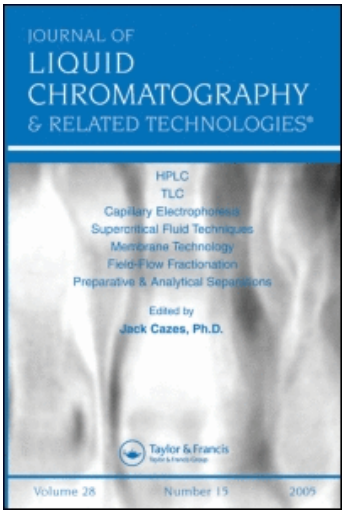

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Isolation and Determination of Soy Bean Trypsin Inhibitor by Using HPL-Affinity Chromatography

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ISOLATION AND DETERMINATION OF SOY BEAN TRYPSIN INHIBITOR BY USING HPL-AFFINITY CHROMATOGRAPHY

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ABSTRACT

Trypsin was immobilized on silica support. The bonded phase thus obtained was used to analyze trypsin inhibitors from soybeans. Deactivation study of soybean trypsin inhibitor was conducted after heating at 75°C as such and also in the presence of N-acetyl cysteine and sodium sulfite.

INTRODUCTION

Trypsin inhibitors constitute more than 6% of the proteins of soybeans (7). Antinutritional and toxicological effects of these inhibitors are well known (6). The protease inhibitory activity of soy protein is largely inactivated by application of heat to soy flour (1,3). Enzyme linked immunosorbent assays or enzyme immunoassays (ELISA or EIA) are generally used to analyze protease inhibitors (2). Trypsin has been

immobilized on the epoxysilanized Partisil-10 and used to analyze soybean trypsin-inhibitors by HPL-affinity chromatography during the present investigation.

EXPERIMENTAL

Materials

Bovine trypsin was purchased from ICN Biochemicals Inc. (Cleveland, OH). Soybean trypsin inhibitor was obtained from Behring Diagnostics (La Jolla, CA). Potassium dihydrogen phosphate, calcium chloride, p-toluene sulfonyl-L-arginine methyl ester (TAME) and analytical grade hydrochloric acid were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Epoxy Partisil-10 packing material was obtained from Whatman Inc. (Clifton, NJ).

Sample Preparations

Solutions of trypsin inhibitor was prepared by dissolving 50mg of trypsin in 2ml of water. The extract of soy seeds was prepared by crushing the seeds to flour, suspending 100mg of flour in 5ml tris-HCl buffer, pH 8.5 or in deionized water, and stirring for one hour at room temperature. The suspension was then centrifuged at 4500Xg for ten minutes and stored as aliquots at -20°C. Soybeans for extract preparation were purchased from the local market.

Preparation of Affinity Material

Trypsin was immobilized by reacting 0.9g of trypsin with 13g of epoxy Partisil-10 in potassium dihydrogen phosphate buffered solution (pH 5). The column was packed by slurrying packing material in methanol and applying a pressure of 5000psi (5).

Assay for Trypsin Activity

Trypsin-bound affinity phase was assayed with p-toluene solfonyl-L-arginine methyl ester (TAME) as a substrate according to the method described by Hummel (3).

A spectrometer, Spectronic 200 (Bausch & Lomb, Rochester, NY), was used to measure increase in absorbance at 247m u.

HPLC Analysis

HPLC was performed using a chromatogram consisting of a variable wavelength UV detector, a spectroflow monitor SF-770 (Kratos Analytical, Ramsey, NJ), a programmable solvent delivery system, Series 3B (Perkin-Elmer Corp., Norwalk, CT), a manual injection valve 50 ul loop (Valco Instrument Co., Houston, TX) and a chart recorder (Laboratory Data Control, Riviera Beach, FL). The column was run at constant pH (4.5) using 0.05M KH_2PO_4 solution in water as a mobile phase.

RESULTS AND DISCUSSION

Stationary Binding Properties of Trypsin Partisil-10 Phase

In Figure 1 a Scathard plot is given for binding of trypsin inhibitor to trypsin Partisil-10 phase. The curve indicates that non-specific adsorption is negligible. The adsorbent sites are, however, not homogeneous as the curve is non-linear. All bound trypsin molecules retain the properties to bind protease. The average association constant, K $2x20^6M^{-1}$ determined from the intercepts, is similar to value $3x10^6M^{-1}$ as reported by Kasche *et al.* (4) for binding of chymotrypsin to soybean trypsin - LiChropher. This indicates that the immobilization does not markedly change the specificity of ligand, and that all bound ligand retain their biospecific function. The association constant for the most and least specific adsorbent sites differed by about a factor 10 as estimated from limiting slope.

Figure 1 indicates that about 2.8nM of trypsin was bound to 1mg of the solid support. The enzyme assay run showed that the bonded phase had 72 units/mg of activity.

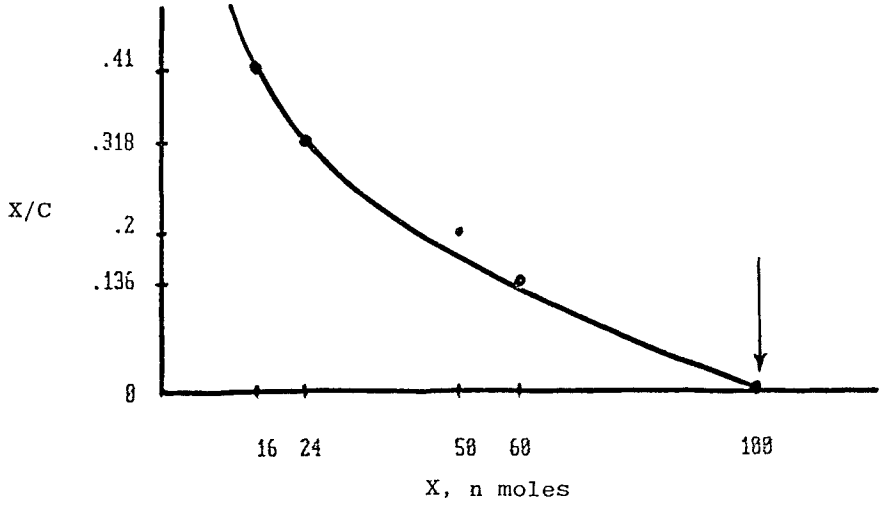

Fig. 1 Scathard plot for the interaction between Trypsin-Partisil-10 phase and soybean trypsin inhibitor. Particles (10mg Trypsin-Partisil-10 containing 100n moles of trypsin) were incubated with various amounts of free soybean trypsin inhibitor for 60 minutes at pH 8 (Tris-HCl, I=0.05.0,2M NaCl) and 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 the suspension. The arrow gives the amount of trypsin bound in the particles.

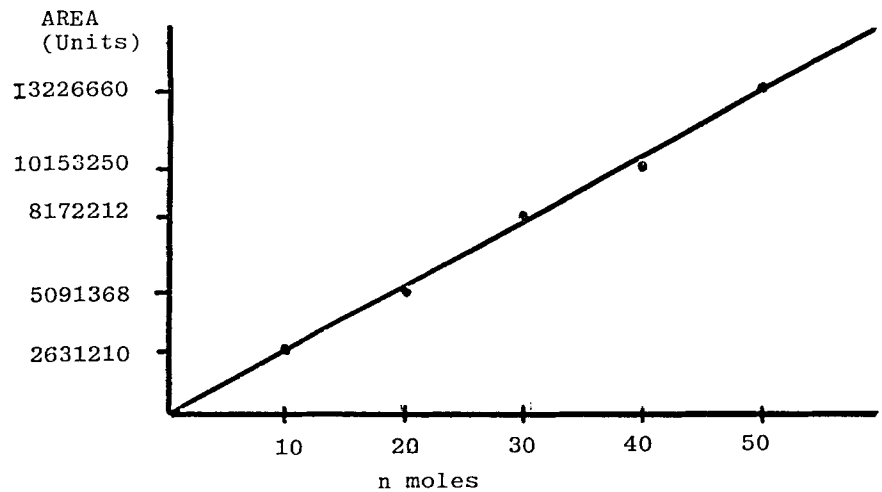

Fig. 2 Area vs. concentration calibration curve of the soybean trypsin from commercial source. Areas at various concentration levels were determined chromatographically by using the conditions given in Fig. 4.

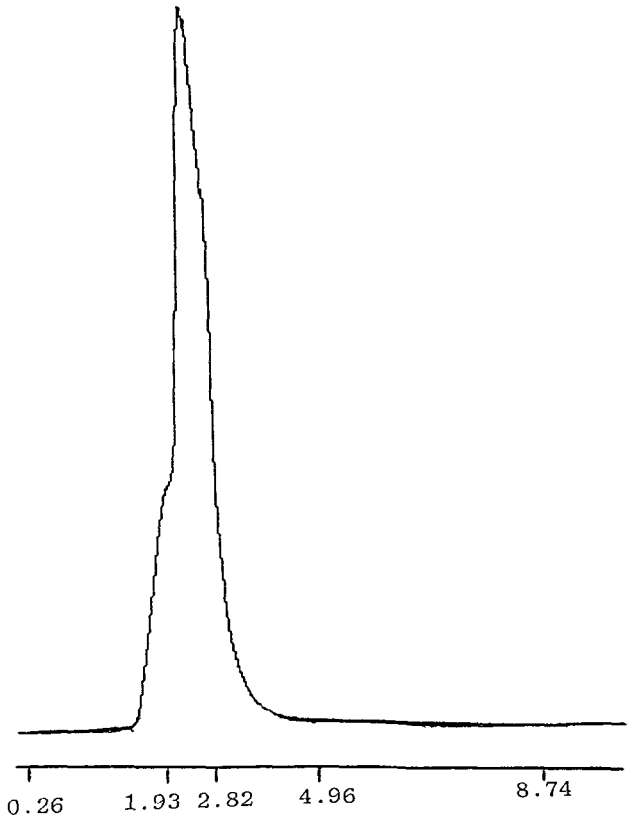

Fig. 3 and 4 Column: 5cmx4.6mm packed with trypsin-silanized Partisil-10 (pore size 300°A, particle diameter 10 um) in which 75mg/g trypsin was immobilized. Elution buffer: 0.05M KH_2PO_4 (pH 4.5). Flow rate: 0.35ml/minute. Figures 3 and 4 represent the elution of trypsin inhibitors from soybean extract and commercial source respectively. Sample size: 15 ul

HPL-Affinity Chromatography

Figure 2 represents the area vs. concentration calibration curve of a commercial trypsin inhibitor which was used to study the binding of trypsin inhibitor to trypsin-bound affinity phase (Figure 1). The amount of free trypsin inhibitor can be determined by using regression equation 1.

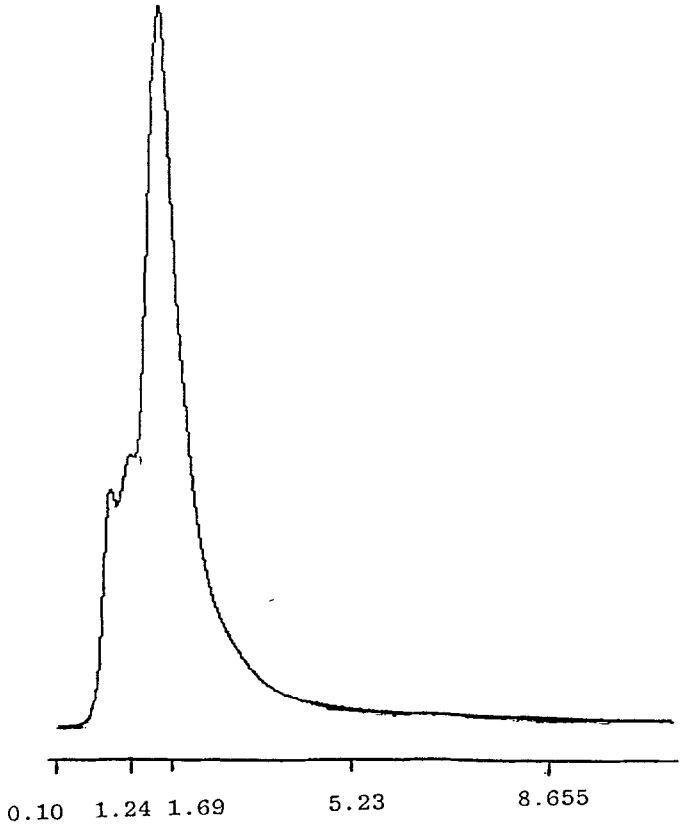

Fig. 4

$$\text{Amount} = 3.79\times10^{-6}\times\text{Area}+2.101\times10^{-1} \quad \text{(Equation 1)}$$

Figures 3 and 4 exhibit the elution of trypsin inhibitors from soybean extract and commercial source respectively on a 5cm trypsin-Partisil-10 column. Two major classes of protease inhibitors have been reported (1). The resolution of inhibitors was achieved on 12cm trypsin-Partisil-10 columns (Figures 5 and 6).

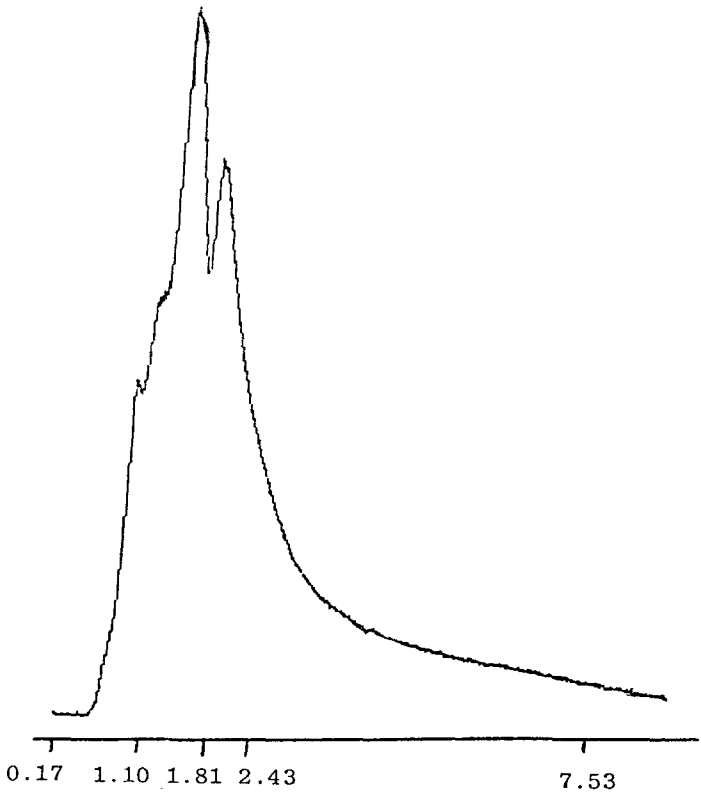

Fig. 5 and 6 Column: 12cmx4.6mm packed with trypsin-silanized Partisil-10 (pore size 300°A, particle diameter 10 um) in which 75mg/g trypsin was immobilized. Elution buffer:0.05M KH_2PO_4 (pH 4.5). Flow rate: 1.25ml/minute. Figures 5 and 6 represent the elution of trypsin inhibitors from soybean extract and commercial source. Sample size: 15 ul

Figure 7 represents the area vs. concentration calibration curve of trypsin inhibitor from soybean extract. The areas at various concentrations for this purpose were determined chromatographically by using the conditions given in the Figure 3. This graph was used to study deactivation of trypsin inhibitor under various conditions (Table1). The regression equation 2 was used for this purpose.

$$\text{Amount} = 1.8177\times10^{-4}\times\text{Area}+4\times10^{-1} \quad \text{(Equation 2)}$$

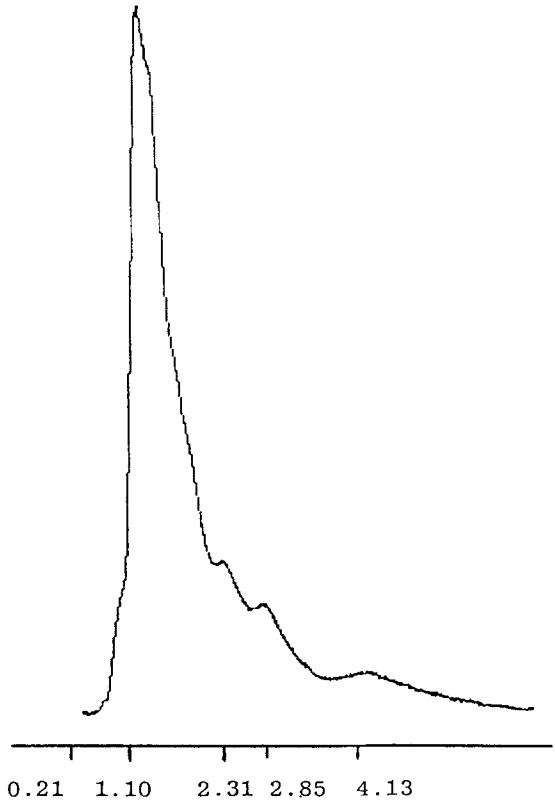

Fig. 6

Table 1 shows the results of analysis of trypsin inhibitor deactivation at 75°C under different treatments (1). The variation in deactivation of trypsin inhibitors in both the samples after heating at 75°C as such, and in presence of sodium sulfite in particular, may be due to the presence of two kinds of trypsin inhibitors in different amounts (Figures 5 and 6). In short, this trypsin immobilized silanized phase offers a great opportunity to analyze trypsin inhibitors in soy-product samples and also in other food materials during food processing in a very short time.

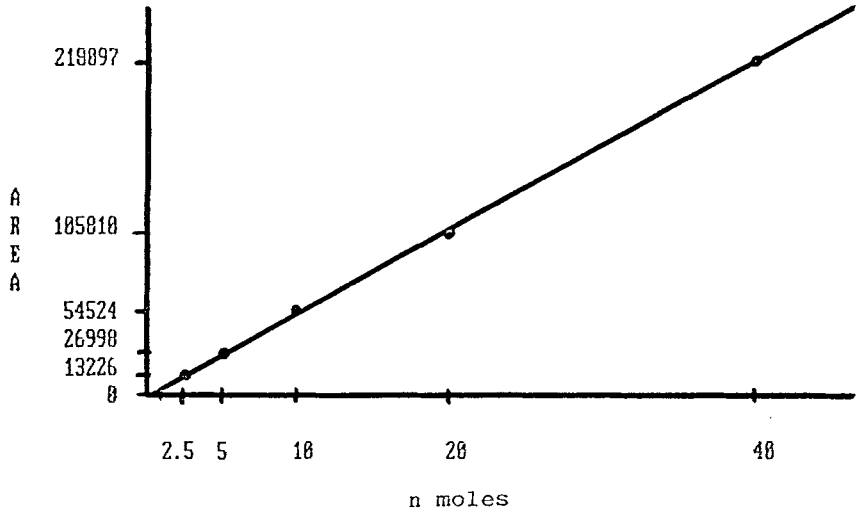

Fig. 7 Area vs. concentration calibration curve of soybean trypsin inhibitor from soybean extract. Areas at different concentration levels were determined chromatographically by using conditions given in Fig. 3. The inhibitor was precipitated from extract by using ammonium sulfate.

TABLE 1

Analysis of Trypsin Inhibitor Deactivation

Commercial Soybean Inhibitor

Treatment (at 75°C)	% of residual inhibitor	% deactivated
None	37.6	62.4
N-acetylcysteine	0.6	99.4
Sodium sulfite	2.6	97.4

Trypsin Inhibitor from Soybean Extract

Treatment (at 75°C)	% of residual inhibitor	% deactivated
None	53.5	46.5
N-acetylcysteine	0.3	99.97
Sodium sulfite	0.11	99.89

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