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PROTEINASE INHIBITORS FROM *LONCHOCARPUS CAPASSA* (APPLE-LEAF) SEED

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Key Word Index—*Lonchocarpus capassa*, Leguminosae; apple-leaf tree; proteinase inhibitors; inhibitor activities; MWs; partial amino acid sequence.

Abstract—Four proteinase inhibitors (DE-1 to DE-4) were purified from *L. capassa* seed by chromatographic procedures involving Sephadex G-50 and DEAE-cellulose. They comprise each 80 amino acids (MW *ca* 10000) including fourteen half-cystine residues. The partial amino acid sequence of inhibitor DE-4 was determined; 60 of the 80 residues have been sequenced. The MW, cystine content and partial sequence of DE-4 resemble those of the Bowman–Birk-type proteinase inhibitors. The properties of inhibitors DE-1 and DE-4 are very similar. Each contains a potent inhibitor for porcine trypsin but they inhibit bovine α-chymotrypsin only weakly.

INTRODUCTION

Laskowski and Laskowski [1] showed that proteinase inhibitors are widely distributed in Leguminosae seeds. On the basis of their MWs and cystine contents the proteinase inhibitors may be divided into two types, *viz.* the Bowman–Birk-type and Kunitz-type inhibitors. The Bowman–Birk-type inhibitors have MWs of 8000–10000 and a high cystine content (usually seven disulphides) [2–8]. In contrast the Kunitz-type inhibitors have MWs of *ca* 20000 and a low cystine content (usually two disulphides) [9–19].

Leguminosae are usually divided in three subfamilies, namely, Mimosoideae, Caesalpinioideae and Lotoideae (Papilionoideae) [20]. A number of proteinase inhibitors from the Lotoideae which resembles either the Bowman–Birk-type or the Kunitz-type have been isolated and characterized. The present communication describes the purification and some of the properties of four proteinase inhibitors from *Lonchocarpus capassa* (apple-leaf) seed which belongs to the subfamily Papilionoideae.

RESULTS

The elution profile obtained for the crude extract on Sephadex G-50 in 0.2 M ammonium hydrogen carbonate solution is shown in Fig. 1. Several peaks were evident, of which only the S_3 peak exhibited trypsin and chymotrypsin inhibitor activities. Peak S_3 was lyophilized and further fractionated on DEAE-cellulose using a linear sodium chloride gradient (0–0.4 M over 2 l.) in 0.05 M Tris–HCl, pH 8. This gave one major and various minor peaks of which only peaks C_7, C_8, C_9 and C_{10} possessed tryptic and chymotryptic activities. The active peaks were rechromatographed using similar conditions as in the first separation. The chromatograms each showed major peaks and afforded protease inhibitors DE-1, DE-2, DE-3 and DE-4. The purification of the proteinase inhibitors is summarized in Table 1. Disc electrophoresis both in the absence and presence of dodecyl sulphate showed that the proteinase inhibitors were probably homogeneous. Some of the properties of the inhibitors are summarized in Table 2 and their amino acid compositions are given in Table 3.

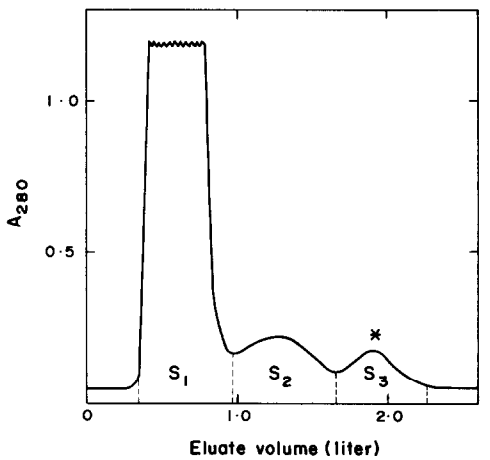

Fig. 1. Gel filtration of the crude extract of the seeds of *L. capassa*. Crude extract (2 g) was loaded on Sephadex G-50 column (3.8 × 150 cm) and eluted with 0.2 M ammonium hydrogen carbonate solution at a flow rate of 50 ml/hr. The column temperature was 20° and the eluate was monitored at 280 nm. The asterisk indicates trypsin and chymotrypsin activities.

Limited proteolysis of proteinase inhibitors at acidic pH with catalytic amounts of enzymes has been reported and is generally accepted for various inhibitors [21–23]. Such limited hydrolysis occurs at the reactive site of the inhibitor. Limited hydrolysis of DE-4 with trypsin at pH 3, and subsequent reduction and S-carboxymethylation and gel filtration yielded three peaks (S_1, S_2

Table 1. Summary of the purification of proteinase inhibitors DE-1, DE-2, DE-3 and DE-4

Steps	Protein (mg)		Total inhibitor activity* [units($\times 10^5$)]	Specific inhibitor activity [units/mg($\times 10^{-3}$)]	Yield (%)
Crude preparation	2000	T	14.80	0.74	100
		C	2.80	0.14	100
Sephadex G-50	95	T	11.12	11.70	75.1
		C	1.91	2.01	68.2
DEAE-cellulose					
DE-1	7	T	1.35	19.29	9.1
		C	0.32	4.57	11.4
DE-2	9	T	1.71	19.00	11.6
		C	0.33	3.67	11.8
DE-3	10	T	2.16	21.60	14.6
		C	0.34	3.40	12.1
DE-4	15	T	3.31	22.07	22.4
		C	0.72	4.80	25.7

*T, trypsin inhibitor; C, chymotrypsin inhibitor.

Table 2. Some of the properties of proteinase inhibitors DE-1, DE-2, DE-3 and DE-4

Property	DE-1	DE-2	DE-3	DE-4
Disc electrophoresis	One band	One band	One band	One band
SDS-gel electrophoresis	One band*	One band*	One band*	One band*
MW by:				
(i) Gel filtration	11000	12400	12500	12300
(ii) SDS-gel	10000	11000	11000	11000
Inhibitor activities	Trypsin Chymotrypsin†	Trypsin Chymotrypsin†	Trypsin Chymotrypsin†	Trypsin Chymotrypsin†
Free SH	none	none	none	none

*Showed also a very weak minor band.
†Weak activity.

and S_3). The amino acid composition shows that peak S_1 is uncleaved inhibitor, and peak S_2 and S_3 represent fragments containing 55 and 25 amino acid residues, respectively (Table 4). Chymotrypsin at pH 3 yielded also three peaks S_4, S_5 and S_6. Peak S_4 represents uncleaved inhibitor and peaks S_5 and S_6 comprised, respectively, 52 and 28 amino acid residues (Table 4).

Figure 3 shows the partial amino acid sequence of protease inhibitor DE-4. Sequencing the amino-terminal segment of inhibitor DE-4 and fragments S_2, S_3, S_5 and S_6 by means of the sequencer showed that S_3 and S_5 were derived from the *N*-terminal segment of DE-4. Consequently, S_2 and S_6 must be aligned from *C*-terminal segment of DE-4. The sequence of the *N*-terminal fragment of DE-4, the partial sequence of S_2 and the partial sequence of S_6 yielded the partial amino acid sequence of DE-4, of the 80 residues, 60 were sequenced (Fig. 3).

Inhibition of porcine trypsin and bovine α-chymotrypsin at pH 8 by increasing levels of inhibitor DE-1, DE-2, DE-3 and DE-4 is shown in Fig. 4.

DISCUSSION

The properties of the four protease inhibitors (DE-1 to DE-4) from *Lonchocarpus capassa* seed are presumably very similar. Their amino acid compositions were very similar and their inhibitor activity characteristics towards trypsin and chymotrypsin were alike. The protease inhibitors each contain 80 amino acids including 14 half-cystine residues. Since no sulphydryl groups could be detected in the intact inhibitors, they are cross-linked by seven intramolecular disulphide bridges.

A comparison of the amino acid sequences of Bowman–Birk-type proteinase inhibitors is presented in Fig. 5. The sequences were aligned with respect to the position of the cystine residues. The partial sequence of DE-4 from *L. capassa* seed was found to be highly homologous to the Bowman–Birk-type proteinase inhibitors. On the basis of its MW, cystine content and partial amino acid sequence, DE-4 represents a Bowman–Birk-type proteinase inhibitor. Bowman–Birk-type double-headed proteinase inhibitors contain two different and independent active sites, usually for trypsin and chymotrypsin (Fig. 5). The reactive sites of DE-4 for trypsin were found to be Arg^{26}-Ser^{27} and for chymotrypsin Phe^{53}-Ser^{54}. The properties of inhibitors DE-1, DE-2 and DE-3 resemble those of DE-4 and, therefore, they also belong to the Bowman–Birk-type proteinase inhibitors.

Table 3. Amino acid composition of proteinase inhibitors DE-1, DE-2, DE-3 and DE-4 given as mols of residue per mol inhibitor

Amino acid	DE-1	DE-2	DE-3	DE-4
Aspartic acid	11.3 (11)	12.1 (12)	11.3 (11)	12.5 (13)
Threonine	4.1 (4)	3.7 (4)	3.9 (4)	3.9 (4)
Serine	13.4 (13)	14.4 (14)	12.5 (13)	14.1 (14)
Glutamic acid	6.7 (7)	6.8 (7)	6.8 (7)	6.7 (7)
Proline	5.3 (5)	5.5 (6)	6.7 (7)	5.6 (6)
Glycine	2.8 (3)	2.7 (3)	2.4 (2)	2.1 (2)
Alanine	1.6 (2)	1.3 (1)	1.2 (1)	1.1 (1)
Half-cystine*	13.4 (14)	13.6 (14)	13.5 (14)	13.6 (14)
Valine	2.7 (3)	2.4 (2)	2.5 (3)	2.3 (2)
Methionine	1.9 (2)	2.0 (2)	2.2 (2)	2.0 (2)
Isoleucine	0.2 (0)	0.1 (0)	0.1 (0)	0.1 (0)
Leucine	2.4 (2)	2.4 (2)	2.3 (2)	2.1 (2)
Tyrosine	1.2 (1)	1.1 (1)	1.1 (1)	1.0 (1)
Phenylalanine	2.2 (2)	2.2 (2)	2.2 (2)	2.0 (2)
Lysine	4.4 (4)	3.8 (4)	4.4 (4)	4.2 (4)
Histidine	2.1 (2)	1.8 (2)	2.7 (3)	2.2 (2)
Arginine	4.0 (4)	3.7 (4)	4.2 (4)	4.0 (4)
Tryptophan†	0	0	0	0
Total	80	80	80	80

*Determined as cysteic acid by the method of ref. [27].
†Determined by the method described in ref. [28].

L. capassa seeds contain several potent Bowman–Birk-type proteinase inhibitors for porcine trypsin. The titration data of Fig. 4 showed that inhibitors DE-1, DE-2, DE-3 and DE-4 each stoichiometrically inhibited trypsin in a molar ratio of 1:1 and the enzyme was completely inhibited. Chymotrypsin was also inhibited by DE-1, DE-2, DE-3 and DE-4 but the binding of the enzyme was very much weaker.

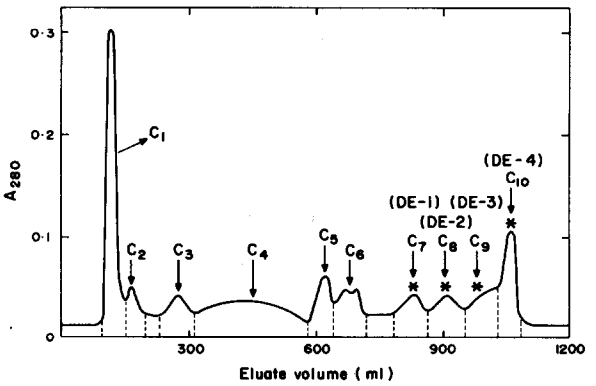

Fig. 2. Chromatography of peak S_3 on DEAE-cellulose. Peak S_3 (0.2 g) was loaded on DEAE-cellulose column (0.9 × 15 cm) and eluted by a linear sodium chloride gradient (0–0.4 M over 2 l) in 0.05 M Tris–HCl at pH 8 at a flow-rate of 50 ml/hr. The column temperature was 20° and the eluate was monitored at 280 nm. The asterisks indicate trypsin and chymotrypsin activities.

EXPERIMENTAL

Materials. Lonchocarpus capassa (apple-leaf) seeds were supplied by the Transvaal Provincial Nursery, Hartbeeshoek. The sources of trypsin, α-chymotrypsin and chemical reagents have been described previously [18].

Methods. The physicochemical methods, the *N*-terminal sequence of reduced and S-carboxymethylated proteinase inhibitor (DE-4) and the sequences of the fragments obtained by limited hydrolysis with trypsin and chymotrypsin were determined with the Beckman sequencer as described [18].

The esterolytic activities of trypsin and α-chymotrypsin were measured spectrophotometrically according to the method of ref. [24] as described earlier [18]. The rates of hydrolysis at 30° of *N*-

Table 4. Amino acid composition of the fragments obtained by limited hydrolysis of DE-4 with trypsin (S_2 and S_3) and chymotrypsin (S_5 and S_6) given as mols of residue per mol fragment

Amino acid	S_2	S_3	S_5	S_6
S-Carboxymethylcysteine	10.0 (10)	4.0 (4)	9.5 (10)	4.0 (4)
Aspartic acid	8.4 (8)	4.5 (5)	6.7 (7)	5.8 (6)
Threonine	2.8 (3)	0.9 (1)	2.5 (3)	1.0 (1)
Serine	8.4 (8)	6.0 (6)	9.9 (10)	4.4 (4)
Glutamic acid	3.8 (4)	2.8 (3)	4.9 (5)	1.5 (2)
Proline	4.4 (4)	2.0 (2)	3.5 (4)	2.1 (2)
Glycine	1.9 (2)	0.3 (0)	0.3 (0)	1.8 (2)
Alanine	1.1 (1)	0.1 (0)	0.8 (1)	0.1 (0)
Valine	2.2 (2)	0.2 (0)	1.3 (1)	1.2 (1)
Methionine	1.9 (2)	0.1 (0)	0.9 (1)	1.0 (1)
Isoleucine	0	0	0	0
Leucine	1.9 (2)	0.2 (0)	1.1 (1)	1.0 (1)
Tyrosine	1.2 (1)	0.1 (0)	0.1 (0)	0.9 (1)
Phenylalanine	2.3 (2)	0.3 (0)	1.2 (1)	1.1 (1)
Lysine	3.4 (3)	1.1 (1)	2.0 (2)	2.0 (2)
Histidine	1.4 (1)	0.8 (1)	1.7 (2)	0.1 (0)
Arginine	2.4 (2)	2.0 (2)	3.9 (4)	0.2 (0)
Total	55	25	52	28

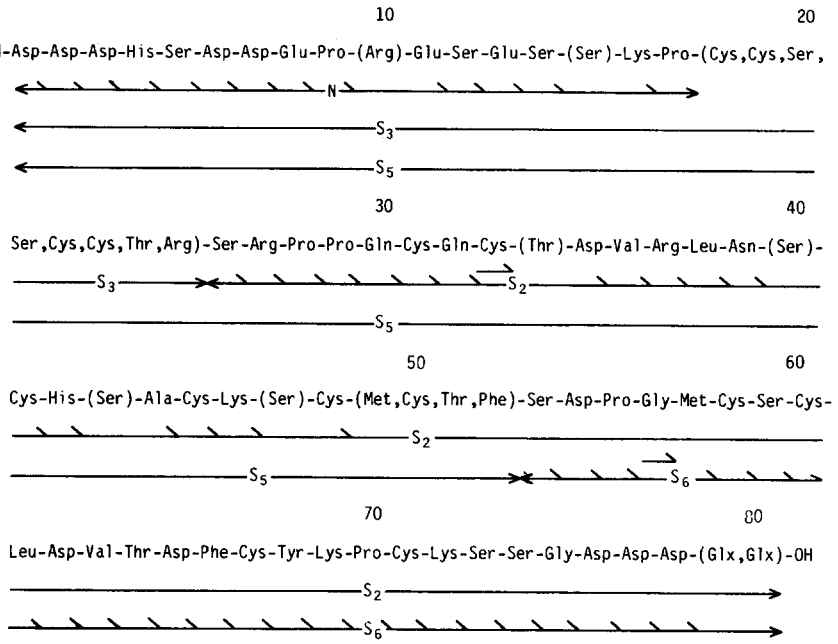

Fig. 3. The amino acid sequence of proteinase inhibitor DE-4 from *L. capassa* seed. N(residues 1–17) shows the sequence of *N*-terminal segment of DE-4. S_3 (residues 1–25) and S_2 (residues 26–80) show the fragments obtained by limited hydrolysis of DE-4 with trypsin at pH 3 and the partial sequence of S_2 (residues 26–52). S_5 (residues 1–52) and S_6 (residues 53–80) indicate the fragments obtained by limited hydrolysis of DE-4 with chymotrypsin at pH 3 and the partial sequence of S_6. The half-arrows show the residues which were sequenced.

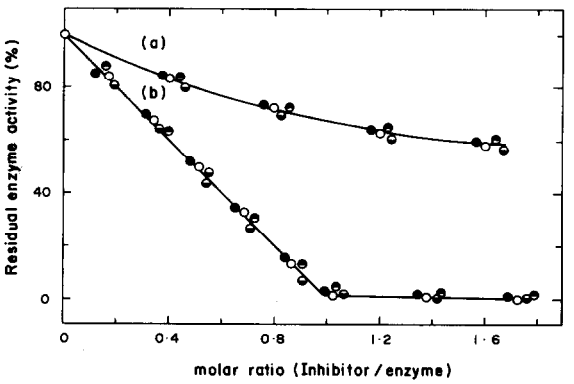

Fig. 4. Inhibition of (a) porcine trypsin and (b) bovine α-chymotrypsin by increasing amounts of Bowman–Birk-type proteinase inhibitors from *L. capassa* seed. DE-1 ○——○, DE-2 ●——●, DE-3 ◓——◓ and DE-4 ◒——◒.

α-benzoyl-L-arginine ethyl ester by porcine trypsin and of *N*-acetyl-L-tyrosine ethyl ester by bovine chymotrypsin, were recorded as a change in *A* at 253 nm and 237 nm, respectively. The inhibitors' activities were estimated from the residual enzymatic activities as described previously [18]. The concn of the enzymes was corrected for inactive materials as determined by active-site titrations [25].

One unit of enzyme activity was defined as that amount of enzyme causing a change in the amount of substrate of 1 μmol/min at 30°. One unit of inhibitor activity was defined as that amount of inhibitor which inhibited 1 unit of enzyme activity. Specific inhibitor activity was expressed as inhibitor units per mg inhibitor.

Limited hydrolysis with trypsin and chymotrypsin. The procedure used for limited hydrolysis at pH 3 proteinase inhibitors with trypsin and chymotrypsin was the same as described by Birk *et al.* [26].

Preparation of the crude inhibitor. Ground *L. capassa* seeds were extracted with 10% NaCl soln (1 l) overnight at 10°. The suspension was then macerated for 5 min in a Waring blender. The extract was clarified by centrifugation at 16000 *g* brought to 75% satn with $(NH_4)_2SO_4$ and ppt recovered by centrifugation. The ppt was redissolved in 10% NaCl soln, dialysed against H_2O and lyophilized. The yield of the extract was 9.7 g.

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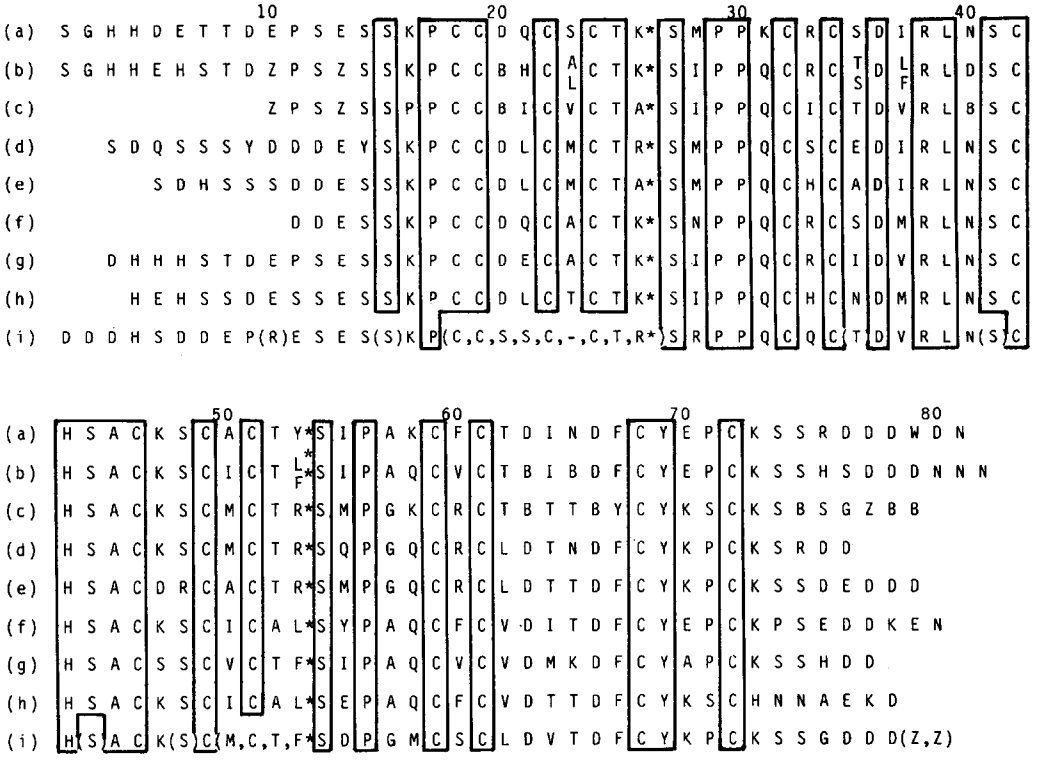

Fig. 5. Comparison of the amino acid sequences of various Bowman–Birk-type double-headed proteinase inhibitors: (a) adzuki bean I [2]; (b) lima bean I [3]; (c) garden bean I [4]; (d) soybean D-II [5]; (e) soybean C-II [6]; (f) soybean BBI [7]; (g) *Macrotyloma axillare*, DE-3 [8]; (h) *Macrotyloma axillare*, DE-4 [8] and (i) *L. capassa*, DE-4 (this paper). The reactive sites are shown by asterisks. The IUPAC one-letter notation for amino acids is used (*Eur. J. Biochem.* **5**, 151, 1968).

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