Affinity Chromatography of Bovine Trypsin

A RAPID SEPARATION OF BOVINE α - AND β -TRYPSIN

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Affinity adsorbents for bovine trypsin were prepared by covalently coupling *p*-(*p*'-aminophenoxypropoxy)benzamidinium to cellulose and to agarose. Trypsin binds to both adsorbents at pH 6–8 and is released at low pH values or in the presence of *n*-butylamine hydrochloride. Pure β -trypsin may be eluted from crude trypsin bound at pH 8.0 to the cellulose adsorbent by stepwise elution with an acetate buffer, pH 5.0. Both α - and β -trypsin may be isolated by chromatography of crude trypsin on the agarose derivative in an acetate buffer, pH 4.0. These two methods for purifying the trypsin are specific to the particular adsorbents. They are rapid and convenient in use. Both methods leave a mixture of the two enzymes bound to the adsorbent and release occurs only at low pH values. The effects of pH, composition and ionic strength of buffer and other variables on both purification methods are described. Affinity adsorbents of soya-bean trypsin inhibitor and of *N*- α -(*N*'-methyl-*N*'-sulphanilyl)sulphanilylagmatine bound to agarose were prepared, but were found to be of limited usefulness in the purification of trypsin.

An adsorbent for the affinity chromatography of enzymes is prepared by covalently attaching an enzyme-specific ligand to an insoluble support. Separation of an enzyme from a mixture is commonly achieved by applying a crude enzyme preparation to the column in a buffer chosen so that the desired enzyme binds firmly to the adsorbent while other proteins are unretarded on elution. The bound enzyme is then commonly eluted in a single sharp peak by an abrupt buffer change (Cuatrecasas & Anfinsen, 1971*a,b*; Friedberg, 1971). This technique has been applied to the separation of trypsin from inert protein by using both small and large ligands (Feinstein, 1970*a,b*; Ureh, 1971; Kasai & Ishii, 1972; Hixson & Nishikawa, 1973). Bovine trypsin, however, exists in two major forms, α -trypsin and β -trypsin, which have been isolated by chromatography on sulphoethyl-Sephadex (Schroeder & Shaw, 1968; Berezin *et al.*, 1969). β -Trypsin has a single polypeptide chain, whereas α -trypsin has two chains resulting from an autocatalytic cleavage of the Lys⁽¹³¹⁾-Ser⁽¹³²⁾ bond. The two enzymes exhibit different kinetic characteristics in their reactions with some synthetic substrates (Hruska *et al.*, 1969; Roberts *et al.*, 1971; Jameson *et al.*, 1973; Roberts & Elmore, 1974). We report the separation of α - and β -trypsin by two simple and rapid chromatographic procedures involving no gradients, and the factors that affect the separations have been investigated. A preliminary account of this work has been published (Jameson & Elmore, 1971). Since this work

was completed, a separation of the two forms of trypsin by gradient elution from chicken ovomucoid inhibitor linked to Sepharose has been reported (Robinson *et al.*, 1971).

Experimental

Materials

Bovine trypsin (thrice-crystallized, salt-free, freeze-dried) and bovine α -chymotrypsin (thrice-crystallized, salt-free, freeze-dried) were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Soya-bean trypsin inhibitor (five times crystallized) was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. *p*'-Nitrophenyl *p*-guanidinobenzoate hydrochloride was synthesized by the method of Chase & Shaw (1967) and the corresponding 4-methylumbelliferyl ester hydrochloride was prepared as described by Jameson *et al.* (1973). *S*-(2-Aminoethyl)-*N*-benzoyl-L-cysteine methyl ester hydrochloride was synthesized by the method of Elmore *et al.* (1967) and 4-methylumbelliferyl *p*-(*NNN*-trimethylammonium)cinnamate chloride by the method of Jameson *et al.* (1973). *p*-(*p*'-Aminophenoxypropoxy)benzamidinium as the benzene-sulphonate was prepared by a method based on that of Baker & Erickson (1968), but the yield of the intermediate *p*-hydroxybenzamidinium (66%) was almost twice that reported by these workers. Dioxan (150 ml for 0.1 mol of *p*-cyanophenol)

replaced chloroform as solvent; reaction with ethanol-HCl was allowed to proceed for 10 days instead of 18 h; unchanged *p*-cyanophenol was removed by distillation under reduced pressure before reaction with NH_3 . *p*-Hydroxybenzamidinium was isolated as the hydrochloride, m.p. 222–224°C [Partridge & Short (1947) quote m.p. 223–224°C], and the latter was then converted into the benzenesulphonate, m.p. 183–186°C [Baker & Erickson (1968) quote m.p. 180–181°C; Partridge & Short (1947) quote m.p. 187°C]. The remainder of the preparation of *p*-(*p*'-aminophenoxypropoxy)benzamidinium benzenesulphonate was carried out as described by Baker & Erickson (1968) except that the hydrogenation of the *p*-nitro precursor was carried out in the presence of 10% palladium on charcoal at atmospheric pressure and the product was crystallized twice from acetonitrile by treatment with activated charcoal. The product had m.p. 180–182°C [Baker & Erickson (1968) quote m.p. 173–174°C]. *NN*-Dimethylformamide was purified by fractional distillation from P_2O_5 . Thionyl chloride was purified by distillation from quinoline followed by distillation from linseed oil. Sepharose 4B and Sepharose 2B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and cellulose powder (Whatman CC31) was purchased from W. and R. Balston Ltd., Maidstone, Kent, U.K.

Sodium N-benzyloxycarbonylsulphanilate. Sulphanilic acid (44.3 g) was dissolved in water (1000 ml) and allowed to react with benzyl chloroformate (47.0 g) in ether (200 ml) at pH 8 (in a pH-stat) with mechanical stirring. The precipitated solid was filtered off and washed with ether. Another crop was obtained by extracting the filtrate with ether and evaporating the aqueous phase under reduced pressure. The total product (78%) was recrystallized from methanol; it did not melt below 300°C (Found: C, 50.8; H, 3.8. $\text{C}_{14}\text{H}_{12}\text{NNaO}_5\text{S}$ requires C, 51.1; H, 3.7%).

N-Benzyloxycarbonylsulphanilyl chloride. Dry sodium *N*-benzyloxycarbonylsulphanilate (20.0 g) was dissolved in dry *NN*-dimethylformamide (20 ml), and dry benzene (200 ml) followed by thionyl chloride (24.0 g) were added. The mixture was stirred for 1 h, poured into an ice-water mixture (200 ml) and then neutralized with NaHCO_3 . The benzene layer was washed with water (3 × 200 ml), dried (with MgSO_4) and evaporated to dryness under reduced pressure. The residual product (93%) had m.p. 111–113°C, unchanged after recrystallization from benzene-light petroleum (b.p. 60–80°C) (Found: C, 51.5; H, 3.9; S, 10.0. $\text{C}_{14}\text{H}_{12}\text{ClNO}_4\text{S}$ requires C, 51.6; H, 3.7; S, 9.8%).

Sodium N-(N'-benzyloxycarbonylsulphanilyl)-N-methylsulphanilate. *N*-Methylsulphanilic acid (10.0 g) was dissolved in a mixture of acetonitrile and water (1:1, v/v) by neutralizing it with NaOH. A solution of *N*-benzyloxycarbonylsulphanilyl chloride

(17.4 g) in acetonitrile (30 ml) was added and the reaction mixture was maintained at pH 6 (with a pH-stat) with vigorous mechanical stirring. When the reaction was complete (about 3 h), the mixture was evaporated to dryness under reduced pressure and the resulting solid was recrystallized twice from methanol-ether with one treatment with activated charcoal and removal of material insoluble in methanol. The resulting sodium salt (20.6 g) was a pale-brown solid. One sample was converted into the *potassium salt* through the free acid. The potassium salt was recrystallized from *NN*-dimethylformamide-ether and then from water. It was dried *in vacuo*, when it had m.p. 175–178°C (decomp.) and was homogeneous by t.l.c. on silica in methanol containing 1% (v/v) acetic acid (Found: C, 43.9; H, 4.5. $\text{C}_{21}\text{H}_{19}\text{KN}_2\text{O}_7\text{S}_2 \cdot 3\text{H}_2\text{O}$ requires C, 44.3; H, 4.4%). Another sample of the sodium salt was converted into the *dicyclohexylammonium salt*, which was recrystallized twice from methanol-ether and then had m.p. 187–188°C and was homogeneous by t.l.c. on silica in methanol containing 1% (v/v) acetic acid (detected by I_2 vapour) (Found: C, 59.9; H, 6.8. $\text{C}_{33}\text{H}_{43}\text{N}_3\text{O}_7\text{S}_2$ requires C, 60.2; H, 6.6%).

N-(N'-Benzyloxycarbonylsulphanilyl)-N-methylsulphanilyl chloride. Sodium *N*-(*N'*-benzyloxycarbonylsulphanilyl)-*N*-methylsulphanilate (10.0 g) was suspended in a mixture of dry *NN*-dimethylformamide (20 ml) and dry benzene (200 ml) and treated with thionyl chloride (20.0 g). The mixture was stirred for 4 h and poured into a slurry of ice and water (750 ml). The aqueous solution was neutralized with NaHCO_3 , more benzene (100 ml) was added and the upper phase was separated. The benzene solution was washed with water (3 × 300 ml), dried (with MgSO_4) and evaporated under reduced pressure to a small volume. Addition of light petroleum (b.p. 60–80°C) gave the product (82%), which had m.p. 147–148°C after recrystallization from benzene-light petroleum (b.p. 60–80°C) and was shown to be homogeneous by t.l.c. on silica in benzene (Found: C, 51.9; H, 3.9; N, 5.6. $\text{C}_{21}\text{H}_{19}\text{ClN}_2\text{O}_6\text{S}_2$ requires C, 51.0; H, 3.9; N, 5.7%).

N-α-[N'-(N''-Benzyloxycarbonylsulphanilyl)-N'-methylsulphanilyl]agmatine hydrochloride. Agmatine sulphate was converted into the dihydrochloride by passage through a column of Dowex 1 (X-4; Cl⁻ form) ion-exchange resin (Bio-Rad Laboratories, Richmond, Calif., U.S.A.). *N*-(*N'*-Benzyloxycarbonylsulphanilyl)-*N*-methylsulphanilyl chloride (1.48 g) dissolved in acetonitrile (30 ml) was added to a stirred solution of agmatine dihydrochloride (609 mg) in water (30 ml) and the apparent pH was kept at 9.5 by addition of 2M-NaOH (in a pH-stat). When reaction ceased (30 min), the solution was evaporated under reduced pressure, yielding a gum which was then extracted with warm propan-2-ol. Evaporation

of the filtered extract under reduced pressure gave the *product* (93%) as an amorphous white solid. It had an ill-defined melting point and decomposed in the range 100–180°C depending on the rate of heating. The homogeneity of the material was demonstrated by t.l.c. on silica in chloroform-methanol-water (11:8:2, by vol.) and in pyridine-2-methylbutan-2-ol-water (7:7:6, by vol.) (detected by I₂ vapour) (Found: C, 48.9; H, 5.3; N, 12.9. C₂₆H₃₃ClN₆O₆S₂·H₂O requires C, 48.6; H, 5.5; N, 13.0%).

N- α -(*N'*-Methyl-*N'*-sulphanilyl)sulphanilylagmatine dihydrochloride. *N*- α -[*N'*-(*N''*-Benzyloxycarbonylsulphanilyl) - *N'* - methylsulphanilyl]agmatine hydrochloride was hydrogenolysed at atmospheric pressure in ethanol in the presence of 10% palladium on charcoal. The filtered solution was acidified with conc. HCl and evaporated to dryness under reduced pressure, giving the *product* (96%) which was dried *in vacuo* to an amorphous solid with a poorly defined m.p. of about 106°C (decomp.). It resisted attempts at crystallization but was shown to be essentially homogeneous by t.l.c. on silica in methanol-chloroform-water (11:8:2, by vol.) (Found: C, 41.0; H, 5.5; S, 12.1. C₁₈H₂₈Cl₂N₆O₄S₂ requires C, 41.0; H, 5.4; S, 12.2%). Addition of an excess of ethanolic 1,4-dihydroxy-9,10-anthraquinone-2-sulphonic acid (rhodizonic acid) to an ethanolic solution of the dihydrochloride caused the *rhodizone salt* to crystallize. This was washed with ethanol and recrystallized from 2-methoxy-ethanol-acetone, when it had an m.p. of about 192°C (decomp.), which was dependent on the rate of heating (Found: C, 49.8; H, 4.6; S, 11.5. C₃₂H₃₅N₆O₁₁S₃ requires C, 49.5; H, 4.6; S, 12.4%).

p-(*p'*-Aminophenoxypropoxy)benzamidine - Sepharose and *N*- α -(*N'*-methyl-*N'*-sulphanilyl)sulphanilylagmatine-Sepharose conjugates. *p*-(*p'*-Aminophenoxypropoxy)benzamidine and *N*- α -(*N'*-methyl-*N'*-sulphanilyl)sulphanilylagmatine were coupled covalently to Sepharose by a procedure based on that of Cuatrecasas *et al.* (1968). In a typical preparation, Sepharose 4B (50ml of settled gel) was stirred with water (50ml) and CNBr (5g) was added. The pH was kept at 11 by addition of 4M-NaOH (in a pH-stat) and a little ice was added to prevent a marked rise in temperature. When alkali uptake ceased, the gel was rapidly filtered off, washed with cold 0.1M-NaHCO₃ (1000ml) and suspended in 0.1M-sodium acetate buffer (50ml) adjusted to pH 5.0 with acetic acid. A solution of *p*-(*p'*-aminophenoxypropoxy)benzamidine benzenesulphonate (22.2mg) in aq. 50% (v/v) methanol (5ml) was added and the mixture was gently agitated at room temperature for 24h. The gel was filtered off, washed with more acetate buffer, and the content of *p*-(*p'*-aminophenoxypropoxy)benzamidine in the filtrate was determined spectrophotometrically at 263nm. The gel

was washed slowly and successively in a short column with 1M-sodium acetate-acetic acid, pH 5.0 (1000ml), 0.2M-NH₄Cl-NH₃, pH 9.5 (1000ml), and water (1000ml). It was stored as a slurry in 0.02% (w/v) NaN₃ at 4°C. Coupling of the benzamidine derivative to Sepharose 2B and to Sephadex G-200 was carried out in a similar manner, but the latter shrank considerably and acquired poor flow characteristics. Coupling experiments were carried out with smaller quantities of CNBr and of *p*-(*p'*-aminophenoxypropoxy)benzamidine benzenesulphonate. Normally, over 90% of the added benzamidine derivative coupled to the gel except when less than 20mg of CNBr/ml of gel was used in the activation step (Table 1). Coupling of *N*- α -(*N'*-sulphanilyl)sulphanilylagmatine to Sepharose 4B was performed similarly. The acetate buffer used for the reaction between *N*- α -(*N'*-methyl-*N'*-sulphanilyl)sulphanilylagmatine (10mg) and Sepharose 4B (10ml), which had been activated with CNBr (1g), usually had a pH of 4.5, but similar results were obtained at pH 3.0. Unchanged agmatine derivative was determined spectrophotometrically at 270nm and the results showed that 94–95% of it had become attached covalently to the gel. A test for the presence of primary aromatic amino groups, which was carried out on the products by attempted diazotization and coupling with 2-naphthol, was negative. When the reaction between activated Sepharose and the agmatine derivative was carried out in NaHCO₃ buffer at pH 9.0, however, a positive test for primary aromatic amino groups was obtained, indicating that in this case some coupling had occurred on the sulphonamido or guanidino N atoms.

p-(*p'*-Aminophenoxypropoxy)benzamidine - cellulose conjugate. Cellulose powder (Whatman CC31; 30g) was stirred with 0.5M-NaOH (1200ml) for 1h, washed with water until the washings were no longer alkaline, stirred with 0.5M-HCl for 1h and rewashed with water. Fines were removed and the remaining cellulose (66ml packed vol.) was activated with CNBr (2.5g), washed and allowed to react with the benzamidine benzenesulphonate (22.2mg) in 0.1M-sodium acetate buffer, pH 5.0. The product was washed as described for the Sepharose derivatives; 96% of the benzamidine derivative had become attached covalently to the cellulose.

Soya-bean trypsin inhibitor-Sepharose conjugate. Sepharose 4B (39ml of settled gel) was activated with CNBr (4.0g), washed with cold 0.1M-NaHCO₃ (800ml) and suspended in 0.1M-NaHCO₃ buffer, pH 9.0. A solution of soya-bean trypsin inhibitor (176mg) in water (4ml) was added and the mixture was gently stirred overnight at 4°C. After the product was washed, spectrophotometric assay of the washings at 280nm showed that the gel contained 2.9mg of trypsin inhibitor/ml (65% coupling efficiency).

Methods

Solutions of trypsin were kept at 0–4°C; stock enzyme solutions for kinetic measurements were made up in 1M-HCl containing 20mM-CaCl₂. Measurements of pH were made at room temperature. Concentrations of buffer constituents are given as final concentrations.

Column chromatography. Columns were maintained at 4°C. Eluent was delivered by means of a peristaltic pump and eluent gradients were linear. The column effluent was monitored at 280nm with a Uvicord u.v. analyser (LKB-Produkter AB, Stockholm, Sweden) and checked manually in selected cases on a spectrophotometer. The effluent was divided into timed fractions and delivered into test tubes maintained at 4°C in a water bath. Fractions that were to be freeze-dried were concentrated where necessary by ultrafiltration at 0°C through a Diaflo UM-10 membrane (Amicon Corp., Lexington, Mass., U.S.A.) and desalted by gel-filtration chromatography in 1M-HCl by using a short column of Sephadex G-25.

Measurement of protein concentrations. Trypsin concentrations were obtained from measurements of the extinction at 280nm; values of $E_{1\text{cm}}^{1\%}$ and molecular weight were taken as 15.6 (Baines *et al.*, 1964) and 23800 (Cunningham, 1954) respectively.

Determination of the operational molarity of trypsin solutions. In early experiments, the total content of active (α - and β -)trypsin in solutions was determined by titration with *p*'-nitrophenyl *p*-guanidinobenzoate at pH8.3 (Chase & Shaw, 1967). In addition, titrations were performed at pH4.0 (Hruska *et al.*, 1969) in attempts to determine the relative proportions of α - and β -trypsin. In most cases, however, trypsin solutions were titrated spectrofluorimetrically with 4-methylumbelliferyl *p*-guanidinobenzoate at pH8.3 and 25°C (Jameson *et al.*, 1973). As previously reported, β -trypsin was acylated by the latter reagent in a few seconds, but the reaction with α -trypsin required over 10min and this difference was exploited for the determination of the amounts of each form of enzyme present. Although the acylation of α -trypsin by 4-methylumbelliferyl *p*-guanidinobenzoate obeyed pseudo-first-order kinetics fairly well under the conditions used, it was preferred to calculate the amount of α -trypsin present by a curve-fitting procedure which required no assumptions about the order of the reaction. Fluorescence-intensity readings were taken at intervals varying from a few seconds to several minutes depending on the rate of change of fluorescence intensity. After the first few seconds, changes in fluorescence intensity (corrected for the non-enzymic hydrolysis of 4-methylumbelliferyl *p*-guanidinobenzoate) were due only to the acylation of α -trypsin. A computed curve was fitted to these readings by

the method involving orthogonal polynomials by using the program described by Elmore *et al.* (1963) and since rewritten in FORTRAN for the ICL 1907 computer. The value of the fluorescence intensity at zero time obtained by back-extrapolation of the computed curve was taken as a measure of the content of β -trypsin in the sample, and the content of α -trypsin could then be obtained from the difference in fluorescence intensity at zero time and when acylation was complete.

Determination of the operational molarity of α -chymotrypsin solutions. Solutions of α -chymotrypsin were titrated with 4-methylumbelliferyl *p*-(NNN-trimethylammonium)cinnamate as described by Jameson *et al.* (1973).

Measurement of the trypsin-binding capacity of affinity adsorbents. A known excess (2–4-fold) of bovine trypsin was applied to a small column (about 0.9cm×3cm) in a pH8.0 buffer, usually of 0.05M-triethanolamine hydrochloride–triethanolamine containing 10mM-CaCl₂. The column was washed with this buffer until elution of protein had essentially ceased; the concentration of trypsin in the pooled effluent was determined by measuring E_{280} and by spectrofluorimetric titration with 4-methylumbelliferyl *p*-guanidinobenzoate. The amount of trypsin bound by the affinity adsorbent could then be calculated. The bound enzyme was eluted with a solution of 0.5M-*n*-butylamine hydrochloride in the triethanolamine buffer or alternatively with a solution of 0.1M-citric acid containing 1M-NaCl. The trypsin content of the pooled effluent was determined as above giving the trypsin-binding capacity of the column directly. Four values of the trypsin-binding capacity were thus obtained. Although serious discrepancies between the four values were observed only when a very large excess of trypsin was used, it was considered that the spectrophotometric determination of the protein content of the effluent obtained in the second step was most reliable because (a) the spectrophotometric method applied to the first effluent would overestimate the amount of unbound trypsin if inert protein were present and (b) titration of either fraction with 4-methylumbelliferyl *p*-guanidinobenzoate would underestimate trypsin if any autolysis occurred during the binding and elution procedures.

Identification of N-terminal amino acids. Dansylation of trypsin samples was carried out in 0.1M-NaHCO₃ and in 0.25M-NaHCO₃ containing 4M-urea as described by Gray (1967) and Hartley (1970). The solvent in both cases contained 50% (v/v) acetone. In the latter case, the solution of dansylated protein was desalted by gel filtration before hydrolysis. Dansyl-amino acids in the acid hydrolysates were separated by t.l.c. on polyamide layers as described by Hartley (1970).

Steady-state kinetic measurements. These were carried out with a recording pH-stat by using *S*-(2-aminoethyl)-*N*-benzoyl-L-cysteine methyl ester hydrochloride and α -*N*-benzoyl-L-arginine ethyl ester hydrochloride as substrates. Detailed descriptions of the experimental and computational methods have been given elsewhere (Elmore *et al.*, 1963; Baines *et al.*, 1964).

Results

Affinity adsorbents

The insoluble support chosen for most of the affinity adsorbents was agarose (Cuatrecasas *et al.*, 1968). Cellulose has also been found to be satisfactory, an observation independently reported by Lowe & Dean (1971) and Uren (1971). The structures of synthetic inhibitors were devised so that the enzyme-specific moieties were held well away from the supporting matrix (Cuatrecasas, 1970). *p*-(*p*'-Aminophenoxypropoxy)benzamidine was selected as a compound structurally related to trypsin inhibitors with K_i values in the range 2–8 μ M (Baker & Erickson, 1968). The spacing group comprises an aromatic ring and a flexible ether-linked aliphatic chain. The successful use of affinity adsorbents based on *p*-(*p*'-aminophenoxypropoxy)benzamidine must be due to these structural features and/or their low K_i values. Table 1 lists the activation conditions, coupling yields, content of inhibitor and trypsin-binding capacity for affinity adsorbents containing this inhibitor. *N*- α -(*N*'-Methyl-*N*'-sulphanilyl)sulphanilylagmatine was chosen as an analogue of the known trypsin inhibitor, α -*N*-toluene-*p*-sulphonylagmatine (Rule & Lorand, 1964) for which the K_i value is of the order of 1 mM. The two sulphanilyl groups provide a linear rigid arm by which the agmatine moiety is linked to the matrix. Soya-bean trypsin inhibitor was chosen as a representative of the

natural trypsin inhibitors and the addition of a spacer was considered unnecessary.

Chromatographic behaviour of soya-bean trypsin inhibitor–Sephacrose conjugate

Bovine serum albumin was only slightly retarded when applied to a column of soya-bean trypsin inhibitor–Sephacrose conjugate in 50 mM-Tris–HCl buffer containing 10 mM-CaCl₂, pH 7.8, but bovine trypsin was bound to the adsorbent in this buffer and was subsequently eluted with 1 M-*n*-butylamine hydrochloride, a competitive inhibitor of trypsin ($K_i = 1.7$ mM; Inagami, 1964), at pH 3.0. A saturation experiment showed that the trypsin-binding capacity of the adsorbent, as measured by titration of the effluent with *p*'-nitrophenyl *p*-guanidinobenzoate, was 2.4 mg of trypsin/ml of gel. Bovine α -chymotrypsin bound to the adsorbent in pH 8.2 borate buffer (10 mM-Na₂B₄O₇, 2 mM-CaCl₂, 50 mM-NaCl, adjusted with HCl) and also in pH 6.0 10 mM-sodium cacodylate–HCl buffer containing 2 mM-CaCl₂. In both cases, the α -chymotrypsin was eluted with 0.5 M-*n*-butylamine hydrochloride in 0.1 M-acetic acid. These results resemble those reported by Feinstein (1970b). When chromatography was carried out in 0.1 M-sodium formate-formic acid buffer, pH 3.5, some selectivity for trypsin emerged; α -chymotrypsin was virtually unretarded, whereas most of the trypsin activity was retarded and appeared as a very broad peak immediately after a peak of inert protein. No evidence of the resolution of α - and β -trypsin was obtained by gradient elution of trypsin bound to the affinity adsorbent in pH 7.8 50 mM-Tris buffer containing 10 mM-CaCl₂. In these experiments, pH gradients (obtained by mixing the above Tris buffer with an equal volume of 0.05 M-sodium citrate buffer, pH 3.5), concentration gradients of *n*-butylamine hydrochloride (to 1 M at pH 7.8)

Table 1. Characteristics of the affinity adsorbents derived from *p*-(*p*'-aminophenoxypropoxy)benzamidine: relative amounts of CNBr used in activation of supports, efficiency of the inhibitor coupling reactions, inhibitor contents of the adsorbents and their actual trypsin-binding capacities

For details see the text. Available trypsin-binding capacity is calculated as a percentage of a theoretical trypsin-binding capacity equivalent to the inhibitor content of the adsorbent.

Support	CNBr used in activation (mg/ml of support)	Yield of inhibitor-coupling reaction (% coupled)	Inhibitor content adsorbents (μ mol/ml)	Trypsin-binding capacity of adsorbents (mg of enzyme/ ml of adsorbent)	Available trypsin-binding capacity of adsorbents (%)
Sephacrose 4B	100	94	0.940	3.46	15.4
Sephacrose 4B	100	44*	0.435	1.26	12.2
Sephacrose 4B	20	91	0.182	0.52	12.0
Sephacrose 2B	5	65	0.647	1.4, 1.1	8.9, 7.1
Cellulose powder	38	96	0.717	0.49	2.8

* The reason for this unexpectedly low value is not known.

and a combined gradient were all used. These results prompted us to investigate the possible use of insolubilized synthetic trypsin inhibitors, although other natural trypsin inhibitors have been shown to provide useful affinity adsorbents for trypsin (Robinson *et al.*, 1971; Feinstein, 1970*a,b*).

Chromatographic behaviour of N- α -(N'-methyl-N'-sulphanilyl)sulphanilylagmatine-Sephacrose conjugate

This agmatine derivative exhibited more selectivity for trypsin than did the soya-bean trypsin inhibitor-Sephacrose conjugate. Bovine α -chymotrypsin (5mg) applied to a column (0.6cm $\times$ 7cm) of N- α -(N'-methyl-N'-sulphanilyl)sulphanilylagmatine-Sephacrose conjugate and eluted with a pH 8.0 Tris buffer (10mm-Tris-HCl containing 2mm-CaCl₂, $I = 0.022$ mol/l) was only very slightly retarded, but bovine trypsin (5mg), chromatographed under the same conditions, yielded two peaks of protein. The first was largely inert protein and contained only 0.4mg of active trypsin as determined by titration with *p*'-nitrophenyl *p*-guanidinobenzoate. The second peak was very broad, with a long trailing edge, and contained 1.2mg of trypsin. Elution with 0.1M-acetic acid then yielded a third peak containing 0.6mg of trypsin. Titration with *p*'-nitrophenyl *p*-guanidinobenzoate at pH 4.0 showed that the last two peaks contained about the same proportions of α - and β -trypsins. Similar results were obtained when the trypsin was applied to the column in a pH 6.0 buffer (10mm-sodium cacodylate-HCl containing 2mm-CaCl₂, $I = 0.022$ mol/l) and again finally eluted with 0.1M-acetic acid. Replacement of the acetic acid elution step with a NaCl concentration gradient yielded all the enzyme remaining bound in a single peak (1.2mg of trypsin) at $I = 0.18$ mol/l. Ionic strength also markedly influenced the binding of trypsin to the affinity column in three buffers at pH 8.0. These buffers (10mm-Tris, 10mm-Na₂B₄O₇ and 10mm-sodium barbiturate, adjusted to pH 8.0 with HCl, H₃BO₃ and HCl respectively and all containing 2mm-CaCl₂) were adjusted to the required ionic strength with NaCl. For $I \geq 0.07$ mol/l, most of the trypsin was eluted in a single unretarded peak, whereas at $I = 0.02$ mol/l, two peaks were obtained and some trypsin remained bound as described above.

N- α -(N'-Methyl-N'-sulphanilyl)sulphanilylagmatine-Sephacrose conjugate clearly functions as a specific affinity adsorbent for bovine trypsin, but only at low ionic strengths. This undesirable property may be due to the high K_i values of agmatine derivatives or to the rigid linear structure of the inhibitor, or both. The elution of two main peaks, which both contain α - and β -trypsin, after the enzyme has been applied to the column in pH 6.0 or 8.0 buffers of $I = 0.02$ mol/l and eluted at low pH or high ionic strength is an additional unsatisfactory feature. This

behaviour may stem from the linkage of the agmatine derivative to Sepharose by more than one type of covalent bond, effectively forming multiple types of insolubilized inhibitor. Supporting this theory, it is known that *N*-substituted imidocarbonates, isourea derivatives and *N*-substituted carbamates are all formed when amines are coupled to CNBr-activated polysaccharides (Axén & Ernback, 1971). The additional possible complication that coupling might have involved the guanidino atoms is considered unlikely, since chemical tests showed that the affinity adsorbent did not contain free amino groups.

Chromatographic behaviour of p-(p'-aminophenoxy-propoxy)benzamidine-Sephacrose conjugate

When crude trypsin was applied to a column of this affinity adsorbent in 50mm-triethanolamine hydrochloride-triethanolamine buffer, pH 8.0, containing 10mm-CaCl₂, the enzyme was bound and a peak of inactive protein was soon eluted (Fig. 1*a*). Subsequent elution with 0.2M-KCl containing 1mm-HCl yielded only an insignificant quantity of protein. Development with 10mm-HCl containing 0.5M-*n*-butylamine hydrochloride eluted the bound trypsin in a single peak, which contained 86% of active enzyme and represented 80% of the original enzyme applied to the column. Subsequent experiments showed *n*-butylamine hydrochloride (≥ 0.25 M) to be an effective eluent in the pH range 2.0-8.0. When the inhibitor was applied in a concentration gradient in a pH 8.0 50mm-triethanolamine hydrochloride-triethanolamine buffer containing 10mm-CaCl₂, the bound trypsin was eluted in a single broad peak when the concentration of *n*-butylamine hydrochloride was approx. 0.13M. Various final eluents of pH 2.0-2.5 eluted the bound trypsin as effectively as solutions of *n*-butylamine hydrochloride. These included: 0.1M-citric acid-1M-NaCl, pH 2.0; 0.1M-formic acid, pH 2.4; 0.2M-formic acid-sodium formate buffer containing 0.5M-NaCl, pH 2.5. Less predictably, the bound trypsin was also eluted by water, although continuously rather than as a discrete peak. The elution by water of proteins bound to affinity adsorbents is uncommon but not unknown (Vahlquist *et al.*, 1971; Blumberg *et al.*, 1970).

The specificity of the affinity column was demonstrated by its failure to bind α -chymotrypsin significantly in a pH 6.0 buffer (50mm-sodium succinate-HCl containing 10mm-CaCl₂) (Fig. 1*b*), or in a pH 8.0 buffer (50mm-triethanolamine hydrochloride-triethanolamine containing 10mm-CaCl₂). The content of active α -chymotrypsin in the first peak was 94% of the total enzyme applied.

The trypsin-binding capacity of the affinity column was usually 1-3mg/ml of gel (Table 1), but only a small proportion (7-16%) of the insolubilized

Fig. 1. Chromatography of bovine trypsin and α -chymotrypsin on p -(p' -aminophenoxypropoxy)benzamidine-Seph-
arose conjugate

(a) Trypsin (20mg) was applied to a column (1.5 cm $\times$ 25 cm) of affinity adsorbent in 50mM-triethanolamine hydrochloride-triethanolamine buffer containing 10mM- CaCl_2 , pH8.0. The eluent was changed to 1mM-HCl containing 0.2M-KCl at the first arrow and then to 10mM-HCl containing 0.5M- n -butylamine hydrochloride at the second arrow. —, E_{280} ; $\circ$, active trypsin concentration as measured by titration with 4-methylumbelliferyl p -guanidinobenzoate. Flow rate, 24ml/h; fraction size, 6.0ml. (b) Chymotrypsin (20mg) was applied to the column in 50mM-sodium succinate-HCl buffer containing 10mM- CaCl_2 , pH6.0. The eluent was changed to 50mM-sodium succinate-HCl containing 0.5M-NaCl, pH6.0, at the first arrow and then to 0.2M-formic acid-sodium formate containing 0.5M-NaCl, pH2.5, at the second arrow. Flow rate, 20ml/h; fraction size, 4.9ml. Symbols are as in (a).

on p -(p' -aminophenoxypropoxy)benzamidine-Seph-
arose conjugate is a useful means of ridding trypsin
of other proteins, including α -chymotrypsin, and it
could be used to free α -chymotrypsin of contaminating
trypsin.

Chromatographic behaviour of p -(p' -aminophenoxy- propoxy)benzamidine-cellulose conjugate

Trypsin bound strongly to this adsorbent in buffers
composed either of 50mM-triethanolamine hydro-
chloride-triethanolamine, pH8.0, or of 0.2M-
 NH_4HCO_3 - NH_3 , pH8.4, both buffers containing
10mM- CaCl_2 . The binding of the enzyme was
unaffected by the inclusion of 0.5M-NaCl in the former
buffer. Complete elution of the bound enzyme could be
effected by solutions of low pH including: 0.1M-
citric acid-1M-NaCl, pH2.0; 0.1M-citric acid-0.1M-
NaCl, pH2.1; 20mM-HCl; 50mM-citric acid-50mM-
NaCl, pH2.4. In contrast, although dilute formic
acid easily eluted trypsin from p -(p' -aminophenoxy-
propoxy)benzamidine-Seph-
arose conjugate, 0.3M-
formic acid was only partially effective when the
enzyme was bound to the corresponding inhibitor-
cellulose conjugate.

Chromatography of bovine trypsin on p -(p' -
aminophenoxypropoxy)benzamidine-cellulose con-
jugate under conditions resembling those described
in Fig. 1 yielded a very similar elution profile, and the
main difference lies in the binding capacities of the
two affinity adsorbents. p -(p' -Aminophenoxyprop-
oxy)benzamidine-cellulose conjugate contained at
least as much inhibitor as did the corresponding
inhibitor-Seph-
arose conjugate, but the proportion
of coupled inhibitor available for binding trypsin was
much lower in the former case (Table 1). It is possible
that binding of enzyme may be subject to serious
steric hindrance as a result of the impermeable
crystalline structure of cellulose.

Isolation of β -trypsin by using p -(p' -aminophenoxy- propoxy)benzamidine-cellulose conjugate

Experiments with a pH gradient showed that it
was possible to elute pure β -trypsin specifically after
crude trypsin had been bound by the affinity adsorbent.
The effects of eluent composition are important and
are discussed below. From these early observations,
a stepwise elution scheme was developed which
offers a very convenient and rapid method
for isolating bovine β -trypsin (Fig. 2). Crude bovine
trypsin (20mg) was applied to a column of p -(p' -
aminophenoxypropoxy)benzamidine-cellulose con-
jugate in 50mM-triethanolamine hydrochloride-
triethanolamine buffer containing 10mM- CaCl_2 at
pH8.0. Elution with this buffer yielded a peak of
inert protein. An abrupt change of eluent to 50mM-
sodium acetate-acetic acid, pH5.0, gave a peak of

inhibitor was actually available for enzyme binding.
This phenomenon appears to be unrelated to the rela-
tive amount of CNBr used in the activation process
and is therefore unlikely to be due to a substantial
decrease in the gel pore size, which could arise if
cross-linking of the gel occurred. Chromatography

β -trypsin (5.1–5.3 mg). Assay with 4-methylumbelliferyl *p*-guanidinobenzoate showed that α -trypsin was absent and that the β -trypsin was 90–94% active. Its identity with β -trypsin, isolated by the method of Schroeder & Shaw (1968), was confirmed by measurement of the K_m and k_{cat} values by using *S*-(2-aminoethyl)-*N*-benzoyl-L-cysteine methyl ester as the substrate (Table 2) and by demonstrating the presence of only one *N*-terminal residue, isoleucine. After β -trypsin had been removed from the column, a further quantity of enzyme (5.2–5.3 mg), which was shown to contain 18% α -trypsin by titration with 4-methylumbelliferyl *p*-guanidinobenzoate, was eluted with a solution of 50 mM-citric acid containing 50 mM-NaCl, pH 2.3.

*Isolation of α - and β -trypsin by using *p*-(*p*'-aminophenoxypropoxy)benzamidine–Sephadex conjugate*

The behaviour of trypsin on this affinity adsorbent depends strongly on the composition of the eluent, and this is discussed below. A successful separation of α - and β -trypsin is shown in Fig. 3. In this experiment, crude bovine trypsin (50 mg) was applied to a column (1.5 cm $\times$ 25 cm) of *p*-(*p*'-aminophenoxypropoxy)benzamidine–Sephadex 4B conjugate in 50 mM-sodium acetate–acetic acid buffer, pH 4.0, and eluted at 55 ml/h. The three incompletely resolved peaks successively eluted were shown by titration with 4-methylumbelliferyl *p*-guanidinobenzoate to consist of inert protein, α -trypsin and β -trypsin respectively. The fraction containing β -trypsin was very dilute and it was necessary to concentrate the solution by ultrafiltration at 0°C and to desalt it on Sephadex G-25 before it was freeze-dried. The yields of α - and β -trypsin isolated were 3.8 and 12.4 mg respectively and the enzymes were obtained in states of purity ($\geq 97\%$) at least as good as those attainable by chromatography on sulphoethyl-Sephadex (Schroeder & Shaw, 1968). The identity of our preparations with those obtained by Schroeder & Shaw (1968) was again confirmed by measurement of K_m and k_{cat} with *S*-(2-aminoethyl)-*N*-benzoyl-L-cysteine methyl ester as substrate (Table 2). A firmly bound fraction of enzyme (3.4 mg)

was eluted with 0.1 M-citric acid containing 1 M-NaCl and contained 20% α -trypsin.

This procedure conveniently gives both α - and β -trypsin, but the latter is obtained as a very dilute solution; the method was therefore used only when α -trypsin was specifically sought. Since this form of the enzyme lost activity significantly in a few hours at pH 4.0 and 0°C, a high flow rate and rapid processing of the effluent were necessary to obtain good yields. β -Trypsin was much more stable in solution. The same relative stabilities were observed with freeze-dried samples of α - and β -trypsin prepared by the method of Schroeder & Shaw (1968) and stored at –20°C for approx. 2 years. Titration with 4-methylumbelliferyl *p*-guanidinobenzoate showed that β -trypsin had retained much of its activity, but α -trypsin was virtually inactive.

Fig. 2. Isolation of bovine β -trypsin by affinity chromatography of crude trypsin on *p*-(*p*'-aminophenoxypropoxy)-benzamidine–cellulose conjugate

Crude trypsin (20 mg) was applied to a column (2.2 cm $\times$ 14.3 cm) of affinity adsorbent in 50 mM-triethanolamine hydrochloride–triethanolamine buffer containing 10 mM-CaCl₂, pH 8.0. The eluent was changed to 50 mM-sodium acetate–acetic acid, pH 5.0, at the first arrow and to 50 mM-citric acid containing 50 mM-NaCl, pH 2.3, at the second arrow. —, E_{280} ; ○, active trypsin concentration as measured by titration with 4-methylumbelliferyl *p*-guanidinobenzoate. Flow rate, 19 ml/h; fraction size, 5.0 ml.

Table 2. k_{cat} and K_m values for the hydrolysis of *S*-(β -aminoethyl)-*N*-benzoyl-L-cysteine methyl ester at pH 8.0 and 25.0°C by α - and β -trypsin isolated both by affinity chromatography and by ion-exchange chromatography on sulphoethyl-Sephadex

Values are means $\pm$ s.d.

Enzyme	Source	k_{cat} (s ⁻¹)	$1 \times 10^4 K_m$ (M)	No. of runs
α -Trypsin	Affinity chromatography	10.3 $\pm$ 0.2	0.77 $\pm$ 0.03	9
	Ion-exchange chromatography*	8.8 $\pm$ 0.1	1.10 $\pm$ 0.04	10
β -Trypsin	Affinity chromatography	32.8 $\pm$ 0.5	1.07 $\pm$ 0.05	9
	Ion-exchange chromatography*	35.0 $\pm$ 0.6	1.04 $\pm$ 0.04	12

* Values taken from Roberts & Elmore (1974).

Effect of elution conditions on the resolution of α - and β -trypsin on p -(p' -aminophenoxypropoxy)benzamidine-cellulose and -Sephrose conjugates

The development of the foregoing successful methods for the isolation of α - and β -trypsin was the culmination of an extensive study of different elution conditions which revealed that the resolution of the mixture of enzymes depended on factors such as pH, ionic strength, nature of the buffer species and flow rate. The choice of support for the insolubilized inhibitor is another variable. The results of some of our experiments are summarized in Tables 3 and 4.

Discussion

The results of some of our experiments could not have been predicted in advance and cannot be fully explained in retrospect. For example, the order of elution of α - and β -trypsin from the p -(p' -phenoxypropoxy) benzamidine-cellulose complex depends on the nature of the eluting buffer (Fig. 2, Table 3). Some of the experiments summarized here indicate that successful separation of closely related proteins by affinity chromatography is largely serendipitous in the present state of our knowledge. This situation might be modified in the light of the critical appraisal (O'Carra *et al.*, 1974) of the current state of the art of affinity chromatography. It is probable that the behaviour of α - and β -trypsin on the various inhibitor conjugates is not due to bioaffinity alone but involves compound affinity as defined by O'Carra *et al.* (1974). The predominantly hydro-

Fig. 3. Separation of bovine α - and β -trypsin by affinity chromatography on p -(p' -aminophenoxypropoxy)benzamidine-Sephrose conjugate

Crude trypsin (50mg) was applied to a column (1.5cm $\times$ 25cm) of affinity adsorbent in 50mM-sodium acetate-acetic acid buffer, pH4.0. The eluent was changed to 0.1M-citric acid containing 1M-NaCl, pH2.0, at the arrow. —, E_{280} ; $\circ$, active trypsin concentration as measured by titration with 4-methylumbelliferyl p -guanidinobenzoate; $\bullet$, percentage of α -trypsin in the total trypsin present. Flow rate, 55 ml/h; fraction size, 9.2 ml.

Table 3. Effect of elution conditions on the resolution of α - and β -trypsin on p -(p' -aminophenoxypropoxy)benzamidine-cellulose conjugate

Loading buffer	Eluting buffer	Chromatographic result
50mM-Triethanolamine hydrochloride-triethanolamine containing 10mM- CaCl_2 , pH8.0	50mM-Sodium acetate-acetic acid, pH4.0	β -Trypsin contaminated with α -trypsin
	50mM-Sodium cacodylate-HCl, pH5.8	Similar to Fig. 2, but yield of β -trypsin was low
	50mM-Ammonium acetate-acetic acid, pH5.0	Very broad peak of β -trypsin
	pH gradient generated by adding 50mM-sodium acetate, pH4.0, to 50mM-sodium cacodylate-HCl, pH7.0 then 0.1M-citric acid-1M-NaCl.	β -Trypsin peak contained traces of α -trypsin in trailing edge. Citric acid-NaCl gave a peak containing both α - and β -trypsin.
0.2M-Ammonium formate- NH_3 , pH8.4	Two gradients generated by adding 50mM-ammonium formate-formic acid, pH4.0, to the loading buffer then adding 20mM-HCl to the formate buffer, pH4.0.	Two broad peaks only partially resolved. The first contained both α - and β -trypsin (pH4.1), the second contained β -trypsin (pH3.9).

Table 4. *Effect of elution conditions on the resolution of α - and β -trypsin on p -(p' -phenoxypropoxy)benzamidinium-Sepharose conjugate*

Loading buffer	Eluting buffer	Chromatographic result
50 mM-Sodium acetate-acetic acid, pH 3.5	Loading buffer	Unresolved peak containing inert protein and α -trypsin with β -trypsin in long trailing edge
50 mM-Sodium acetate-acetic acid, pH 4.0	Loading buffer	Similar to Fig. 3
50 mM-Sodium acetate-acetic acid, pH 4.5	Loading buffer	Similar to Fig. 3 but peaks were broader
50 mM-Sodium acetate-acetic acid, pH 5.0	Loading buffer	Peaks were broadened to give a continuum
50 mM-Sodium acetate-acetic acid, pH 4.0 containing 0.5 M-NaCl	Loading buffer	Similar to that obtained with pH 5.0 buffer
50 M-Ammonium formate-formic acid, pH 4.0	Loading buffer	No discrete peak of β -trypsin
	Loading buffer + 1 mM-cetyltrimethyl ammonium bromide	Elution volume of α -trypsin decreased
50 mM-Sodium succinate-HCl, pH 6.0, containing 10 mM-CaCl ₂	pH gradient generated by adding 0.2 M-formic acid-NaOH, pH 2.5, to loading buffer; both buffers contained 0.5 M-NaCl	Two incompletely resolved peaks were eluted at pH 3.0 and pH 2.8; the first peak contained β -trypsin, the other contained a mixture of α - and β -trypsin

phobic character of the spacer arm may cause some of the subsites of the enzyme to be occupied and bring part of the surface of the enzyme into juxtaposition with the support thereby facilitating additional interactions by hydrogen bonding. If so, the differences in chromatographic behaviour of the inhibitor conjugate derived from cellulose and Sepharose can be partially explained. Nevertheless, binding of trypsin presumably involves the active site, since the enzyme can be displaced by the reversible inhibitor, *n*-butylamine.

'Firmly-bound' trypsin

This description is applied to trypsin which remained bound to conjugates derived from p -(p' -aminophenoxypropoxy)benzamidinium after elution with acetate buffers, pH 4.0 or 5.0, or with 20 mM-butylamine hydrochloride. The retention of some 'firmly bound' trypsin appeared to be independent of the relative amount of CNBr used to activate the support and of the nature of the support. The 'firmly bound' trypsin was eluted, however, with solutions of citric acid, formic acid or HCl at pH 2 or with solutions of *n*-butylamine hydrochloride (≥ 0.2 M). It always proved to be a mixture of the two forms of trypsin, containing 15–20% α -trypsin. All attempts to resolve the 'firmly bound' α - and β -trypsin by gradient elution with both pH gradients and *n*-butylamine concentration gradients were unsuccessful.

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