IMMOBILIZED PANCREATIC LIPASE AND COLIPASE FOR PURIFICATION AND BINDING STUDIES

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1. Introduction

For fat digestion to occur a 1:1 lipase–colipase complex is required at the oil–water interphase [1–4]. Therefore, studies of lipase–colipase interactions which occur in solution may be important. Direct studies of lipase colipase binding in the absence of substrate have been few and and as the dissociation constant is weak, 5×10^{-7} M [5], binding cannot be detected by gel filtration unless very high concentrations are used [6]. Standard purifications of the two proteins include at least 6 time-consuming steps [7–9].

Although immobilization of enzymes has undergone rapid growth [10], systems employing immobilized proteins for the isolation of other proteins have mostly been restricted to the serine proteases and their inhibitors [11,12]. In this study we have immobilized porcine pancreatic lipase and colipase individually on Sepharose using a conventional technique [13]. Affinity columns of the immobilized cofactor or enzyme provide rapid means for purification of the analogous protein as well as a sensitive technique for studying the characteristics of their binding.

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2. Materials and methods

2.1. *Source of materials*

Sepharose 4B (Pharmacia Fine Chemicals), Tributyrin (E. Merck), DFP-di-isopropylfluorophosphate (Fluka, AG), acetonitrile (Fisher) and sodium azide (British Drug Houses) were used. Sodium taurodeoxycholate was synthesized by standard procedures and was 97% pure by thin-layer chromatography. Porcine pancreatic lipase and colipase and horse pancreatic lipase were purified by conventional techniques [7,9] to provide material for immobilization.

2.2. *Lipase and colipase assays*

Lipase and colipase activities were determined by titration [14,15]. Reactions were carried out at room temperature in 10–15 ml buffer on 500 μl of tributyri For lipase assays the buffer contained 2 mM Tris–maleate pH 7.0, 150 mM NaCl, 1 mM $CaCl_2$ and 0.02% sodium azide (lipase buffer). For colipase assays, lipase buffer plus 4 mM sodium taurodeoxycholate was used (colipase buffer).

2.3. *Immobilization*

Sepharose 4B was activated according to the technique in [13]. To 10 ml activated gel (50 mg CNBr/ml settled gel) was added lipase (10 mg) or colipase (19 mg) in 15 ml 0.1 M $NaHCO_3$ and

incubated overnight under gentle agitation at 4°C. Uncoupled protein was washed from the incubated gel by 1 liter of 0.1 M $NaHCO_3$, pH 8.4. The coupling yield for lyophilized matrix-bound lipase or colipase was determined by amino acid analysis [16]. The molar values for HIS and ARG in pure porcine lipase [7] or colipase [9] were used to calculate the amount of bound protein. When converting the protein content of dried gel to swollen gel it was assumed that 40 mg dried gel corresponded to 1 ml swollen Sepharose.

2.4. *Affinity chromatography*

The raw material for colipase purification was obtained from Novo Industri A/S (Copenhagen). This powder contained 2.5–4% colipase and was dissolved (75 mg/ml) in lipase buffer, pH 5.8 and chromatographed directly on columns of immobilized lipase. For lipase purification, defatted pancreatic powder [7] was extracted in 100 mM Tris (pH 9.0), 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM DFP, centrifuged for 30 min at 12 000 × *g* and subjected to gel filtration on Sephadex G-100 to separate lipase from colipase. The lipase pool from Sephadex could then be purified directly on colipase–Sepharose. Samples (0.5–3.0 ml) were applied to the columns, pre-equilibrated with lipase buffer, and elution was regulated by a peristaltic pump. Colipase polyacrylamide-gel electrophoresis [14] and lipase SDS–gel electrophoresis [17] were as described.

3. Results and discussion

All buffers used in affinity experiments and during storage contained 0.02% sodium azide to prevent microbial growth. To prevent proteolytic digestion of the affinity columns during purification of crude pancreatic extracts, DFP (1 mM) must be present in the eluting buffers. Under the conditions of immobilization ≃ 13.2 nmol lipase were bound per ml Sepharose (0.66 mg/ml) and the coupled protein retained about 25% molar colipase-binding capacity. Binding capacity was estimated by measuring the maximum amount of protein which could be retarded from an excess applied to the columns. Approximately 43 nmol colipase/ml Sepharose (0.43 mg/ml) were coupled and retained at least 20% of its molar binding capacity. A moderate sized column of colipase–Sepharose (100 ml), prepared under the above conditions, could purify about 40 mg lipase at a time. Immobilized lipase gave less than 1% of its normal activity in the tributyrin assay system. Between chromatographic separations, columns were washed with 3–4 column vol. colipase buffer, pH 9.0 to remove tightly-bound lipase or colipase which constituted less than 7% total binding capacity.

The low concentration of protein immobilized in these experiments (10–40 nmol/ml Sepharose) may explain why retardation occurred instead of the more usual tight binding. The dissociation constant

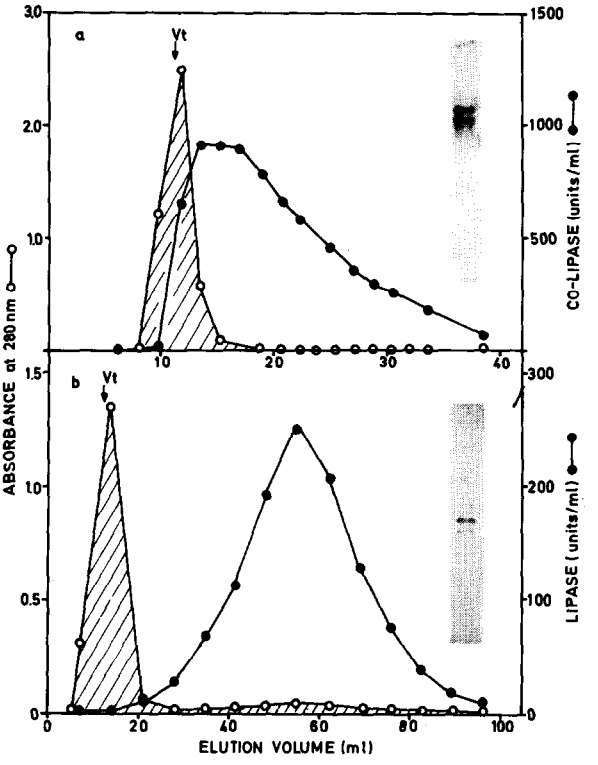

Fig.1. Chromatography of pancreatic extracts on columns (7.9 ml bed vol.) of lipase–Sepharose (0.66 mg lipase/ml packed gel) (a) and colipase–Sepharose (0.43 mg colipase/ml packed gel) (b). The columns were equilibrated at pH 5.8 and the separations made at room temperature. 1a. Affinity chromatography of Novo Industri powder from porcine pancreas (11 mg in 150 μl lipase buffer), flow rate 0.3 ml/min, 1.60 ml/fraction. 1b. Affinity chromatography of a lipase fraction of porcine pancreas (isolated by gel filtration), 14 mg protein in 3 ml lipase buffer, 0.5 ml/min, 6.8 ml/fraction.

for the binding of lipase to colipase can be estimated from binding capacity and the degree of retardation in zonal affinity chromatography experiments [18,19].

At pH 7.0, 150 mM NaCl and 23°C, a lipase–colipase K_d of 1.2×10^{-6} M was calculated with lipase–Sepharose and 1.4×10^{-6} M with colipase–Sepharose. These values compare well with the calorimetric value ($K_d = 5 \times 10^{-7}$ M) [5] and indicate that the system can be quantified.

The biological specificity of immobilized porcine pancreatic lipase and colipase are shown in fig.1. Colipase I and II were purified by single step affinity chromatography of Novo Industri product (fig.1a). A similar purification effect was obtained with heat-treated (80°C, 10 min) aqueous extracts of pancreatic acetone powders. Figure 1b shows the purification of porcine pancreatic lipase by affinity chromatography following gel filtration of crude extract. Because of the natural binding of lipase to colipase in solution a preliminary gel filtration step greatly improves purification of lipase on the immobilized colipase column. The presence of one strong band and one weak band on the SDS gel indicates that the Sephadex fraction was enriched in one of the two allelic variants of pancreatic lipase [7]. The effect of bile salts on lipase binding to immobilized colipase is shown in fig.2. The inhibition of binding by bile salts confirms earlier calorimetric data [5] which indicated inhibition. Figure 2 also shows the binding of horse lipase to porcine colipase and the absence of any interaction between free and bound colipase. The qualitative effects of pH, ionic strength and different detergents on lipase colipase binding during affinity chromatography have been compared with equilibrium binding results obtained by aqueous two-phase partition [20] and found to be in agreement. It is therefore possible that lipase colipase binding observed during affinity chromatography may be representative of the biological situation. The system may also help answer the intriguing question of why lipase and colipase are separate molecules.

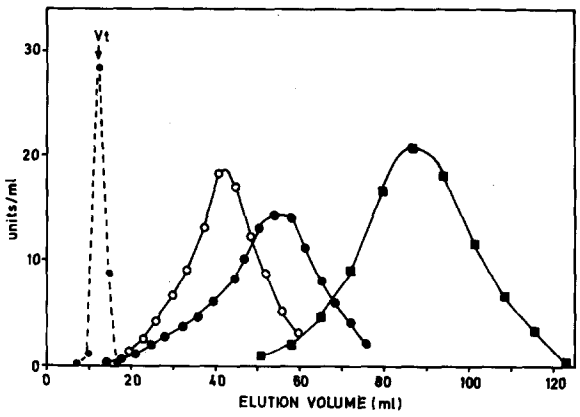

Fig.2. Chromatography of pancreatic lipases and colipase on a colipase–Sepharose column (0.43 mg colipase/ml packed gel, 7.9 ml bed vol.), pH 5.8, room temperature. Four situations are shown: (●- - - - -●) porcine colipase (5 μg sample in 0.5 ml) eluted with lipase buffer; (○——○) porcine pancreatic lipase (50 μg sample in 0.5 ml) eluted with colipase buffer (lipase buffer plus bile salt + 4 mM sodium taurodeoxycholate); (●——●) porcine pancreatic lipase (50 μg sample in 0.5 ml) eluted with lipase buffer; and (◆——◆) horse pancreatic lipase (81 μg in 0.5 ml) eluted with lipase buffer.

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