revised manuscript received March 20th, 1989)

Mild hydrophobic interaction chromatography is attracting increasing interest, for instance in the purification of enzymes acting on hydrophobic substrates, such as steroids or lipids. This technique exploits the moderately hydrophobic character of certain polymers, essentially various polyethers so far, to obtain high purification ratios together with the recovery of specific activities which generally are better than those obtained by traditional hydrophobic interaction chromatography (HIC)[1–8].

In previous papers[6,7] we reported the purification of $\Delta_{5\rightarrow4}$ 3-oxosteroid isomerase from *Pseudomonas testosteroni* by chromatography on stationary phases obtained by the immobilization of polyethylene glycol (PEG) on Sepharose 6B by means of spacer arms totally devoid of hydrophobic moieties strong enough to play a role in the separation process and therefore to lead to erroneous interpretations.

In this paper, we report preliminary results concerning the ability of this technique to meet a more complicated challenge, *i.e.*, to obtain good purification ratios and high recoveries for three closely related enzymes, simply by stepwise elution and starting from the same crude extract.

EXPERIMENTAL

Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). Polyethylene glycol monomethyl ether ($\bar{M}_w \approx 750$) and N,N'-carbonyldiimidazole were supplied by Aldrich (F.R.G.). Pluronic L 64 was purchased from Serva (Heidelberg, F.R.G.). The crude extract of *Pseudomonas testosteroni* was prepared as described previously[6]. The three enzymes tested were $\Delta_{5\rightarrow4}$ 3-oxosteroid isomerase (E.C. 5.3.3.1), 3α-hydroxysteroid dehydrogenase (α-HSD) (E.C. 1.1.1.50 and 3β,17β-hydroxysteroid dehydrogenase (β-HSD) (E.C. 1.1.1.51). Their enzymatic activities were assayed with Δ_5-androstene-3,17-dione, androsterone and testosterone, respectively, according to previously published procedures[9,10]. Protein concentrations were measured according to the Amido Black technique[11]. The term potassium phosphate implies a mixture of K_2HPO_4 and KH_2PO_4 in the ratio 306.9:168.6 (w/w).

0021-9673/89/$03.50

RESULTS

Stationary phases were prepared according to a procedure described previously[7] by immobilization of two different polyethers on to Sepharose 6B, after preliminary activation of this matrix with N,N'-carbonyldiimidazole. The two polyethers concerned are polyethylene glycol monomethyl ether, $CH_3O(CH_2CH_2O)_nH$ ($\bar{M}_w \approx 750$), and Pluronic L 64, a copolymer of polyethylene glycol (PEG) and polypropylene glycol (PPG), $HO(CH_2CH_2O)_{n/2}[CH_2CH(CH_3)O]_p(CH_2CH_2O)_{n/2}H$, with $p = 30$ and $n = 27$ ($\bar{M}_w \approx 3000$), These two polyethers differ essentially in their hydrophilic–lipophilic balance (HLB), their lipophilic character increasing with their content of PPG moieties.

As hydrophobic interactions are highly dependent on ionic strength, the strategy of mild hydrophobic interaction chromatography implies a careful selection of this parameter during both the adsorption and desorption steps. A high ionic strength (yet as low as possible in order to minimize the retention of slightly hydrophobic contaminants) is required for the adsorption step, whereas a low ionic strength (yet as high as possible in order to minimize the release of strongly interacting contaminants) is necessary during the desorption.

Fig. 1A shows the elution profile obtained with Sepharose–PEG, starting with 20% potassium phosphate in the mobile phase. The three enzymes are separated from unretarded contaminants. However, the desorption step results in a poor purification of α-HSD and no separation between β-HSD and isomerase. If the experiment is

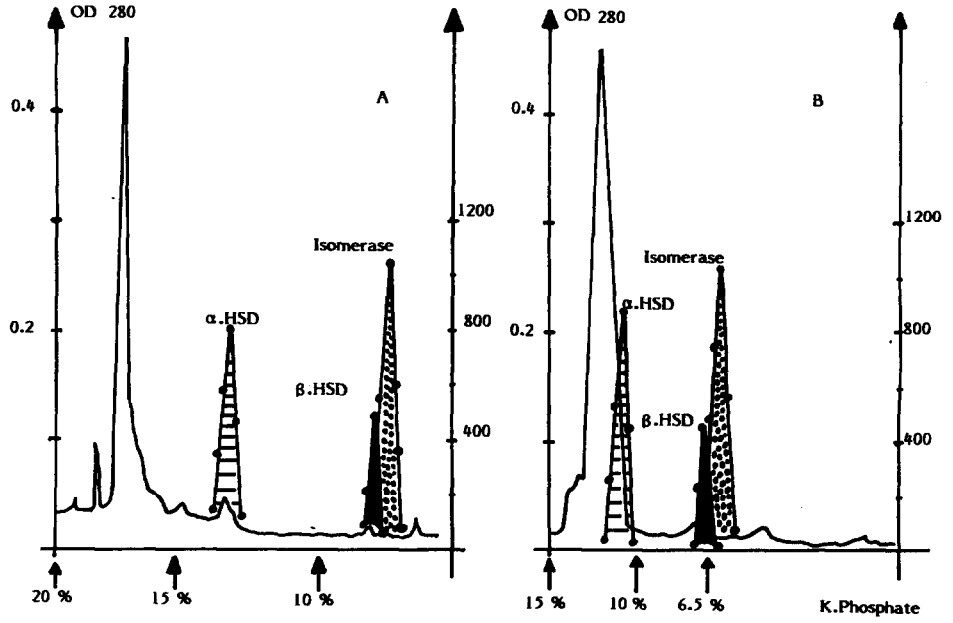

Fig. 1. Stepwise elution of the *Pseudomonas testosteroni* crude extract (0.4 ml) with various concentrations of potassium phosphate, pH 7.0 (ionic strength changes are indicated by arrows). Stationary phase: Sepharose–polyethyleneglycol monomethyl ether. Column, 38 cm × 1 cm I.D.; flow-rate, 26 ml/h; fractions taken every 5 min; room temperature. The enzymatic activities are indicated in arbitrary units.

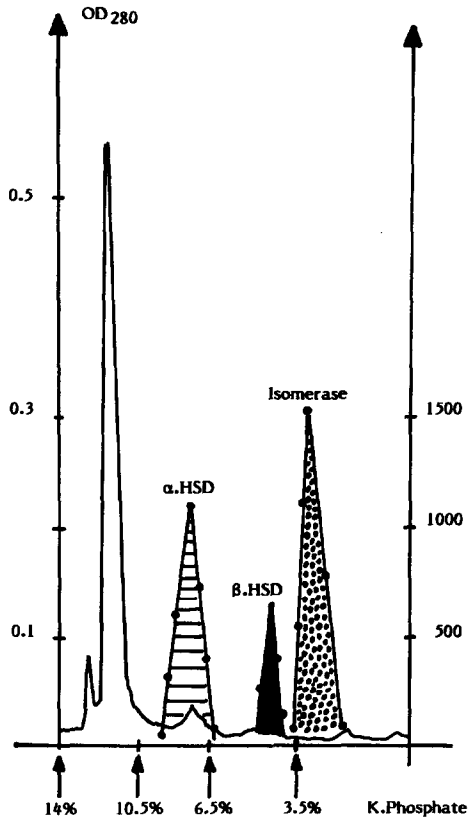

Fig. 2. Stepwise elution of the *Pseudomonas testosteroni* crude extract. Stationary phase: Sepharose–Pluronic L64. Other conditions as in Fig. 1.

carried out with a slightly more hydrophobic stationary phase, *i.e.*, Sepharose–Pluronic, the minimum ionic strength permitting the adsorption of the three enzymes without retardation of the contaminants is weaker (14% potassium phosphate), as expected (Fig. 2). Unlike the Sepharose–PEG, the desorption step on Sepharose–Pluronic leads to total separation of the three enzymes from one another, with good recoveries of enzymatic activities (αHSD 60%; β-HSD 40%; isomerase 65%) and almost quantitative removal of the contaminants in each fraction (Table I). Under

TABLE I

CONTAMINANTS IN THE FRACTIONS CONTAINING THE THREE DIFFERENT ENZYMES

Results are % of the total proteins in the crude extract.

Stationary phase	*Enzyme*		
	α-HSD	*β-HSD*	*Isomerase*
Sepharose–PEG	5.7	2.8	0.5
Sepharose–Pluronic	1.5	2.0	Not detectable

these ionic strength conditions, the experiment carried out on Sepharose-PEG leads to no retention at all of α-HSD and complete overlapping of the peaks corresponding to β-HSD and isomerase (Fig. 1B).

These results indicate that slight modifications of the hydrophobic character of the stationary phase and the ionic strength conditions during chromatography can result in different separation characteristics. Stationary phases synthesized by immobilization of more hydrophobic polyethers, such as polypropylene glycol (PPG) or polytetramethylene glycol (PTMG), are currently under investigation. They should bring additional potential to this field of mild hydrophobic interaction chromatography.

ACKNOWLEDGEMENT

We thank T. Geoffroy (UA-494) for technical assistance.

REFERENCES

1 S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 120 (1976) 321–333.
2 T. G. I. Ling and B. Mattiasson, *J. Chromatogr.*, 254 (1983) 83–89.
3 N. T. Miller, B. Feibush and B. L. Karger, *J. Chromatogr.*, 316 (1985) 519–536.
4 Y. Shibusawa, U. Matsumoto and M. Takatori, *J. Chromatogr.*, 398 (1987) 153–164.
5 J. P. Chang, Z. El Rassi and Cs. Horváth, *J. Chromatogr.*, 319 (1985) 396–399.
6 R. Mathis, P. Hubert and E. Dellacherie, *J. Chromatogr.*, 319 (1985) 43–50.
7 R. Mathis, P. Hubert and E. Dellacherie, *J. Chromatogr.*, 347 (1985) 291–296.
8 R. Mathis, A. Mourey and P. Hubert, *Appl. Environ. Microbiol.*, 54 (1988) 1307–1308.
9 P. Talalay and F. S. Wang, *Biochim. Biophys. Acta*, 18 (1955) 300–301.
10 P. I. Marcus and P. Talalay, *J. Biol. Chem.*, 218 (1956) 661–674.
11 W. Schaffner and C. Weissmann, *Anal. Biochem.*, 56 (1973) 502–514.