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LARGE-SCALE PURIFICATION OF STAPHYLOCOCCAL LIPASE BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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SUMMARY

A fast and very efficient method for the purification of staphylococcal lipase has been developed. It is based on ammonium sulphate precipitation of the culture supernatant, ultrafiltration with an Amicon filter and hydrophobic interaction chromatography on octyl-Sepharose CL-4B. This procedure yields in a 108-fold purification of this lipase, which shows two bands in sodium dodecyl sulphate–polyacrylamide gels. Both bands are active towards different substrates.

INTRODUCTION

Many investigators[1–3] have observed that bacterial or fungal lipases adsorb to hydrophobic ligands. These ligands were either coated onto glass beads or coupled to some other solid supports. Hepatic and lipoprotein lipases are known to adsorb to heparin-Sepharose and can be eluted by a salt gradient[4,5]. The interaction of lipoprotein lipase with heparin-like polysaccharides has recently been studied more closely[6]. Wang[7] was able to purify a bile-salt activated lipase by affinity chromatography from a cholic acid-coupled Sepharose CL-4B column.

Kosugi and Suzuki[2] found that at least an octyl residue has to be coupled to the agarose in order for the lipase to be bound. It Seemed, therefore, reasonable to use octyl-Sepharose CL-4B for the purification of staphylococcal lipase. The present paper describes the optimal conditions for adsorption of staphylococcal lipase to octyl-Sepharose, removal of the bulk of the protein by a desoxycholate gradient and a good recovery of this bacterial lipase in pure form.

EXPERIMENTAL

The strain *Staphylococcus aureus* (TEN 5) was isolated by C. G. Gemmell, Royal Infirmary, University of Glasgow. It was cultivated and the lipase was precipitated from the culture supernatant as described earlier[8].

The ammonium sulphate precipitate was dissolved in a small volume of Tris–HCl buffer, 0.05 *M*, pH 8.0, containing 0.15 *M* KCl. This solution was then subject to

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ultrafiltration at constant volume with an Amicon XM-100 membrane until the filtrate was free of protein. The above buffer was used for this process.

The lipase solution was then diluted in the above Tris–HCl buffer so that its protein content was 1 mg/ml. One gram of wet octyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) was used per 10 mg protein. The slurry was then stirred gently at 4–8°C for 20 h. The gel was transferred onto a Büchner funnel and washed with five volumes of Tris–HCl buffer, 0.05 *M*, pH 8.0, containing 0.15 *M* KCl. Subsequently, the gel was washed stepwise with five volumes of different Tris–HCl buffers: 0.05 *M*; 0.01 *M* and 0.001 *M* of pH 8.0. The washings were then repeated with five volumes of the same Tris–HCl buffers but of pH 9.0. Finally the gel was equilibrated with 0.05 *M* Tris–HCl buffer, pH 8.0, and put into a column (60 × 2.5 cm). A linear sodium desoxycholate (SDOC) gradient (0–1%; 10-fold bed volume) was then applied to remove contaminating material. A constant flow-rate of 20 ml/h at 4°C was maintained. *Staphylococcus aureus* lipase was eluted by a linear Triton X-100 gradient (0–1%; 10-fold bed volume) in 0.05 *M* Tris–HCl of pH 8.0, containing 2.0 *M* KCl.

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis

This was performed according to Laemmli[9]. A 10–40 μg amount of protein was loaded per trough. Phosphorylase (mol.wt. 95,000; E.C. 2.4.1.1.), bovine serum albumin (mol.wt. 67,000), fumarase (mol.wt. 49,000; E.C. 4.2.1.2.), carboanhydrase (mol.wt. 30,000; E.C. 4.2.1.1.) and lysozyme (mol.wt. 14,500; E.C. 3.2.1.17.) were used as reference proteins and purchased from Boehringer (Mannheim, G.F.R.).

Protein determinations

These were performed as described by Petersen[10] or using the more rapid procedure of Bradford[11].

Detection and removal of Triton X-100

The concentration of Triton X-100 was determined as outlined by Gareval[12]. Its removal has been described previously[13].

Enzymatic methods

Lipase activity was measured as described by Rick[14]. The substrate dispersion was prepared in distilled water and contained 5 m*M* triolein (Serva, Heidelberg, G.F.R.), 5% gum arabic (E. Merck, Darmstadt, G.F.R.), 4 m*M* SDOC (E. Merck) and 0.1 *M* NaCl. Released oleic acid was titrated continuously with 0.02 *M* NaOH at 30°C. One unit of lipase is defined as that amount of protein which liberates 1 μmole of oleic acid per minute under the above conditions.

Esterase activity was determined as reported earlier[15] except that mercaptoethanol was omitted and the buffer was adjusted to pH 7.4. One unit of esterase is defined as the amount of protein which causes a change of extinction of 0.001 per minute at 400 nm and 30°C.

Esterase and lipase activities in polyacrylamide gels were detected using 20 m*M* solutions of either α-naphthyl acetate (Sigma, München, G.F.R.), *p*-nitrophenyl acetate (E. Merck) or α-naphthyl nonanoate (Schuchardt, München, G.F.R.) in Tris–HCl buffer, 0.1 *M*, pH 7.8, containing 0.1 *M* NaCl, 4.0 m*M* SDOC and 0.1% fast red TR[16].

Staphylococcus aureus α- and β-hemolysins were determined as described previously[17–19].

RESULTS

Staphylococcus aureus (TEN 5) was cultivated as described earlier[8]. Lipase was precipitated from the culture supernatant at 50% ammonium sulphate saturation, 4°C and pH 8.0. Ultrafiltration was performed as described in Experimental. No lipase was lost during this procedure (Table I).

TABLE I

SUMMARY OF THE PURIFICATION OF *S. AUREUS* (TEN 5) LIPASE (SEE FIG. 2)

Preparation	*Protein (g)*	*Yield (%)*	*Lipase (U × 10^4)*	*Yield (%)*	*Specific activity [U × (mg protein)$^{-1}$]*	*Hemolytic activities (HU × 10^4)*	*Purification (x-fold)*
Culture supernatant	4.6	100	9.8	100	21.4	57.6	1
Ammonium sulphate precipitate	2.2	48	10.3	100	47.0	61.44	2.2
Ultrafiltration	2.0	44	10.5	100	52.4	55	2.5
Hydrophobic interaction chromatography.							
Supernatant after adsorption	1.32	29	0.76	7.7	5.8	56	–
Sodium desoxycholate gradient	0.07	1.5	0.1	≈1.0	–	0	–
Triton X-100 gradient							
peak 1	0.04	0.8	0.3	≈3.0	–	0	–
peak 2	0.04	0.8	8.7	83	2.3×10^3	0	108.8

Different salts and different concentrations thereof (*e.g.*, NaCl, KCl and ammonium sulphate) and different buffers at different pH values and concentrations were tried in order to establish the best conditions for adsorption of staphylococcal lipase. Tris–HCl buffer, 0.05 *M*, pH 8.0, containing 0.15 *M* KCl was the best solvent. One gram of wet octyl-Sepharose CL-4B was taken per 10 mg protein. Adsorption at 4–8°C for 20 h and slow stirring was better than adsorption for shorter periods of time and at room temperature. Under the latter conditions more protein was adsorbed.

In some experiments the extensive washings were omitted. Later, however, it was obvious that the purified lipase contained small amounts of several proteins as evidenced on SDS–polyacrylamide gels. These seemed to adsorb only slightly and could be removed by extensive washings of the octyl-Sepharose after adsorption.

Table I shows that α- and β-hemolysins are not adsorbed to octyl-Sepharose under these conditions. At most 5% of the original amount of protein is eluted from the column by the two gradients employed. The linear sodium desoxycholate gradient (0–1%) in Tris–HCl buffer 0.05 *M*, pH 8.0 (Fig. 1), eluted about half of the adsorbed

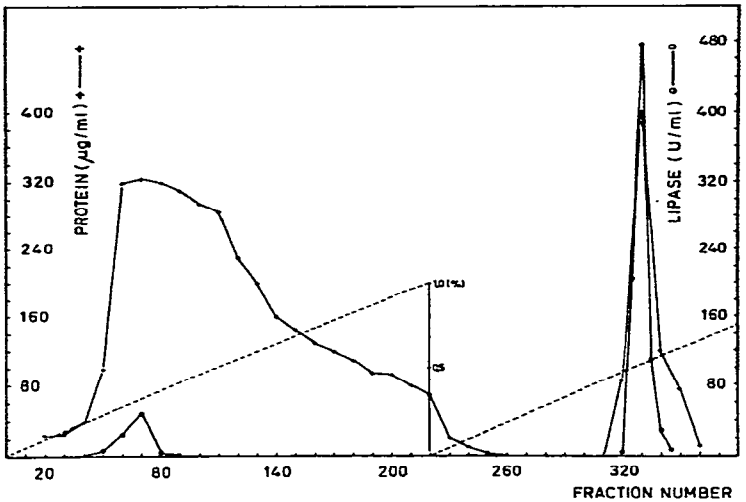

Fig. 1. Isolation of staphylococcal lipase by hydrophobic interaction chromatography on octyl-Sepharose CL-4B. Column: 30 × 2.5 cm. Fraction: 5.5 ml per tube. The first gradient is a SDOC gradient (0–1%; fractions 1–220) in 0.05 *M* Tris–HCl buffer, pH 8.0; flow-rate 30 ml/h. The second gradient is a linear Triton X-100 gradient in the same buffer (0–1%; fractions 221–440); flow-rate 20 ml/h. Lipase activity (○) in U/ml and protein (+) in µg/ml.

protein and only approximately 3% of the lipase activity (Table I). A second gradient, consisting of Triton X-100 (0–1%) in 0.05 *M* Tris–HCl, pH 8.0, eluted all the lipase and a protein of mol.wt. 34,000 together (SDS gels not shown); lipase and this component seemed to interact with octyl-Sepharose to the same extent and possess similar hydrophobic properties.

Experiments with different Triton X-100 gradients (*e.g.*, steepness of the gradient, flow-rates, temperatures) or different ratios of octyl-Sepharose CL-4B to protein to be adsorbed did not result in the separation of these two proteins.

The above Triton X-100 elution procedure combined with a pH gradient (pH 8.0–9.0) resulted in the coelution of the lipase with the lower-molecular-weight impurity at lower detergent concentration (0.3% Triton X-100).

Porath *et al.*[20] and Sasaki *et al.*[21] have shown that hydrophobic interaction also depends on the ionic strength. A decreasing KCl gradient (0.2–0.0 *M*) was superimposed upon the increasing Triton X-100 gradient in Tris–HCl buffer, 0.05 *M*, pH 8.0. The first fractions containing protein showed mainly the mol.wt. 34,000 component. Lipase was eluted in later fractions (data not shown).

In subsequent experiments the Triton X-100 gradient (0–1%) in Tris–HCl buffer, 0.05 *M*, pH 8.0, was made up to 1 *M*, 2 *M* and 3 *M* with respect to KCl. As is shown in Fig. 2 the Triton X-100 gradient containing 2 *M* KCl was able to separate the mol.wt. 34,000 protein from lipase. The latter is eluted at a Triton X-100 concentration of 0.75%, whereas the former is eluted at 0.55%. Fig. 3 shows SDS–polyacrylamide gels of different fractions of a large scale purification of lipase (*cf.*, Fig. 2). There is a double band for the purified staphylococcal lipase. Both bands reacted with typical lipid substrates (*e.g.*, α-naphthyl nonanoate) or esterase substrates (*e.g.*, *p*-

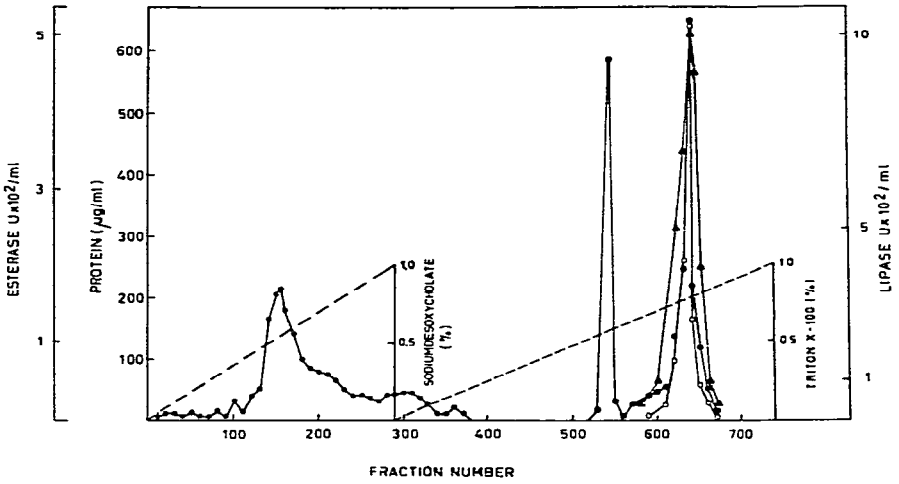

Fig. 2. Isolation of staphylococcal lipase by hydrophobic interaction chromatography on octyl-Sepharose CL-4B. Column: 60 × 2.5 cm. The first gradient is a SDOC gradient (0–1 %; fractions 1–290) in 0.05 *M* Tris–HCl, pH 8.0; flow-rate 30 ml/h; fraction volume 7 ml. The second gradient consists of a linear increase in Triton X-100 in 0.05 *M* Tris–HCl buffer, pH 8.0, containing 2.0 *M* KCl (fractions 291–755); flow-rate 20 ml/h; fraction volume 4.5 ml. Protein concentration in µg/ml (●), lipase activity in U/ml (○) and esterase activity in U/ml (▲).

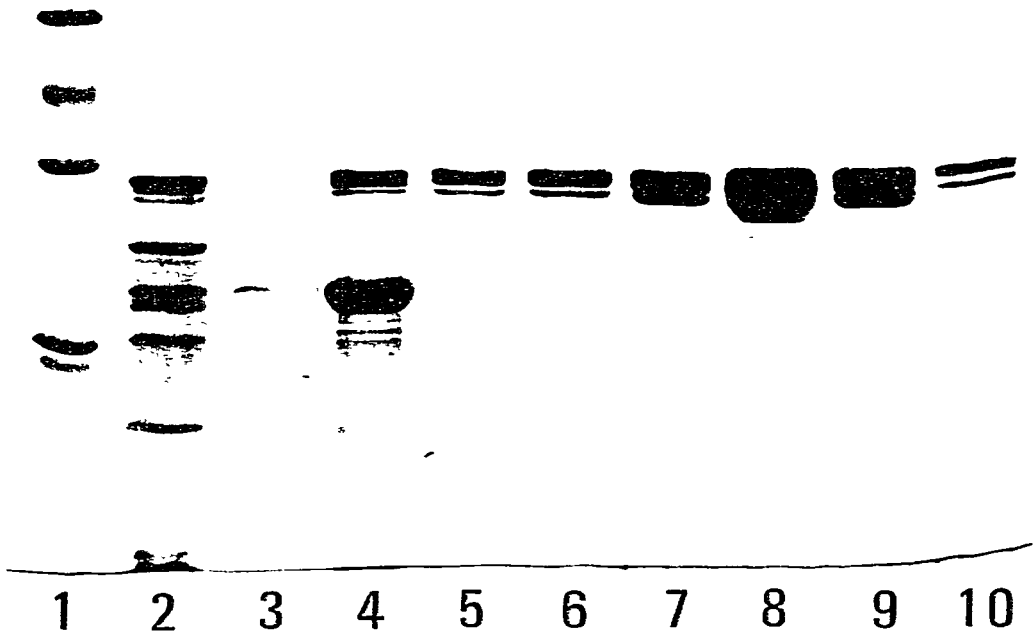

Fig. 3. SDS–polyacrylamide gel electrophoresis of different fractions of the purification of lipase. Tracks: 1, 1.5–2.5 µg of each marker protein; 2, 260 µg ammonium sulphate-precipitated material; 3–1p, 20 µl of the following fractions of the column eluent of Fig. 2: 530, 540, 605, 610, 620, 630, 640 and 650.

nitrophenyl acetate) even after polyacrylamide gel electrophoresis in the presence of SDS (data not shown).

Table I gives an overall account of the purification of staphylococcal lipase. The apparent molecular weight of the lipase can be extrapolated as 43,000 and 44,000 respectively (Fig. 3).

DISCUSSION

The adsorption was not an all or none process. The extensive washings removed traces of impurities from the octyl-Sepharose after lipase had been adsorbed. Quite a considerable number of faint bands were seen in SDS–polyacrylamide gels of the purified lipase when these washings were omitted. These proteins therefore only interact slightly with octyl-Sepharose under these adsorption conditions.

α- and β-hemolysins of *S. aureus* strains do react with a variety of membranes[22] and should therefore have hydrophobic regions. They are inhibited from interacting with octyl-Sepharose CL-4B under our experimental adsorption conditions. This is an indication that specific adsorption conditions can be worked out in order to purify certain hydrophobic proteins.

It was noticed earlier that this lipase has a rather high apparent molecular weight, in physiological buffers[8,23,24] ranging from 100,000 to 600,000. Because of this, an Amicon membrane (XM-100) with a high exclusion range of 100,000 could be used to remove some proteins, especially coloured material, without loss of lipolytic activity (Table I).

Both protein bands (Fig. 3) showed enzymatic activities towards either lipid or ester substrates even after SDS–polyacrylamide gel electrophoresis. There is no visible difference in staining of the two bands with the various substrates. Several explanations can be put forward to explain this.

(a) The lipase may be incompletely glycosylated. Lipase of the fungus *Geotrichum candidum* contains 7.5% sugar[25].

(b) Pancreatic phospholipase A_2 is secreted as a proenzyme from which a heptapeptide is split off. The proenzyme also shows limited enzymatic activity[26,27]. Furthermore, several toxins are secreted as protoxins which are converted into toxins in the course of an infection[28–30].

(c) A mutation of a single amino acid in the histidine binding protein of *Salmonella typhimurium* caused a molecular weight difference of about 2000 Daltons in SDS–polyacrylamide gels[31].

(d) Wiley and Rogolsky[32] separated two antigenically distinct forms of staphylococcal exfoliatins on SDS–polyacrylamide gels. One protein is under chromosomal and the other under plasmid control. These two toxins differ slightly in molecular weight.

Further experiments should reveal which of these explanations are true in the case of this staphylococcal lipase.

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