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HIGH PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHIC RESOLUTION OF TRYPSINS ON SOYBEAN TRYPSIN INHIBITOR BONDED PHASE

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ABSTRACT

An affinity phase was synthesized by immobilizing soybean trypsin inhibitor on epoxysilanized Partisil-10 by direct chemical reaction and resolution of various pancreatic trypsins was studied.

INTRODUCTION

Previously, Kaul et al. (2) immobilized soybean trypsin inhibitor on Octyl-Sepharose phase by hydrophobic interaction to purify trypsin. Kasche et al. (3) studied high performance liquid affinity chromatographic resolution of proteases such as trypsins on soybean trypsin inhibitor - immobilized LiChrospher support. Soybean trypsin was immobilized

by glutardialdehyde method (5). This paper reports the use of epoxy silanized Partisil to immobilize soybean inhibitor by direct chemical reaction to achieve similar chromatographic performances.

EXPERIMENTAL

Materials

Bovine trypsin, α -chymotrypsin and α -chymotrypsinogen were purchased from ICN Biochemicals, Inc., (Cleveland, OH). Soybean trypsin inhibitor was obtained from Behring Diagnostics (La Jolla, CA). Potassium dihydrogenphosphate, calcium chloride, p-toluenesulfonyl-L-arginine methyl esters (TAME) and analytical grade hydrochloric acid were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI).

Packing Material

Epoxy Partisil-10 phase obtained from Whatman Inc. (Clifton NJ).

Sample Preparation

Solutions of trypsin, α -trypsinogen and α -trypsin were prepared by dissolving 20 mg of each in 5 ml of 0.01M KH_2PO_4 buffer.

Synthesis of Affinity Phase

Soybean trypsin was immobilized by reacting 2g of soybean trypsin inhibitor with 12 g of epoxy Partisil-10 in 0.1M KH_2PO_4 solution at 35°C. The column was packed by slurring the affinity phase in methanol and applying a pressure of 5000 psi. The unreacted epoxy groups were deactivated with glycerol (4).

Assay of Soybean Trypsin Inhibitor Activity

Soybean trypsin inhibitor bound affinity phase was assayed with p-toluenesulfonyl-L-arginine methyl ester (TAME) as the substrate according to the method described by Hummel (1). Spectrometer, Spectronic 200

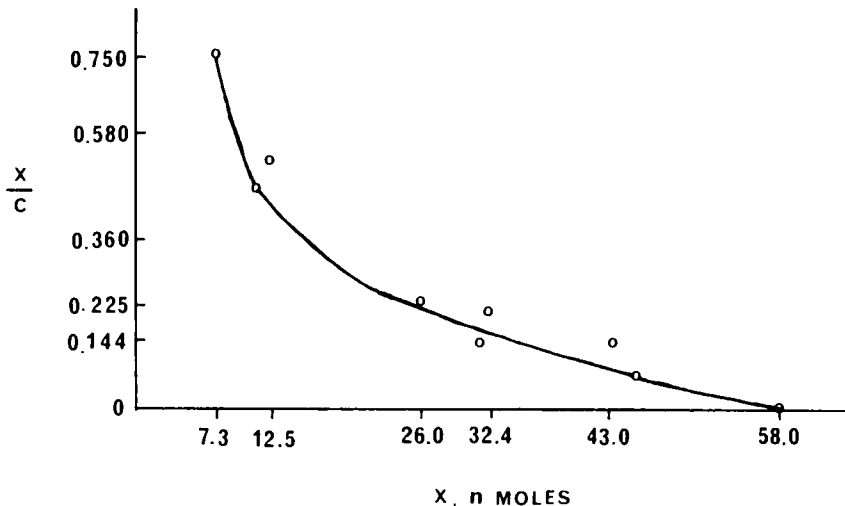


Fig. 1. Scatchard plot for the interaction between soybean trypsin inhibitor-immobilized Epoxy-Partisil-10 and chymotrypsinogen. Particles (36mg Epoxy-Partisil-10 containing 60 n moles soybean trypsin inhibitor) were incubated with various amounts of free chymotrypsinogen for 60 minutes at pH 8 (tris-HCl, I = 1.05, NaCl) at 25°C. The particles were kept suspended by agitation. After equilibration the bound amount of enzyme, X, was determined from the added free enzyme content, C, in the filtrate of suspension. The arrow gives the amount of trypsin bound in the particles.

(Bauch & Lomb, Rochester, NY) was used to measure increase in absorbance at 247 m μ .

HPLC Analysis

HPLC was performed using a variable wavelength UV detector, Spectroflow monitor SF-770 (Kratos Analytical, Ramsey, NJ); a programmable solvent delivery system, Series 3B (Perkin-Elmer Corp., Norwalk, Conn.); a manual injection valve, with 50 μ l loop (Valco Instruments Co., Houston, TX) and a chart recorder (Laboratory Data Control, Riviera Beach, FL).

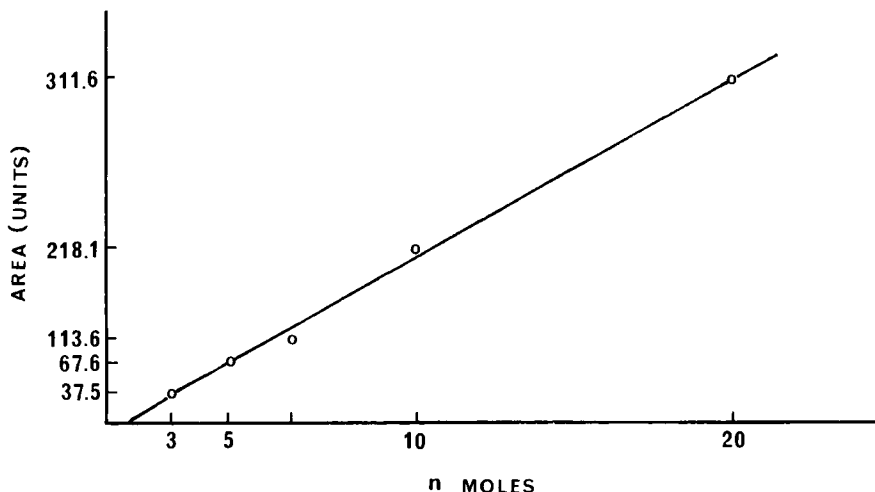


Fig. 2. Plot of n moles of chymotrypsin vs area. This graph was used to determine free enzyme in Figure 1.

The column was run at constant pH 4.5 using 0.05M KH_2PO_4 solution as a mobile phase.

RESULTS AND DISCUSSION

Stationary Binding Properties of Soybean Inhibitor-Partisil-10

In Figure 1, a Scathard plot is given for binding of α -chymotrypsinogen to soybean trypsin inhibitor-Partisil-10. The curve indicates that non-specific adsorption is negligible. The adsorption sites are, however, not homogeneous as the curve is nonlinear. All bound trypsin inhibitor molecules retain the properties to bind protease. The apparent association constant, $K_{\text{app}} = 2 \times 10^6 \text{M}^{-1}$, determined from the intercepts is similar to the value $3 \times 10^6 \text{M}^{-1}$ as reported by Kasche et al.(3) for the binding α -chymotrypsin to soybean trypsin inhibitor-LiChrospher.

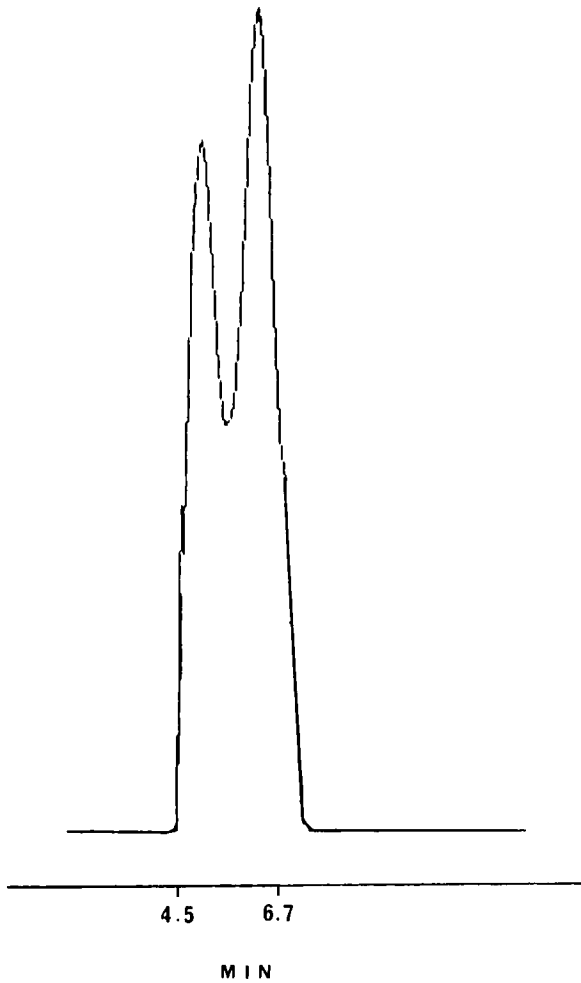


Fig. 3. Separation of α - and β -trypsin. Column: 6 cm x 4.6 mm (I.D.) packed with epoxysilanized Partisil-10 (pore size 300 A, particle diameter 10 μ m) in which 40 mg/g soybean trypsin inhibitor was immobilized by direct chemical reaction. Elution buffer: 0.05 M KH_2PO_4 , pH 4.5; Flow rate: Sample size: 15 μ l mixture containing 75 μ g of α - and β -chymotrypsins.

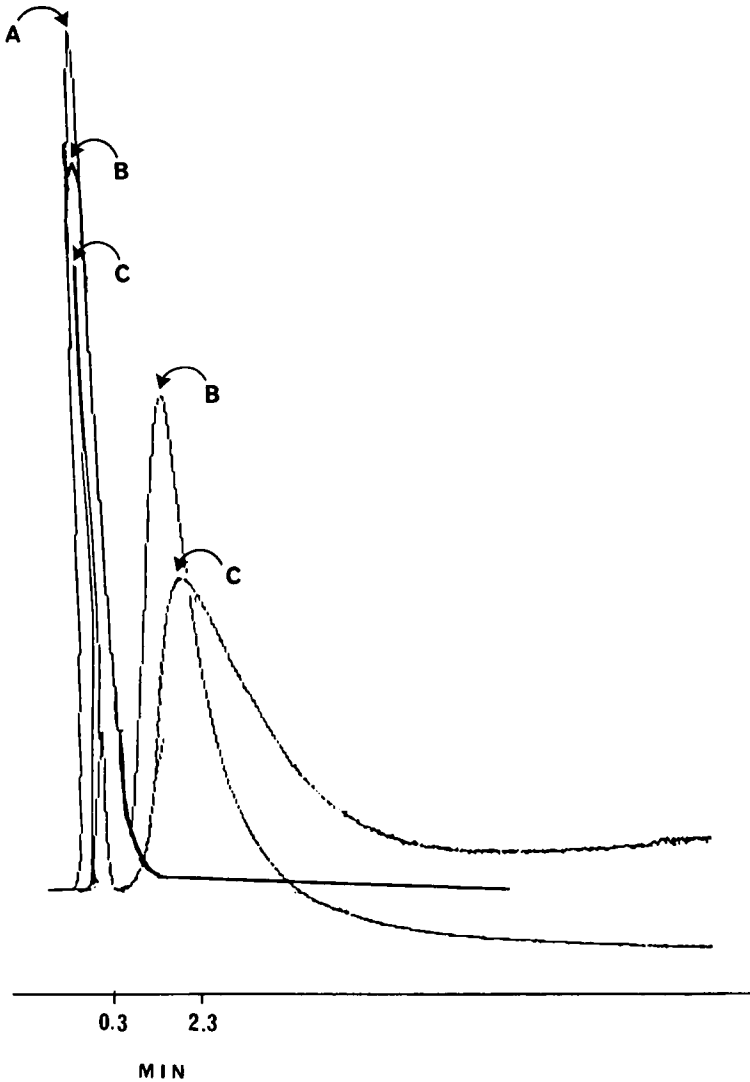


Fig. 4. Separation of α -chymotrypsin and chymotrypsinogens at constant pH 4.5. Column: Same as described in Figure 3. Flow rate: A, chymotrypsinogen, 2 ml/min; B, α -chymotrypsin, 2 ml/min; C, α -chymotrypsin, 1 ml/min. Sample size: 10 μ l with 50 μ g of protein.

This indicates that the immobilization does not markedly change the specificity of the ligand, and that all bound ligands retain their biospecific function. The association constants for the most and least specific adsorbent sites differed by a factor 10, as estimated from limiting slope.

Figure 2 represents a graph of concentration vs area of α -chymotrypsinogen. The curve is linear. The equation 1 was derived to determine the amounts of trypsinogen in various injections (Figure 1).

$$\text{Amount} = 3.554 \times 10^{-2} \times \text{area} + 1.0219 \quad (\text{Eq. 1})$$

HPL-Affinity Chromatography

Isocratic resolution of α - and β -trypsin is exhibited in the Figure 3. Kasche et al. (3) reported similar separation by using a pH gradient on trypsin-immobilized-aminosilanized LiChrospher column. Figure 4 represents isocratic resolution of α -chymotrypsin from chymotrypsinogen which is similar to as earlier reported by Kasche et al. (3) on trypsin-inhibitor-immobilized aminosilanized column. Similar peak broadening of α -chymotrypsin at lower flow rate was observed by these workers. The peak broadening at lower flow rate may be due to longer time of passage of the mobile phase through the column to allow more diffusion of all eluate into the stationary phase.

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