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One-step purification of trypsin and α-chymotrypsin by affinity chromatography on Eupergit–aprotinin, a novel carrier for purification of serine proteases

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ABSTRACT

Aprotinin isolated from bovine lungs was covalently immobilized on Eupergit C. The highly selective affinity adsorbent was used to purify trypsin and α-chymotrypsin from pancreatic extract in a single step. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis indicated that both enzymes were highly purified. The maximum binding capacity of Eupergit–aprotinin for bovine trypsin was calculated to be *ca.* 7.5 mg per gram of gel (wet resin).

INTRODUCTION

The natural proteinase inhibitor from bovine organs, aprotinin, is widely used as a valuable tool in biochemical and biomedical research[1–3]. Moreover, it is a very potent drug for the treatment of different clinical conditions, *e.g.*, acute pancreatitis and hyperfibrinolytic haemorrhage. In addition, aprotinin is very well suited to inhibit undesired proteolytic reactions in tissue culture[4]. An important application is the purification of various proteinases by affinity chromatography using immobilized aprotinin[5–8]. This proteinase inhibitor is an excellent candidate as a ligand for affinity chromatography[4]. In contrast to other serine proteinase inhibitors used as affinity ligands, aprotinin is able to bind trypsin and α-chymotrypsin with very high affinity.

This paper describes the immobilization of aprotinin to Eupergit C, a chemically and mechanically stable carrier, and the purification of trypsin and α-chymotrypsin from a pancreatic extract in a single step.

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EXPERIMENTAL

Pancreatin was purchased from Merck (Darmstadt, F.R.G.). All other chemicals used were commercially available. Eupergit C was a gift from Röhm Pharma (Weiterstadt, F.R.G.) and aprotinin was kindly provided from Pentapharm (Basle, Switzerland).

Aprotinin was covalently linked to Eupergit C via its amino groups to the epoxy groups of the carrier according to the standard procedure of the manufacturer[9].

Affinity chromatography

A 1-g amount of Eupergit–aprotinin (wet resin, equivalent to 1.15 ml of gel) equilibrated in 50 m*M* Tris–HCl (pH 8.0) containing 0.5 *M* sodium chloride and 1 m*M* calcium chloride (buffer A) was incubated with 5 ml of pancreatic extract (22 mg/ml) for 60 min at room temperature. The gel was poured into a chromatographic column and washed several times with the same buffer until the adsorption at 280 nm reached an adsorption baseline of 0.05. The bound proteases were eluted with 0.1 *M* hydrochloric acid (pH 1.0) containing 25 m*M* calcium chloride and collected in tubes containing 2 *M* Tris–HCl (pH 7.5). The eluent was dialysed against 50 m*M* Tris–HCl containing 1 m*M* calcium chloride and concentrated in collodion bags (SM 13200). The purity was checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)[10].

After equilibration of the gel with buffer A the gel was stored at 4°C in 0.02 *M* sodium phosphate buffer (pH 7.2) containing 0.16 *M* sodium chloride and 0.02% thimerosal (thiomersal) as a preservative.

Protein determination

Protein concentrations were determined with a bicinchoninic acid protein reagent (Pierce, Rockford, IL, U.S.A.).

RESULTS

Different amounts of aprotinin from bovine lungs immobilized on Eupergit C indicated an almost proportional trypsin binding ability up to 15 mg of aprotinin per gram of gel (wet resin) (Fig. 1). Larger amounts of aprotinin (> 15 mg) linked to 1 g of Eupergit C resulted in an unproportionally small amount of trypsin bound to the adsorbent.

In further studies, 5 mg of aprotinin were immobilized per gram of Eupergit C (wet resin). Trypsin and α-chymotrypsin were purified from pancreatic extract in a single step. Both enzymes emerged in a single protein peak from the column (Fig. 2). SDS-PAGE analysis indicated two major proteins (trypsin and α-chymotrypsin) and some low-molecular-weight autolysis fragments of these enzymes (Fig. 3).

DISCUSSION

For affinity chromatography aprotinin was immobilized on different supports, *e.g.*, Sepharose, poly(vinyl alcohol) and porous glass as reported earlier[5–8]. This

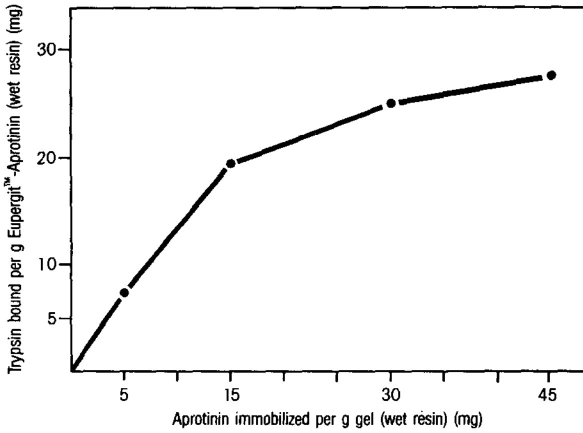

Fig. 1. Effect of different amounts of immobilized aprotinin on Eupergit to the trypsin-binding capacity of the gel.

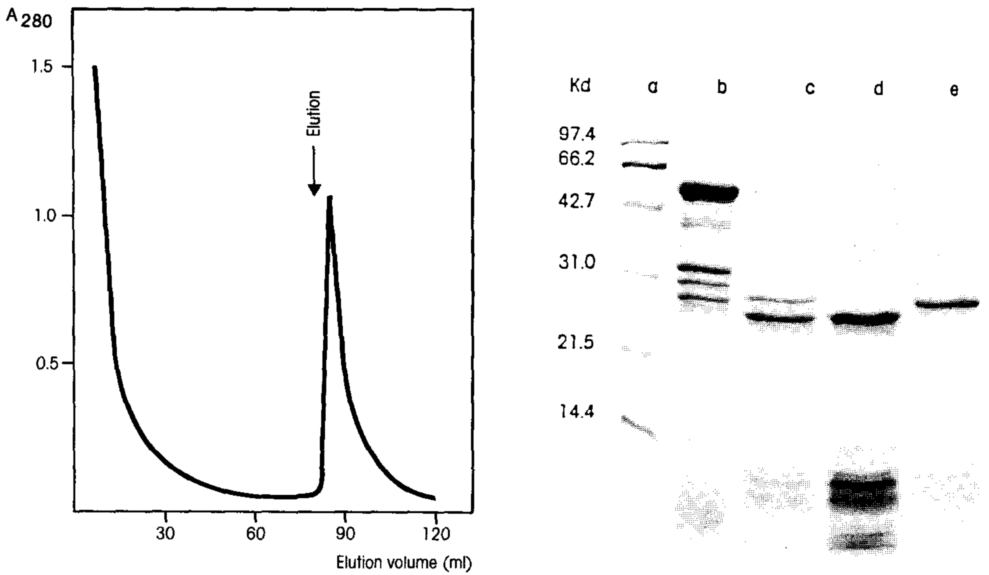

Fig. 2. Affinity chromatography of trypsin and α-chymotrypsin with Eupergit–aprotinin [5 mg of aprotinin per gram of gel (wet resin)]. Pancreatic extract (5 ml) was incubated with 1 g of gel (wet resin) and poured in a column (3 cm × 0.7 cm I.D.). Trypsin and α-chymotrypsin were eluted with 0.1 *M* hydrochloric acid (pH 1.0) containing 25 m*M* calcium chloride.

Fig. 3. SDS-PAGE analysis of Eupergit–aprotinin fractions. Lanes: (a) molecular weight standards; (b) porcine pancreatic extract; (c) trypsin and α-chymotrypsin of pancreatic extract eluted from the affinity adsorbent (Eupergit–aprotinin); (d) commercially available trypsin from Sigma eluted from the affinity adsorbent; (e) commercially available α-chymotrypsin from Sigma eluted from the affinity adsorbent. Gel concentration, 15%. Kd = kilodalton.

study was undertaken to investigate the purification of trypsin and α-chymotrypsin using Eupergit C–aprotinin, a novel carrier for affinity chromatography with different advantages resulting from its chemical (stable in all commonly used buffers between pH 1 and 9, no leakage of the ligand) and physical properties (high flow-rates of *ca.* 1000 $ml/cm^2 \cdot h$ at 0.5 bar).

This work showed that aprotinin covalently linked to Eupergit C is a highly selective affinity adsorbent. It has been used to purify trypsin and α-chymotrypsin from pancreatic extract in a single step. SDS-PAGE indicated that both enzymes were highly purified. The binding capacity of 1 ml of Eupergit–aprotinin [5 mg of aprotinin per gram of gel (wet resin)] for bovine trypsin was calculated to be *ca.* 7.5 mg.

The column has been used several times without any apparent decrease in capacity or selectivity. The stability of the gel is still under examination. Owing to its excellent chemical, mechanical and binding properties, Eupergit C–aprotinin is an ideal adsorbent for the purification of trypsin and α-chymotrypsin in both laboratory and industrial processes. It remains to be established whether aprotinin linked to Eupergit C could also be used for the purification of other serine proteases.

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