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SYNOPSIS: Gel filtration and disc electrophoresis were used as simple and fast techniques for the investigation of the interaction and stoichiometry between UTI and trypsin. UTI appears to possess only a single trypsin binding site. The nature of the interaction between the inhibitor and enzyme appears to be dependent on the concentration ratio of the reactants. When UTI is in excess molar concentration, a single binary complex with trypsin of mol. wt. 95,000 is observed. In the presence of a molar excess of enzyme, this macromolecule is no longer observed, but proteins of mol. wt. 41,000 and 20,000 result. The possibility that UTI may be hydrolyzed to a partially degraded active fragment by the excess enzyme resulting in the formation of a modified inhibitor enzyme complex is proposed.

The urinary trypsin inhibitor (UTI) is a protein normally present in human urine which inactivates trypsin and which has been reported to possess anticoagulant and antifibrinolytic activity.[1,2] UTI, which has recently been obtained as an apparently homogeneous protein, is a glycoprotein, containing 10% carbohydrate, and has an estimated mol. wt. of 70,000.[3] The interaction between UTI and trypsin was investigated by gel filtration and disc electrophoresis to study the nature of trypsin inactivation by UTI and to serve as a model for the inactivation of other enzymes.

MATERIALS AND METHODS

UTI was prepared from pooled urine from non-proteinuric females in the third trimester of pregnancy according to the method of Proksch and Routh.[3] Twice recrystallized bovine trypsin (TRL-7LA) and trypsin which had been inactivated by treatment with diisopropylfluorophosphate (DIP-trypsin; TDIP-9GA) were purchased from the Worthington Biochemical Corp., Freehold, New Jersey. The nonenzymatic proteins used to calibrate the Sephadex G-100 column were purchased as a kit (8190A) from Mann Research Laboratories, Inc., New York, New York.

TRYPSIN AND TRYPSIN INHIBITOR ASSAY

Trypsin was assayed by the method of Roth in which the enzymatic hydrolysis rate of $N_1\alpha$-benzoyl-DL-arginine-β-naphthylamide hydrochloride (BANA) (Calbiochem, Los Angeles, California) is measured fluorometrically.[4] The trypsin inhibitor content of a given fraction was determined from its ability to reduce the hydrolytic activity of trypsin. The inhibition of trypsin by the purified UTI preparation was linear with increasing concentration of the inhibitor between 0 and 95% inhibition. The curve was non-linear between 95% and 100% inhibition.

Trypsin concentration was determined using the optical factor of 0.67 mg A^{-1} at 280 nm.[5]

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COLUMN CHROMATOGRAPHY

The interaction between UTI and trypsin was investigated by gel filtration chromatography according to the method of Andrews.[6] A 2.5 x 100 cm column was packed with Sephadex G-100 (40-120 micron diameter, Pharmacia Fine Chemicals, Piscatway, N. J.) to a bed height of 85 cm. The column was eluted with a flow rate of 5 ml hr^{-1} cm^{-2} by the reverse flow technique with a constant hydrostatic pressure of 16 cm. The proteins used as standards to calibrate the column were horse apo-ferritin, human gamma globulin, bovine serum albumin, ovalbumin, chymotrypsin, cytochrome *c* (Mann Research Lab) and soybean trypsin inhibitor (Worthington Biochemical Corp.)

ELECTROPHORESIS

Disc electrophoresis was performed using the simplified technique of Clark.[7] The protein bands were identified by fixation and staining with Coomassie brilliant blue dye in 12.5% trichloroacetic acid.[8]

RESULTS

The naturally occurring protease inhibitors may vary with respect to their number of enzyme binding sites. The lower molecular weight inhibitors, such as the soybean trypsin inhibitor (SBTI) and Kunitz's pancreatic inhibitor, are monovalent.[9,10] Other inhibitors with a higher molecular weight, such as duck-egg ovomucoid, may simultaneously bind two moles of trypsin.[11] The stoichiometry of the interaction between UTI and trypsin was investigated by gel filtration chromatography on a Sephadex G-100 column, calibrated with respect to molecular weight.

As shown in Fig. 1a, UTI was eluted mainly in a single fraction with a peak of 196 ml. This corresponds to the elution of a protein of mol. wt. 70,000 (Fig. 2). When trypsin was added to UTI in either equal or reduced molar concentrations an additional peak appeared (Fig. 1b). This frac-

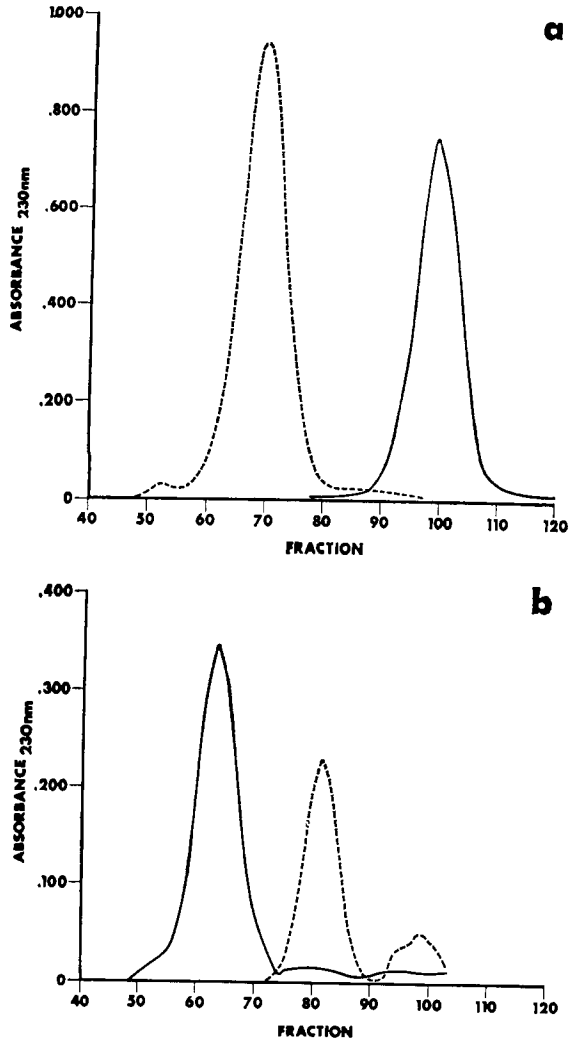

Figure 1. *The Elution of UTI and UTI Trypsin Complexes from Sephadex G-100.* Fig. 1a. The dotted line represents the elution of 13 mg of UTI. The solid line represents the elution of 4.1 mg of DIP-trypsin. Fig. 1b. The solid line represents the elution of nearly equal molar amounts of UTI and trypsin. UTI (3.0 mg) was dissolved in 0.5 ml of buffer (0.05 M Tris-HCl, 0.1 M KCl, pH 7.5), and 1.0 mg of trypsin was dissolved in another 0.5 ml of buffer. The two solutions were mixed and allowed to incubate for 10 min. The sample was then applied to the column. The dotted line represents the elution of 2.0 mg of UTI and 1.0 mg of trypsin (an approximate 1.5 molar excess of enzyme) prepared as previously described. The fraction volumes on the abscissa were 3 ml.

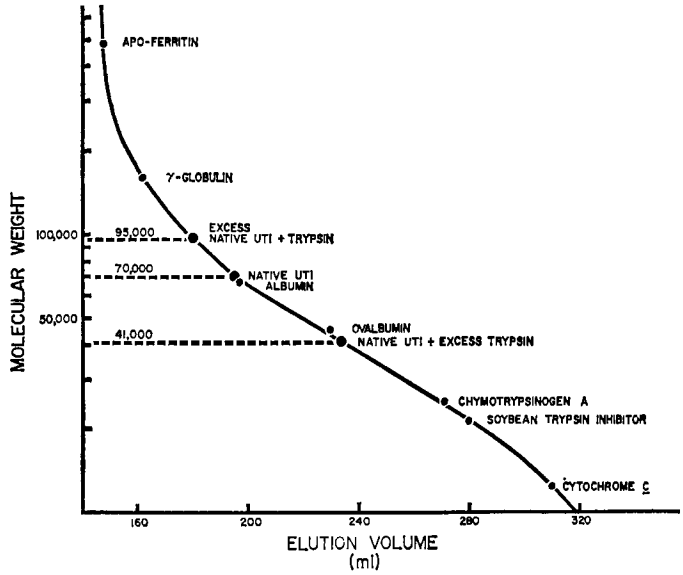

Figure 2. *Calibration Curve of the Sephadex G-100 Column for the Estimation of the Molecular Weights of the UTI Trypsin Complexes.*

tion, with an elution maximum at 179 ml, possessed an apparent mol. wt. of 95,000 (Fig. 2). Since trypsin has a mol. wt. of 24,000 and UTI has an approximate mol. wt. of 70,000, a protein of 95,000 mol. wt. would correspond to the elution of a binary complex. These fractions did not possess any additional inhibitor capacity, indicating, therefore, that UTI possesses only a single binding site for trypsin.

When UTI was incubated with a molar excess of trypsin for 10 minutes before application to the column, a different pattern was observed (Fig. 1b). No protein was eluted in either the 70,000 or 95,000 mol. wt. zones; instead, two new peaks appeared. The first protein was eluted in a symmetrical peak at 233 ml, which corresponded to a molecule of 41,000 mol. wt. (Fig. 2). This region did not possess either trypsin or inhibitor activity. The second fraction was eluted in a broad non-symmetrical zone at 295 ml, corresponding to a protein of approximately 20,000 mol. wt. As shown in Fig. 1a, inactive DIP-trypsin was eluted in this region. A slight amount of trypsin activity was observed in this area. These fractions probably represent the elution of the excess enzyme and any hydrolytic products.

The complexes between UTI and trypsin were further studied by disc electrophoresis. At the pH of the running gel buffer, pH 8.1, UTI migrated toward the anode and trypsin moved toward the cathode. A complex between the two proteins would thus be expected to possess an intermediate electrophoretic mobility. The electrophoresis of UTI with various amounts of trypsin is presented in Fig. 3. UTI (Fig. 3a) appears to contain only a single band of protein. The addition of approximately one-half an equivalent molar amount of trypsin (Fig. 3b) resulted in the appearance of another protein band which was less anionic than UTI. This new protein band probably represents the 95,000 mol. wt. fraction. The addition of a molar excess of trypsin (Fig. 3c) resulted in the complete disappearance of the UTI band, indicating that the UTI preparation contains only inhibitory protein. There was no indication of the 95,000 mol. wt. macromolecule. Instead, only a broad diffuse band of reduced electrophoretic mobility proteins may be observed near the top of the gel. The disc electrophoresis study confirms the previous gel filtration experiments in that the interaction between UTI and

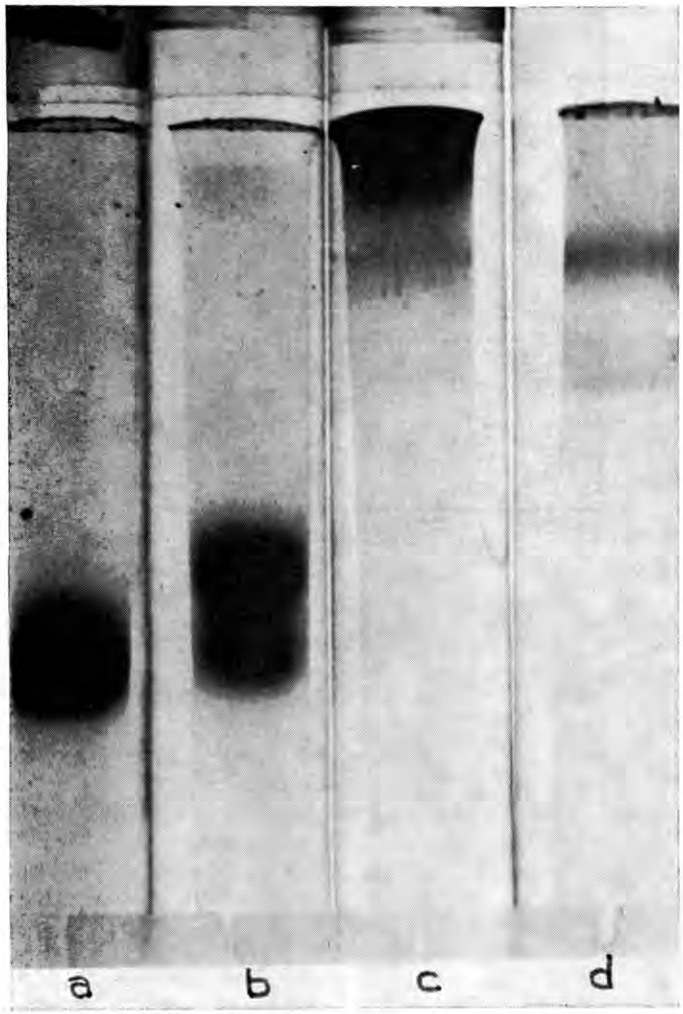

Figure 3. *Disc Electrophoresis of UTI and UTI Trypsin Complexes.* a. 50 μg UTI; b. 45 μg UTI, 9 μg trypsin (approximately ½ equivalent molar amount of enzyme); c. 50 μg UTI, 40 μg trypsin (approximately 2.5 equivalent molar amount of enzyme); d. 60 μg trypsin. UTI and trypsin were separately dissolved in the Roth trypsin assay buffer, pH 7.8. The solutions were then mixed and allowed to incubate for 5 min. An equal amount of 10% sucrose was then added and the samples were separated electrophoretically for 45 min. with a constant voltage of 20 volts cm^{-1}.

trypsin apparently varies with the relative enzyme concentration.

Discussion

When trypsin, mol. wt. 24,000, is added to equal or greater molar amounts of UTI, mol. wt. 70,000, a complex of mol. wt. 95,000 is observed. This new macromolecule does not possess either trypsin or inhibitor activity. UTI apparently has only a single trypsin binding site.

After UTI was incubated with a molar excess concentration of trypsin, different results were obtained. UTI completely disappeared and there was no subsequent appearance of the 95,000 mol. wt. complex. Instead, two different protein fractions were observed. One fraction was present as a broad nonsymmetrical peak of approximate mol. wt. 20,000, and appeared in part to be due to the elution of excess enzyme. The other band which was symmetrical corresponded to the elution of a macromolecule of 41,000 mol. wt. which did not contain any trypsin or inhibitor activity. The addition of increasing amounts of UTI to a molar excess of trypsin results in a linear stiochiometric decrease in proteolytic activity between 0 and 95% inhibition. UTI, therefore, is active in the presence of excess enzyme. The only macromolecule larger than trypsin which was observed after incubating UTI with a molar excess of enzyme was the fraction of mol. wt. 41,000. The nature of the interaction between UTI and trypsin appears to be dependent on the concentration ratio of the two reactants. The data suggest that UTI may be partially digested by the excess trypsin and that this is followed by the subsequent formation of a complex between the partially hydrolyzed inhibitor and enzyme. Since trypsin has a mol. wt. of 24,000, such a modified inhibitor would possess an approximate mol. wt. of 17,000.

The modification of protease inhibitors by trypsin has been previously observed. Laskowski and Ozawa have reported that soybean trypsin inhibitor may be proteolytically cleaved by trypsin to an active modified form.[12] This reaction, however, occurred only under acid conditions, and required only catalytic amounts of enzyme. The proposed hydrolysis of UTI in the present experiment, however, occurred under alkaline conditions and required a molar excess of trypsin. The significance of the proposed property of UTI to undergo extensive proteolysis and retain activity is at present uncertain. Such a system, however, could represent part of a mechanism for the regulation of antiprotease activity in which the inhibitor activity may be moderated by proteolytic reactions influenced by relative enzyme concentrations. Such a possibility will be explored in future investigations.

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