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Hydrophobic interaction chromatography of *Chromobacterium viscosum* lipase on polyethylene glycol immobilized on Sepharose

J.A. Queiroz[a,*], F.A.P. Garcia[b], J.M.S. Cabral[c]

[a]*Departamento de Química, Universidade da Beira Interior, 6200 Covilhã, Portugal*
[b]*Departamento de Eng[a] Química, Universidade de Coimbra, 3000 Coimbra, Portugal*
[c]*Laboratório de Eng[a] Bioquímica, Instituto Superior Técnico, 1000 Lisbon, Portugal*

Abstract

The purification of *Chromobacterium viscosum* lipase was studied using a polyethylene glycol–Sepharose gel. An elemental analysis method is also reported for the determination of polyethylene glycol bonded to Sepharose CL-6B, using carbon as the reference element. The influence of the mobile phase composition on the chromatographic behaviour of *Chromobacterium viscosum* lipase was studied and it was found that the retention of lipase depends on the salt used and increased with increasing ionic strength and pH. The retention of lipase was, however, unaltered on changing the molecular mass of polyethylene glycol bonded to Sepharose. By using 15% (w/w) potassium phosphate in the eluent at pH 7, most of the lipase was retained on the column and by washing with 10 m*M* phosphate buffer recoveries of 75% protein and 79% lipolytic activity were achieved.

Keywords: *Chromobacterium viscosum*; Stationary phases, LC; Mobile phase composition; Lipase; Enzymes

1. Introduction

Hydrophobic interaction chromatography (HIC) has become a popular technique for purifying proteins. Proteins are separated in HIC based on differences in their content of hydrophobic amino acid side-chains on their surface [1]. The separation takes place by differential interaction with hydrophobic substituents on gels and the strength of the binding depends not only on the type of ligand and matrix but also on the type and concentration of salt, pH, temperature and additives [2].

However, negative side-effects have also been noted. The binding is, in many cases, too strong to be useful in a chromatographic process and may even be practically irreversible. In order to elute a strongly bound protein, organic solvents, detergents or chaotropic agents may be required [3], which may lead to protein denaturation. In this respect, the use of mild hydrophobic stationary phases prepared by immobilization of polyethylene glycol (PEG) on agarose gels appears to be a promising alternative, since this would permit an adequate binding strength without the

* Corresponding author.

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above-mentioned drawbacks. In fact, chromatographic supports consisting of covalently bonded PEG on Sepharose have been used not only for the fractionation of standard protein mixtures or simple extracts but also with complex mixtures [4–6].

The determination of the ligand concentration on affinity gels is usually required not only for the complete physico-chemical characterization of the stationary phases but also for the optimization of the ligand coupling reaction and when investigating the effect of degree of substitution on separation. Previously described methods for determining the PEG content of PEG–Sepharose supports are based mainly on spectrophotometry [7], NMR spectroscopy [8] or gas chromatography [9]. In this work, we tried to develop a simple procedure for the determination of the degree of substitution in PEG–Sepharose by elemental analysis using carbon as the reference element. Speed of analysis, ease of use and minimization of experimental errors are some advantages of elemental analysis over other techniques.

This paper describes also the influence of mobile phase composition on the chromatographic behaviour of *Chromobacterium viscosum* lipase. The effectiveness of some salting-out salts (at different concentrations) at various pH values in increasing lipase–adsorbent interactions is described.

2. Experimental

2.1. Materials

Sepharose CL-6B was obtained from Pharmacia (Uppsala, Sweden), PEG 10 000 from Merck (Darmstadt, Germany) and 1,3-butadiene diepoxide from Aldrich (Milwaukee, WI, USA). All other reagents were of analytical-reagent grade.

2.2. Lipolytic preparation

A lipolytic preparation of *Chromobacterium viscosum* lipase from Toyo Jozo (Tokyo, Japan) with a high nominal specific activity (3880 U/mg) was used.

2.3. Protein determination

The concentration of protein in the samples was determined by the method of Bradford [10].

2.4. Activity measurement

Lipase activity was measured in an oil–water emulsion medium [11]. Amounts of 20 g of olive oil, 20 g of Triton X-100 and 60 ml of distilled water were mixed and stirred for 30 min. A volume of 5 ml of the resulting emulsion and 2 ml of water were preincubated at 37°C for temperature stabilization. The reaction was started by adding 0.5 ml of lipase solution, allowed to progress for 20 min and stopped by adding 16 ml of acetone–ethanol (1:1). The liberated fatty acids were then assayed by titration with 50 m*M* NaOH.

2.5. Synthesis of PEG-Sepharose

Sepharose CL-6B gel was activated by coupling 1,3-butadiene diepoxide to it according to Sundberg and Porath [12]. PEG 10 000 was subsequently bonded to the epoxy-activated gel according to Hedman and Gustafsson [13]. The PEG 10 000–Sepharose CL-6B gel thus obtained was then treated with 1 *M* sodium hydroxide overnight at room temperature for the purpose of inactivating residual free epoxy groups.

2.6. Elemental analysis method

A Fisons EA1108 elemental analyser was used. The scheme of the elemental analyser coupled to a data processing unit has been described previously [14,15]. The samples (0.5–2 mg) were held in tin capsules, placed inside the autosampler drum and then dropped sequentially into the combustion reactor. The resulting combustion mixture entered the gas chromatographic column where the individual components were separated, eluted and measured with a thermal con-

ductivity detector. The instrument was calibrated with sulfanilamide standard.

2.7. Chromatographic method

The gel (about 5 ml) was packed in a column (10 × 1 cm I.D.) and equilibrated with the desired mobile phase at a flow-rate of 7.5 ml/h. After the lipolytic extract (300 μl, 3 mg) had been applied, the elution profile was obtained by continuous measurement of the absorbance at 254 nm. Fractions of 1 ml were collected and the lipolytic activity and protein concentration were determined.

3. Results and discussion

3.1. Determination of PEG bonded to Sepharose by elemental analysis

By the elemental analysis procedure described above, simultaneous automatic C, H, N, S analyses were obtained. However, in order to determine the PEG content on PEG 10 000–Sepharose CL-6B, only carbon was used as the reference element, not only for simplification but mainly because C is the major component in the samples and thus is determined with the smallest relative error [16].

The percentage of carbon in the substituted gel ($\%C_G$) given by the elemental analyser corresponds to

$$\%C_G = \frac{C_S + C_P}{W_S + W_P} \quad (1)$$

where C_S and C_P are the total masses of carbon in Sepharose CL-6B and PEG 10 000, respectively and W_S and W_P are the total masses of Sepharose CL-6B and PEG 10 000, respectively.

In this way, the degree of substitution is obtained from the percentage of carbon in the gel-bonded PEG, in the gel-free PEG and in the PEG and can be calculated from the following general equation:

$$\%C_G = \frac{\%C_S}{1+x} + \frac{\%C_P}{1+x^{-1}} \quad (2)$$

Table 1
Results obtained for carbon analysis

Sample	C (%) (average)[a]	Standard deviation[a]
Sepharose CL-6B	42.8890	0.2173
Epoxy-activated Sepharose CL-6B	43.2398	0.0959
PEG 10 000	53.6543	0.0767
PEG–Sepharose	45.6366	0.1601

[a] $n \geq 4$.

where the degree of substitution (x) is equal to W_P/W_S and is expressed as grams of PEG per gram of dry gel.

Table 1 shows the results obtained for the percentage of carbon and the standard deviation for each sample (with at least four runs per sample).

The amount of PEG 10 000 bonded to Sepharose CL-6B was evaluated from Eq. 2 (using the percentage of carbon in the gel-bonded PEG, $\%C_G$, in the epoxy-activated Sepharose CL-6B, $\%C_S$, and in the PEG 10 000, $\%C_P$). The final result obtained for this gel was 0.2990 g of PEG per gram of dry gel with a relative standard deviation of 5.7%.

To check the reproducibility of the method, more than twenty artificial mixtures of different and known amounts of PEG 10 000 and Sepharose CL-6B were prepared (Fig. 1). The $\%C_G$ obtained by calculation (assuming the values of $\%C_S$ and $\%C_P$ given in Table 1) corresponds to

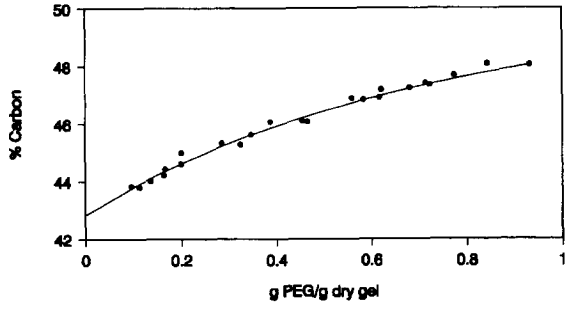

Fig. 1. Carbon results, calculated (——) and obtained (●), for different mixtures of Sepharose CL-6B and PEG 10 000.

the theoretical values (line) and are compared with the values given by the elemental analyser (points). In this way, as it can be seen that good agreement between the calculated and obtained values was achieved with a correlation coefficient of 0.9956.

3.2. Chromatographic separation of lipase

The stationary phase used in the chromatographic experiments was prepared by covalent immobilization of PEG on Sepharose CL-6B via 1,3-butadiene diepoxide. This spacer arm was used instead of the normally used 1,4-butanediol diglycidyl ether in order to avoid erroneous interpretations of the results, since we have demonstrated the ability of this epoxy-activated spacer arm as a hydrophobic ligand in the interaction with *Chromobacterium viscosum* lipase [17]. Control experiments carried out using underivatized Sepharose CL-6B or modified with 1,3-butadiene diepoxide (without PEG) did not result in any retention of lipase on the column.

The effect of some salts and their concentration and the influence of pH on lipase–adsorbent interactions were studied. The effect of the molecular mass of PEG, bonded to Sepharose, was also investigated.

The effect of some salting-out salts on the chromatographic behaviour of *C. viscosum* lipase was studied and it was found (Fig. 2) that the retention depends on the salt used. According to Melander *et al.* [18], in the absence of special binding effects, an increase in salt molality or a change of salt in the mobile phase to one of greater molal surface tension increment will result in increased retention of proteins by HIC. In fact, for sodium chloride, for example, a small percentage of lipase was retained on the column and it was the salt used with the smallest molal surface tension increment [1]. Potassium phosphate was selected for further experiments since the recovery and degrees of purification were slightly better when potassium phosphate was used instead of ammonium sulphate or sodium sulphate in the mobile phase. When 5% (w/v) dextran T-500 was used in the mobile phase, no retention was achieved between the lipase and the stationary phase.

As hydrophobic interactions are highly depen-

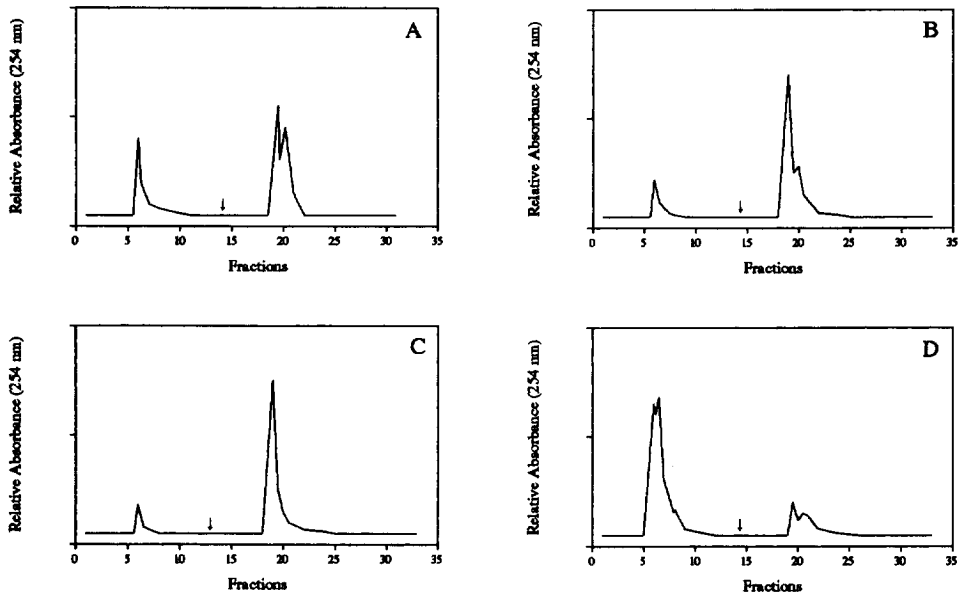

Fig. 2. HIC on PEG 10 000–Sepharose CL-6B column. Buffer: (A) 15% (w/w) potassium phosphate; (B) 20% (w/v) $(NH_4)_2SO_4$; (C) 15% (w/v) Na_2SO_4; (D) 4 *M* NaCl in 10 m*M* phosphate (pH 7). Desorption (↓) is obtained with 10 m*M* phosphate buffer (pH 7).

dent on ionic strength [18,19], the strategy of mild HIC implies careful selection of this parameter. The effect of salt concentration in the eluent buffer on the retention of lipase was investigated by using potassium phosphate in the mobile phase. On increasing the concentration of potassium phosphate from 0 to 15% (w/w) (Fig. 3), the amount of bound lipase increased and the results indicated that lipase can be desorbed from the stationary phase under mild conditions without the risk of protein denaturation. The opposite occurs when the hydrophobic interaction of *C. viscosum* lipase takes place with a phenyl-Superose column, where the elution is only obtained with a gradient from 0 to 65% (v/v) ethylene glycol [3].

Electrostatic interactions may also be important for protein retention in HIC [20]. For the analysis of the effect of pH, solutions of 15% (w/w) potassium phosphate were prepared with different proportions of K_2HPO_4 and KH_2PO_4 to obtain different pH values. As shown in Fig. 4, at pH 7.0 and 8.5, i.e., around and above, respectively, the isoelectric point of lipase (p*I* 6.9 [21]), a greater interaction between the lipase and PEG can be obtained than at pH 5.0 or 6.0 (where the lipase has a positive net charge). This suggests that some ionic interactions are present between lipase and PEG, which agree with the considerations made for general aqueous two-phase systems [22].

In order to study the effect of the molecular mass of PEG on the fractionation process, PEG 400 and 6000 were immobilized on Sepharose CL-6B using the same procedure above described for the PEG 10 000–Sepharose CL-6B. The chromatograms obtained and with different concentrations of potassium phosphate [0–15% (w/w)] in the mobile phase are identical with those obtained for the PEG 10 000 and shown in Fig. 3.

As discussed above, the adsorption increases with increasing ionic strength. Desorption can then be performed by just lowering the ionic strength and, in fact, by washing the column with phosphate buffer (10 m*M*), after retention of lipase, good recoveries were obtained: about 75% for protein and 79% for the lipolytic activity (Table 2). A 1.1-fold increase in specific activity was obtained and no further purification could be

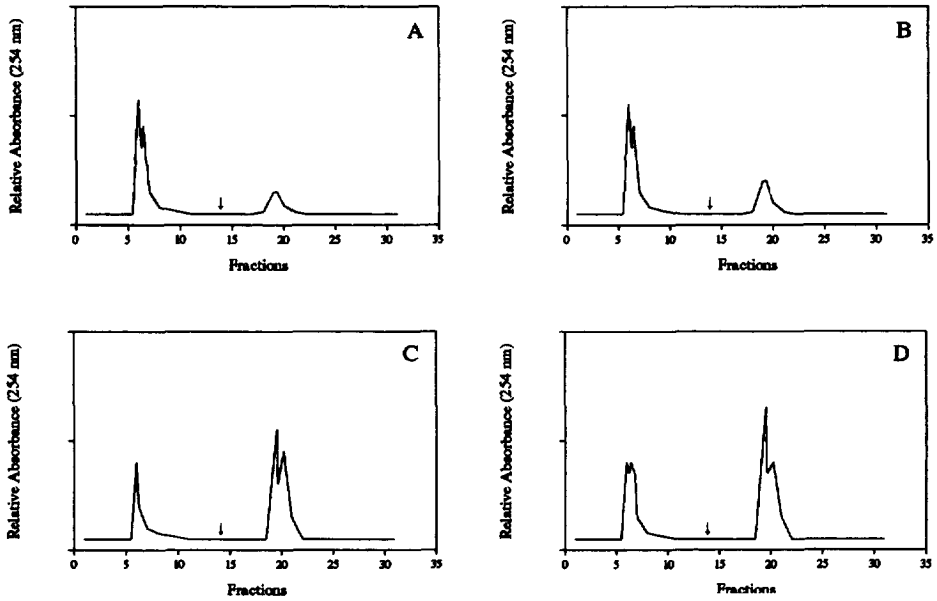

Fig. 3. HIC on PEG 10 000–Sepharose CL-6B column. Buffer: (A) 10 m*M* phosphate (pH 7), and containing (B) 5%, (C) 10% and (D) 15% (w/w) potassium phosphate. Desorption (↓) is obtained with 10 m*M* phosphate buffer (pH 7).

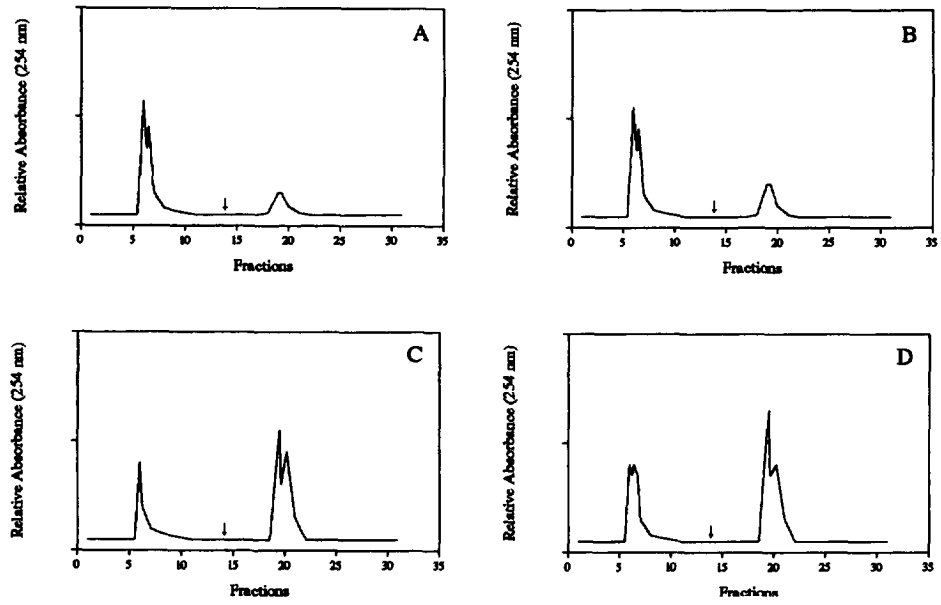

Fig. 4. HIC on PEG 10 000–Sepharose CL-6B column. Buffer: 15% (w/w) potassium phosphate at pH (A) 5.0, (B) 6.0, (C) 7.0 and (D) 8.5. Desorption (↓) is obtained with 10 m*M* phosphate buffer (pH 7).

achieved, probably owing to the high purity of the initial lipolytic mixture used.

4. Conclusions

The method developed for the determination of ligand in the gel-bonded PEG by elemental analysis was characterized by the quantitative determination of the carbon content in the substituted gel. The carbon percentage obtained can easily be converted into the amount of PEG on Sepharose through the use of Eq. 2. The distinctive feature of this method is the variation in the percentage of carbon from one gel to another. The method can also be applied to PEG having other molecular masses or to other agarose-based supports. Some advantages over other methods include the use of a known and simple analytical technique (such as elemental analysis), the good reproducibility and the fact that pretreatment of samples is not needed. In this way, this method offer an original application of a technique already available for the quantification

Table 2
Activity yield and purification factor for *C. viscosum* lipase obtained by HIC on PEG 10 000–Sepharose CL-6B

Sample	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor (-fold)
Original lipolytic preparation	1.060	2900	2736	100	–
HIC on PEG–Sepharose	0.782	2291	2930	79.0	1.1

of polyethers immobilized on polysaccharide supports.

The interaction of lipase with chemically bonded PEG stationary phase seems to be selective and to take place with the $-(CH_2)_2-$ units of the immobilized PEG. The extent of retention of lipase is affected by the salt used and increases with increasing ionic strength in the eluent buffer and with higher pH values. The properties of the gel used seem to provide an adequate approach to lipase isolation and separation based on their hydrophobic properties.

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