A Trypsin and Chymotrypsin Inhibitor from Chick Peas (*Cicer arietinum*)

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1. A trypsin and chymotrypsin inhibitor was isolated by extraction of chick-pea meal at pH 8.3, followed by $(\text{NH}_4)_2\text{SO}_4$ precipitation and successive column chromatography on CM-cellulose and calcium phosphate (hydroxyapatite). 2. The inhibitor was pure by polyacrylamide-gel and cellulose acetate electrophoresis and by isoelectric focusing in polyacrylamide gels. 3. The inhibitor had a molecular weight of approx. 10000 as determined by ultracentrifugation and by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. A molecular weight of 8300 was resolved from its amino acid composition. 4. The inhibitor formed complexes with trypsin and chymotrypsin at molar ratios of 1:1. 5. Limited proteolysis of the inhibitor with trypsin at pH 3.75 resulted in hydrolysis of a single -Lys-X- bond and in consequent loss of 85% of the trypsin inhibitory activity and 60% of the chymotrypsin inhibitory activity. Limited proteolysis of the inhibitor with chymotrypsin at pH 3.75 resulted in hydrolysis of a single -Tyr-X- bond and in consequent loss of 70% of the trypsin inhibitory activity and in complete loss of the chymotrypsin inhibitory activity. 6. Cleavage of the inhibitor with CNBr followed by pepsin and consequent separation of the products on a Bio Gel P-10 column, yielded two active fragments, A and B. Fragment A inhibited trypsin but not chymotrypsin, and fragment B inhibited chymotrypsin but not trypsin. The specific trypsin inhibitory activity, on a molar ratio, of fragment A was twice that of the native inhibitor, suggesting the unmasking of another trypsin inhibitory site as a result of the cleavage. On the other hand, the specific chymotrypsin inhibitory activity of fragment B was about one-half of that of the native inhibitor, indicating the occurrence of a possible conformational change.

The presence of proteinase inhibitors in legume seeds is a well-known fact (Laskowski & Laskowski, 1954; Birk, 1968; Pusztai, 1967). The inhibitors most thoroughly investigated were those from soya beans, lima beans and groundnuts (Birk, 1974). The importance of legume seeds as a rich source of proteins for animal and human consumption also promoted the studies of inhibitors. Nutritional investigations of soya-bean proteins showed that the trypsin inhibitors were not responsible for the low nutritional value of raw soya beans found for birds and mammals, but they caused pancreatic hypertrophy (Gertler *et al.*, 1967) and governed the balance of biosynthesis and secretion of pancreatic enzymes (Konijn *et al.*, 1970a,b; Madar *et al.*, 1974).

Studies on the inhibitory sites of several of the trypsin inhibitors from plant sources indicated that they were 'double-headed', exhibiting separate independent sites for trypsin and chymotrypsin inhibition. Examples of this were the Bowman-Birk soya-bean inhibitor (AA) and the lima-bean inhibitor (Birk *et al.*, 1967; Krahn & Stevens, 1970), in which the reactive sites were also the inhibitory sites (Stevens, 1971). In some inhibitors, of which the

groundnut inhibitor was an example, the trypsin-reactive site was not its trypsin-inhibitory site. Moreover, it was rather likely that the trypsin-reactive site of the groundnut inhibitor coincided with or was in the vicinity of the chymotrypsin-inhibiting site (Birk, 1974).

The presence of trypsin- and chymotrypsin-inhibiting activity in chick-pea (*Cicer arietinum*) extracts was known for a long time (Laskowski & Laskowski, 1954; Chernikov *et al.*, 1966), but very little information was available on the nature and mode of action of the pure inhibitor (Birk, 1974; Levinsky *et al.*, 1975). It was the aim of the present work to study the biochemical properties of the inhibitors from chick peas.

Experimental

Assay of trypsin- and α -chymotrypsin-inhibiting activity

Inhibition of proteolysis was determined by Kunitz's casein digestion method at pH 7.6 (Kunitz, 1947; Laskowski, 1955). Inhibiting activity was de-

fined in trypsin-inhibiting units and chymotrypsin-inhibiting units (Kunitz, 1947; Birk *et al.*, 1963). Inhibition of the esterolytic activity of trypsin and chymotrypsin was determined on tosyl-L-arginine methyl ester, 0.01 M in reaction mixture, and acetyl-L-tyrosine ethyl ester, 0.01 M in reaction mixture respectively, at pH 8.1, 25°C in a Gilford 2400 spectrophotometer. The reaction mixture was composed of either 25 μ l of enzyme solution (containing 2.5 μ g of trypsin) or 30 μ l of enzyme solution (containing 3 μ g of chymotrypsin), plus 5–20 μ l of aq. 0.1% solution of inhibitor, which were preincubated for 5 min at room temperature in 1 ml of 46 mM-Tris/11.5 mM-CaCl₂ buffer, pH 8.1. The reaction was started by the addition of 100 μ l of the enzyme plus inhibitor solution to 3 ml of substrate solution. Inhibition of esterolysis was expressed in inhibition units, which were defined as μ mol of substrate hydrolysed by 1 mg of enzyme per min of reaction.

Protein content was estimated by E_{280} measurements. A protein solution giving $E_{1\text{cm}}^{1\%} = 1.00$ was defined as possessing 1 absorbance unit/ml. Specific inhibitory activity was defined as the number of trypsin-inhibiting or chymotrypsin-inhibiting units per absorbance unit.

Preparation of the inhibitor

Commercial chick peas served as starting material. The seeds were ground finely to flour. All operations were performed at room temperature. A portion of chick-peas flour (400 g) was suspended in 4 litre of 0.2 M-sodium phosphate buffer, pH 8.3. The suspension was stirred for 2 h. The undissolved residue was removed by centrifugation at 23 300 g (r_{av} 13.8 cm) for 20 min at 2°C in a Sorvall Superspeed RC2-B model centrifuge. The supernatant was filtered and the resulting yellowish solution (3225 ml) was designated 'initial extract'. The latter was then treated with 60% satd. (NH₄)₂SO₄. The resulting precipitate was collected by centrifugation at 23 300 g (r_{av} 13.8 cm) at 2°C for 20 min in a Sorvall Superspeed RC2-B model centrifuge, dissolved in a minimum volume of water and then dialysed swiftly against water (12 changes, every hour, against 10 litre of water, each). It was treated with 2 M–5 mM-acetic acid, pH 4.0, and applied to a column (4.0 cm $\times$ 55 cm) of CM-cellulose equilibrated with 5 mM-sodium acetate buffer, pH 4.0. Elution was performed first with 1 litre of 5 mM buffer followed by a gradient to 0.16 M-NaCl in starting buffer (mixing chamber volume 1 litre) and then by a second gradient to 0.32 M-NaCl in starting buffer (mixing chamber volume 1 litre). The flow rate was 450 ml/h, and 15 ml fractions were collected. The active fraction that emerged with the last gradient contained most of the inhibitor. It was then desalted by swift dialysis against water and kept as a freeze-dried preparation (yield about 350 mg). For further purifi-

cation, the freeze-dried preparation was dissolved in 20 ml of 0.05 M-sodium phosphate buffer, pH 6.8, and applied to a column (4 cm $\times$ 22 cm) of calcium phosphate (hydroxyapatite) (Tiselius *et al.*, 1956; Levin, 1962). Elution was first performed with 400 ml of 0.05 M-phosphate buffer, pH 6.8, and then with 400 ml of 0.25 M of the same buffer. The flow rate was 100 ml/h and approx. 6 ml fractions were collected. The fraction containing the inhibitor was dialysed swiftly 10 times against water and then kept as a freeze-dried preparation in a desiccator. It was designated 'chick-peas trypsin and chymotrypsin inhibitor'.

Characterization of the inhibitor

Disc-electrophoresis analyses were performed in 15% (w/v) polyacrylamide gel at pH 4.5 with a current of 7.5 mA/tube for 60 min (Reisfeld *et al.*, 1962). Isoelectric focusing in polyacrylamide gels was performed with a pH gradient in the range of 3–10 (Wrigley, 1968). Cellulose acetate-electrophoresis analyses were performed with 0.08 M-collidine acetate buffer, pH 6.9, at constant voltage (400 V) during 20 min in a Beckman Microzone cell model R-101. Molecular-weight determinations were performed by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969) as well as in the ultracentrifuge (Spinco model E) by the Yphantis method (Yphantis, 1960), by using a partial specific volume resolved from amino acid composition.

For amino acid analysis, 1 mg samples of inhibitor were hydrolysed with 2 ml of 6 M-HCl at 110°C for 22 and 48 h in the presence of a known amount of DL-norleucine. The hydrolysed samples were analysed by a single column separation programme in a Bio-Cal BC-200 amino acid analyser. Tryptophan was determined by acid hydrolysis as above in the presence of 1% thioglycolic acid (Matsubara & Sasaki, 1969) and analysed on a short column for basic amino acids (Spackman, 1963).

The N-terminal amino acid was determined by the dinitrophenyl method (Porter & Sanger, 1948) and by the cyanate method (Stark & Smyth, 1967). The C-terminal amino acid was determined with carboxypeptidase B and carboxypeptidase A (Fraenkel-Conrat *et al.*, 1955).

The formation of complexes between the inhibitor and trypsin or chymotrypsin was resolved from dose-response curves and by cellulose acetate electrophoresis in 0.08 M-collidine acetate buffer, pH 7.0.

Reactive-site studies of the inhibitor were performed by incubation of a 2% (w/w) solution of the inhibitor with either trypsin (1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamido-butan-2-one-treated) or α -chymotrypsin at pH 3.75, 25°C for 40 h, followed or not by incubation at pH 8.0 with carboxypeptidase B or carboxypeptidase A respectively, as described by

Finkenzstadt & Laskowski (1965) and Osawa & Laskowski (1966). The inhibitory activities of the differently treated preparations were then assayed on esterolytic activities of trypsin and α -chymotrypsin as described above.

Maleylation of the lysine residues of the inhibitor was carried out by maleic anhydride (Butler *et al.*, 1968). Assay of the inhibitory activity of the maleylation product was then performed on the esterolytic

activities of trypsin and α -chymotrypsin, as described above.

Treatment with CNBr and pepsin

Cleavage of the inhibitor by CNBr at the methionine residue followed by digestion with pepsin was performed as described by Odani & Ikenaka (1973). The only change in the procedure was that the final reaction mixture was diluted with 50 ml of water and

Fig. 1. Elution profile for the purification of the trypsin and chymotrypsin inhibitor from chick peas, by successive column chromatography

(a) A CM-cellulose column (55 cm $\times$ 4 cm) was equilibrated with 5 mM-sodium acetate buffer, pH 4.0; $(\text{NH}_4)_2\text{SO}_4$ precipitate (Table 1) dissolved in 550 ml of water was treated with 2 M- to 5 mM-acetic acid, pH 4.0, and applied to the column; elution was primarily performed with 1 litre of 5 mM-sodium acetate buffer, pH 4.0, and then followed by a gradient to 0.16 M-NaCl concentration in the same buffer, total volume of 1 litre, and then by a second gradient to 0.32 M-NaCl concentration in the buffer, total volume of 1 litre. The flow rate was 450 ml/h and 15 ml fractions were collected. The straight line denotes NaCl concentration, as determined by titration with 0.1 M- AgNO_3 . (b) Chromatography of active fraction from CM-cellulose column on a column (22 cm $\times$ 4.0 cm) of hydroxyapatite; stepwise elution was performed with different concentrations of sodium phosphate buffer, pH 6.8. The flow rate was 100 ml/h, and 6 ml fractions were collected. —, Protein (E_{280}); ----, trypsin-inhibiting activity.

was freeze-dried only once instead of three times. The freeze-dried material was then passed through a column (1.5 cm $\times$ 200 cm) of Bio-Gel P-10 previously equilibrated with 0.1 M-formic acid. The elution was monitored by measuring the E_{280} of the effluent fractions. Inhibitory activities of the effluent fractions were determined on the substrates tosyl-L-arginine methyl ester and acetyl-L-tyrosine ethyl ester against trypsin and chymotrypsin respectively, as described above.

Reagents

Casein and DL-norleucine were obtained from Nutritional Biochemicals, Cleveland, OH, U.S.A.;

tosyl-L-arginine methyl ester, acetyl-L-tyrosine ethyl ester, carboxypeptidase B, carboxypeptidase A, α -chymotrypsin, trypsin (1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one-treated, pepsin and maleic anhydride from Sigma Chemical Co., St. Louis, MO, U.S.A.; NaCl and HCl from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.); $(\text{NH}_4)_2\text{SO}_4$ from Mallinckrodt Chemical works (St. Louis, MO, U.S.A.); CM-cellulose from Serva (Heidelberg, Germany); Bio-Gel P-10 from Bio-Rad Laboratories (Richmond, CA, U.S.A.); trypsin from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.); CNBr from Fluka (Buchs, Switzerland); acetic acid from Frutarom Ltd. (Haifa, Israel).

Table 1. *Purification of trypsin and chymotrypsin inhibitor from chick peas*

Details are given in the Experimental section. One trypsin-inhibiting unit is defined as μmol of substrate hydrolysed by 1 mg of enzyme per min of reaction.

Procedure	Volume (ml)	Concentration (units/ml)	$10^{-3} \times$ Total units	Specific activity (units/ E_{280})	Yield (%)	Purification (fold)
Initial extract	3225	3892	25100	175	100	1
$(\text{NH}_4)_2\text{SO}_4$ precipitate	550	16600	18260	200	73	1.2
CM-cellulose effluent	1092	3408	7446	14348	30	82
Hydroxyapatite effluent	97	26900	5216	53521	21	306

Table 2. *Amino acid composition of chick-peas inhibitor and of its fragments A and B*

The results from the amino acid analyser are expressed as residues per molecule. The inhibitor (100 nmol) was hydrolysed in evacuated sealed tubes in 6M-HCl at 110°C for 22h and analyses were performed with a Bio-Cal BC-200 amino acid analyser. Tryptophan was estimated by acid hydrolysis as above in the presence of 1% thioglycolic acid and analysed on a short column for basic amino acids (Spackman, 1963). Values in parentheses are nearest whole integers. Similar results were obtained for 48 h hydrolysates.

Amino acid	Content (residues/molecule)		
	Native inhibitor	Fragment A	Fragment B
Aspartic acid	9.0 (9)	8.0 (8)	1.0 (1)
Glutamic acid	5.6 (6)	2.9 (3)	2.8 (3)
Glycine	6.0 (6)	1.6 (2)	1.7 (2)
Alanine	3.8 (4)	1.8 (2)	2.3 (2)
Valine	3.2 (3)	1.2 (1)	0.8 (1)
Leucine	1.8 (2)	Traces	1.2 (1)
Isoleucine	3.4 (3)	1.4 (1)	2.0 (2)
Serine	6.8 (7)	4.0 (4)	3.2 (3)
Threonine	5.8 (6)	4.4 (4)	2.3 (2)
Half-cystine	12.0 (12)	5.8 (6)	4.0 (4)
Methionine	0.8 (1)	0.0 (0)	0.0 (0)
Proline	5.0 (5)	2.3 (2)	2.5 (3)
Phenylalanine	1.0 (1)	1.1 (1)	0.0 (0)
Tyrosine	1.8 (2)	1.0 (1)	1.0 (1)
Histidine	1.0 (1)	0.0 (0)	1.0 (1)
Lysine	7.0 (7)	4.4 (4)	3.0 (3)
Arginine	2.4 (2)	1.2 (1)	1.1 (1)
Tryptophan	0.0 (0)	0.0 (0)	0.0 (0)
Cysteic acid	0.0 (0)	0.9 (1)	1.0 (1)

EXPLANATION OF PLATE I

Electrophoretic analyses of the trypsin and chymotrypsin inhibitor from chick peas

(a) Electrophoresis of the inhibitor (100 μ g/tube) was performed in 15% (w/v) polyacrylamide gel at pH4.5 with a current of 7.5 mA/tube for 60 min (Reisfeld *et al.*, 1962). Gels were stained with 1% Naphthalene Black 12B solution in 7% (v/v) acetic acid. Migration was downwards towards the cathode. (b) Electrophoresis of the inhibitor and of its complexes with trypsin and with α -chymotrypsin was performed on cellulose acetate strips at 28 V/cm for 20 min in collidine acetate buffer, pH7. Spots were detected by staining with Naphthalene Black 12B. Sample (1), chymotrypsin; sample (2), inhibitor + chymotrypsin (molar ratio 1:1); sample (3), inhibitor; sample (4), inhibitor + trypsin (molar ratio 1:1); sample (5), trypsin.

Results

Purification of the inhibitor

The subsequent purification of the trypsin and chymotrypsin inhibitor on CM-cellulose and hydroxyapatite columns is shown in Figs. 1*a* and 1*b*. The different steps leading to the isolation of the inhibitor are summarized in Table 1. A portion (400 g) of chick-pea flour yielded 200 mg of the inhibitor.

Characterization and properties

The molecular weight of the inhibitor, calculated from the ultracentrifugal analysis and by using a partial specific volume ($\bar{v}$) of 0.72 (as derived from the amino acid composition), was 10016. A value of approx. 10000 mol.wt. was obtained by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The specific extinction of the freeze-dried preparation of the inhibitor at 280 nm was $E_{1\%}^{1\text{cm}} = 2.5$. The amino acid composition (Table 2) showed that it was rich in half-cystine, contained one methionine residue and lacked tryptophan. The inhibitor contained no cysteine. The *N*-terminal amino acid was glycine and the *C*-terminal amino acid was lysine. The inhibitor appeared in two electrophoretically distinguishable forms (Plates 1*a* and 1*b*).

The enzyme-inhibitor complex was formed by reaction of 1 mol of inhibitor with 1 mol of trypsin or α -chymotrypsin, as could be seen from the dose-response curves (Figs. 2*a* and 2*b*) and by the electrophoretic analysis on cellulose acetate strips (Plate 1*b*).

The effect of limited proteolysis of the inhibitor at pH 3.75 with either trypsin or α -chymotrypsin was summarized in Table 3. Both trypsin and α -chymotrypsin caused considerable losses in the two inhibitory activities of the inhibitor. Digestions at pH 8 with carboxypeptidase B or carboxypeptidase A, after the above treatments with trypsin or chymotrypsin respectively, did not cause further loss in inhibitory activity. The latter digestion resulted in release of the newly formed *C*-terminal amino acids lysine and tyrosine respectively, suggesting that the trypsin-reactive site was -Lys-X- and that the chymotrypsin-reactive site was -Tyr-X-. The involvement of a lysine residue in the trypsin-reactive site of the inhibitor was also demonstrated by the maleylated inhibitor, which lost 85% of its trypsin-inhibitory activity, whereas the chymotrypsin-inhibitory activity was fully retained.

Treatment of the inhibitor with CNBr followed by pepsin resulted in three distinct proteinaceous fractions which were separated on a Bio-Gel P-10 column. Fraction I emerged in 60–70 ml of the column eluate, had a similar amino acid composition to that of the intact inhibitor and possessed inhibitory activities both against trypsin and chymotrypsin. Fraction A (72–79 ml) inhibited trypsin but not chymotrypsin,

and fraction B (82–88 ml) inhibited chymotrypsin and did not affect trypsin. The additive amino acid composition of fractions A and B (mol. wt. 4382 and 3268 respectively, as calculated from amino acid compositions) was very similar to that of the intact inhibitor (Table 2). Thus the above interaction of the inhibitor with CNBr followed by pepsin, resulted in cleavage of the double-headed trypsin and chymotrypsin inhibitor into two fragments, one a trypsin inhibitor and the other a chymotrypsin inhibitor. Moreover, the amount of fragment A used to achieve almost full inhibition of trypsin was one-quarter of

Fig. 2. Dose-response curves of inhibition of trypsin and chymotrypsin by the inhibitor from chick peas

Inhibition of trypsin (a) and chymotrypsin (b) was determined on tosyl-L-arginine methyl ester (0.01 M in reaction mixture) and on acetyl-L-tyrosine ethyl ester (0.01 M in reaction mixture) respectively, at pH 8.1, 25°C in a Gilford 2400 spectrophotometer. Details were given in the Experimental section. Inhibition was expressed as percentage of residual trypsin or chymotrypsin activity.

Table 3. *Effect of incubation of the inhibitor with either trypsin or α -chymotrypsin at pH 3.75 followed or not by digestion at pH 8.0 with carboxypeptidase B or A respectively, on the inhibitory activity against trypsin and chymotrypsin when assayed on tosyl-L-arginine methyl ester and acetyl-L-tyrosine ethyl ester respectively*

The residual inhibitory activity after each treatment is expressed as % of the control.

Incubation of the inhibitor	Residual inhibitory activity against	
	Trypsin (% of control)	Chymotrypsin (% of control)
At pH 3.75, 40h (control)	100	100
With trypsin at pH 3.75, 40h	15	40
With trypsin at pH 3.75 for 40h, followed by carboxypeptidase B at pH 8.0 for 48h	15	40
With chymotrypsin at pH 3.75, 40h	30	0
With chymotrypsin at pH 3.75 for 40h, followed by carboxypeptidase A at pH 8.0 for 48h	30	0

that of the intact inhibitor indicating a twofold increase in the specific activity against trypsin, whereas the cleavage decreased the specific chymotrypsin inhibitory activity of fragment B by about 50%.

Discussion

The amino acid composition of the trypsin and chymotrypsin inhibitor from chick peas was similar to the more typical trypsin inhibitors from legume seeds, such as the lima-bean inhibitors, inhibitor AA from soya beans and the groundnut inhibitor, which were rich in cystine and lacked tryptophan (Birk & Gertler, 1971; Birk, 1974). Electrophoretic analyses of the inhibitor in polyacrylamide gels and on cellulose acetate strips (Plates 1a and 1b) showed the presence of two distinct inhibitory bands. These might be considered as true iso-inhibitors since they were isolated by conventional chromatographic procedures and were not exposed to possible proteolysis by affinity chromatography on immobilized trypsin or chymotrypsin columns.

The chemical treatments of the inhibitor, namely maleylation, which decreased the trypsin inhibitory activity by 85%, but did not affect the chymotrypsin inhibitory activity, as well as treatment with CNBr, which led to the ultimate fragmentation of the inhibitor into two separate inhibitors, clearly indicated that the inhibitor was a double-headed independent inhibitor and therefore resembled inhibitor AA from soybeans. On the other hand, limited proteolysis of the inhibitor with trypsin, which opened a single-Lys-X- bond at the trypsin-inhibitory site, decreased the trypsin-inhibitory activity by 85%, but also affected to a considerable extent the chymotrypsin-inhibitory activity (Table 3). In a similar manner, the limited proteolysis with chymotrypsin, which was directed towards a single-Tyr-X- bond at the chymotrypsin-inhibitory site, not only inactivated completely the chymotrypsin-inhibitory activity, but also affected

the ability of the inhibitor to inhibit trypsin. These findings suggested a different three-dimensional structure of the chick-peas inhibitor as compared with inhibitor AA. It seemed that opening of a single reactive bond at either of the two inhibitory sites allowed a conformational change, such as folding of the newly formed N- or C-terminal chain, which might have led eventually to masking of the other, intact, inhibitory site.

Of special interest was the finding on the potentiation of the specific trypsin-inhibitory activity of fragment A, which might have resulted from unmasking of an additional trypsin-inhibitory site hidden in intact chick-peas inhibitor. Further investigation of this phenomenon as well as elucidation of the covalent structure of the inhibitor should furnish the still-missing information as to whether and to what extent this inhibitor was homologous with inhibitor AA from soya beans and with the lima-bean inhibitors. It would also shed more light on the evolutionary aspects of this unique group of biologically active low-molecular-weight proteins in legume seeds and would contribute additional evidence for or against the universality of the hypothesis that the 'double-headed' inhibitors arose by a process of gene duplication.

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