PURIFICATION OF TRYPSIN BY AFFINITY CHROMATOGRAPHY ON OVOMUCOID-SEPHAROSE RESIN

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One of the major problems in enzymology is the difficulty in purifying enzymes. The purification is usually done through multiple steps which takes a long time and results in low yields. The usefulness of affinity chromatography in overcoming these difficulties was demonstrated by Fritz et al. [1]. They cross-linked trypsin, α-chymotrypsin and kallikrein to copolymer of ethylenemaleic acid (EMA), an insoluble carrier introduced by Levin et al. [2], and used the insoluble enzymes to isolate the corresponding natural inhibitors of these proteases. In later study, Fritz et al. [3] fractionated kallikrein and plasmin by chromatography on resin of kallikrein inhibitor cross-linked to EMA. Cuatreccasas et al. [4] cross-linked synthetic inhibitors of several enzymes to the uncharged insoluble carrier Sepharose according to the method of Axén et al. [5] and used the synthetic inhibitor-Sepharose resins to purify several enzymes by single step of affinity chromatography.

Chicken ovomucoid is an egg white protein which is capable of inhibiting bovine trypsin [6]. In this study the method of affinity chromatography was adapted to purification of trypsin by use of chicken ovomucoid-Sepharose resin.

Eighty-five ml of Sepharose 2B (Pharmacia) were activated with CNBr according to Cuatrecases et al. [4] and were coupled to 200 mg chicken ovomucoid (Worthington Biochemical Corp.). The coupling was done by stirring for 24 hr at room temperature the activated Sepharose and the ovomucoid in 250 ml of 0.10 M $NaHCO_3$, pH 8.5. After coupling, the ovomucoid-Sepharose was washed on sintered glass funnel with the following: six times each with 250 ml of 0.10 M $NaHCO_3$, pH 8.5; H_2O; 0.20 M KCl-HCl, pH 2.0; H_2O and with 0.10 M triethanolamine (TEA) buffer, pH 8.1, containing 0.02 M $CaCl_2$. The ovomucoid-Sepharose was then suspended in TEA buffer and packed in a column 35 × 1.7 cm. The column was washed in the cold with 500 ml of TEA buffer. A likewise treatment of washing was done to untreated Sepharose 2B and a column was packed. Twenty mg of crystalline bovine trypsin (Worthington Biochemical Corp.) were dissolved in 2.0 ml of TEA buffer and applied to the top of the column. The elution was started with TEA buffer and followed with 0.20 M KCl-HCl, pH 2.0. Fractions of 2.8 ml were collected. Protein concentration was determined by absorbancy at 280 nm. The activity of trypsin was assayed by the method of Erlanger et al. [7]. The substrate was *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPA, Mann Research Laboratories) and its rate of hydrolysis was followed by the aborbancy increase at 410 nm. Trypsin was chromatographed also on Sepharose 2B column and α-chymotrypsin was chromatographed on the ovomucoid-Sepharose column.

The results are shown in fig. 1. When trypsin was chromatographed on Sepharose 2B (fig. 1A) a single peak of protein emerged from the column containing the enzymatic activity. No more protein or enzymatic activity were eluted with the second step of elution with KCl. As trypsin was chromatographed on ovomucoid-Sepharose resin (fig. 1B), a protein peak emerged at the same volume as in the previous case with the Sepharose column. The protein did not have any detectable tryptic activity. The second step of elution on ovomucoid-Sepharose resulted in eluting a second peak of protein which contained all the tryptic activity. The results with α-chymotrypsin

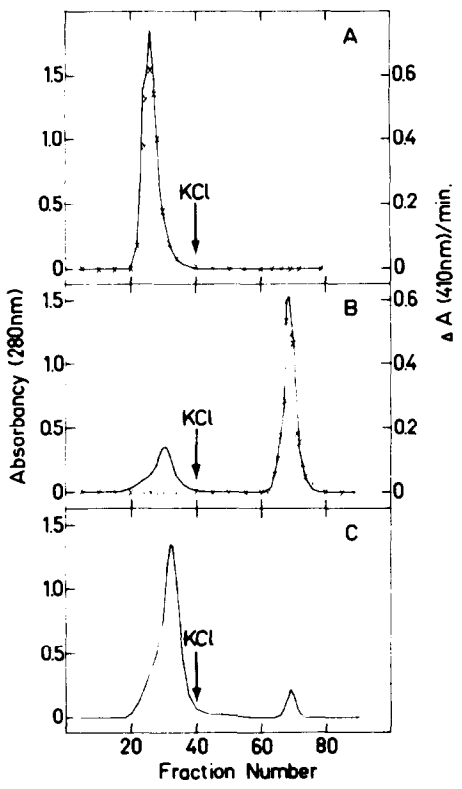

Fig. 1. Column chromatography of trypsin and α-chymotrypsin on Sepharose and ovomucoid-Sepharose. First step of elution with 0.10 M triethanolamine buffer, pH 8.1, containing 0.02 M $CaCl_2$. Second step of elution with 0.20 M KCl-HCl, pH 2.0. Solid line – absorbancy at 280 nm; XXX – hydrolysis of BAPA. (A). Trypsin chromatography on Sepharose. (B). Trypsin chromatography of ovomucoid-Sepharose. (C). α-Chymotrypsin chromatography on ovomucoid-Sepharose.

are shown in fig. 1C. A first large peak was eluted with TEA buffer and a minor one with KCl.

Chicken ovomucoid is an inhibitor of bovine trypsin but it does not inhibit bovine α-chymotrypsin [6]. It binds trypsin in a 1 : 1 molar ratio to give a very stable complex which dissociates only when the pH is lowered from neutral to acidic pH. By cross-linking it to Sepharose we obtained an ovomucoid-Sepharose resin which exhibits the same specificity as soluble ovomucoid. It binds trypsin but it does not bind α-chymotrypsin. The protein devoid of tryptic activity which was eluted with TEA buffer could be an inactive protein which contaminated the trypsin preparation or it could also contain α-chymotrypsin which is present in commercial trypsin preparations [8]. The specific activity of trypsin purified by affinity chromatography on ovomucoid-Sepharose is about 10% higher than the specific activity of the starting crystalline trypsin from commercial source. The choice of Sepharose as an insoluble carrier of protein inhibitors of proteases appears to be prefered to charged insoluble carriers like EMA. Since Sepharose is devoid of charged groups it does not undergo ion-exchange and therefore the absorption of enzymes is due only to the cross-linked corresponding inhibitor. The ovomucoid-Sepharose was reused for several experiments after regeneration by passing TEA buffer through the column. This method appears to be very useful for purifying trypsin of commercial sources, to free it from contaminations of other proteins. Whereas the method of Kostka and Carpenter [8] results in inactivating the chymotrypsin activity in trypsin preparation, it also inactivates slightly trypsin activity and also the preparation still contains contaminants.

The isolation of proteases is usually long and complicated process. The multiplicity of different steps in addition to autolysis results in low yields. In the case of pancreatic proteases like trypsin, chymotrypsin and carboxypeptidase, many times the most pure commercial preparation of one protease contains contaminations by other proteases and inert proteins. According to Bender et al. [9] commercial trypsin preparations contain 49–60% of active trypsin. The protein inhibitors of protease exhibit highly selected specific inhibitory activity against proteases [10]. They form a very stable complex with the corresponding proteases at neutral pH but dissociate at low pH values. These properties of protein inhibitors combined with the ease of coupling proteins to Sepharose suggest their use for isolation and purification of proteases. This method could also be used the other way around, namely to isolate protein inhibitors by affinity chromatography on proteases-Sepharose resins.

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