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PROTEINASE INHIBITORS FROM *ERYTHRINA LYSISTEMON* SEED

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Key Word Index—*Erythrina lysistemon*; Leguminosae; common coral tree; proteinase inhibitors; inhibitor activities; molecular weights; *N*-terminal sequences.

Abstract—Four proteinase inhibitors (DE-1 to DE-4) were purified from *E. lysistemon* seed by chromatographic procedures involving Sephadex G-50, DEAE-cellulose and DEAE-Sepharose. They comprise 166–167 amino acid residues (MW 18 200) including four half-cystine residues and resemble the Kunitz-type proteinase inhibitors. The *N*-terminal primary structure of DE-3 showed also homology with those of the Kunitz-type inhibitors. For DE-1, DE-2 and DE-4 no free *N*-terminal amino acid was found. Whereas DE-1 inhibits bovine α-chymotrypsin strongly and has no action on porcine trypsin, DE-2 inhibits trypsin strongly and α-chymotrypsin only weakly. DE-3 contains a potent inhibitor for both trypsin and α-chymotrypsin, but DE-4 inhibits trypsin strongly and binds α-chymotrypsin only weakly.

INTRODUCTION

Proteinase inhibitors occur widely in the animal and plant kingdoms and they have been isolated from various Leguminosae seeds [1]. Members of the Leguminosae are usually divided into three subfamilies, namely, Mimosoideae, Caesalpinioideae and Lotoideae (Papilionoideae) [2]. A number of proteinase inhibitors from the Lotoideae, which includes most of the common food and fodder legumes of agricultural importance, and several from the Caesalpinioideae have been isolated and characterized [3]. Recently, Odani *et al.* [4] and Kortt and Jermyn [5] reported on proteinase inhibitors from legume seeds which belong to the subfamily Mimosoideae.

The genus *Erythrina*, a legume of subfamily Papilionoideae [6], consists of about 108 species of trees and shrubs distributed throughout tropical to warm-temperature regions of the world [7, 8]. It has been established that seeds from southern African species of *Erythrina*, viz. *E. acanthocarpa*, *E. caffra*, *E. humeana*, *E. latissima* and *E. lysistemon* contain large concentrations of proteinase inhibitors. The purification and characterization of two proteinase inhibitors (DE-1 and DE-3) from the seed of *E. latissima*, have recently been described [9]. The present communication describes the purification and some of the properties of four proteinase inhibitors from the seeds of *E. lysistemon*.

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. Various peaks were evident with only peak S_2 exhibiting trypsin as well as chymotrypsin inhibitor activities. Peak S_2 was lyophilized and further fractionated on DEAE-cellulose using a linear sodium chloride gradient (0–0.2 M over 2 l.) in 0.05 M Tris–HCl at pH 8. This revealed three major proteinase inhibitor peaks (Fig. 2). Peaks C_1, C_2 and C_3 were each rechromatographed on DEAE-Sepharose columns (0.9 × 15 cm) using a linear sodium chloride gradient (0–0.2 M over 1 l.) at a flow of 12 ml/hr in 0.05 M Tris–HCl pH 8. The chromatograms revealed for C_1 a major peak (DE-1), for C_2 a major peak (DE-2) and two minor peaks, and for C_3 two major peaks (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

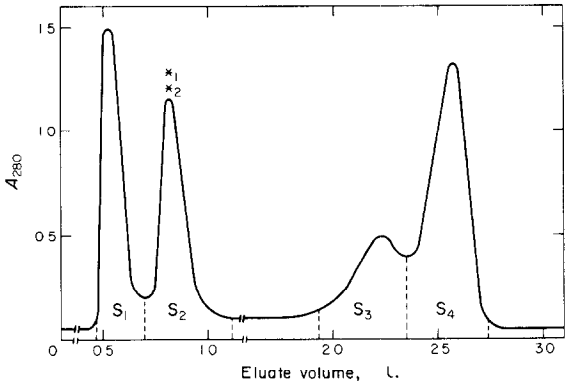

Fig. 1. Gel filtration of the crude extract of the seeds of *E. lysistemon*. Crude extract (2 g) was loaded on Sephadex G-50 column (3.8 × 150 cm) and elution effected with 0.2 M ammonium hydrogen carbonate solution at a flow rate of 50 ml/hr. The column temperature was 20° and the eluate was monitored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

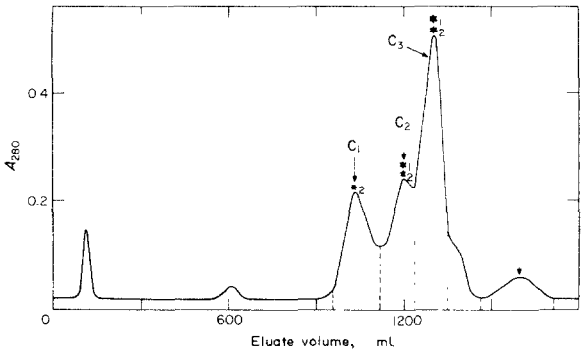

Fig. 2. Chromatography of peak S_2 on DEAE-cellulose. Peak S_2 (0.25 g) was loaded on DEAE-cellulose (0.9 × 15 cm) column and elution effected by a linear sodium chloride gradient (0–0.2 M over 2 l.) in 0.05 M Tris–HCl at pH 8 at a flow rate of 50 ml/hr. The column temperature was 20° and the eluate was monitored at 280 nm. *1 indicates trypsin inhibitor activity and *2 chymotrypsin inhibitor activity.

proteinase inhibitors DE-1, DE-2, DE-3 and DE-4 were probably homogeneous. Some of the properties of the inhibitors are summarized in Table 2 and their amino acid composition is given in Table 3. The *N*-terminal primary structure of reduced and *S*-carboxymethylated proteinase inhibitor DE-3, determined using the Beckman sequencer, is given in Fig. 4(e). However, Edman degradation using the sequencer failed to yield any *N*-terminal amino acid for intact proteinase inhibitors DE-1, DE-2 and DE-4. 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. 3.

DISCUSSION

The proteinase inhibitors from Leguminosae may be divided into two general groups depending on their MW and cystine content, namely, the Bowman–Birk-type inhibitors and the Kunitz-type inhibitors. The Bowman–Birk-type inhibitors have MWs of 8000–10 000 and a high cystine content (usually seven disulphides) [10–17]. The Kunitz-type inhibitors have MWs *ca* 20 000 and a low cystine content (usually two disulphides). The Kunitz soybean trypsin inhibitor [18–20] is a typical example of this group. The inhibitors from winged bean (*Psophocarpus tetragonolobus*) [21, 22], the seeds of *Albizzia julibrissin* [4], *Erythrina latissima* [9] and *Acacia elata* [5] belong also to the Kunitz-type.

The MWs of proteinase inhibitor DE-1, DE-2, DE-3 and DE-4 from *E. lysistemon* by gel filtration and dodecyl sulphate gel electrophoresis were of the order of 18 000, and hence the inhibitors comprise 166–167 amino acid residues including four half-cystine residues (Tables 2 and 3). Since no sulphydryl groups could be detected in the intact inhibitors, they are cross-linked by two intramolecular disulphide bonds. The MWs and low disulphide content of the *E. lysistemon* inhibitors resemble the Kunitz-type proteinase inhibitors. Furthermore, in Fig. 4 the *N*-terminal primary structure of inhibitor DE-3 is compared with those of Kunitz soybean trypsin inhibitor [18–20] and Kunitz-type inhibitors from the seeds of *Albizzia julibrissin* (A-II and B-II) [4] and *E. latissima* [9]. The high degree of homology is quite obvious.

The inhibitory activity characteristics of the four Kunitz-type proteinase inhibitors from *E. lysistemon* seed were varied and different. For DE-1 the titration curve (Fig. 3a) was at first linear and extrapolation of

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 × 10^3)	Specific inhibitor activity (units/mg protein)	Yield (%)
Crude preparation	2000	T 2700	1350	100.0
		C 2940	1470	100.0
Sephadex G-50	405	T 1976	4880	73.2
		C 2336	5770	79.5
DEAE-cellulose and DEAE-Sepharose				
DE-1	48	T 0	0	0
		C 478	9960	16.3
DE-2	52	T 383	7360	14.2
		C 190	3660	6.5
DE-3	42	T 420	10 000	15.6
		C 286	6800	9.7
DE-4	45	T 437	9710	16.2
		C 76	1700	2.6

T—trypsin inhibitor; C—chymotrypsin.

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	18 100	16 800	18 400	17 700
(ii) SDS-gel	19 100	18 300	18 800	18 700
Inhibitor activities	Chymotrypsin	Trypsin Chymotrypsin	Trypsin Chymotrypsin	Trypsin (Chymotrypsin)
Free SH	None	None	None	None
N-Terminal amino acids	None found	None found	Valine	None found

*Sodium dodecyl sulphate.

Table 3. Amino acid composition of proteinase inhibitors DE-1, DE-2, DE-3 and DE-4

Amino acid	DE-1	DE-2	DE-3	DE-4	Kunitz soybean trypsin inhibitor [18–20]
Aspartic acid	18.1(18)	15.4(15)	16.2(16)	15.2(15)	26
Threonine	10.9(11)	7.9(8)	8.5(9)	7.6(8)	7
Serine	12.6(13)	15.9(16)	13.6(14)	16.6(17)	11
Glutamic acid	19.0(19)	19.9(20)	25.4(25)	20.3(20)	18
Proline	12.3(12)	11.7(12)	9.9(10)	11.3(11)	10
Glycine	12.1(12)	15.7(16)	13.8(14)	15.4(15)	16
Alanine	11.9(12)	4.3(4)	6.2(6)	3.9(4)	8
Half-cystine*	3.7(4)	4.1(4)	3.7(4)	4.0(4)	4
Valine	12.0(12)	10.4(10)	11.7(12)	10.5(11)	14
Methionine	0.2(0)	1.0(1)	0.1(0)	1.1(1)	2
Isoleucine	6.1(6)	9.1(9)	5.8(6)	8.8(9)	14
Leucine	15.7(16)	13.6(14)	15.6(16)	13.6(14)	15
Tyrosine	6.0(6)	9.8(10)	7.7(8)	9.6(10)	4
Phenylalanine	4.9(5)	6.1(6)	4.1(4)	5.8(6)	9
Lysine	7.8(8)	10.2(10)	11.8(12)	10.7(11)	10
Histidine	2.0(2)	1.3(1)	2.0(2)	2.0(2)	2
Arginine	7.6(8)	7.7(8)	6.5(7)	7.3(7)	9
Tryptophan	1.8(2)	2.1(2)	2.0(2)	2.1(2)	2
Total	166.0	166.0	167.0	167.0	181

Samples were hydrolysed for 24 hr. Values are given as mol of residue/mol of proteinase inhibitors.

*Determined as cysteic acid by the method of Hirs [29].

the data indicated that 1 mol of the inhibitor reacts with *ca* 2 mol of α-chymotrypsin. At higher molar ratios α-chymotrypsin was completely inhibited. Trypsin was not inhibited by DE-1. The titration curves (Figs. 3b and d) for DE-2 and DE-4 were similar. Data showed that inhibitors DE-2 and DE-4 stoichiometrically inhibited trypsin in the molar ratio of 1 : 1, α-chymotrypsin was also inhibited but did not follow a stoichiometric relationship and the inhibitor capacities were different, inhibitor DE-2 showed a higher inhibitory capacity than DE-4. Inhibitor DE-3 contains a very potent inhibitor for both trypsin and α-chymotrypsin but the titration curves were somewhat different (Fig. 3c). Trypsin was inhibited in a stoichiometric relation by inhibitor DE-3. The inhibition was almost complete at a molar ratio of the inhibitor to trypsin of 1 : 1. The inhibition of α-chymotrypsin by increasing levels of DE-3 was linear at first and extrapolation of the data indicated that one mol of the inhibitor binds with 2 mol of α-chymotrypsin.

A number of papers have appeared, recently, on the Kunitz-type proteinase inhibitors from various legume sources [4, 5, 9, 21, 23]. The inhibitory activity characteristics of the proteinase inhibitors from *E. lysistemon* seed in most cases resemble those of Kunitz-type proteinase inhibitors from other legume seed.

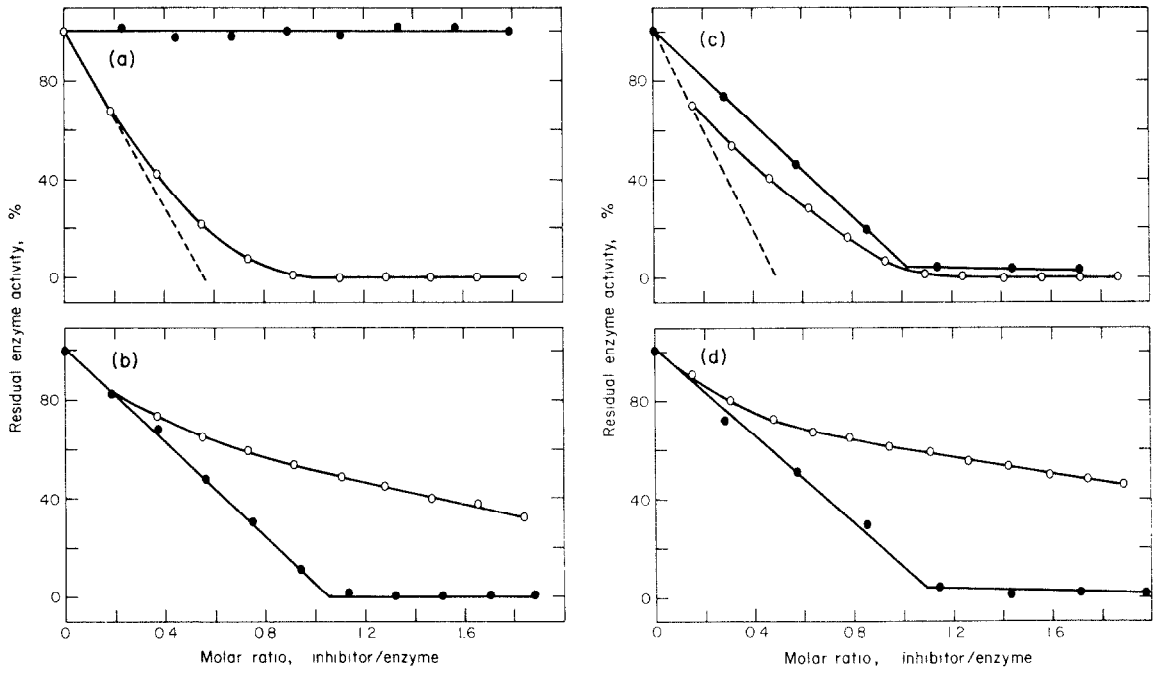

Fig. 3. Inhibition of porcine trypsin and bovine α-chymotrypsin by increasing amount of Kunitz-type proteinase inhibitors from *E. lysistemon* seed. (a) DE-1, (b) DE-2, (c) DE-3 and (d) DE-4. Inhibition of trypsin ●—●. Inhibition of α-chymotrypsin ○—○. MW of 18 200 for the four inhibitors was used.

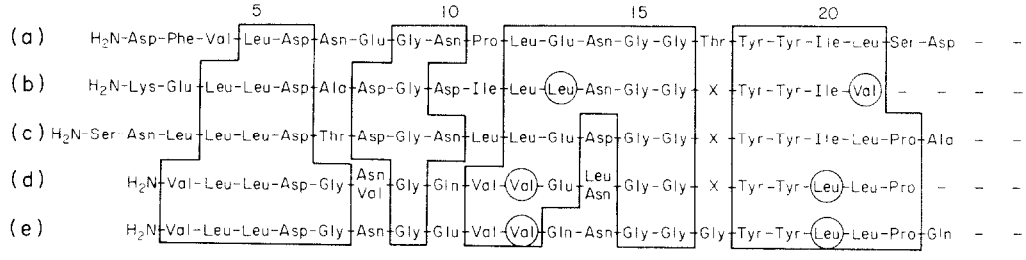

Fig. 4. Comparison of the *N*-terminal primary structures of Kunitz-type proteinase inhibitors from various sources. (a) Kunitz soybean trypsin inhibitor [18–20]; (b) *Albizzia julibrissin* A-II [4]; (c) *Albizzia julibrissin* B-II [4]; (d) *Erythrina latissima* DE-3 [9] and (e) *Erythrina lysistemon* DE-3 (this paper). The circles in a boxed region indicate variant amino acid, × indicates unidentified amino residues.

EXPERIMENTAL

Materials. *Erythrina lysistemon* seeds were supplied by the Forest Research Institute, Ketjen Street, Pretoria West. Procine trypsin (3 × crystallized) was supplied by Miles Laboratories (Pty) Ltd, Cape Town. Bovine α-chymotrypsin was obtained from Worthington. *N*-α-Benzoyl-L-arginine ester hydrochloride (BzArgOEt) and *N*-acetyl-L-tyrosine ethyl ester (AcTyrOEt) was obtained from BDH Chemicals and Merck, respectively. Diethylaminoethylcellulose (DEAE-cellulose) was a microgranular preparation (DE-52) from Whatman. Sephadex G-50 (fine) and DEAE-Sepharose CL-6B were obtained from Pharmacia.

Physicochemical methods. Sephadex G-50, DEAE-cellulose and DEAE-Sepharose columns were prepared as recommended by the manufacturers and the eluates were monitored at 280 nm with a Beckman spectrophotometer. Estimation of MWs by gel filtration was carried out as described in ref. [23] using a Sephadex G-50 column (0.9 × 150 cm). Markers used were soybean trypsin inhibitor (20 100), myoglobin (17 800), ribonuclease (13 700) and *Naja nivea* toxin α (7900). Disc electrophoresis at pH 8.9 using a 15% gel was performed according to the method of ref. [24]. SDS gel electrophoresis at pH 7.2 using a 10% gel was carried out as described in ref. [25].

Proteinase inhibitor assays. Assays used were based on the methods developed in ref. [26]. The rates of hydrolysis at 30° of *N*-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 and 237 nm, respectively. Both enzymes were kept as stock soln of 3 mg/ml in 1 mM HCl. The substrates were used at concns of 1 mM for both *N*-α-benzoyl-L-arginine ethyl ester and *N*-acetyl-L-tryrosine ethyl ester in 0.05 M Tris–HCl, 0.01 M $CaCl_2$, pH 8 and 0.05 M KPi, pH 7, containing 10%

MeOH, respectively. Inhibition of trypsin and chymotrypsin by increasing levels of the inhibitors was assessed by incubating the enzymes with suitable quantities of the inhibitors in 0.1 M Tris–HCl pH 8, for 5 min at room temp. as described [22], and thereafter assaying for enzyme activity remaining. The concn of the enzymes was corrected for inactive materials as determined by active-site titrations [27]. 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 one unit of enzyme activity. Specific inhibitor activity was expressed as inhibitor units/mg inhibitor.

Chemical analysis methods. Amino acid analyses were performed with an automatic Beckman amino acid analyzer. Samples were hydrolysed with 6 M HCl for 24 hr in sealed evacuated tubes; phenol was added to prevent destruction of tyrosine [28]. Half-cystine was determined as cysteic acid by the method of ref. [29]. For the determination of tryptophan the samples were hydrolysed with 3 M *p*-toluene sulphonic acid as described in ref. [30]. Free sulphydryl groups were assayed in intact proteinase inhibitor samples in 6 M guanidinium chloride according to ref. [31].

N-Terminal amino acid sequence. The *N*-terminal sequence of reduced and *S*-carboxymethylated proteinase inhibitor samples was determined with a Beckman sequencer as described [32].

Preparation of the crude proteinase inhibitor. Ground defatted *E. lysistemon* seeds (100 g) were extracted with 0.5 M NaCl solution (1 l.) overnight at 10°. The suspension was then macerated for 5 min in a Waring Blender. The extract was clarified by centrifugation at 10 000 rpm, brought to 70% satn with $(NH_4)_2SO_4$ and the ppt recovered by centrifugation. The ppt was redissolved in 0.05 M NaCl soln, dialysed against H_2O and lyophilized. The yield of the extract was 15.1 g.

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