Journal of Chromatography, 347 (1985) 291–296
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 038

Note

Polyethylene oxide: a ligand for mild hydrophobic interaction chromatography?

R. MATHIS, P. HUBERT* and E. DELLACHERIE
Laboratoire de Chimie-Physique Macromoléculaire, C.N.R.S. U.A. 494, 1 rue Grandville, 54042 Nancy Cédex (France)
(Received July 16th, 1985)

Since the initial paper by Er-el *et al.*[1] in 1972, much work has been carried out on the fractionation of protein mixtures by means of hydrophobic interaction chromatography. Denaturation problems encountered during the elution under the severe conditions frequently required have made it necessary to seek less hydrophobic ligands liable to afford retention of the activity, together with good purification ratios.

In this respect, the use of mild hydrophobic stationary phases prepared by immobilization of polyethylene oxide either on soft gels[2,3] or, very recently, on more rigid supports, suitable for high-performance liquid chromatographic (HPLC) studies[4,5], appears to be a promising alternative. However, as mild hydrophobic interactions are involved, much care should obviously be taken not to introduce strongly interacting hydrophobic spacer arms on the gels, as these would lead to erroneous interpretations of the phenomena observed. In our opinion, the previously mentioned papers[2–5] do not give this particular point all the attention we think it deserves. In fact, we recently demonstrated[6] that the purification of $\Delta_{5\rightarrow4}$-3-oxosteroid isomerase by elution of the initial complex mixture on polyethylene oxide-bound Sepharose 6B was mainly due to hydrophobic interactions with the spacer arm itself, *i.e.*, 1,4-butanediol diglycidyl 1-ether.

In this paper, we report the synthesis of various stationary phases, obtained by unambiguous immobilization of polyethylene oxide on to Sepharose 6B by means of spacer arms totally devoid of hydrophobic moieties strong enough to interfere in the fractionation process.

EXPERIMENTAL

Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). Polyethylene glycol monomethyl ether (MW 750) and 1,4-butanediol diglycidyl 1-ether were supplied by Janssen Chimica (Belgium). Epichlorohydrin and boron trifluoride etherate were purchased from Fluka (Buchs, Switzerland). Ethylene glycol diglycidyl ether, (±)-1,3-butadiene diepoxide and N,N′-carbonyldiimidazole (CDI) were obtained from Aldrich Chimie (F.R.G.). As previously[6], the term potassium phosphate implies a mixture of K_2HPO_4 and KH_2PO_4 with a ratio of 306.9 g of K_2HPO_4 to 168.6 g of KH_2PO_4.

0021-9673/85/$03.30

$\Delta_{5\to4}$-3-oxosteroid isomerase (E.C. 5.3.3.1) (referred to as isomerase in the following text) was extracted from *Pseudomonas testosteroni* acetonic powder as described previously[6]. The chromatographic conditions (flow-rate, sample loading, isomerase activity measurements, total protein concentration, etc.) were as previously reported[6].

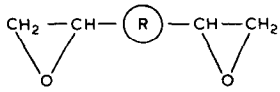
The amount of polyethylene glycol monomethyl ether immobilized on the various gels prepared was measured according to the procedure described by Drevin and Johansson[7] using a Girdel 300 gas chromatograph, an Icap 5 integrator and the same GC packings and experimental conditions, except for the oven temperature (60°C).

RESULTS AND DISCUSSION

Synthesis of the stationary phases

Previously reported results[6] showed that the fractionation of isomerase from the initial protein mixture can be obtained by elution on a gel merely substituted by 1,4-butanediol diglycidyl 1-ether. Various stationary phases were prepared in order to study the influence of the spacer arm structure on the hydrophobic interactions observed and to find out the extent to which polyethylene oxide can act as a mild hydrophobic ligand.

Three "blank gels" were obtained by reaction of Sepharose 6B, under conditions similar to those described by Sundberg and Porath[8], with various diepoxides represented schematically by the general formula



(a)

Table I gives the amounts of epoxy groups immobilized on the three different gels prepared. The final stationary phases were complete after deactivation of the epoxy groups by treatment at pH 14 for about 36 h at room temperature.

Two stationary phases were prepared by the immobilization on Sepharose 6B of polyethylene glycol monomethyl ether [$HO\text{-}(CH_2CH_2O)_n\text{-}CH_3$, MW 750], using two different synthetic routes:

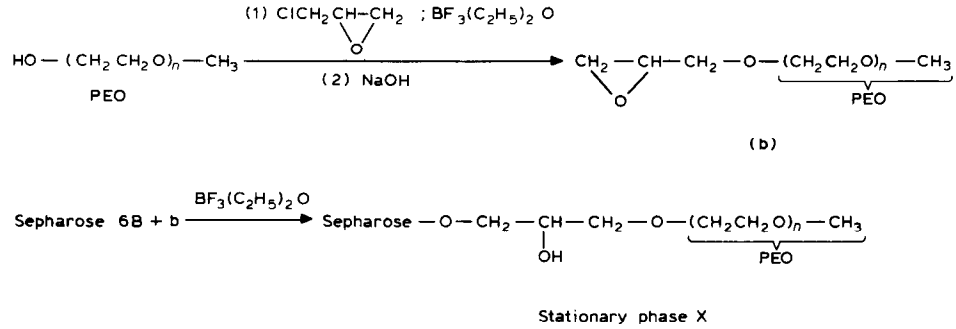
TABLE I

IMMOBILIZATION OF DIEPOXIDES OF GENERAL FORMULA (a) ON TO SEPHAROSE 6B

Parameter	*Stationary phase*		
	A	*B*	*C**
Reagent	(±)-1,3-Butadiene diepoxide	Ethylene glycol diglycidyl ether	1,4-Butanediol diglycidyl 1-ether
®	None	$CH_2\text{–}O\text{–}(CH_2)_2\text{–}O\text{–}CH_2$	$CH_2\text{–}O\text{–}(CH_2)_4\text{–}O\text{–}C$
Epoxy groups per gram of dry gel (μmol/g)	910	100	850

* Gel C is identical with that described in ref. 6.

Route I. The stationary phase X was prepared according to the following procedure, previously described by Hjertén *et al.*[9] and Ulbrich *et al.*[10]:

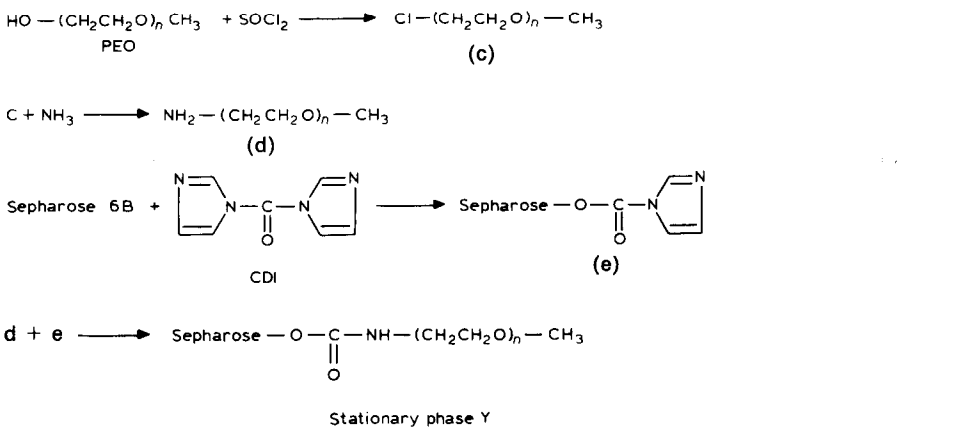


Immobilization of ligands on chromatographic supports is usually carried out after preliminary activation of the matrix. In route I a reverse procedure was used, so as not to introduce on to the Sepharose 6B undesirable unreacted spacer arms that might interfere in the fractionation process.

Polyethylene glycol monomethyl ether (PEO) was first treated with epichlorohydrin in the presence of $BF_3(C_2H_5)_2O$ as a catalyst at 55°C for 4 h with vigorous stirring. The intermediate obtained was subsequently reacted with NaOH at room temperature for 4 h to yield the epoxy-activated derivative b. This compound contained 800 μmol of epoxy groups per gram of polymer, corresponding to about 0.6 mol of epoxy groups per mol of PEO.

This partially epoxy-activated polymer was reacted with Sepharose 6B under rotation for 1 h at 50°C in dry dioxane. The gel was then extensively washed with dioxane, dioxane–water mixtures and finally with water in order to remove unreacted PEO. The amount of PEO immobilized on stationary phase X was 70 μmol per gram of dry gel as determined according to the procedure described by Drevin and Johansson[7].

Route II. The stationary phase Y was prepared according to the following procedure:



In a preliminary step, Sepharose 6B was activated with carbonyldiimidazole (CDI) according to the procedure reported by Bethell *et al.*[11]. The amount of imidazolyl carbamate groups immobilized on Sepharose 6B (e) was 1700 μmol per gram of dry gel.

In order to avoid the formation of water-sensitive carbonate groups, which the reaction of (e) with PEO itself would yield, the hydroxy function of this polymer was converted into NH_2 by successive treatment of PEO with thionyl chloride and then NH_3 at 100°C under pressure, according to a procedure reported earlier[12].

Subsequent reaction of (d) with (e) was carried out in dry dioxane at 40°C under rotation for 48 h. The gel obtained was extensively washed with dioxane, dioxane–water mixtures and finally with water to remove unreacted amino–PEO. Unreacted imidazolyl carbamate groups were subsequently deactivated by treatment at pH 3 overnight. Stationary phase Y thus obtained contained about 520 μmol of PEO per gram of dry Sepharose, as determined according to Drevin and Johansson[7]. Nitrogen microanalysis (1.2%) led to a higher result (800 μmol/g).

Chromatographic experiments

The synthesis of three different gels (A, B and C), differing in the number of CH_2 units, allowed us to investigate the role of the spacer arm structure on the hydrophobic interaction observed.

As was pointed out earlier[6], purification of isomerase from the initial complex mixture can be achieved with a good recovery of its activity (*ca.* 75%) together with elimination of most of the contaminants (> 99.5%) by elution at high ionic strength (10% potassium phosphate) on Sepharose 6B merely substituted by 1,4-butanediol diglycidyl 1-ether (gel C). Our assumption was that the fractionation is based on hydrophobic interactions with the $(CH_2)_4$ units of this immobilized spacer arm.

Total retention of isomerase can also be achieved on gel B, provided that the experiment is carried out at even higher ionic strength (15% *vs.* 10% potassium phosphate). This difference is probably due to the stronger hydrophobic character of the $(CH_2)_4$ moieties compared with that of $(CH_2)_2$, together with the substitution ratio of interacting groups, which is higher on gel C (850 μmol/g) than on gel B (100 μmol/g).

In contrast, no fractionation of isomerase from the bulk of contaminants can be obtained on gel A [$(CH_2)_0$].

Chromatographic experiments carried out on either gel X or Y, obtained by immobilization of PEO on Sepharose 6B by means of spacer arms ($-CH_2-CHOH-CH_2$ or $-CO-NH$) not liable to interfere in the hydrophobic interaction, gave very similar results. Fig. 1 shows the elution profile obtained on either stationary phase X or Y. Isomerase is strongly retained on gels equilibrated with 15% potassium phosphate and subsequently eluted stepwise with 10% potassium phosphate, once the bulk of contaminants has been removed. This result can be compared with those obtained on gels B and C, and indicates that polyethylene oxide exhibits a lower hydrophobic character than that of $(CH_2)_4$ moieties, but similar to that of $(CH_2)_2$ units. On the other hand, an increase in the amount of PEO bound on the stationary phase does not seem to influence the strength of the hydrophobic interaction involved, as no major changes in the elution profiles are observed whether the experiment is carried out on gel X or gel Y (500 *vs.* 70 μmol/g).

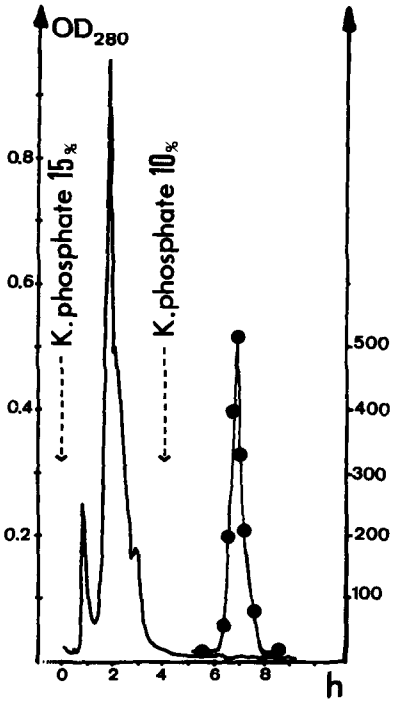


Fig. 1. Stepwise elution of the isomerase-containing sample (0.4 ml) successively with 15% and 10% potassium phosphate (pH 7.0) on gel X (see text). Other experimental conditions as described under Experimental. ●, Isomerase enzymatic activity in arbitrary units.

Further experiments are currently in progress to explore the field in more detail. Nevertheless, the present results already clearly demonstrate without any ambiguity the capacity of polyethylene oxide to act as a mild hydrophobic ligand, able to afford a convenient alternative to traditional hydrophobic interaction chromatography.

ACKNOWLEDGEMENTS

We thank T. Geoffroy (U.A. 494), M. F. Guingamp, M. Frayard and Y. Muller (I.U.T. de Biologie Appliquée de Nancy) for technical assistance.

REFERENCES

1 Z. Er-el, Y. Zaidenzaig and S. Shaltiel, *Biochem. Biophys. Res. Commun.*, 49 (1972) 383–390.
2 U. Matsumoto and Y. Shibusawa, *J. Chromatogr.*, 187 (1980) 351–362.
3 T. G. I. Ling and B. Mattiasson, *J. Chromatogr.*, 254 (1983) 83–89.
4 N. T. Miller, B. Feibush and B. L. Karger, *J. Chromatogr.*, 316 (1985) 519–536.
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 I. Drevin and B. L. Johansson, *J. Chromatogr.*, 295 (1984) 210–214.
8 L. Sundberg and J. Porath, *J. Chromatogr.*, 90 (1974) 87–98.
9 S. Hjertén, J. Rosengren and S. Påhlman, *J. Chromatogr.*, 101 (1974) 281–288.
10 V. Ulbrich, J. Makès and M. Jurecek, *Collect. Czech. Chem. Commun.*, 29 (1964) 1466–1475.
11 G. S. Bethell, J. S. Ayers, M. T. W. Hearn and W. S. Hancock, *J. Chromatogr.*, 219 (1981) 353–359.
12 P. Hubert, *Thèse de Doctorat*, Nancy, France, 1979.