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DETERMINATION OF ANTI-PROTEASE HOMOGENEITY

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SUMMARY

Virgin and modified [single peptide bond between arginine (63) and isoleucine (64) is cleaved] soyabean anti-trypsin was separated by chromatofocusing using a narrow pH range. The separation on anion-exchange and reversed-phase chromatography was less satisfactory. Anti-proteases isolated by affinity chromatography from *Boophilus decoloratus* were monitored for the formation of any modified protein, with chromatofocusing.

INTRODUCTION

Affinity chromatography has been shown to be a useful and specific technique, which is especially applicable in the isolation of small quantities of biologically active compounds[1,2]. This process has been used to isolate inhibitors, with proteases such as, trypsin and chymotrypsin coupled to a solid like Sepharose 4B[3,4]. Although successful isolations of anti-proteases with protease-bound Sepharose have been reported[2,3,5,6] it has also been shown that the inhibitor is modified upon formation of a complex with the protease on the affinity column to a lesser or larger degree[4,5]. It is known that catalytic amounts of trypsin establish a true thermodynamic equilibrium between virgin (reactive site, peptide bond intact) and modified (this bond hydrolyzed) soyabean trypsin inhibitor[7,8]. Both virgin and modified inhibitors usually still inhibit their protease[4,5]. Elaborate chemical[9] or electrophoretic[7] methods are usually used to distinguish between virgin and modified inhibitors.

We have studied the separation of virgin and modified soyabean anti-trypsin with reversed-phase, anion-exchange and chromatofocusing high-performance chromatography. These methods were then applied to monitor the purity of anti-proteases isolated from eggs of the tick *Boophilus decoloratus* by affinity chromatography.

EXPERIMENTAL

All of the proteins used in this study were purchased from Boehringer Mannheim (Randburg). All reagents, including high-performance liquid chromatography (HPLC) solvents, were either analytical or HPLC grade and were obtained from Merck (Johannesburg). Pharmalyte, Sephadex G-100 and PD 10 columns containing Sephadex G-25 were obtained from Pharmacia (Protea Physical and Nuclear Instrumentation, Johannesburg, South Africa). Eggs of the tick *Boophilus decoloratus* were collected as described previously[10].

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HPLC systems

For reversed-phase chromatography a standard commercial system of the Beckman-Altex dual pump gradient system Model 322 with a α 20-μl loop and a 7.5 × 0.46 cm I.D. Ultrapore RPSC Beckman-Altex (C_3, 5 μm) column was used.

For the anion and chromatofocusing separations a Pharmacia fast protein liquid chromatograph (FPLC) system was used, consisting of two P-500 pumps, a V-7 valve injector with a 100-μl loop, a GP-250 gradient programmer, a UV-1 monitor, a FRAC-100 fraction collector and a REC-482 recorder. Anion-exchange chromatography was performed with a Mono Q column (50 × 5 mm I.D.) while a Mono P column (200 × 5 mm I.D.) was used for the chromatofocusing separations.

Assay for the presence of anti-trypsin

Bovine trypsin was assayed in 0.1 *M* phosphate buffer, pH 7.4, with benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) as a substrate according to the method described previously[11], measuring the increase in absorbance at 405 nm at 25°C on a Beckman DU 8 spectrophotometer. The anti-protease was assayed by measuring the decrease in activity of a known amount of trypsin in the presence of the anti-protease.

Preparation of trypsin–Sepharose 4B

Trypsin was coupled to pre-swollen cyanogen bromide (CNBr)-activated Sepharose 4B by the procedure prescribed by Pharmacia[12]. Trypsin-bound Sepharose was suspended in 0.1 *M* phosphate buffer, pH 7.4 and packed into a C10/10 Pharmacia column.

Electrophoresis

Small-pore polyacrylamide gels (PAGE) in tubes were prepared according to the formulation of Davis, except that the gel buffer pH was 9.3[13]. PAGE was also performed on slab gels (75 × 75 × 3 mm) with gel concentration T = 4.2% and cross-linkage C = 3.23%[14]. The gel buffer was the same as for the small-pore polyacrylamide gels. Polyacrylamide gel isoelectric focusing (IEF) was performed using slab gels [T = 5.17%, C = 3.23%, pH = 4.0–6.5 and 3–10, ampholine concentration = 6.7% (v/v); Pharmalyte Pharmacia[15]]. Staining was achieved with Coomassie Brilliant Blue R 250[16].

Soyabean anti-trypsin

Commercially available virgin soyabean anti-trypsin (Boehringer) was purified on a Mono Q column, with buffer A consisting of a 0.02 *M* bis-Tris [bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane] pH 7.2 and buffer B of 0.02 *M* bis-Tris, pH 7.2, containing 0.3 *M* sodium chloride.

Preparation of a mixture of virgin and modified anti-typsin

A slightly modified method of Mattis and Laskowski Jr.[9] was used. The pH of the incubation mixture was adjusted from pH 3.5 to 8.5 using a PD 10 column with 0.01 *M* Tris–0.1 *M* sodium chloride pH 8.5 buffer as eluent. This solution was concentrated and desalted using a Millipore agitator fitted with an immersible CX-10 ultrafiltration unit. After passage through a G-100 Sephadex column to remove the trypsin as trypsin–inhibitor complex, the trypsin–inhibitor mixture was freeze-

dried. The inhibitor obtained was assumed to be approximately 10% virgin and 90% modified[9].

Toxin and trypsin inhibitor from B. decoloratus eggs

Crude egg extracts were prepared in 2 g batches (wet mass) as described previously[10,18]. The egg suspension was centrifuged at 85,000 *g* for 5 h at 5°C in a Beckman L5-65 centrifuge. The supernatant (*ca.* 10 cm^3) was applied to a Pharmacia K26/40 column containing Sephadex G-100 in distilled water with a flow-rate of 30 cm^3/h; fractions of 3 cm^3 were collected, and absorbancy at 280 nm was determined. The fractions were then tested for guinea-pig toxicity[10] as well as the presence of the anti-protease.

The toxic fractions were pooled and freeze-dried. This fraction was divided into two equal parts. The first part was purified by chromatofocusing on a Mono P column; starting buffer, 0.025 *M* diethanolamine–HCl, pH 9.5; eluting buffer, Polybuffer 96 (5.2%, v/v), Pharmalyte [8–10.5 (1.0%, v/v)]–HCl, pH 8.0.

The second part was applied to the affinity column in a 0.1-*M* phosphate buffer, pH 7.4. The column was developed with the same buffer until no more proteins were eluted, as measured by the absorbance at 280 nm. Any adsorbed proteins were eluted with a solution at pH 1.2 containing 0.01 *M* calcium chloride and 0.5 *M* sodium chloride[17].

The fractions from the Sephadex G-100 showing trypsin inhibition were pooled, freeze-dried and divided into three parts. The first was further purified by affinity chromatography. This material remained on the column for 1 h while the second fraction was loaded and left on the affinity column for 24 h. The third fraction was loaded directly onto a Mono P column; starting buffer, 0.025 *M* imidazole–HCl, pH 5.6; eluting buffer, Polybuffer 74–HCl (10%, v/v), pH 3.5.

The peak obtained from the affinity chromatography which showed trypsin inhibition was dialyzed, freeze-dried and then analyzed by chromatofocusing on a Mono P column.

RESULTS AND DISCUSSION

Separation of virgin and modified soyabean anti-trypsin

In order to prepare modified soyabean anti-trypsin, it was necessary to purify the commercial product supplied by Boehringer Mannheim. This product could conveniently be purified on a Mono Q anion-exchange column, since it is known to have an isoelectric point (p*I*) of 4.5. Different gradients over 15 min with NaCl in the bis-Tris buffer gave satisfactory resolution. This material was pure, as confirmed by tube and slab gel PAGE as well as by IEF, pH 4.0–6.5.

It was previously shown that trypsin cleaves a single peptide bond between arginine (63) and isoleucine (64) on binding with virgin soyabean anti-trypsin[18]. Both virgin and modified anti-protease in general show inhibition of their respective proteases[4,5]. Virgin and modified soyabean anti-trypsin were previously separated by tube PAGE at pH 9.7, and this was explained solely on the basis of charge effects[9]. On the modified inhibitor, the amino group of Ile (64) is largely uncharged at this pH, whereas the Arg (63) carboxyl group is completely ionized, thus giving rise to a net charge difference of *ca.* −1 between virgin and modified inhibitors. The total

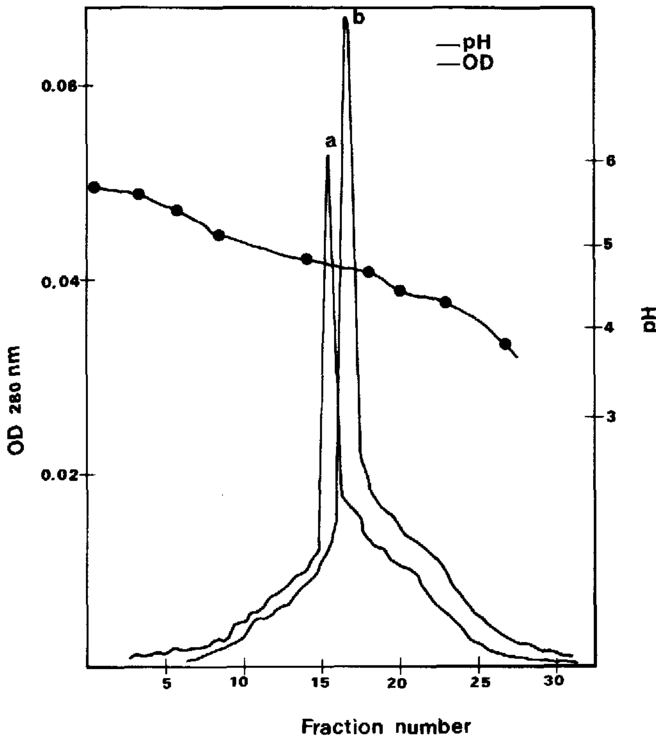

Fig. 1. Elution pattern of modified and virgin soyabean anti-trypsin after chromatofocusing on a Mono P column. Starting buffer: 0.025 *M*, imidazole–HCl pH 5.6. Eluting buffer: Polybuffer 74–HCl (10%, v/v) pH 3.5. (a) 0.1 mg modified soyabean antitrypsin in 100 μl starting buffer. (b) 0.1 mg modified soyabean anti-trypsin in 100 μl starting buffer. Span: 0.05 A, chart-speed: 0.5 cm/min, flow-rate: 1 ml/min, fraction volume: 1 ml.

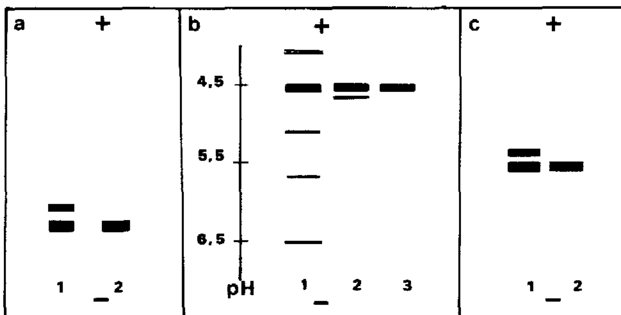

Fig. 2. Electrophoretic character of the virgin and modified soyabean anti-trypsin, (a) PAGE slab gel: (1) Modified and virgin soyabean anti-trypsin, 20 μg. (2) Virgin soyabean anti-trypsin, 20 μg. (b) IEF gel: (1) standard calibration proteins, human carbonic anhydrase B, bovine carbonic anhydrase B, β-lactoglobulin A, Soyabean anti-trypsin, glucose oxidase. (2) Modified and virgin soyabean anti-trypsin, 25 μg. (3) Virgin soyabean anti-trypsin, 25 μg. (c) PAGE tube gels: (1) Modified and virgin soyabean anti-trypsin, 35 μg. (2) Virgin soyabean anti-trypsin 35 μg.

charge on the virgin is *ca.* -12[7,19] and the charge difference due to peptide bond hydrolysis is about 8% at pH 9.7.

The method of Mattis and Laskowski Jr.[9], which was reported to yield 90% modified and 10% virgin soyabean anti-trypsin was used to prepare such a mixture. Under the same electrophoretic conditions as used by these authors we obtained practically identical results (Fig. 2) and assumed that the mixture prepared, contained about 90% modified and 10% virgin inhibitor. Slab gel PAGE and IEF electrophoresis showed the presence of a more negatively modified anti-trypsin, moving faster than the virgin compound.

Modified and virgin soyabean anti-trypsin were chromatographed on a Mono P chromatofocusing column as shown in Fig. 1 with a narrow pH range from 5.6 to 3.5. The separation is statisfactory, the modified anti-trypsin showing the shorter retention time. From the chromatogram it can be seen that the modified protein sample still contained a little virgin anti-trypsin.

It was also attempted to separate the mixture on a Mono Q anion-exchange column, with a salt gradient at pH 9.3 in an 0.02 *M* ethanolamine buffer. Although the modified and virgin inhibitor could not be resolved, a difference in retention times could be demonstrated. This separation could possibly be improved if the small charge difference that exists between the two compounds could be exploited maximally for separation by using electrophoretic titration curves[20].

Isolation of a toxin from B. decoloratus eggs

A protein, toxic to guinea pigs, could be isolated from the supernatant of the crude tick egg extract by successive chromatography, firstly on Sephadex G-100 and then on a Mono P column as shown in Fig. 3. Peak 1 from Sephadex G-100 chromatography showed no trypsin inhibition but was toxic to guinea pigs. Chromatography of peak 1 on the Mono P column yielded four peaks, the first of which was again toxic to guinea-pigs, but which now shows trypsin inhibition of the slow tight-binding type as defined by Morrison[21]. Rechromatography of this peak on a Mono P column shows only one peak, and the purity of the toxin was confirmed by IEF electrophoresis in which a p*I* of 9.2 could be determined. The fact that the toxic protein (molecular mass 40,000)[22] only inhibited trypsin after chromatofocusing suggests that it was probably bound to a protein which dissociates under chromatographic conditions. This was supported by the observation that the toxin was eluted from the Sephadex G-100 column with a group of proteins with k values corresponding to molecular mass of between 60,000 and 70,000.

Since the pure toxin showed trypsin inhibition, it was decided to isolate it by affinity chromatography. Both, peak 1 from the Sephadex G-100 and peak 1 from the Mono P column, were subjected to chromatography on a trypsin-bound Sepharose column, but were not retained on this column material, even after incubation on the column for 12 h. Chromatofocusing on a Mono P column (Fig. 3) and IEF electrophoresis (Fig. 4) showed that the toxin was unaltered by these procedures. The fact that the toxin (molecular mass 40,000), was not bound to trypsin on the affinity column, can possibly be explained by unfavourable steric interactions with the trypsin-bound Sepharose. However, the toxin is a slow tight-binding inhibitor of trypsin, which inhibits the trypsin at concentrations similar to that of the enzyme[22]. Therefore, in principle, it should have been retained by the affinity column.

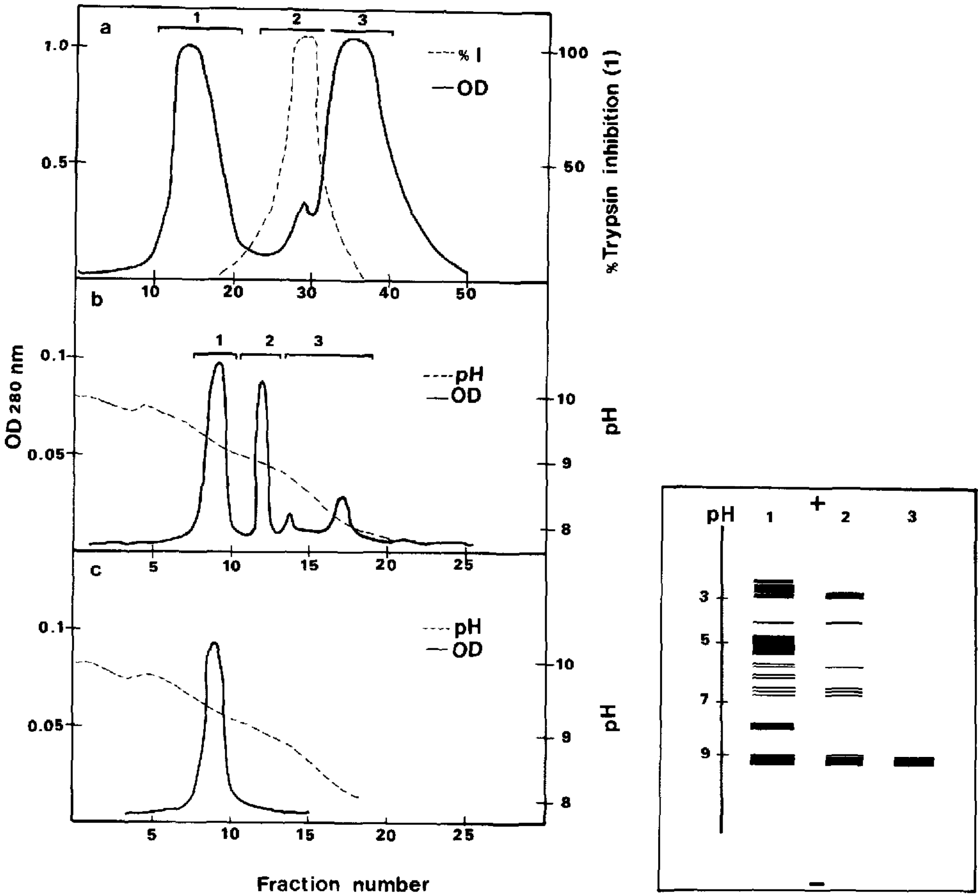

Fig. 3. Isolation of toxin and anti-protease from *B. decoloratus* eggs. (a) Gel-permeation chromatography on Sephadex G-100 column. Concentration of sample, 2 g/10 ml. Column dimensions: K26/40, flow-rate: 30 cm^3/h, fraction volume: 3 ml, eluent: distilled water. (b) Elution curve of Peak 1 from G-100 on Mono P, chromatofocusing column. Starting buffer: 0.02 *M* diethanolamine–HCl, pH 10. Eluting buffer: Poly-buffer 96 HCl (5.2%) pH 8.0 and 1 ml Pharmalyte pH 8–10.5. Span: 0.1 A, chart-speed: 0.5 cm/min, flow-rate: 1 ml/min, fraction volume: 1 ml. (c) Toxic fraction from (b) on Mono P chromatofocusing column, conditions same as for (b).

Fig. 4. Analytical isoelectric focusing (IEF) of the isolation of *B. decoloratus* egg toxin. (1) Crude *B. decoloratus* extract, 35 μg. (2) Toxic peak from Sephadex G-100 column, 35 μg. (3) Toxic peak after chromatofocusing on a Mono P column, 30 μg.

Isolation of an anti-protease from B. decoloratus eggs

The presence of a trypsin inhibitor was observed in peak 2, obtained from the Sephadex G-100 chromatography as shown in Fig. 3. This trypsin-inhibiting fraction, together with similar fractions, incubated on the Sepharose-trypsin column for 1 and 24 h respectively, were then analyzed on a Mono P column, as shown in Fig. 5. It was observed by this chromatofocusing that peak 2 contained a number of components, one with a p*I* of about 5.45, which shows trypsin inhibition. After incubation of 1 h (Fig. 5b) on the Sepharose-trypsin column, a new peak appeared, which also

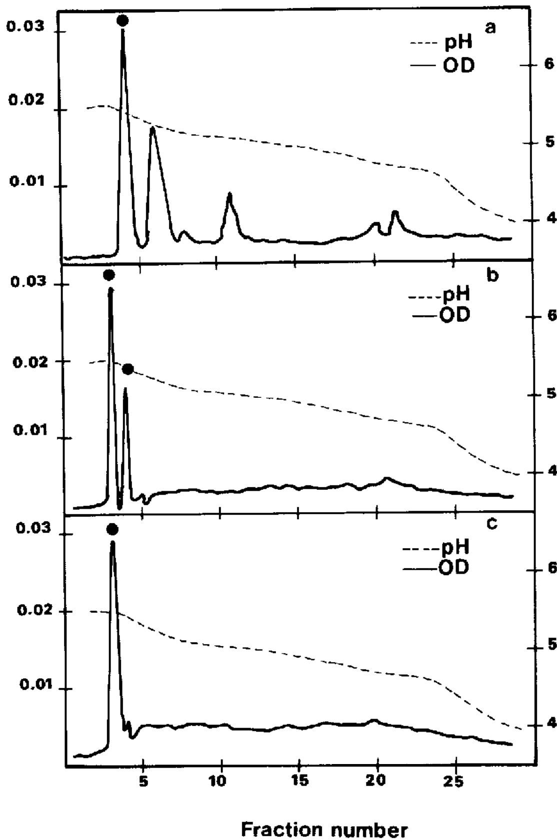

Fig. 5. Elution pattern of *B. decoloratus* anti-protease on a Mono P chromatofocusing column. Starting buffer: 0.025 *M* imidazole–HCl pH 5.6. Eluting buffer: Polybuffer 74–HCl (10%, v/v) pH = 3.5. (a) Anti-protease: peak 2 from G-100, concentration: 0.1 mg/100 μl starting buffer. (b) Anti-protease after incubation on trypsin–Sepharose 4B column for 1 h. Concentration: 0.1 mg/100 μl starting buffer. (c) Anti-protease after incubation on trypsin Sepharose 4B column for 24 h. Concentration: 0.1 mg/100 μl starting buffer. Span: 0.05 A, flow-rate: 1 ml/min, chart-speed: 0.5 cm/min, fraction volume: 1 ml. ●, Trypsin-inhibiting activity.

shows anti-trypsin activity, with a p*I* of about 5.5. It was assumed that this peak represents the modified anti-trypsin. The ratio of virgin to modified anti-trypsin after 1 h was about 1:2.

On incubation for 24 h (Fig. 5c) on the affinity column, most of the virgin anti-trypsin was converted to the modified compound.

The extent to which modification takes place during affinity chromatography will depend, among others, on the type of anti-protease under consideration[4,5] and the contact time on the column. Affinity chromatography of anti-proteases should not necessarily be abandoned because it also yields modified anti-trypsin. Since the extent of modification can now be established by chromatofocusing, conditions could now be chosen to yield the minimum or, on the other hand, only modified anti-protease, if this type of modification does occur. The modified anti-trypsin could, for instance, possibly be catalyzed to a thermodynamic mixture of virgin and modified

inhibitor, in the presence of catalytic amounts of trypsin, which could then be separated[7,9].

Reversed-phase chromatography

Chromatography of virgin and modified soyabean anti-trypsin on a Ultrapore RPSC column with a 0.01 *M* trifluoroacetic acid (TFA) in acetonitrile gradient from 20–70% over 65 min. (solvent A: 0.01 *M* TFA; solvent B: 0.01 *M* TFA in acetonitrile showed identical retention times for both proteins. It appears that the cleavage of the peptide bond does not alter the hydrophobicity of the modified anti-trypsin to any significant degree.

CONCLUSIONS

Separation of virgin and modified soyabean anti-trypsin could be accomplished by chromatofocusing on a Mono P column with a narrow pH range in the region of the p*I* of these compounds. This type of procedure could therefore be used to monitor the extent of modified anti-protease formation which could take place during affinity chromatography.

Anion-exchange chromatography of virgin and modified anti-trypsin was less successful, although the separation conditions could possibly be still improved. Chromatography of virgin and modified soyabean anti-trypsin on a reversed-phase Ultrapore RPSC (C_3) column gave identical retention times for both proteins. Chromatofocusing is ideally suited to the differentiation between proteins with a small difference (*ca.* 0.1 pH unit) in the p*I* values. A time-dependent modification of the virgin anti-trypsin from *B. decoloratus* on a Sepharose-4B–trypsin column could indeed be demonstrated by chromatofocusing.

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