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ISOLATION AND CHARACTERIZATION OF TRYPSIN INHIBITORS FROM COWPEA (*VIGNA UNGUICULATA*)

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Key Word Index—*Vigna unguiculata*; Leguminosae; cowpea; protease inhibitors; characterization; subunit structure.

Abstract—The trypsin inhibitor fraction from cowpea (*Vigna unguiculata*) has been purified and characterized. Although the total trypsin inhibitor as purified by affinity chromatography on immobilised trypsin was shown to be heterogeneous by gel electrophoresis and isoelectric focusing as well as by function, it was relatively homogeneous in MW (*ca* 17 000) on gel filtration. The total trypsin inhibitor was divided into inhibitors active against trypsin only and active against trypsin and chymotrypsin by affinity chromatography on immobilised chymotrypsin. The 'trypsin-only' inhibitor was the major component of the total trypsin inhibitor. It was shown by isoelectric focusing and gel electrophoresis to contain several isoinhibitors. Determination of the combining weight of this inhibitor and investigation of the complexes formed with trypsin by gel filtration indicated the presence of two protease binding sites per inhibitor molecule. The chymotrypsin/trypsin inhibitor was also shown to be composed of several isoinhibitors. On the basis of gel electrophoresis and gel filtration in dissociating and non-dissociating media both inhibitors were considered to be dimeric molecules with the subunits linked by disulphide bonds; this implies that the 'trypsin-only' inhibitor has one binding site per subunit.

INTRODUCTION

The presence of a trypsin inhibitor in the cowpea (*Vigna unguiculata*, previously known as *V. sinensis*) was first reported in 1947 by Borchers *et al.* [1] and later by Jaffé [2] and others [3]. Initial characterization of the cowpea 'trypsin' inhibitor was carried out by Ventura and Filho [4], who reported that it was essentially a chymotrypsin inhibitor with a MW of *ca* 17 000 although in a later paper this value was revised to be between 13 000–10 000 [5], and had a molar stoichiometry of 1:1 with α-chymotrypsin and 1:1 with trypsin. Later work in this field was carried out by Gennis and Cantor [6] on the isolation and characterization of two protease inhibitors from black-eyed peas (probably *Vigna unguiculata*). These authors found that one of the inhibitors inhibited both trypsin and chymotrypsin simultaneously, whilst the other inhibited two molecules of trypsin simultaneously. The inhibitors had a similar subunit MW of *ca* 8000 on SDS-polyacrylamide gel electrophoresis, and each gave a single band on isoelectric focusing. Investigations of inhibitor subunit interactions showed there was a complex set of multiple equilibria between monomer, dimer and tetramer at either pH 8 or 3, with the dimer normally the predominant species, with an apparent MW of 16–17000, as also found initially by Ventura [4]. Because of this multiple equilibrium of inhibitor types, many different complexes were considered possible between an inhibitor and the two proteases. Each inhibitor was presumed to have two binding sites per subunit and half-site reactivity was invoked to explain the failure to find any evidence of two molecules of protease binding per subunit. Gennis and Cantor carried out amino acid analyses on the two protease inhibitors [8], and these results were significantly different from those obtained for the amino acid composition of the protease inhibitor extracted by Ventura, but were similar to each other.

The present paper describes the purification and characterization of the trypsin inhibitor fraction from cowpea, and its division into inhibitors active against trypsin only and active against both trypsin and chymotrypsin. In this paper the total trypsin inhibitor fraction is designated total trypsin inhibitor, the trypsin inhibitor fraction active against trypsin only is designated trypsin inhibitor, and the fraction active against both trypsin and chymotrypsin is designated chymotrypsin–trypsin inhibitor. The stoichiometry of the trypsin inhibitor complexes with trypsin has been examined by several techniques. On the basis of these and other results a model for the subunit structure of this protease inhibitor is proposed.

RESULTS AND DISCUSSION

Purification of total trypsin inhibitor

The total trypsin inhibitor from dried mature seeds of the cowpea was purified from an albumin extract by affinity chromatography on a trypsin–Sepharose conjugate. The functional purity of the eluted total trypsin inhibitor was checked by rebinding to a trypsin affinity column, when all the protein bound and no non-specific binding of proteins or other materials could be detected. The UV absorbance spectrum of the purified

trypsin inhibitor showed no absorbance which could not be assigned to protein; further, on chromatography of this material on DE-cellulose no fractions were detected that did not possess trypsin inhibitory activity. The yield of inhibitor was *ca* 3 mg per g of seed meal.

Analysis of total trypsin inhibitor

Polyacrylamide gel electrophoretic band patterns of total trypsin inhibitor from several varieties of *V. unguiculata* were complex, but virtually identical between varieties, as shown by densitometric scanning (Fig. 1). Isoelectric focusing of the total trypsin inhibitor from 7 different cowpea varieties also showed a complex band pattern which was qualitatively the same between varieties. Essentially all the inhibitor focused between the pH range 4–5, as indicated by the standard protein pI markers run on the same slab (Fig. 5).

SDS gel electrophoresis of the total trypsin inhibitor was performed in a variety of acrylamide gels using both a continuous and discontinuous buffer system. Electrophoresis of samples not treated with 2-mercaptoethanol gave a band pattern consisting of a major band of MW 13 000 ± 500 (mean of 6 determinations) and two minor bands representing MWs of 16 000 and 18 000 (Fig. 2). In the presence of 2-mercaptoethanol the band at MW 13 000 was unchanged, but the bands at higher MWs were very much lessened and indistinct bands at lower MW (*ca* 8000) appeared (Fig. 2). Carboxymethylation of the protein after reduction led to the disappearance of the higher MW components.

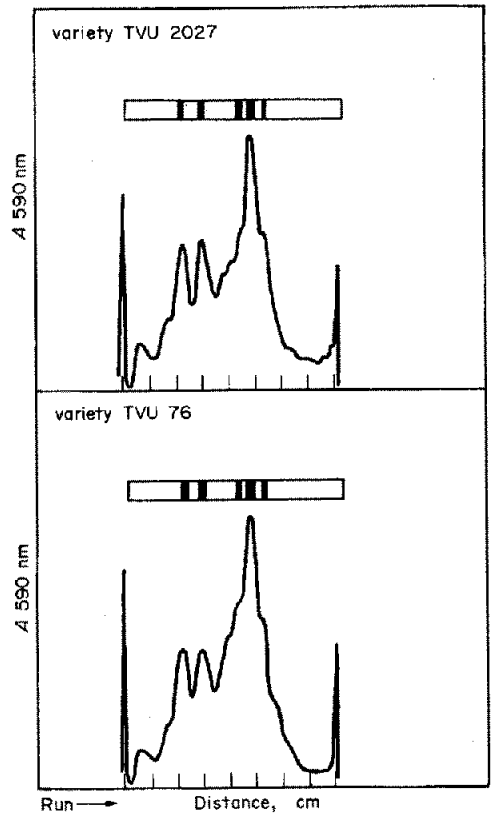

Fig. 1. Electrophoresis of total trypsin inhibitor from two varieties of cowpea at pH 8.3 in 7% polyacrylamide gels. Densitometric scans of stained gels.

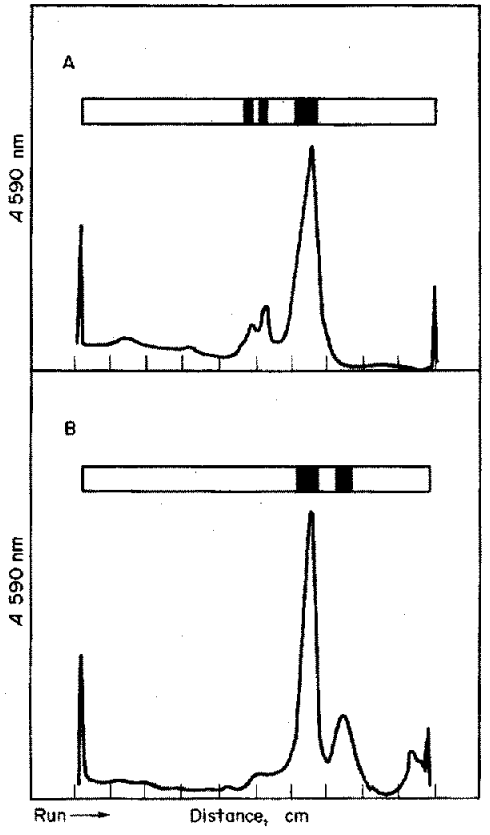

Fig. 2. SDS-polyacrylamide gel electrophoresis of total trypsin inhibitor of cowpea. Densitometric scans of stained gels (12% acrylamide tube gels). A: Unreduced protein; B: protein treated with 2-mercaptoethanol.

Gel filtration

A MW value of 17 000 ± 800 (mean of 3 determinations) was determined for the total trypsin inhibitor by gel filtration on Sephadex G-75 (Fig. 3). Although the inhibitor chromatographed as a single peak it was slightly broader than those of the standard proteins indicating a small degree of heterogeneity in MW. This behaviour on gel filtration may be contrasted to that of the black-eyed pea inhibitor isolated by Gennis and Cantor [7] which separated into 3 peaks due to a slow monomer–dimer–tetramer equilibrium, and to that of lima bean trypsin inhibitor [7] which ran as a very broad peak indicating a fairly rapid monomer–dimer equilibrium. Thus the 17 000 MW value of the cowpea trypsin inhibitor may represent a dimer, but not a dimer in equilibrium with monomer or other oligomers, at least at pH 7–9.

The subunit structure of the total trypsin inhibitor was investigated by gel filtration in 6 M guanidine hydrochloride, when the fully reduced and carboxymethylated inhibitor eluted as 3 components (Fig. 4). On the basis of its chromatographic behaviour in comparison to that of standard proteins (legumin, vicilin, IgG, SBTI, ribonuclease and insulin) under similar conditions, the components could be assigned MW 8000, 5000 and 3000. No peak, or trace of a

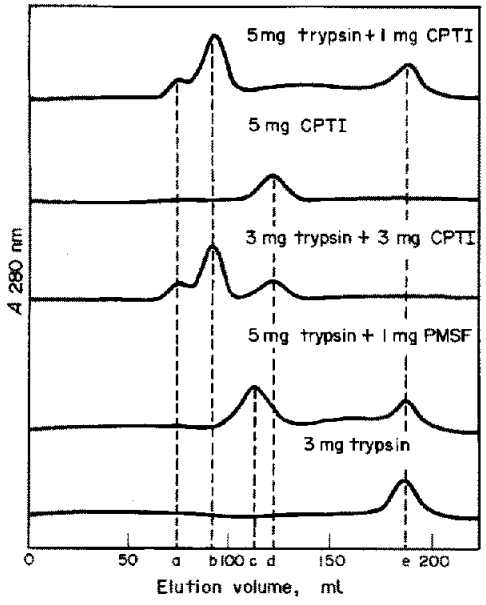

Fig. 3. Gel filtration of cowpea total trypsin inhibitor (CPTI), trypsin and mixtures of the two on a column of Sephadex G-75. a,b = CPTI–trypsin complexes; c = trypsin; d = CPTI; e = autolysed trypsin; PMSF = phenylmethylsulphonyl fluoride. Cowpea trypsin inhibitor gave identical profiles.

peak, was seen at elution volumes corresponding to MW components at 16–18 000 or 13 000.

Amino acid composition and N*-terminal analysis*

The results shown in Table 1 give the amino acid composition of the major fraction of the total trypsin inhibitor (from *V. unguicultata*) as purified by ion-exchange chromatography on DEAE-cellulose. It has a high cystine content, as do some other plant protease inhibitors in which the cystine is involved in very extensive disulphide cross-linking of the polypeptide chain [9]. The amino acid composition of the inhibitor was similar to those reported by Gennis and Cantor [8] for inhibitors isolated from black-eyed peas.

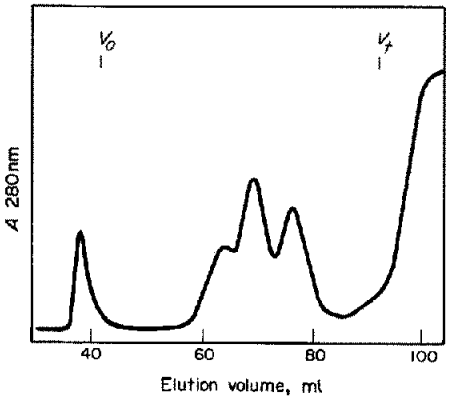

Fig. 4. Gel filtration of reduced and carboxymethylated cowpea total trypsin inhibitor on a column of Sephacryl S-200 in 6 M guanidine hydrochloride, 1 mM dithiothreitol. Material eluting at V_o; blue dextran. Material eluting at V_t; non-protein material.

Table 1. Amino acid composition of the major fraction of cowpea total trypsin inhibitor

Amino acid	Mol %
Lysine	6.2±0.2
Histidine	3.8±0.1
Arginine	4.8±0.3
Cystine	16.0±0.5
Aspartic acid	13.8±0.2
Threonine	3.7±0.1
Serine	13.5±0.4
Glutamic acid	7.5±0.3
Proline	7.4±0.6
Glycine	2.5±0.0
Alanine	3.5±0.3
Valine	7.7±0.5
Methionine	1.2±0.1
Isoleucine	3.6±0.1
Leucine	2.5±0.2
Tyrosine	1.0±0.1
Phenylalanine	1.3±0.1

Values and errors from duplicate determinations, uncorrected for decomposition.

The *N*-terminal amino acids of the total trypsin inhibitor were serine and asparagine; the presence of two amino terminal groups is a probable indicator of a degree of heterogeneity.

Inhibition of trypsin

The combining weight of the total trypsin inhibitor (i.e. the amount of inhibitor equivalent to 1 mol trypsin) was determined by measuring the inhibition of the hydrolysis of BAPNA by a known amount of trypsin in the presence of varying amounts of purified inhibitor solution of known concentration. The degree of inhibition was compared with that produced by soyabean trypsin inhibitor of known equivalence to trypsin in order to calculate a combining weight for cowpea total trypsin inhibitor independent of the activity of the trypsin. The resulting data gave an equivalence of 0.40 mg of inhibitor to 1 mg of trypsin and hence a combining weight of 9400. Determination of the combining weight of the total trypsin inhibitor by assaying uncomplexed trypsin with the active site titrant reagent NPGB gave a value of 9200.

Inhibition of chymotrypsin

The total trypsin inhibitor was also shown to be capable of inhibition of chymotrypsin; using BTEE as substrate virtually complete inhibition could be achieved. By using known quantities of inhibitor and enzyme, the equivalence was shown to be 4.2 mg of inhibitor to 1 mg of chymotrypsin. Thus the chymotrypsin inhibitory activity is *ca* one-tenth that of the trypsin inhibitory activity; this is about the same ratio as found in the seed, indicating that all the chymotrypsin inhibitory activity can be accounted for by the trypsin inhibitor [10].

Since the total trypsin inhibitor was found to contain a small amount of chymotrypsin inhibitory activity, and other analyses had indicated its heterogeneity, it was necessary to further purify the inhibitor.

Separation of trypsin and chymotrypsin–trypsin inhibitors

The trypsin and chymotrypsin–trypsin inhibitors were separated by affinity chromatography on a column of chymotrypsin linked to Sepharose. The unbound fraction was active against trypsin but devoid of chymotrypsin inhibitory activity, whereas the fraction which bound and was subsequently eluted at pH 2.1 contained inhibitory activity towards both trypsin and chymotrypsin. The ratio of the two inhibitor fractions was *ca* 1:4 chymotrypsin–trypsin:trypsin.

Analysis of separated inhibitors

The separation of the trypsin and chymotrypsin–trypsin inhibitors was determined by analysis by isoelectric focusing (Fig. 5). On isoelectric focusing both inhibitors still showed heterogeneity although the combined band patterns of the trypsin and chymotrypsin–trypsin inhibitors corresponded exactly to that obtained for the total trypsin inhibitor. The trypsin inhibitor focused at higher pI (4.5–5) than the chymotrypsin–trypsin inhibitor (4–4.2). Separation of the two inhibitors was also accomplished by SDS-gel electrophoresis under non-reducing conditions (Fig. 6). The trypsin and chmyotrypsin–trypsin inhibitors each gave more than one band, but those of the trypsin inhibitor were of lower MW (12–15 000) than those of the chymotrypsin–trypsin inhibitor (16–20 000 MW). The heavy, spread and irregular bands reproducibly given by the trypsin inhibitor on SDS-gel electrophoresis, even at low concentrations (Fig. 6), indicates that this protein is running anomalously on SDS-gel electrophoresis and that the indicated MW may be incorrect; because of this, gel filtration may give a better estimate of the MW. The chymotrypsin–trypsin inhibitor bands correspond to the bands at higher MW seen in total trypsin inhibitor (Fig. 6).

Equivalence of trypsin inhibitor and chymotrypsin–trypsin inhibitor to trypsin

Using the active site titrant substrate NPGB a molecular combining weight of 8100 was determined for the trypsin inhibitor. This combining weight suggests two binding sites for trypsin per 17 000 MW molecule. A molecular combining weight of 12 500 was determined for the chymotrypsin–trypsin inhibitor.

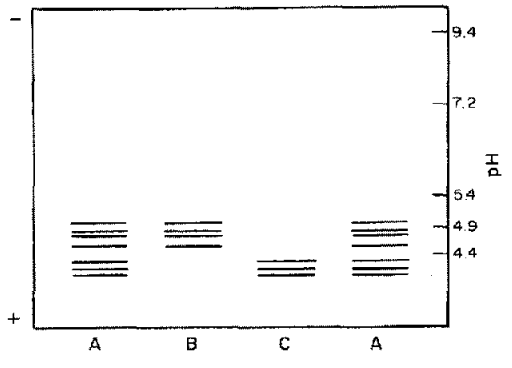

Fig. 5. Isoelectric focusing of cowpea trypsin inhibitors. A = Total trypsin inhibitor; B = trypsin inhibitor; C = chymotrypsin–trypsin inhibitor. pH gradient from standard proteins run on same slab.

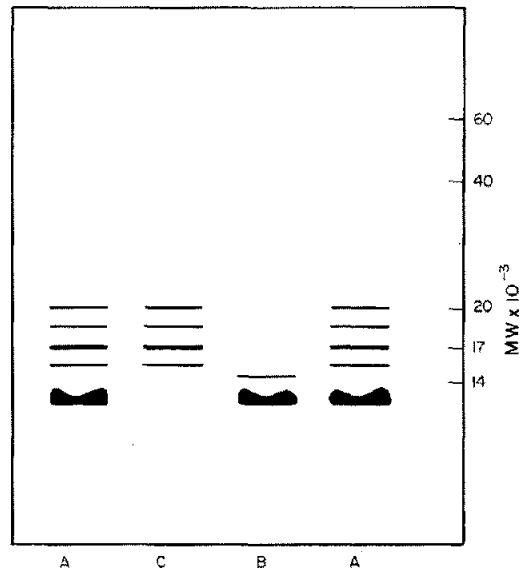

Fig. 6. SDS-gel electrophoresis of cowpea trypsin inhibitors under non-reducing conditions (17% acrylamide slab gel). A = Total trypsin inhibitor; B = trypsin inhibitor; C = chymotrypsin–trypsin inhibitor. MW scale from standard proteins run on same slab.

Determination of complex formation between inhibitor and enzyme

Gel filtration on a Sephadex G-75 column of mixtures of trypsin inhibitor and trypsin gave peaks that could not be due to either molecule alone and thus must represent complexes of the two. Elution profiles for the various complexes are given in Fig. 3 together with the elution profiles for the inhibitor and trypsin. Since trypsin alone, and trypsin in excess to that complexed, was found to digest itself almost completely during passage through the column, trypsin inactivated with phenylmethyl sulphonyl fluoride was used to give an elution profile for intact trypsin molecules.

The complex species present are at indicated MWs of 33 500 and 51 000. The most reasonable explanation for this behaviour is that the former complex represents the addition of one molecule of trypsin to one molecule of inhibitor, the latter addition of two molecules of trypsin to one of inhibitor.

The number of trypsin binding sites was confirmed by determining the inhibitory capacity of the two complexes. After their isolation, the complex between one molecule of inhibitor and two molecules of trypsin (2:1 complex) was found to be inactive in the inhibition of tryptic hydrolysis of NPGB, whereas the 1:1 complex was found to cause inhibition having a molecular combining weight with trypsin of 16 700; the value is *ca* twice that of the trypsin inhibitor. This would appear to indicate that half the number of trypsin binding sites have been blocked resulting in the 1:1 complex being only half as active as the pure inhibitor. Since only one molecule of enzyme was complexed to the inhibitor it is reasonable to conclude that there are two trypsin binding sites per inhibitor molecule.

Subunit structure of cowpea trypsin inhibitors

Although the total trypsin inhibitor of *V. unguiculata* has been shown to be heterogeneous by a number of criteria, it is relatively homogeneous in MW i.e. both trypsin and chymotrypsin–trypsin inhibitors have MWs near 17 000. Gel filtration in a strongly dissociating and reducing medium (6 M guanidine hydrochloride containing dithiothreitol) shows the presence of only lower MW species, indicating that the inhibitors are dissociated into subunits.

SDS gel electrophoresis also shows lower MW species under reducing conditions, whereas under non-reducing conditions bands in the 13 000–18 000 MW range are seen, indicating disulphide bonding between subunits. However, the results of SDS gel electrophoresis imply that reduction is not complete under the conditions used to prepare samples for electrophoresis, since the species observed on SDS-PAGE under reducing conditions have MWs larger than those observed on gel filtration in guanidine hydrochloride and dithiothreitol. Reduction of plant protease inhibitors with a high cysteine content is known to be difficult [9] due to extensive cross-linking of the polypeptide chain(s) through disulphide bonds; this is likely to be the case with the *V. unguiculata* trypsin inhibitors also.

The chymotrypsin–trypsin inhibitor is probably a dimer, with its subunits linked by disulphide bonds since the bands at 16 000–18 000 MW seen due to this inhibitor on SDS-gel electrophoresis under non-reducing conditions are replaced by bands at *ca* 8000 MW under reducing conditions. This inhibitor seems to be similar to that initially reported by Ventura and Filho [4]. It is heterogeneous in charge and MW, being composed of several related species we term isoinhibitors. Since this inhibitor is a comparatively minor component of the cowpea total trypsin inhibitor, we have not yet investigated it further.

The trypsin inhibitor of *V. unguiculata* also contains a number of isoinhibitors since it is heterogeneous in charge and MW. This inhibitor seems to be a disulphide-bonded dimer that is resistant to reduction, but which under strongly dissociating and reducing conditions gives species at 8000, 5000 and 3000 MW. We consider these to represent intact subunits, and large and small fragments of trypsin-cleaved subunits, respectively, since cleavage of trypsin inhibitor polypeptides on trypsin affinity chromatography is a well-established phenomenon [11]. It is possible that some of the heterogeneity observed both in this inhibitor and the chymotrypsin–trypsin inhibitor is due to the method of isolation; however, chymotrypsin affinity chromatography does not introduce any further heterogeneity (Figs. 5 and 6) so proteolysis of these inhibitors other than at their active sites seems unlikely. The dimeric structure proposed for this inhibitor is supported by determination of its complex formation with trypsin, which shows two binding sites per molecule, i.e. one site per subunit. The trypsin inhibitor MW may thus be estimated at *ca* 16 000 on the basis of combining weight and gel filtration; it is probably slightly less than that of the chymotrypsin–trypsin inhibitor. This major inhibitor of the cowpea is distinct from the inhibitor of Ventura and Filho in being inactive against chymotrypsin.

The two inhibitors isolated from *V. unguiculata* seeds in the present work seem to be analogous to those isolated by Gennis and Cantor [6] from black-eyed peas, although the black-eyed pea inhibitors are stated to be homogeneous proteins. Furthermore, certain reported properties of the black-eyed pea inhibitors differ from those found for the cowpea inhibitors (e.g. pI values, oligomerization). These differences may represent differences between varieties or even species rather than discrepancies in experimental results.

EXPERIMENTAL

Materials. Seeds of *V. unguiculata* varieties were obtained from the International Institute of Tropical Agriculture, Ibadan, Nigeria. Cyanogen bromide, trypsin (Type I, from bovine pacreas), and the enzyme substrates α-*N*-benzoyl-DL-arginine-*p*-nitroanilide HCl, (BAPNA) and *N*-benzoyl-L-tryosine ethyl ester (BTEE) were obtained from Sigma. *p*-Nitrophenyl-*p'*-guanidinobenzoate HCl (NPGB) was supplied by United States Biochemical Corp. α-Chymotrypsin was obtained from Worthington. Sepharose 4B, Sephadex G-25 fine, Sephadex G-75 fine and Sephacryl S-200 superfine were supplied by Pharmacia. Buffer components and reagents were obtained from BDH and were of analytical grade when necessary.

Isolation and purification of trypsin inhibitor. Cowpea seed meal (40 g, prepared by grinding seeds for 30 sec in a water-cooled mill) was extracted for 16 hr at 4° in 200 ml 0.1 M NaOAc buffer, pH 4, containing, 0.3 M NaCl, 0.01 M $CaCl_2$. Extract was centrifuged at 9000 *g* for 30 min and clear supernatant was applied to a column of trypsin linked to Sepharose (120 ml vol, *ca* 4 mg protein per ml gel) equilibrated with extraction buffer, and washed until all unbound material had eluted. The trypsin inhibitor was eluted with 0.01 M HCl containing 0.3 M NaCl, 0.01 M $CaCl_2$ at pH 2.1. Separation of the trypsin and chymotrypsin–trypsin inhibitors was achieved by affinity chromatography as described above on a column of chymotrypsin linked to Sepharose 4B. (15 ml vol., *ca* 3 mg protein per ml of gel). Fractions from affinity columns were desalted by chromatography on a column of Sephadex G-25 fine equilibrated with ammonium hydrogen carbonate (0.1 M), and lyophilized. Trypsin, or chymotrypsin, were coupled to Sepharose 4B by the method of ref. [12].

Enzyme assays. Trypsin inhibitory activity was determined by measuring inhibition of the rate of trypsin catalysed hydrolysis of the substrate BAPNA [13] and by active site titration of uncomplexed trypsin using NPGB [14]. α-Chymotrypsin inhibitory activity was determined by measuring inhibition of the rate of chymotrypsin catalysed hydrolysis of BTEE [15].

Gel filtration. MW determinations were carried out on a column of Sephadex G-75 fine packed and equilibrated by upward flow, with Na-Pi buffer (25 mM, pH 7.2, containing 0.1 M NaCl), or, for the determination of enzyme–inhibitor complex formation, ammonium hydrogen carbonate (0.1 M) buffer. The column was calibrated using a series of protein standards (ribonuclease, myoglobin, soyabean trypsin inhibitor, chymotrypsinogen, β-lactoglobulin, ovalbumin and BSA; MW range 12 400–67 000). Gel filtration in 6 M guanidine hydrochloride of reduced carboxymethylated protein samples was carried out on a column of Sephacryl S-200 superfine equilibrated with Tris–HCl buffer (50 mM, pH 8.1) containing 6 M guanidine hydrochloride and 1 mM dithiothreitol [16].

Isoelectric focusing was carried out in 5% acrylamide slab gels in the pH range 3.5–10 according to the methods of ref.

[17]. Protein standards (ferritin, β-lactoglobulin, conalbumin, myoglobin (horse and whale), ribonuclease and cytochrome *c*; pI range 4.4–10.65) were used to determine the pH gradient. Samples for isoelectric focusing were prepared by dissolving the lyophilized material in H_2O at a concn of 2 mg/ml.

SDS gel electrophoresis was carried out at pH 7.2 in 10% acrylamide tube gels according to the method of ref. [18] using the continuous system, and in 17% acrylamide slab gels at pH 8.3 using the discontinuous system of ref. [19]. *S*-Carboxymethylation was carried out using the method of ref. [20]; samples which had been carboxymethylated were subsequently separated on 12% acrylamide gels, using the continuous system.

Non-SDS discontinuous gel electrophoresis was carried out at pH 8.3 in 7% acrylamide tube gels [21].

Amino acid analysis. Amino acid compositions were determined on a Locarte automatic-loading amino acid analyser [22]: prior to analysis duplicate samples of the protein in 6 M HCl were hydrolysed *in vacuo* at 105° for 22 hr. The sulphur amino acid, cystine, was determined after performic acid oxidation of the samples [23].

N-Terminal analysis. This was carried out by the dansyl-Edman method [24].

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