PROTEIN EXPRESSION AND PURIFICATION **12,** 291–294 (1998)
ARTICLE NO. PT970837

NOTE

Affinity Purification of Recombinant Trypsinogen Using Immobilized Ecotin

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Received October 1, 1997, and in revised form October 31, 1997

Affinity purification of inactive precursors (zymogens) of serine proteases on protease inhibitor columns is not feasible, due to the weak interaction between canonical protease inhibitors and protease zymogens. In this study we demonstrate that immobilized ecotin, a unique protease inhibitor from *Escherichia coli,* provides a superior affinity matrix for the purification of trypsinogen and possibly other serine protease zymogens as well. © 1998 Academic Press

Ecotin is a serine protease inhibitor found in the periplasmic space of *Escherichia coli.* The native protein consists of two identical 16-kDa subunits, and one dimer binds two protease molecules to form a tetrameric complex in a network-like arrangement (for a review see 1). Recently, an engineered mutant ecotin deficient in dimerization was shown to be fully active (2). Ecotin has an unusually broad specificity: it strongly inhibits proteases like trypsin, chymotrypsin, elastase, factor Xa, kallikrein, urokinase, and factor XIIa (1). We have observed that ecotin forms a relatively tight complex with trypsinogen, the zymogen form of trypsin, and this complex is stable during purification procedures at neutral pH. In an attempt to exploit this unusual property of this protease inhibitor for affinity chromatography, we immobilized ecotin and used it to purify recombinant trypsinogen expressed in *E. coli.*

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METHODS

Ecotin was overexpressed in *E. coli* BL21 as described by Pál *et al.* (2,3) and purified to homogeneity using a trypsin affinity column (4). Purified ecotin was immobilized by reductive amination using aldehyde activated agarose beads and sodium cyanoborohydride ($NaBH_3CN$). Five milligrams of lyophilized ecotin (dry weight, containing approximately 1 mg protein) was mixed with 3 mL ACTIGEL ALD resin (Sterogene Bioseparations) according to the manufacturer's instructions (pH 7.0; in the presence of 0.1 M $NaBH_3CN$), and the mixture was incubated for 3 h at 22°C and subsequently for 12 h at 4°C. Unreacted aldehyde groups were blocked with 0.1 M ethanolamine and 0.1 M $NaBH_3CN$ (final concentrations). The resin was washed extensively with 2.5 mM hydrochloric acid to remove excess reactants and packed into columns. Using ethanolamine as the blocking agent resulted in very little nonspecific binding during chromatography, which was completely eliminated by the inclusion of 0.2 M NaCl in the loaded sample and the column-washing buffer. In contrast, when ethylenediamine was used to block aldehyde groups, the affinity matrix exhibited relatively strong ion-exchange properties and nonspecific binding of proteins was higher. In a control experiment, no trypsinogen binding was observed to a column containing the aldehyde gel reacted with only 0.1 M $NaBH_3CN$ and 0.1 M ethanolamine in the absence of ecotin.

Rat anionic trypsinogen was expressed in *E. coli* SM138, as described (5). The amount of trypsinogen (referred to as "trypsinogen activity") was estimated using a microplate assay following conversion of the zymogen to trypsin by enterokinase digestion. Column fractions (10 μL) were mixed with 200 μL assay buffer

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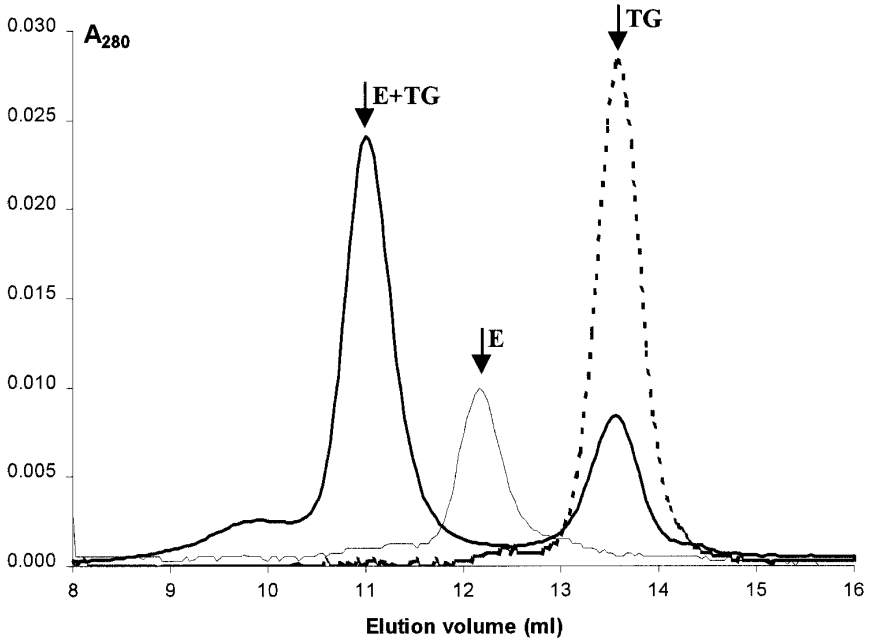

FIG. 1. Interaction between ecotin and trypsinogen. Ecotin (E, 8 μg protein), bovine trypsinogen (TG, 25 μg protein), or a mixture of the two proteins (E + TG, trypsinogen is in excess relative to ecotin) were loaded onto a Superose 12 column (Pharmacia) equilibrated with 50 mM Tris–HCl, pH 8.0, 0.2 M NaCl. Elution was carried out with the same buffer at a 0.5 mL/min rate.

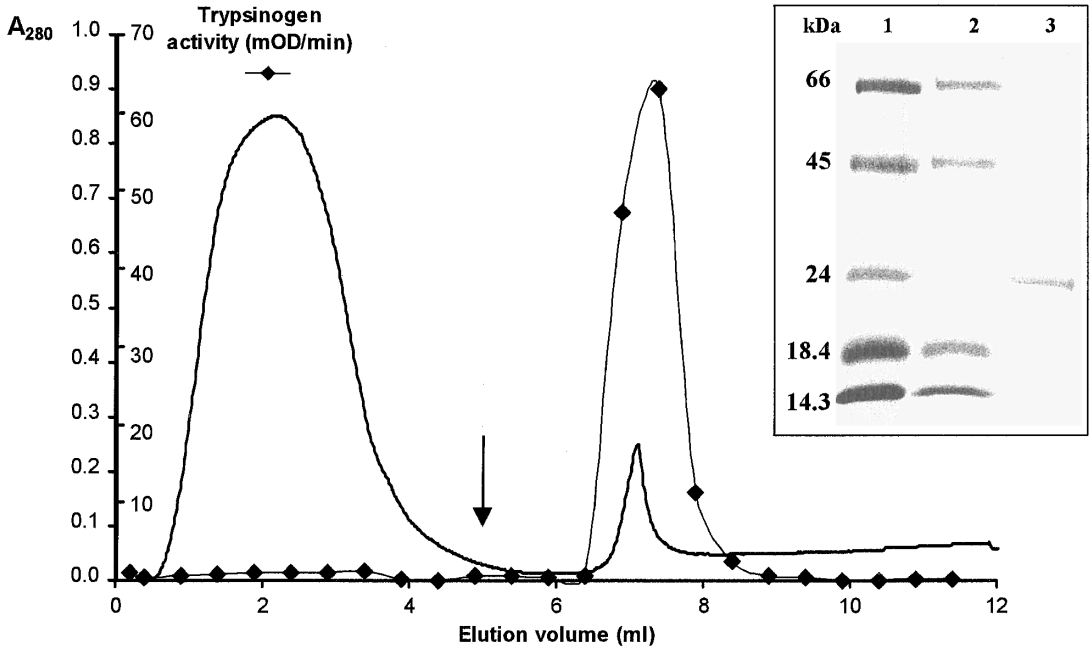

FIG. 2. Separation of trypsinogen on ecotin affinity column. Dalton Mark VI molecular mass markers (3.8 mg protein, Sigma) were loaded onto a 1-mL ecotin column equilibrated with 50 mM Tris–HCl pH 8.0, 0.2 M NaCl. The column was washed with the same buffer and bound trypsinogen was eluted with 50 mM hydrochloric acid (arrow). Trypsinogen activity in column fractions was assayed after enterokinase activation as described under Methods. (Inset) SDS–polyacrylamide gel (4–20% gradient) electrophoresis of marker mix (38 μg protein, lane 1), flowthrough (22 μg protein, lane 2), and eluted trypsinogen (2 μg protein, lane 3). Proteins in marker mix: 66 kDa, bovine serum albumin; 45 kDa, ovalbumin; 24 kDa, bovine trypsinogen; 18.4 kDa, β-lactoglobulin; 14.3 kDa, lysozyme. [The mix also contains pepsin (34.7 kDa) which anomalously comigrates with ovalbumin in this gel system.]

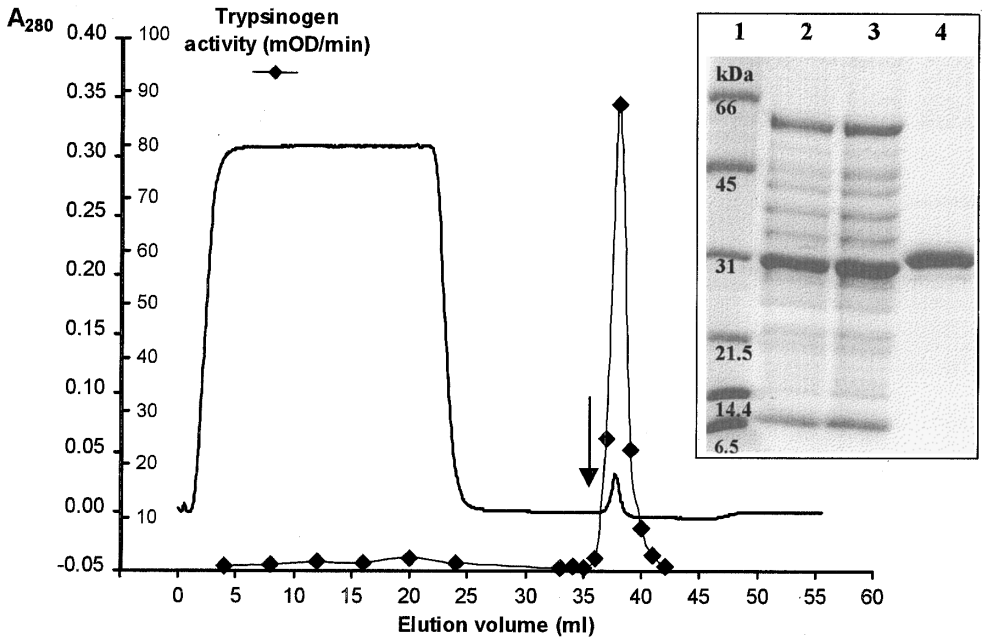

FIG. 3. Purification of recombinant trypsinogen on ecotin affinity column. An *E. coli* SM138 culture expressing wild-type rat anionic trypsinogen was grown to saturation at 37°C and periplasmic fraction was prepared by osmotic shock. Salt concentration and pH was adjusted by adding 50 mM Tris–HCl, pH 8.0, and 0.2 M NaCl (final concentrations), and 20 mL periplasmic fraction (2 mg total protein) was loaded onto a 1-mL ecotin column equilibrated with 50 mM Tris–HCl, pH 8.0, 0.2 M NaCl. The column was washed with the same buffer, and pure trypsinogen was eluted with 50 mM hydrochloric acid (arrow). (Inset) SDS–polyacrylamide gel (4–20% gradient) electrophoresis of molecular mass markers (lane 1), periplasmic fraction (5 μg protein, lane 2), flowthrough (6 μg protein, lane 3), and eluted trypsinogen (5 μg protein, lane 4). Note that in this gel system rat trypsinogen exhibits a larger relative molecular mass (ca. 31 kDa) than predicted (25 kDa). Periplasmic fractions contain low amounts of trypsinogen (3–5 μg/mL), and the corresponding band is not visible in the total extract. The faint band migrating in front of trypsinogen is trypsin generated by self-activation of the zymogen during sample storage. Molecular mass standards (Bio-Rad): 66 kDa, serum albumin; 45 kDa, ovalbumin; 31 kDa, carbonic anhydrase; 21.5 kDa, trypsin inhibitor; 14.4 kDa, lysozyme; 6.5 kDa, aprotinin.

(100 mM Tris–HCl, pH 8.0, 1 mM $CaCl_2$), 28 ng enterokinase (Biozyme), and 200 μM (final concentration) synthetic chromogenic substrate (*N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide, Sigma). Kinetics of release of the chromophor was followed at 405 nm in a SpectraMax 250 plate reader (Molecular Devices). Activity was expressed as rate of substrate cleavage in arbitrary units (milliOD per min). Protein was determined with the Cuprous^{1+} protein assay (Geno Technology) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Using conventional ion-exchange methods for purification of recombinant trypsinogen from *E. coli* we observed that ecotin was copurified with one of the trypsinogen fractions, indicating that the zymogen might bind to the protease inhibitor (data not shown). To confirm the complex formation, we ran samples of ecotin, trypsinogen, and the mixture of the two proteins on a Superose 12 (Pharmacia) analytical gel-filtration column (Fig. 1). When traces of elution profiles are overlaid, the peak representing the tetrameric complex (80 kDa) is clearly separated from the ecotin (32 kDa, dimer) and trypsinogen (24 kDa) peaks.

To utilize this interaction for affinity purification of trypsinogen, we immobilized ecotin by reductive amination as described under Methods. Feasibility of trypsinogen purification using the new affinity matrix was first demonstrated by loading a commercial molecular mass marker mix (Dalton Mark VI, Sigma) containing bovine trypsinogen onto an ecotin column. With the exception of trypsinogen, all proteins were recovered in the flowthrough, and trypsinogen was eluted from the column under acidic conditions (Fig. 2). As shown in Fig. 3, the ecotin column proved to be an excellent tool for the one-step purification of recombinant rat trypsinogen from *E. coli*. Crude periplasmic fraction from *E. coli* SM138 expressing wild-type trypsinogen was loaded onto a 1-mL ecotin column equilibrated with 50 mM Tris–HCl, pH 8.0, 0.2 M NaCl, and the column was extensively washed with the same buffer. Highly pure zy-

mogen was eluted with 50 mM hydrochloric acid. Although data are not shown, we found that the column is capable of binding chymotrypsinogen, trypsin, chymotrypsin, and elastase, too, and these proteins can be eluted with acidic solutions. We have routinely used ecotin columns for purification of recombinant trypsinogen mutants expressed in bacteria or yeast. Combined with conventional ion-exchange techniques, the column proved to be useful for isolation of zymogens and proteases from the pancreas, too. Similarly, isolation of certain coagulation and fibrinolytic factors from blood serum (1) should be feasible.

In summary, immobilized ecotin was used to purify recombinant trypsinogen expressed in *E. coli*. This is the first published affinity chromatography method suitable for purification of a pancreatic zymogen, and it may be generally applicable for the purification of other proteases as well as their zymogens.

ACKNOWLEDGMENTS

This work was supported by Grant 70NANB5H1111 from the Advanced Technology Program (United States Department of Commerce, National Institute of Standards and Technology) and California Defense Conversion Matching Grant C96-0044 from the California Trade and Commerce Agency.

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