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Short communication

Hydrophobic interaction chromatography of *Chromobacterium viscosum* lipase

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Abstract

The influence of mobile phase composition on the chromatographic behaviour of *Chromobacterium viscosum* lipase was studied by using an epoxy-activated spacer arm as a ligand in hydrophobic interaction chromatography. The retention of lipase depends on the salt used and increased with ionic strength, indicating that the interaction of lipase with the stationary phase is of a hydrophobic nature. Using 20% (w/v) ammonium sulphate in the eluent a total retention of lipase on the column was obtained and by washing with 10 m*M* phosphate buffer a recovery of 79% protein and 89% lipolytic activity were achieved.

1. Introduction

Hydrophobic interaction chromatography (HIC) is a very powerful separation technique that is widely used in protein purification. In HIC, the addition of salting-out salts to the equilibration buffer (and sample solution) decreases the availability of water molecules in solution, increases the surface tension and enhances the ligand–protein interactions [1]. Elution and separation, according to differences in the surface hydrophobicity of proteins [2], are in general brought about by decreasing the salt concentration of the eluent.

The main parameters to consider for separation processes using HIC are: the type of ligand and matrix, the type and concentration of salt, pH, temperature, and additives [3].

Strong hydrophobic interactions sometimes result in almost irreversible adsorption or denaturation during elution with harsh conditions (organic solvents, detergents, chaotropic agents, etc.). An example is the hydrophobic interaction between the lipase of *Chromobacterium viscosum* and a phenyl-Superose column, where elution is only obtained with a gradient of 0–65% (v/v) ethyleneglycol [4]. Therefore, ligands with intermediate hydrophobic character are of great interest, as they provide an adequate binding strength without the drawbacks mentioned above. This can be achieved by the use of bisoxiranes. Bisoxiranes have also been used for the introduction of reactive oxirane groups into agarose and for simultaneous stabilization of the gel by simultaneous cross-linking [5].

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This work aims to study the influence of mobile phase composition on the chromatographic behaviour of *C. viscosum* lipase using bisoxirane (1,4-butanediol diglycidyl ether) with intermediate hydrophobicity as a ligand in HIC. The effectiveness of some salting-out salts (in different concentrations) at various pH values to increase lipase–adsorbent interaction is described.

2. Experimental

2.1. Materials

Sepharose CL-6B was obtained from Pharmacia (Uppsala, Sweden) and 1,4-butanediol diglycidyl ether from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Lipolytic preparation

A commercial lipolytic preparation of *Chromobacterium viscosum* lipases (3880 U/mg) from Toyo Jozo was used.

2.3. Protein determination

The concentration of protein in the samples was determined by the method of Bradford [6].

2.4. Activity measurement

Lipase activity was measured in an oil–water emulsion medium [7]. Amounts of 20 g of olive oil, 20 g of Triton X-100 and 60 ml of distilled water were mixed and magnetically 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 an acetone–ethanol (1:1) mixture. The free fatty acids were then assayed by titration with 50 mM NaOH.

2.5. Preparation of gel

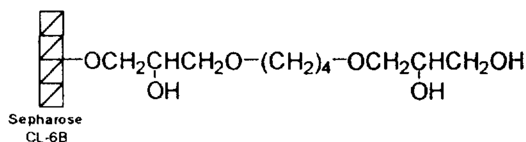
The gel was prepared by coupling 1,4-butanediol diglycidyl ether to Sepharose CL-6B according to Sundberg and Porath [5]. The amount of epoxy groups bound was around 500 μmol/g dry gel. The gel thus obtained was subsequently treated with 1 M sodium hydroxide overnight at room temperature to inactivate the free epoxy groups.

2.6. 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 4.5 ml/h. After the lipolytic extract (300 μl, 3 mg) was 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

In this work the HIC of *C. viscosum* lipase using an epoxy-activated spacer arm with intermediate hydrophobicity as a ligand is studied. The stationary phase was prepared by covalent immobilization of 1,4-butanediol diglycidyl ether on Sepharose CL-6B. This carbohydrate gel was selected because of its chemical stability and its large pore size (to role out as far as possible the interference of gel-permeation phenomena). The schematic structure of the gel obtained is



The effect of some salts, their concentrations, and the influence of pH on lipase–adsorbent interaction was analysed.

Experiments carried out with different salting-

out salts (Fig. 1) indicate that the nature of the ions can play a significant role in the fractionation process. For sodium chloride, e.g., a small percentage of lipase was retained on the column, using a high concentration of the salt in the buffer. According to Melander et al. [8] an increase in salt molality or 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 in HIC. Sodium chloride is the salt used with the smallest molal surface tension increment [2].

The hydrophobic interactions are known to increase with the ionic strength of the medium [8,9]. In this way, as shown in Fig. 2, the progressive increase in ammonium sulphate concentration leads to a parallel increase in the amount of bound lipase. By this procedure, the total retention of lipase on the column was 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 hydrophobic interactions. As can be seen in Fig. 3, no significant influence of pH was observed on the adsorption of lipase to the matrix. The retention of *C. viscosum* lipase was not strongly affected in the pH 4–9 range, whether the net charge of the lipase is negative or positive (p*I* 6.9 [10]). This suggests that the hydrophobic interactions play a major role in the retention of the lipase ra' than ionic interactions. However, Hjertén ι [11] have found that the retention of proteins change more drastically at pH values above 8.5 and/or below 5 than in the pH 5–8.5 range.

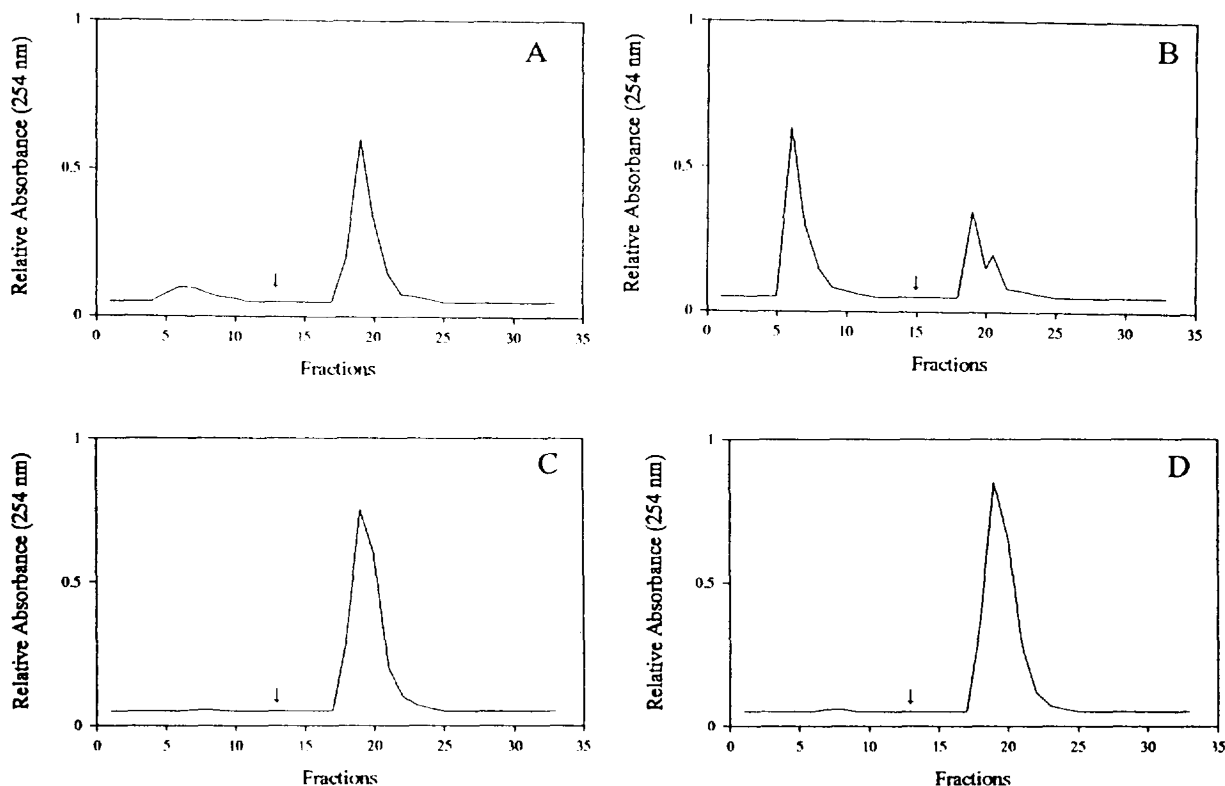


Fig. 1. Hydrophobic interaction chromatography on Sepharose CL-6B column modified with 1,4-butanediol diglycidyl ether (see text). Buffer: (A) 15% (w/w) potassium phosphate, pH 8; (B) 4 *M* NaCl; (C) 15% (w/v) Na_2SO_4; and (D) 20% (w/v) $(NH_4)_2SO_4$ in 10 m*M* phosphate, pH 7. Desorption (↓) is obtained with 10 m*M* phosphate buffer, pH 7.

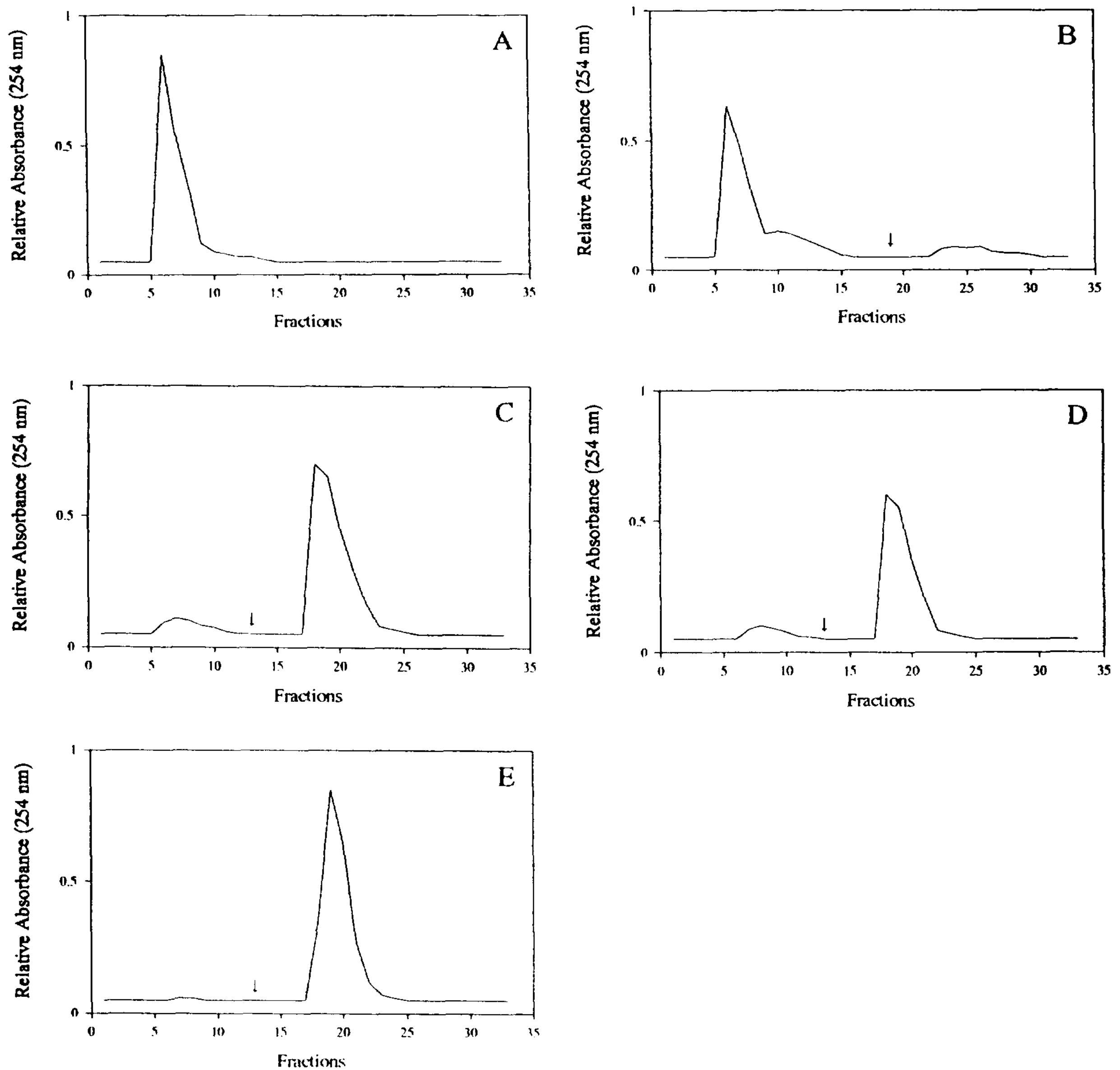


Fig. 2. Hydrophobic interaction chromatography on Sepharose CL-6B column modified with 1,4-butanediol diglycidyl ether (see text). Buffers: 10 mM phosphate, pH 7 (A), containing 10% (B), 15% (C), 17.5% (D) and 20% (E) (w/v) $(NH_4)_2SO_4$. Desorption (↓) is obtained with 10 mM phosphate buffer, pH 7.

However, the magnitude of these alterations in the retention is different for different proteins.

In ideal HIC a decrease in the ionic strength of the buffer might therefore increase desorption. By washing the adsorbent with phosphate buffer (10 mM), after total retention of lipase in the gel, a good recovery yield was obtained: about 79% for protein and 89% for the lipolytic activity. The opposite, as mentioned earlier, 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 of 0–65% (v/v) ethyleneglycol [4]. An 1.3-fold increase of specific activity was obtained

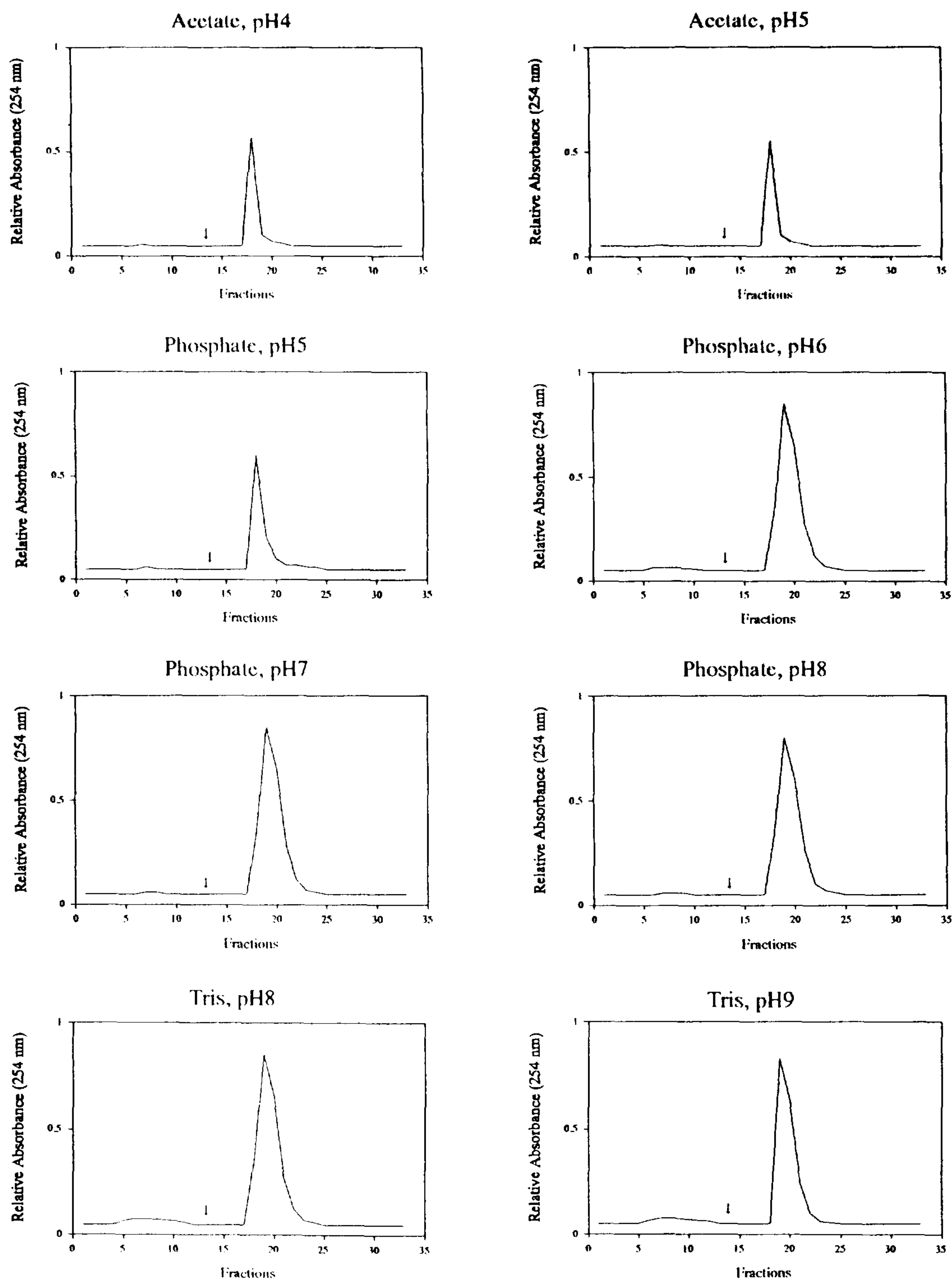


Fig. 3. Hydrophobic interaction chromatography on Sepharose CL-6B column modified with 1,4-butanediol diglycidyl ether (see text). Buffer: 20% (w/v) $(NH_4)_2SO_4$ in 10 mM acetate, phosphate or Tris. Desorption (↓) is obtained with the respective buffer (10 mM).

and no further purification could be achieved probably due to the high purity of the initial lipolytic mixture used.

Control experiments carried out using underivatizated Sepharose CL-6B, or modified by covalent immobilization of 1,3-butadiene diepoxide [without $(CH_2)_4$], did not result in any retention of lipase in the column. This shows that the fractionation of lipase in the gel is not due to the properties of the Sepharose or to the epoxy groups of the ligand. On the other hand, some precipitation of lipase on the top of column (about 10%) may occur, but if a centrifugation step is included before injection of the sample, no change in the chromatographic behaviour of lipase is obtained.

In conclusion, all the experimental results obtained support the hypothesis that the lipase–matrix interaction consisted of hydrophobic binding. In fact, Mathis et al. [12] have assumed that the hydrophobic interaction takes place with the–$(CH_2)_4$–units of the immobilized epoxy-activated spacer arm. 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 by the ionic strength. HIC with the gel used seems to provide an interesting approach for lipase fractionation.

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