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Isolation of Trypsins by Affinity Chromatography*

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ABSTRACT: Bovine α- and β-trypsin were separated by affinity chromatography on a column of chicken ovomucoid covalently bound to Sepharose. The two active fractions were selectively eluted by a pH gradient. The procedure was also applied to the purification of porcine and dogfish trypsins and to the isolation of trypsin from activated bovine pancreatic juice. Neither trypsinogen nor α-chymotrypsin was retarded by this column.

Affinity chromatography has recently been introduced as a method of enzyme purification (Cuatrecasas *et al.*, 1968). The method depends on the affinity of an enzyme toward specific substrate analogs or inhibitors covalently coupled to an insoluble matrix. In contrast to other chromatographic techniques which separate proteins on the basis of their molecular size, charge distribution, or both, affinity chromatography separates enzymes on the basis of their specificity and can be directly applied to the isolation of analogous enzymes from different species. The present investigation was undertaken to design a procedure for the large-scale purification of trypsins by affinity chromatography. After an examination of several protein inhibitors, chicken ovomucoid (CHOM)[1] covalently bound to Sepharose was selected because this protein inhibitor is highly specific in binding trypsin and can be easily prepared in large quantities. The method was successfully applied to the isolation of bovine trypsin, porcine trypsin, and trypsin from pancreatic extracts of the spiny Pacific dogfish. During the course of this investigation Feinstein (1970) reported the purification of bovine trypsin on a similar ovomucoid–Sepharose column. The present method extends the usefulness of this approach by demonstrating its applicability to other species of trypsin. Furthermore the method described herein is also capable of separating α- and β-trypsin, the two predominant species found in conventional preparations of bovine trypsin (Schroeder and Shaw, 1968).

Experimental Section

Materials. The whites of fresh chicken eggs were obtained from Gaffney Suppliers, Inc., Puyallup, Washington. Crystalline bovine trypsin, trypsinogen, and α-chymotrypsin were obtained from Worthington Biochemical Corp. Bovine α- and β-trypsin were prepared according to the method of Schroeder and Shaw (1968). Porcine trypsin was a product of Novo Industri, Copenhagen. Dogfish trypsinogen and trypsin were prepared as described by Tye (1971). Bovine pancreatic juice was collected as described by Keller *et al.* (1958) and was found to have undergone spontaneous activation. After adjustment to pH 3 with HCl, the insoluble material was removed by centrifugation and discarded. SE-Sephadex C-50, DEAE-Sephadex A-50, and Sepharose 4B were all purchased

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[1] The following abbreviations are used: BAEE, α-*N*-benzoyl-L-arginine ethyl ester; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoic acid; CHOM, chicken ovomucoid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TAME, tosyl-L-arginine methyl ester.

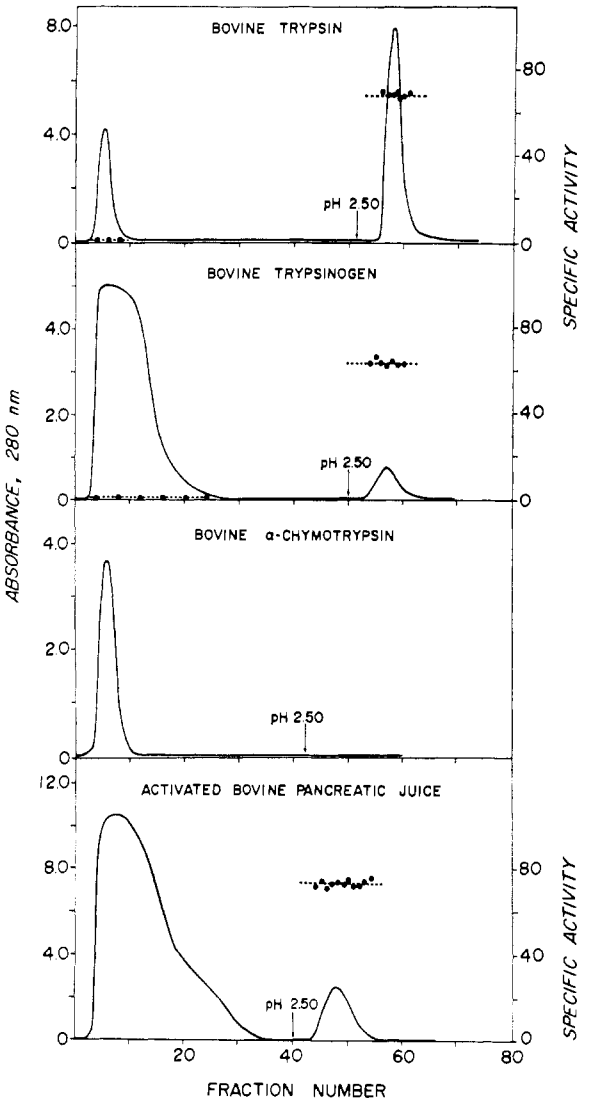

FIGURE 1: Chromatography on a CHOM-Sepharose column (1.5 × 25 cm) of bovine pancreatic enzymes. The column was equilibrated with 0.10 M Tris-chloride–0.05 M calcium chloride–0.50 M potassium chloride (pH 7.50) at 60 ml/hr. The enzymes were dissolved in 10 ml of buffer, applied to the column, and washed until no further protein was eluted. Active trypsin was then eluted with 0.10 M potassium formate–0.50 M potassium chloride (pH 2.50) and 10-ml fractions were collected. The following amounts of enzyme were applied: bovine trypsin, 250 mg; bovine trypsinogen, 60 mg, containing 2–5% trypsin contamination; bovine α-chymotrypsin, 50 mg; activated bovine pancreatic juice, 20 ml. The specific activity toward BAEE (μmole min^{-1} mg^{-1}) is indicated by the black dots.

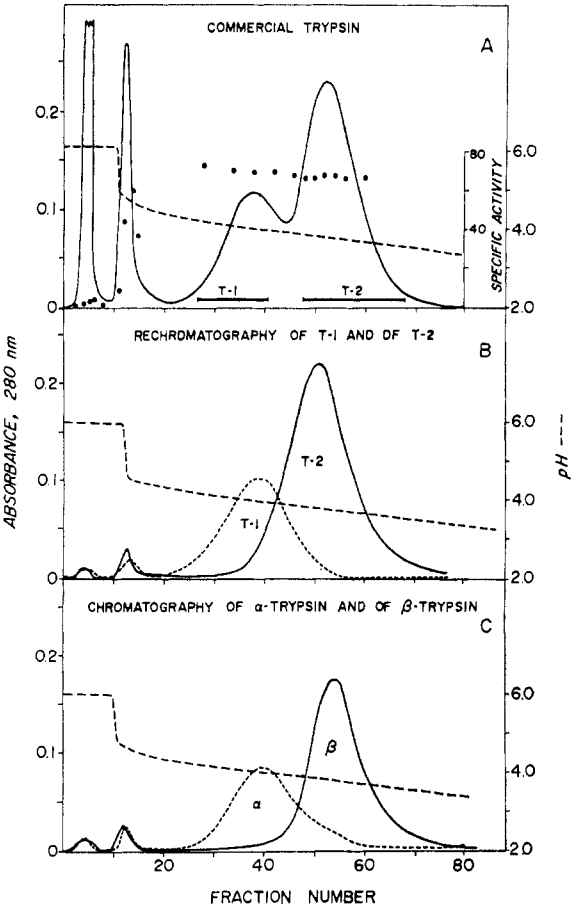

FIGURE 2: Chromatography of bovine trypsin on a CHOM-Sepharose column (0.9 × 66 cm). The column was equilibrated at pH 6.0 and a pH gradient (dashed line) was formed as described in Results. Fractions of 14 ml were collected at a flow rate of 70 ml/hr. The specific activity toward BAEE (μmole min^{-1} mg^{-1}) is indicated by the black dots. In part C the chromatography is repeated separately with α-trypsin (dotted line) and β-trypsin (solid line).

from Pharmacia Fine Chemicals, Inc., and prepared for use according to the manufacturer's recommendations. Benzoyl-L-arginine ethyl ester, tosyl-L-arginine methyl ester, and *N*-acetyl-L-tyrosine ethyl ester were obtained from Cyclo Chemical Co. *p*-Nitrophenyl-*p'*-guanidinobenzoic acid was prepared as described by Chase and Shaw (1967). Benzamidine hydrochloride was purchased from Aldrich Chemical Co. and cyanogen bromide from Eastman Kodak Co. All other chemicals were of reagent grade.

Methods. Amino-terminal sequences were determined with the Beckman sequencer according to the method of Edman and Begg (1967) as modified by M. A. Hermodson (to be published).

Esterase activity was measured with a Radiometer TTT-1 autotitrator and recorder. Standard 0.100 N NaOH was delivered as the titrant into the reaction vessel which was maintained at 26.0°. The substrate, 0.01 M BAEE, dissolved in 0.01 M Tris, 0.05 M $CaCl_2$, and 0.10 M KCl (pH 7.8), was used routinely to measure trypsin activity. Other substrates, 0.001 M TAME and 0.01 M *N*-acetyl-L-tyrosine ethyl ester, were used in the same buffer at the same temperature.

The concentration of active sites was determined with NPGB in a Cary Model 16 spectrophotometer as described by Chase and Shaw (1967).

Dilute protein solutions were concentrated in an Amicon ultrafiltration cell with a UM-10 membrane. The pressure was generated with N_2 gas and the temperature was kept at 4° during the ultrafiltration.

Protein concentration was determined with a differential refractometer (Phoenix Precision Instrument Co.) after calibrating with known NaCl solutions and applying the Δn values given by Stacey (1956). The values used for dn/dc were 0.1861 and 0.1934 ml per g which are the averages of values measured at 546 and 436 nm with a variety of proteins (Doty and Edsall, 1951). The extinction coefficient was determined from the trypsin concentration and the absorbance at 280 nm measured in a Cary Model 16 spectrophotometer.

Preparation of Chicken Ovomucoid (CHOM). Ovomucoid was isolated from chicken egg whites as described by Line-

TABLE I: N-Terminal Sequences of Purified Trypsins.[a]

	PTH-amino Acid Identified					
	Turn Number					
Fraction	1	2	3	4	5	6
T-1	Ile	Val	Gly	Gly	Tyr	Thr
	Ser	Ser		Thr	Ser	Tyr
T-2	Ile	Val	Gly	Gly	Tyr	Thr

[a] Fractions T-1 and T-2 were obtained as shown in Figure 2A. The yield of the stable PTH-amino acids (Ile, Val, Gly, Tyr) at each turn of the Edman degradation was approximately 90%. The unstable PTH-amino acids (Ser, Thr) could only be determined qualitatively. No other PTH-amino acids were found in yields greater than 5%. For T-1 the yield of PTH-Gly at turn 3 was twice the yield of PTH-Gly at turn 4. For T-2 the yield of PTH-Gly at turn 3 was equal to the yield of PTH-Gly at turn 4. For T-1 the yield of PTH-Tyr at turn 5 was equal to the yield of PTH-Tyr at turn 6. The sequence of bovine trypsin includes

NH_2-Ile-Val-Gly-Gly-Tyr-Thr——Lys-Ser-Ser-Gly-Thr-Ser-Tyr——
7 8 9 10 11 12 131 132 133 134 135 136 137

The numbering is that of bovine trypsinogen (Walsh and Neurath, 1964). β-Trypsin is a single polypeptide chain, residues 7–229, whereas α-trypsin is made up of two polypeptide chains, residues 7–131 and 132–229 (Schroeder and Shaw, 1968).

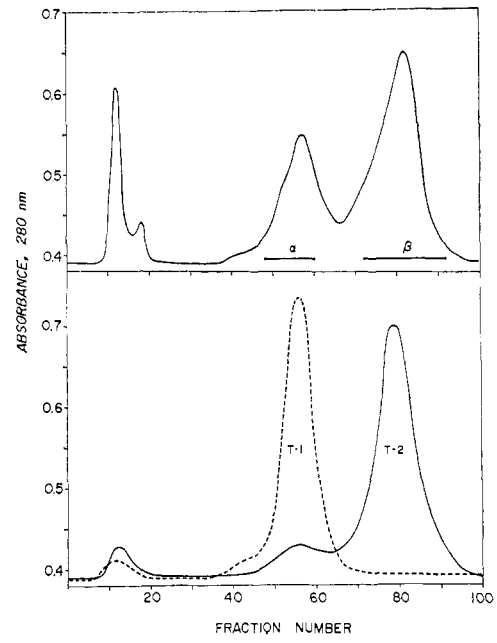

FIGURE 3: Chromatography of bovine trypsin on a SE-Sephadex C-50 column (1.5 × 80 cm) using 0.10 M Tris-chloride–0.02 M calcium chloride–0.001 M benzamidine (pH 7.10) at 12 ml/hr (12 ml/tube). In the upper diagram 60 mg of bovine trypsin was applied to the column in 10 ml of buffer. The bars drawn under each peak indicate the tubes that were pooled and concentrated for the chromatography of α- and β-trypsin in Figure 2C. The lower diagram represents separate chromatograms developed as above with fractions T-1 (dotted line) and T-2 (solid line) obtained from Figure 2B.

weaver and Murray (1947) except that precipitates were collected by centrifugation at 8000*g* rather than by vacuum filtration. This product (40 g) was absorbed on a DEAE-Sephadex column (5.0 × 40.0 cm), equilibrated with 0.05 M sodium phosphate at pH 6.5 as described by Feeney *et al.* (1967). After elution of the unretarded proteins, the trypsin inhibitor was eluted with the same buffer but containing also 0.25 M NaCl. The weight of CHOM recovered after dialysis and lyophilization was 30.6 g. Using the above method, 70 lb of fresh egg white yielded 107 g of salt-free CHOM.

Preparation of Chicken Ovomucoid-Sepharose (CHOM-Sepharose). CHOM-Sepharose was prepared by a procedure similar to that described by Cuatrecasas *et al.* (1968). A suspension of Sepharose 4B (equivalent to 175 ml of settled gel) was "activated" at pH 10.5 by the addition of 35 g of cyanogen bromide (Axén *et al.*, 1967; Porath *et al.*, 1967) in a sintered glass funnel. The suspension was mixed by passing N_2 gas upward through the sintered glass. CHOM (10 g) was added to the activated Sepharose and the suspension gently agitated in 0.1 M $NaHCO_3$ (pH 9.5) for 18 hr at 4°. Excess CHOM was removed by repeatedly washing the product with 0.2 N formic acid and 0.2 N Tris-HCl (pH 7.5). The product, 160 ml of settled CHOM-Sepharose, contained approximately 26 mg of CHOM/ml and bound 7.8 mg of trypsin/ml.

Results

Binding of Trypsin to CHOM-Sepharose. The binding of bovine trypsin to a CHOM-Sepharose column was examined by applying 250 mg of the enzyme at pH 7.5. Figure 1A shows that all of the active trypsin was bound and could be eluted at pH 2.5, conditions known to dissociate the CHOM-trypsin complex. In some experiments, 0.001 M benzamidine was added to prevent autolysis of the enzyme during the loading procedure. This precaution did not affect the binding of trypsin to the column.

Specificity of CHOM-Sepharose. For this method to be generally applicable to the purification of trypsins, the adsorbent must not bind other proteins usually present in crude preparations of trypsin. Neither bovine trypsinogen nor chymotrypsin was retarded by this column at pH 7.5 (Figure 1B,C). In fact, none of the proteins, other than trypsin, found in acidified, activated pancreatic juice (Figure 1D) was adsorbed under these conditions. The specific activity toward BAEE of trypsin isolated in one step from this crude mixture was 64.2 μmoles min^{-1} mg^{-1} compared to 30–60 μmoles min^{-1} mg^{-1} for commercial preparations. The activity of the most highly purified trypsin obtained in this work was 67.3 μmoles min^{-1} mg^{-1}.

pH Gradient Elution of Bovine Trypsin. Selective elution of bovine trypsin from the CHOM-Sepharose column was best achieved by application of a pH gradient. The column was first equilibrated at 4° with 0.02 M MES buffer (pH 6.0) containing 0.05 M $CaCl_2$ and 0.50 M KCl. After applying 80 mg of trypsin dissolved in 10 ml of the same buffer and washing with more buffer, a pH gradient from pH 4.50 to 2.75 was applied. To form the gradient two identical chambers were used: the first contained 0.1 M formic acid–0.50 M KCl, adjusted at room temperature to pH 4.50 with KOH; and the second chamber contained 0.1 M formic acid–0.50 M KCl, adjusted to pH 2.75. Active trypsin emerged as two peaks (Figure 2A). When these fractions (T-1 and T-2) were separately chromatographed on the same column, each emerged as a single peak (Figure 2B).

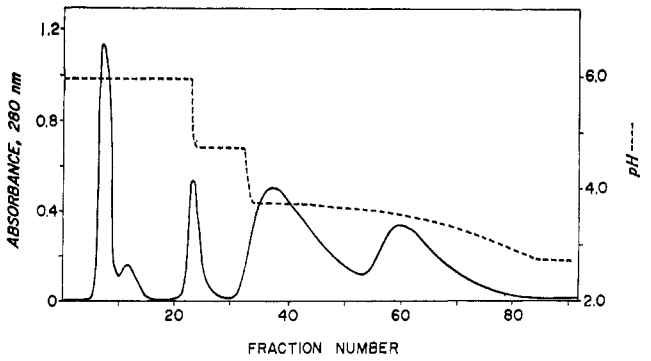

FIGURE 4: Chromatography of bovine trypsin on a CHOM-Sepharose column (1.27 × 75 cm) with a nonlinear pH gradient. The column was equilibrated with the same pH 6.00 buffer as in Figure 2. Fractions of 19 ml were collected at 95 ml/hr. The trypsin (200 mg) was applied in 10 ml of pH 6.00 buffer. The column was then developed with the following series of buffers: (1) 450 ml of pH 6.00 buffer, (2) 200 ml of 0.10 M potassium formate–0.50 M potassium chloride (pH 4.50), and (3) 1000 ml of the pH gradient described in Results. The dashed line represents the measured pH of the eluate.

Under the same conditions of chromatography, α- and β-trypsin emerged at the same effluent volumes as fractions T-1 and T-2 (*cf.* Figure 2B,C). The identities implied by these similarities in chromatographic behavior were confirmed by examining the mobilities of T-1 and T-2 in the system used by Schroeder and Shaw (1968) to separate α- and β-trypsin. Comparison to the elution volumes of α- and β-trypsins (Figure 3A,B) showed that T-1 corresponds to α-trypsin and T-2 to β-trypsin.

The only chemical differences between α- and β-trypsin are the additional amino-terminal seryl residue and carboxyl-terminal lysyl residue present in α-trypsin, both arising from the autolytic cleavage of the Lys_{131}–Ser_{132} bond (Schroeder and Shaw, 1968). The amino-terminal amino acid sequences of T-1 and T-2 were therefore examined to determine if the affinity column had in fact separated these two forms of trypsin from one another. Table I identifies the PTH-amino acids found at each turn of an Edman degradation using the Beckman sequencer. It is evident that T-1 and T-2 have the same amino-terminal sequences as α- and β-trypsin, respectively.

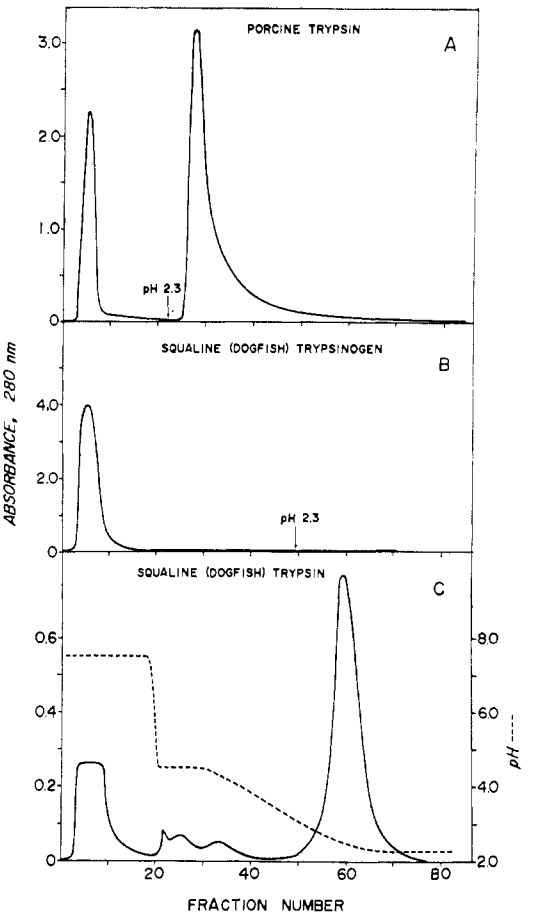

FIGURE 5: A CHOM-Sepharose column (1.5 × 25 cm) was equilibrated with the same buffer and flow rate as in Figure 1. Diagrams A, B, and C represent the chromatography of 215 mg of porcine trypsin, 80 mg of dogfish trypsinogen, and 150 mg of dogfish trypsin, respectively. In the upper two diagrams (A and B), the chromatograms were developed as in Figure 1 with buffer at pH 2.30. In the lower diagram, the column was washed with 100 ml of 0.5 M potassium chloride–0.1 M formate (pH 4.5), then with a gradient as in Figure 2 except that the final buffer was pH 2.30.

TABLE II: Kinetic Constants of Bovine Trypsins.[a]

Substrate	Trypsin Concn by	k_{cat} (min^{-1}) T-1	T-2	Unfractionated Commercial Trypsin
TAME[b]	Absorbance		2.44×10^3	
TAME	NPGB		2.47×10^3	
BAEE[c]	Absorbance	1.51×10^3	1.50×10^3	1.16×10^3
BAEE	NPGB	1.57×10^3	1.54×10^3	1.56×10^3

[a] Fractions T-1 and T-2 were obtained as in Figure 2A. [b] Concentration of TAME = 1 mM. This is outside the range of substrate activation of the type discussed by Trowbridge *et al.* (1963). [c] The K_m value for T-2 (2×10^{-6} M) was determined in the presence of benzamidine in 0.01 M Tris–0.05 M $CaCl_2$–0.10 M KCl (pH 7.8) at 26° using the principle outlined by Dixon and Webb (1964) and detailed by Tye (1971). This may be compared to the value of 4.3×10^{-6} M reported by Baines *et al.* (1964).

As an additional criterion for purity, the active-site concentrations of T-1 and T-2 were determined by titrations with NPGB and compared to the protein concentration. The latter was calculated from the measured absorbancy at 280 nm, the absorbancy index of 1.54 cm^{-1} mg^{-1} ml, separately determined for each fraction, and the molecular weight of 23,891. The number of active sites was 0.96 and 0.98 per mole of T-1 and T-2, respectively, in contrast to 0.74 for the unfractionated preparation. Since according to these values, fractions T-1 and T-2 approached theoretical purity (1.00), the rate constants for the hydrolysis of BAEE and TAME were also determined and are reported in Table II. When related to protein concentration, the activities of T-1 and T-2 toward BAEE were significantly higher than those of unfractionated trypsin and higher than the various values recorded in the literature (*e.g.*, Green and Neurath, 1953; Baines *et al.*, 1964). However, when related to the operational normality, the activities were practically identical, indicating that unfractionated, commercial trypsin contained protein unreactive toward NPGB and BAEE.

Nonlinear Gradient Elution of Bovine Trypsin. Higher resolution of α- and β-trypsin can be achieved by nonlinear pH gradient elution. To form the gradient, three identical chambers were used: the first two contained 0.10 M formic acid–0.50 M KCl, adjusted at room temperature to pH 4.50 with KOH; and the third chamber contained 0.10 M formic acid–0.50 M KCl, adjusted to pH 2.75. The increased resolution of α- and β-trypsin on the CHOM-Sepharose column achieved with this nonlinear gradient is illustrated in Figure 4.

Purification of Trypsins from Various Biological Species. In order to determine the general applicability of the CHOM-Sepharose column to the purification of trypsins from other species, partially purified porcine and dogfish trypsins were chromatographed under the conditions illustrated in Figure 5. In each case the active trypsin was quantitatively bound to the column at neutral pH and was eluted at acid pH. The material not retarded by the column was inactive.

Discussion

Our interest in the evolutionary development of a homologous class of trypsins has led to the design of a method that could be used to isolate trypsins from crude tissue extracts obtained from various species. The tight binding and the high specificity of certain macromolecular inhibitors toward trypsin make them suitable for the construction of affinity chromatography columns. Chicken ovomucoid bound to Sepharose is especially appropriate for this purpose since this protein inhibitor does not bind the homologous enzyme chymotrypsin. This method has been used as a single column process to isolate bovine trypsin from either commercial trypsin or from activated pancreatic juice. The high purity of the trypsin was demonstrated by its content of 0.96–0.98 active site/mole. The same basic procedure yielded highly purified porcine and dogfish trypsins. Since the isoelectric points of these three trypsins vary from pH 4.5 to 10.5, it is clear that specific binding properties of these enzymes are far more important for their adsorption on this column than are nonspecific electrostatic interactions. Thus CHOM-Sepharose should be useful for the purification in gram quantities of any trypsin-like enzyme which is specifically inhibited by CHOM. Conversely, this chromatographic system can be used to remove trypsin contamination from trypsinogen, chymotrypsin, or from enzyme preparations in general. In those cases where CHOM does not inhibit trypsin, *e.g.*, starfish (Winter and Neurath, 1970) or human (Kassel, 1970), other insolubilized protein inhibitors such as pancreatic or soybean could be similarly employed.

Although both bovine α- and β-trypsin are inhibited by CHOM, they can be selectively eluted from the insoluble CHOM derivative by a gradient of decreasing pH. Apparently the α-trypsin-CHOM complex is more readily dissociated by increasing the proton concentration than the complex with β-trypsin.[2] The resolving power of the separation technique is demonstrated by the fact that two proteins of identical molecular weight and amino acid sequence are separated by virtue of small differences in the binding affinity induced by a single internal bond cleavage.

Acknowledgment

We thank Dr. M. A. Hermodson for determining the amino-terminal amino acid sequences of trypsin fractions T-1 and T-2.

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[2] Dr. M. Laskowski, Jr., reported at the First International Research Conference on Proteinase Inhibitors held in Munich, 1970, that the association constant for the formation of the complex of soybean trypsin inhibitor with β-trypsin is about ten times greater than with α-trypsin. This behavior parallels the apparent differences in binding of the two forms of trypsin to CHOM (Figure 2).