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Short communication

Preliminary study on hydrophobic interaction chromatography of *Chromobacterium viscosum* lipase on polypropylene glycol immobilized on Sepharose

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Abstract

The purification of *Chromobacterium viscosum* lipase was performed using a polypropylene glycol–Sepharose gel. 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 ionic strength. Using 20% (w/v) ammonium sulphate in the eluent, a total retention of lipase on the column was obtained. © 1998 Elsevier Science B.V.

Keywords: *Chromobacterium viscosum*; Mobile phase composition; Lipase; Enzymes

1. Introduction

Hydrophobic interaction chromatography (HIC) is a very powerful separation technique that is widely used in protein purification. The addition of salting-out salts to the equilibration buffer (and sample solution) promotes ligand–protein interactions and the separations are based on differences in the surface hydrophobicity of proteins [1,2]. 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 [3].

Many traditional hydrophobic ligands have been covalently immobilized on chromatographic supports, resulting sometimes in almost irreversible adsorption or denaturation of proteins during the elution with harsh conditions. An example is the hydrophobic interaction between the lipase of *Chromobacterium viscosum* and a PhenylSuperose column, where the elution is obtained only with a gradient of 0–65% (v/v) ethylene glycol [4]. The analogy between partitioning in aqueous two-phase systems and chromatographic procedures has been utilized to achieve new mild hydrophobic ligands, which provide an adequate binding strength without the above drawback. The elution is in general brought about by decreasing the salt concentration of the eluent.

The interaction between lipases and polyethylene glycol was previously reported [5] and the results thus obtained encouraged the study of the interaction between lipases and polypropylene glycol (PPG) immobilized on Sepharose.

In this work, PPG was covalently bond on Sepharose CL-6B by the use of a bisoxirane (1,3-butadiene diepoxide). In fact, bisoxiranes have been used not only for the introduction of reactive oxirane groups

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into agarose [6] but also for the introduction of other hydrophobic ligands for the purification of lipases [7]. This paper describes the influence of mobile phase composition on the chromatographic behaviour of *C. viscosum* lipase. 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). PPG 4000 and 1,3-butadiene diepoxide were 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 [8].

2.4. Activity measurement

Lipase activity was measured in an oil–water emulsion medium [9]. 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 an acetone–ethanol (1:1, v/v) mixture. The liberated fatty acids were then assayed by titration with 50 m*M* NaOH.

2.5. Preparation of the gel

Sepharose CL-6B was activated by coupling 1,3-butadiene diepoxide to it according to the method of Sundberg and Porath [6]. PPG 4000 was subsequently bonded to the epoxy-activated gel according to the method of Hedman and Gustafsson [10]. The PPG 4000-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.

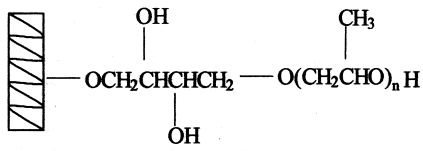
2.6. Chromatographic method

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 6.0 ml/h. After the lipolytic extract (400 μl, 4 mg) had been applied, the elution profile was obtained by continuous measurement of the absorbance at 280 nm. Fractions of 3 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 purification of *C. viscosum* lipase was studied.

The stationary phase used in the chromatographic experiments was prepared by covalent immobilization of PPG 4000 on Sepharose CL-6B. This carbohydrate gel was selected because of its chemical stability. The schematic structure of the gel obtained is



Sepharose CL-6B

Control experiments carried out using underivatized Sepharose CL-6B or modified with 1,3-butadiene diepoxide (without PPG) did not result in any retention of lipase on the column. All the experimental results obtained support the hypothesis

that the lipase–gel interaction consists of hydrophobic binding.

Experiments carried out with different salting-out salts (Fig. 1) indicate that the nature of the ions plays a significant role in the fractionation process. For potassium phosphate and sodium chloride a small amount of lipase was retained on the column even if a high concentration of the salts in the buffer is used. According to Melander et al. [11], an increase in salt molality or 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 HIC. On the other hand, sodium sulphate and ammonium sulphate promote lipase binding to the support to a great extent because they present high increments of molal surface tension.

The hydrophobic interactions are known to increase upon increasing the ionic strength of the medium [11,12]. 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. In fact, for 10% (w/v) of the salt in the buffer no lipase remains bounded to the gel, but on increasing salt concentration to 15% (w/v) the amount of lipase retained is near to 14%. The total retention of lipase in the column is obtained only 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. The retention of *C. viscosum* lipase was not strongly affected in the pH range 5–8.5 and the chromatographic profile is similar to the observed in Fig. 1A. This suggests that hydrophobic interactions play a major role in the retention of the lipase, as previously obtained with 1,4-butanediol diglycidyl ether [7].

As discussed above protein adsorption increases with the ionic strength. Desorption can then be

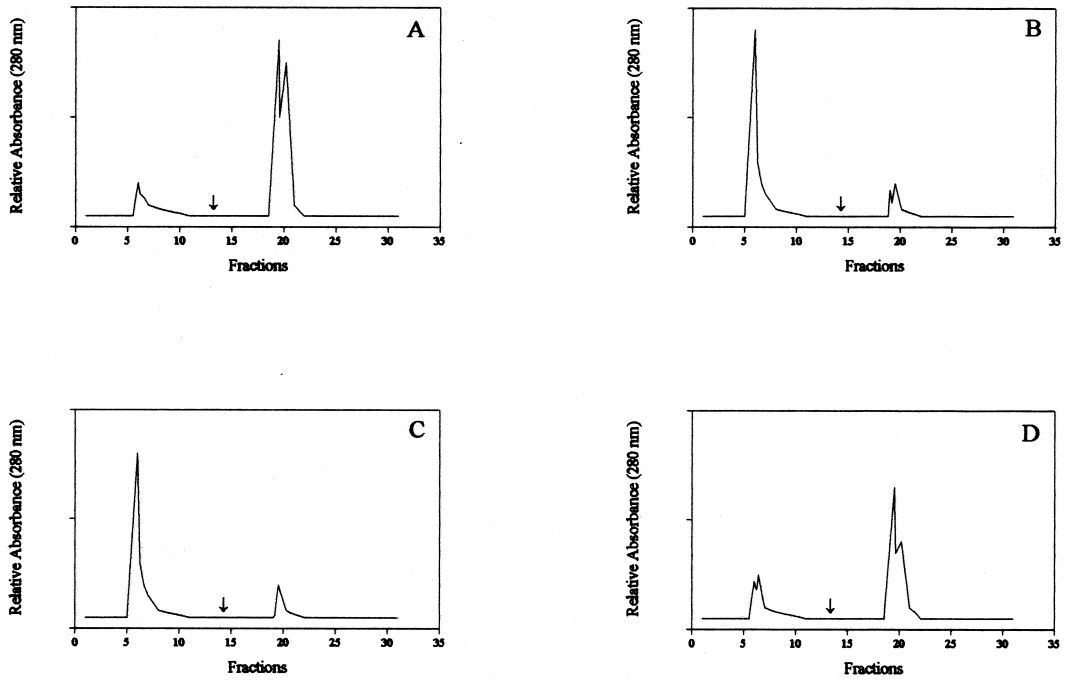


Fig. 1. Hydrophobic interaction chromatography on PPG-Sepharose CL-6B column. Buffer: A, 20% (w/v) $(NH_4)_2SO_4$; B, 15% (w/w) potassium phosphate; C, 4 *M* NaCl and D, 15% (w/v) Na_2SO_4 in 10 m*M* phosphate (pH 7). Desorption (↓) is obtained with 10 m*M* phosphate buffer (pH 7).

Table 1
Activity yield and purification factor for *C. viscosum* lipase obtained by HIC on PPG-Sepharose CL-6B

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Original lipolytic preparation	1.320	3000	2286	100	–
HIC on PPG-Sepharose	0.617	1935	2819	65	1.3

performed by just lowering the ionic strength and, in fact, by washing the support with 10 m*M* phosphate buffer (pH 7) (Fig. 2), relatively high recovery yields were obtained: about 49% for protein and 65% for the lipolytic activity (Table 1). A 1.3-fold increase of specific activity was obtained which is of the same order of magnitude as the purification factor achieved with the same original lipolytic preparation with other ligand for HIC [5,7]. No further purification could be achieved probably owing to the high purity of the initial lipolytic mixture used.

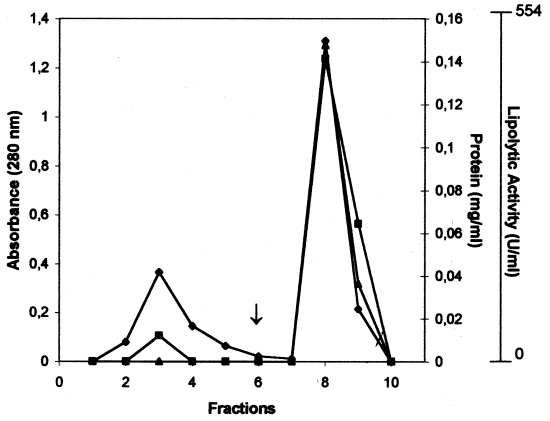


Fig. 2. Hydrophobic interaction chromatography on PPG-Sepharose CL-6B column. Buffer: 20% (w/v) $(NH_4)_2SO_4$ in 10 m*M* phosphate (pH 7). Desorption (↓) is obtained with 10 m*M* phosphate (pH 7). ♦, Absorbance (280 nm); ■, protein (mg/ml); ▲, lipolytic activity (U/ml).

In conclusion, the properties of the gel used seem to provide an adequate approach to lipase fractionation based on their hydrophobic properties. The interaction of lipase with the stationary phase is apparently selective and the extent of lipase is significantly affected by the salt used and increases with increasing ionic strength.

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