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Hydrophobic interaction chromatography of *Chromobacterium viscosum* lipase on polypropylene glycol immobilised on Sepharose

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Abstract

The fractionation of *Chromobacterium viscosum* lipase was performed using a polypropylene glycol–Sepharose gel. The influence of mobile phase composition on the adsorption of lipase on the gel was studied and it was found that the retention of lipase depends on the salt used and increased with increasing the ionic strength. The retention was not strongly affected by changing the pH value of the mobile phase. By using 20% (w/v) ammonium sulphate in phosphate buffer a total retention of lipase on the column was obtained and by simply decreasing the ionic strength of the buffer, desorption of lipase could be achieved. The chromatographic purification of *Chromobacterium viscosum* lipase by hydrophobic interaction chromatography on Sepharose CL-6B modified by covalent immobilisation of 1,4-butanediol diglycidyl ether, polyethylene glycol and polypropylene glycol was also compared.

Keywords: Hydrophobic interaction chromatography; *Chromobacterium viscosum*; Mobile phase composition; Lipases; Enzymes

1. Introduction

Lipases have received much attention recently because of their potential applications in biotechnological processes [1]. Isolation and purification of lipases from different sources (mainly microorganisms and mammals) have been extensively reported. Most of the purification procedures for lipases were based on a combination of several non-specific techniques, such as ammonium sulphate precipitation, gel filtration and ion-exchange chromatography [2]. Hydrophobic interaction chromatography (HIC) has also become a popular technique for purifying lipases [3,4].

In HIC, proteins are separated according to differences in their surface hydrophobicity [5,6]. The main parameters to consider for separation processes using hydrophobic interaction are the type of ligand and matrix, the type and concentration of salt, pH, temperature and additives [7–10]. Since the introduction of HIC and the classical hydrophobic adsorbents (phenyl- and octyl-based gels), few efforts have been concentrated on improving this technique for bioseparation.

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However, these hydrophobic adsorbents in some applications are too hydrophobic to be effective. In many cases, proteins are found to bind irreversibly to the stationary phase or to undergo denaturation due to their interactions with the required mobile or stationary phase or both. Thus, the biological activity of the protein, or the protein itself, is usually irreversibly lost in these cases. For instance, the adsorption of the lipase of *Chromobacterium viscosum* on a phenyl-Superose column implies the elution with a gradient of 0–65% (v/v) ethylene glycol in 50 m*M* phosphate buffer [3]. HIC with mild hydrophobic stationary phases and with the use of decreasing salt gradient can be used to circumvent this difficult.

Ligands with intermediate hydrophobic character are of great interest, as they provide an adequate binding strength and an elution by simply decreasing the salt concentration of the eluent. The analogy between partitioning in aqueous two-phase systems and chromatographic procedures has been utilised to achieve new mild hydrophobic ligands. In fact, chromatographic supports consisting of covalently bonded polyethylene glycol (PEG) on agarose gels appears to be a promising alternative and have been used not only for the fractionation of standard protein mixtures but also with complex mixtures [11–13]. The results obtained for the interaction of lipases with PEG in aqueous two-phase systems [14] and with PEG immobilised on Sepharose [15] encouraged the study of the interaction between lipases and polypropylene glycol (PPG) immobilised on Sepharose.

In a previous paper, a preliminary study on the interaction between lipases and PPG covalently bound on Sepharose by the use of a bisoxirane (1,3-butadiene diepoxide) was reported [16]. In this study we have prepared the gel by directly coupling PPG diglycidyl ether to Sepharose CL-6B and the influence of mobile phase composition on the chromatographic behaviour of *C. viscosum* lipase is described. The effects of different salting-out salts and salt concentration at various pH values on the adsorption of *C. viscosum* lipase are studied.

In the present paper, the chromatographic purification of *C. viscosum* lipase by HIC on Sepharose CL-6B modified by covalent immobilisation of 1,4-butanediol diglycidyl ether, PEG and PPG was also compared.

2. Experimental

2.1. Materials

Sepharose CL-6B was obtained from Pharmacia (Uppsala, Sweden) and PPG diglycidyl ether (average number-average molecular mass, M_n, ca. 380) was obtained from Aldrich (Milwaukee, WI, USA). All other reagents were of analytical grade.

2.2. Lipolytic preparation

A lipolytic preparation of *Chromobacterium viscosum* lipase from Toyo Jozo (Tokyo, Japan) with 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 [17].

2.4. Activity measurement

Lipase activity was measured in an oil–water emulsion medium [18]. 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 stabilisation. 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 an acetone–ethanol (1:1) mixture. The liberated fatty acids were then assayed by titration with 50 m*M* NaOH.

2.5. Preparation of the gel

The gel was prepared by coupling the PPG diglycidyl ether to Sepharose CL-6B according to Sundberg and Porath [19]. Five grams of suction-dried Sepharose CL-6B were washed on a glass filter-funnel with water and then mixed with 5 ml of PPG diglycidyl ether. Five millilitres of 0.6 *M* sodium hydroxide solution containing 10 mg of sodium borohydride were then added. The suspension was mixed by rotation for 8 h at 25°C and the reaction stopped by washing the gel on a glass

filter-funnel with large volumes of water. The amount of epoxy groups bound was around 150 μmol/g dry gel. The PPG–Sepharose CL-6B gel thus obtained was then treated with 1 *M* sodium hydroxide overnight at room temperature for the purpose of deactivating the free epoxy groups.

2.6. Chromatographic method

The chromatographic experiments for lipase fractionation were carried out, at room temperature, in a standard chromatographic system from Pharmacia. 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 6.0 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 280 nm. Fractions of 1 ml were collected and the lipolytic activity and protein concentration were determined.

3. Results and discussion

In this work the possibility of using PPG with intermediate hydrophobicity as ligand in HIC for the fractionation of *C. viscosum* lipase was studied. The chromatographic purification of *C. viscosum* lipase on Sepharose CL-6B modified by covalent immobilisation of 1,4-butanediol diglycidyl ether [20], PEG [15] and PPG was also compared.

The stationary phase used in the chromatographic experiments was prepared by covalent immobilisation of PPG diglycidyl ether directly on Sepharose CL-6B. The schematic structure of the gel thus obtained is

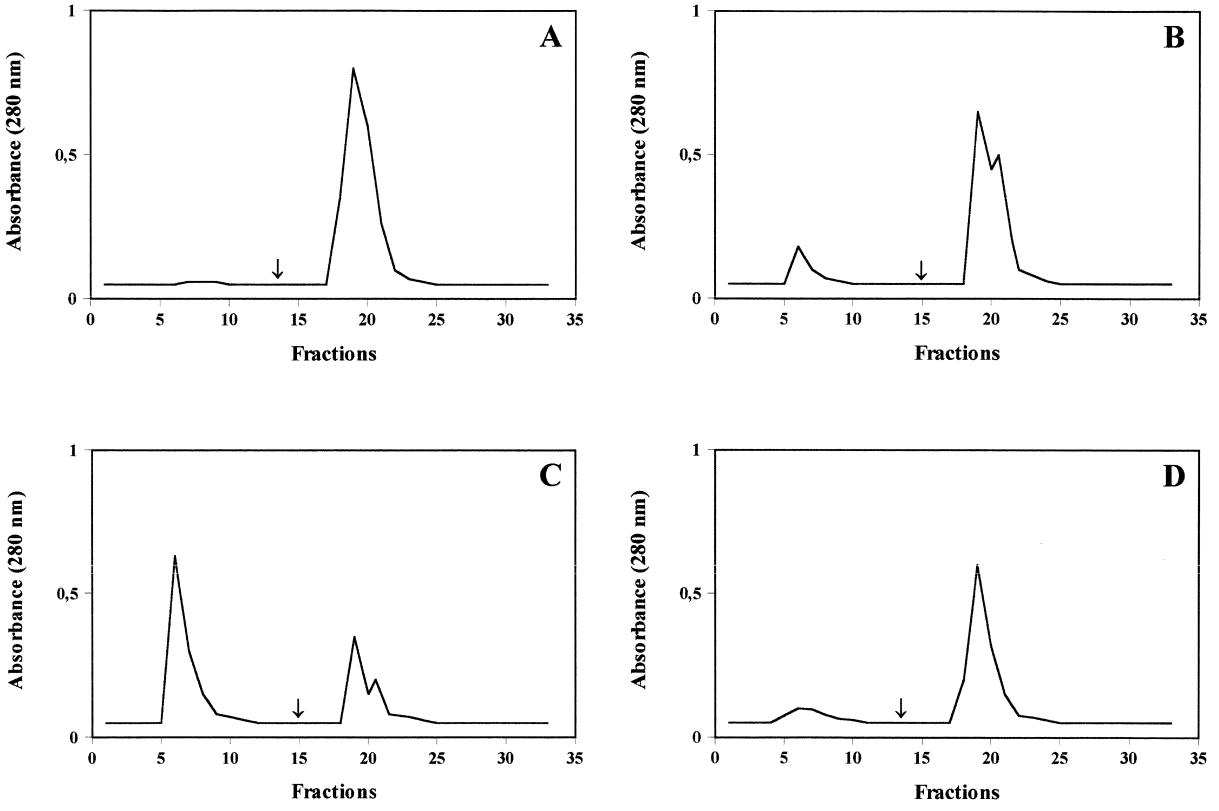
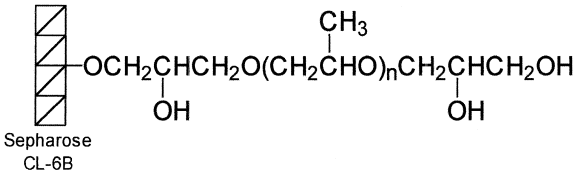


Fig. 1. Hydrophobic interaction chromatography on PPG–Sepharose CL-6B column. Buffers: (A) 20% (w/v) $(NH_4)_2SO_4$; (B) 15% (w/w) potassium phosphate; (C) 4 *M* NaCl and (D) 15% (w/v) Na_2SO_4 in 10 m*M* phosphate, pH 7. Desorption (↓) is obtained with 10 m*M* phosphate buffer, pH 7; sample size injected: 300 μl (3 mg lipolytic extract).



The effects of some salts and their concentration at various pH values on the adsorption of lipase were analysed.

It is well known from other studies [9,21] that changing the salt type is one of the most important parameters for modulating retention and selectivity in HIC. By this way, in order to characterise a hydrophobic column for a given separation, the effect of salt should be determined. Chromatographic experiments carried out with different salting-out salts (Fig. 1) indicate that the nature of the ion plays, in fact, a significant role in the fractionation process. For sodium chloride a small amount of lipase was retained on the column even if a high concentration of the salt in the buffer is used. According to Melander et al. [21], a change of salt in the mobile phase to one of greater molal surface tension increment (in the absence of special binding effects) will result in increased retention of proteins by hydrophobic interaction. On the other hand, sodium sulphate and ammonium sulphate promote lipase binding to the support in a great extent because they present high increments of molal surface tension. Thus, varying the salt in the eluent will result in significant changes not only in the overall retention but also in the selectivity of the separation, either when the extract to be used is a complex mixture of proteins [9].

The hydrophobic interactions are known to in-

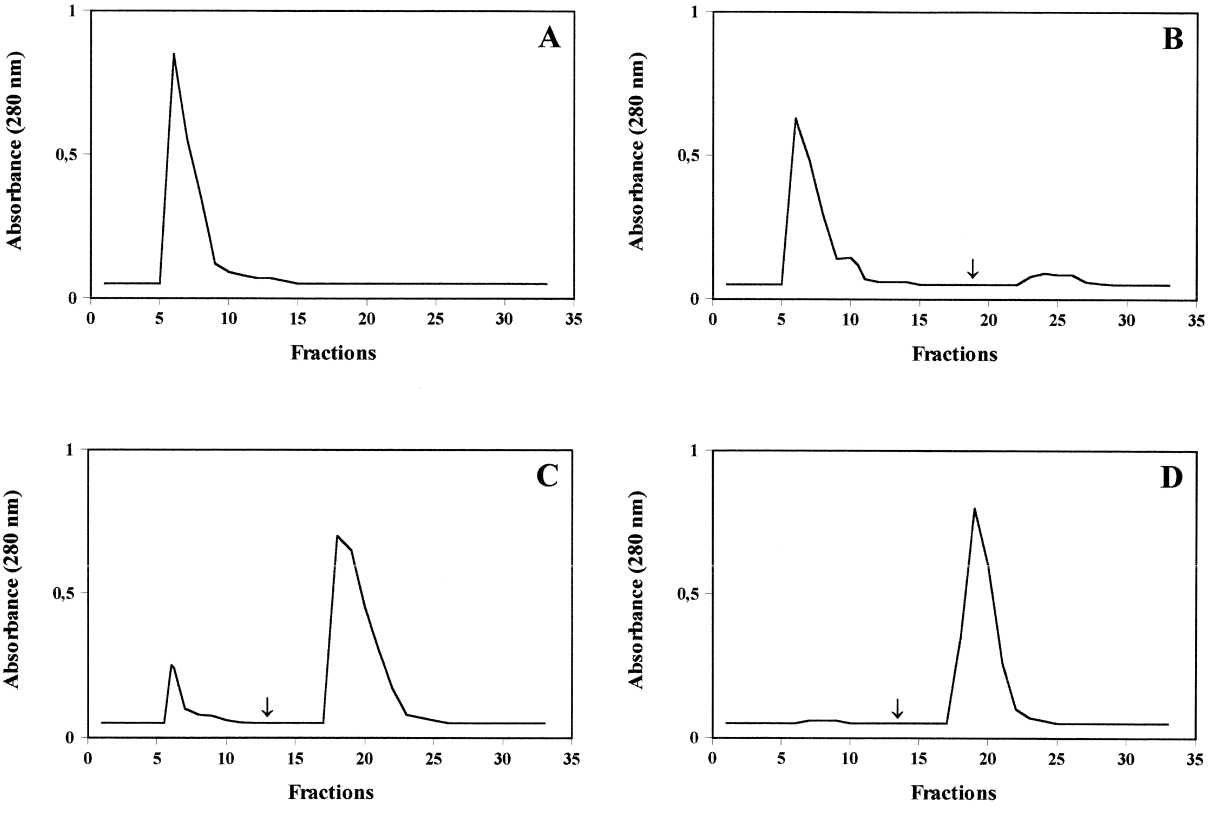


Fig. 2. Hydrophobic interaction chromatography on PPG–Sepharose CL-6B column. Buffers: (A) 0% (w/v); (B) 10% (w/v); (C) 15% (w/v) and (D) 20% (w/v) $(NH_4)_2SO_4$ in 10 m*M* phosphate, pH 7. Desorption (↓) is obtained with 10 m*M* phosphate buffer, pH 7; sample size injected: 300 μl (3 mg lipolytic extract).

crease upon increasing the ionic strength of the medium [22,23]. The effect of salt concentration in the eluent buffer on the retention of lipase was investigated by using ammonium sulphate in the mobile phase. The progressive increase in ammonium sulphate concentration leads to a simultaneous increase in the amount of bound lipase (Fig. 2). In fact, for 10% (w/v) of the salt on the buffer no lipase remains bounded to the gel, but on increasing salt concentration to 15% (w/v) the amount of protein retained was near to 50%. The total retention of lipase in the column was only obtained with 20% (w/v) ammonium sulphate.

The effect of pH on protein retention in HIC is not well defined. For the analysis of pH effect in our system, the buffer concentration was 10 m*M* for all pH values and 20% (w/v) ammonium sulphate was used to induce lipase retention on the column. The retention of *C. viscosum* lipase was not strongly affected in the range of pH 6–9 (Fig. 3). This suggests that hydrophobic interactions play a major role in the retention of the lipase on the PPG–Sepharose column, as previously obtained with the 1,4-butanediol diglycidyl ether ligand [20]. On the other hand, the lipase retained on a PEG–Sepharose column showed a dependence on the pH buffer [15].

As discussed above lipase retention on the PPG–Sepharose column increases with the ionic strength. Desorption can then be performed by just lowering the ionic strength and, in fact, by washing the support with 10 m*M* phosphate buffer, after total retention of lipase, a recovery of 122% lipolytic activity was obtained. This lipolytic activity recovery higher than 100% was also obtained for the partitioning of lipases in aqueous two-phase systems [14]. A 1.8-fold increase of specific activity was obtained

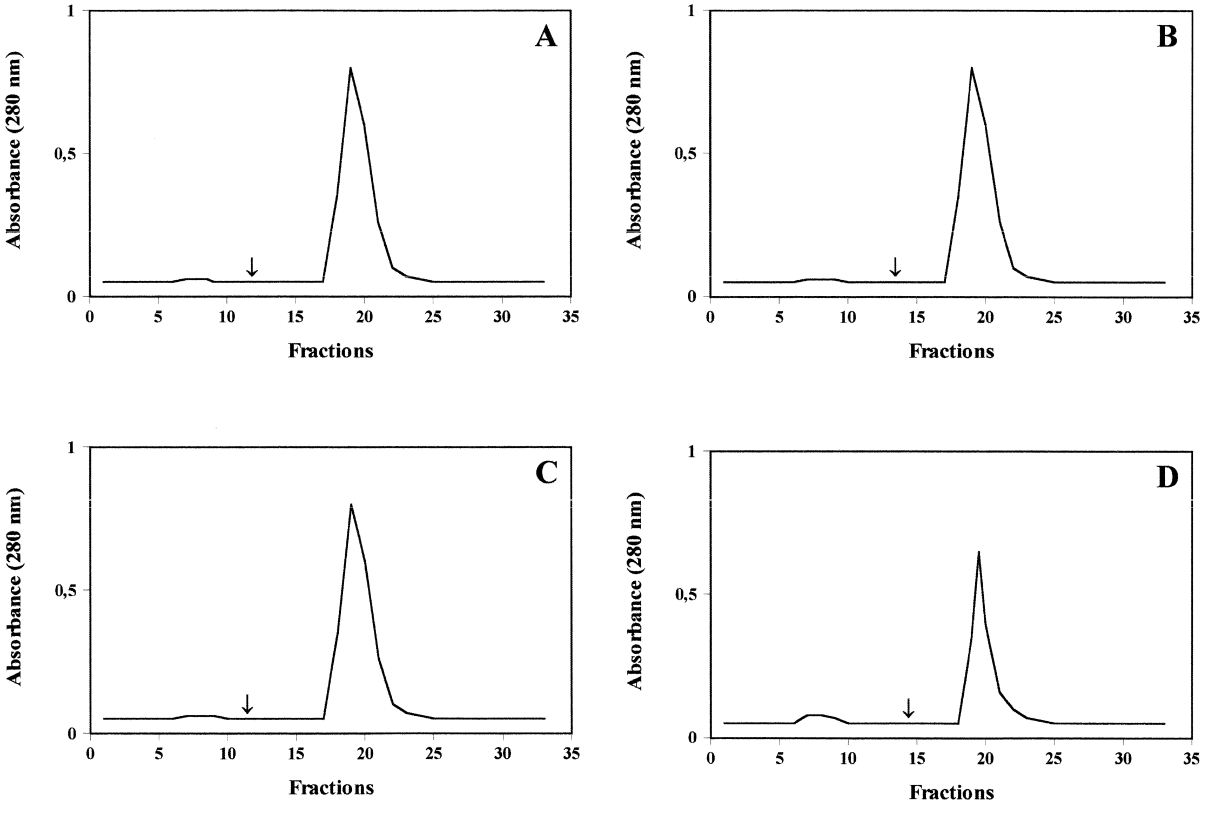


Fig. 3. Hydrophobic interaction chromatography on PPG–Sepharose CL-6B column. Buffers: 20% (w/v) $(NH_4)_2SO_4$ at pH 6.0 (A); 7.0 (B); 8.0 (C) and 9.0 (D) in 10 m*M* phosphate. Desorption (↓) is obtained with 10 m*M* phosphate buffer; sample size injected: 300 μl (3 mg lipolytic extract).

Table 1
Lipolytic activity yield and purification factor for *C. viscosum* lipase obtained by hydrophobic interaction chromatography on Sepharose CL-6B modified by covalent immobilisation of 1,4-butanediol diglycidyl ether [20], polyethylene glycol [15] and polypropylene glycol

Gel name	Immobilised ligand	Lipolytic activity yield (%)	Purification factor
Epoxy–Sepharose	$-(CH_2)_4-$	89	1.3
PEG–Sepharose	$-(CH_2CH_2O)_n-$	79	1.1
PPG–Sepharose	$-(CH_2\underset{\displaystyle CH_3}{\underset{\vert}{C}}HO)_n-$	122	1.8

which is slightly higher than the purification factor achieved with the same original lipolytic preparation with other ligands for HIC (Table 1). No further purification could be achieved owing probably to the high purity of the initial lipolytic mixture used.

In Fig. 4, lipase adsorption onto different Sepharose-based gels was compared in the presence of 20% (w/v) ammonium sulphate in the phosphate buffer. Lipase was adsorbed on all the derivatised gels, but the chromatographic behaviour obtained on the PEG–Sepharose column is slightly different. The interaction of lipase with epoxy– and PPG–Sepharose stationary phases seem to be mainly based on their hydrophobic properties. Some other different types of weak molecular interactions could also be present between lipase and PEG, as obtained by

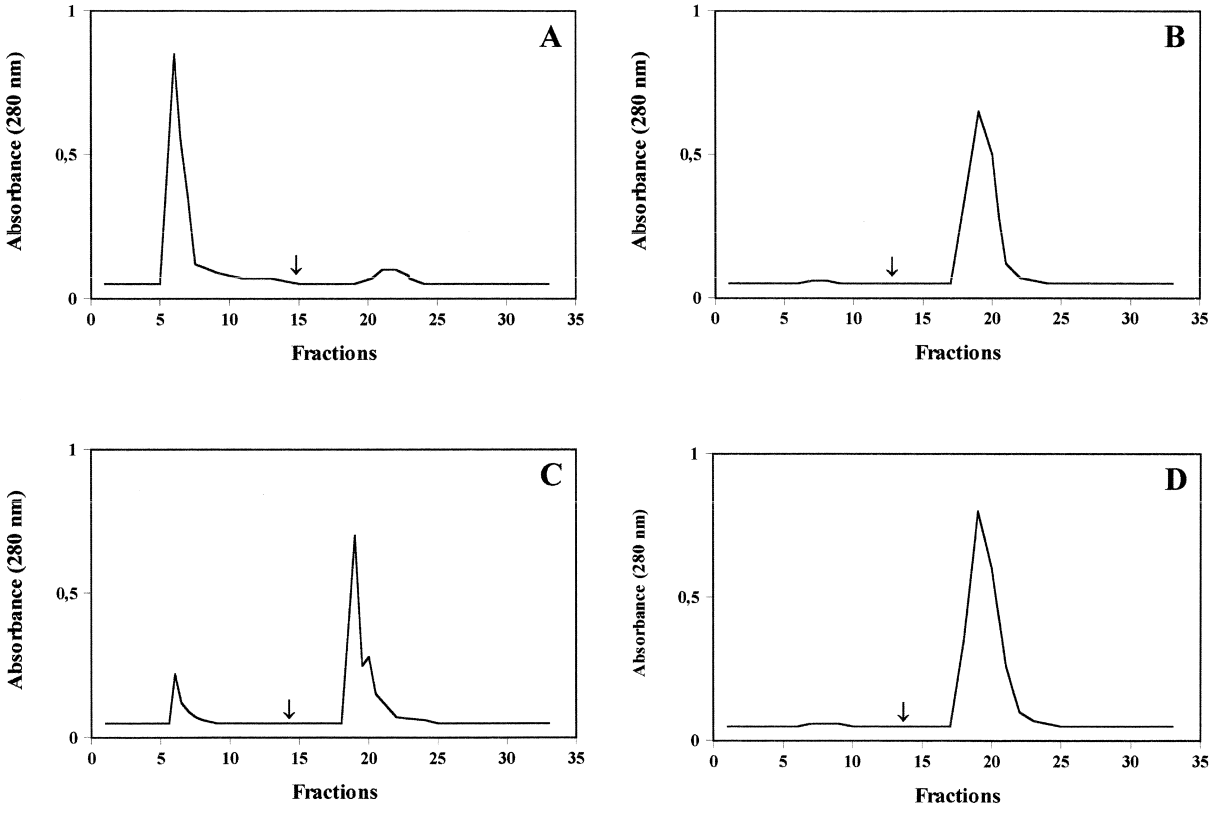


Fig. 4. Hydrophobic interaction chromatography on underivatised Sepharose (A); epoxy–Sepharose (B); PEG–Sepharose (C) and PPG–Sepharose CL-6B column (D). Buffer: 20% (w/v) $(NH_4)_2SO_4$ in 10 m*M* phosphate, pH 7. Desorption (↓) is obtained with 10 m*M* phosphate buffer, pH 7; sample size injected: 300 μl (3 mg lipolytic extract).

Berna et al. [23] on the protein adsorption of human serum onto different agarose-based chromatographic gels.

Control experiments carried out using underivatised Sepharose CL-6B and using Sepharose CL-6B activated with 1,3-butadiene diepoxide (without PPG), with ammonium sulphate in the buffer (Fig. 4A), or PPG–Sepharose, without salt in the buffer, did not result in any retention of lipase in the column. The fractionation of lipase in the gel is not due to the properties of the Sepharose gel itself, and depends neither on the ligand nor on the salt alone, but requires the combined effect of both species.

In conclusion, the properties of the PPG–Sepharose gel used seem to provide an adequate approach to lipase fractionation based on their hydrophobic properties. It was found that the interaction of lipase with the stationary phase is apparently selective and the extent of retention of lipase is significantly affected by the salt used and increases with increasing ionic strength. All the experimental results obtained support the hypothesis that the lipase–gel interaction consists of hydrophobic binding. Finally, the PPG with intermediate hydrophobicity appears to represent a new ligand for HIC not only for *C. viscosum* lipase but may also be applicable to lipases from other sources and seems to provide an interesting approach for the fractionation of hydrophobic proteins.

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