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PROTEINASE INHIBITORS FROM *ERYTHRINA CORALLODENDRON* AND *ERYTHRINA CRISTAGALLI* SEEDS

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Key Word Index—*Erythrina corallodendron*; *E. cristagalli*; Leguminosae; proteinase inhibitors; inhibitor activities; M_rs; N-terminal sequences.

Abstract—Eight and five proteinase inhibitors were purified from *Erythrina corallodendron* and *E. cristagalli* seeds, respectively, by gel filtration followed by ion exchange chromatography on DEAE-cellulose and DEAE-sepharose. Each inhibitor consists of 161–163 amino acids (M_r 18 000) including four half-cystine residues and resembles the Kunitz-type proteinase inhibitors. The N-terminal amino acid sequence of trypsin inhibitor DE-7 from *E. corallodendron* seed resembles those of other *Erythrina* species. For the other inhibitors no free N-terminal amino acid was found. DE-1, -2, -3, -4 and -5 from the seed of *E. corallodendron* contain potent inhibitors for α-chymotrypsin and they have practically no action on trypsin. From the same seed, inhibitors DE-6, -7 and -8 strongly inhibit trypsin and also inhibit α-chymotrypsin to varying degrees. From the seeds of *E. cristagalli*, inhibitors DE-1 and -8 inhibit trypsin strongly and DE-2, -3 and -4 are strongly inhibitory for α-chymotrypsin. On summarizing the inhibitor characteristics of the Kunitz-type proteinase inhibitors from the seeds of eight different species of *Erythrina*, it was obvious that there is a relationship between the alanine content of the inhibitors and their activities. A high alanine content is associated with potent α-chymotrypsin activities and low alanine content with strong trypsin activities.

INTRODUCTION

The trees and shrubs of the genus *Erythrina*, a legume which belongs to the subfamily Papilionoideae, are distributed throughout tropical to warm regions of the world [1, 2]. The alkaloids [3], amino acids [3–5] and lectins [6–10] which occur in the seeds of various species of *Erythrina* have been studied. The lectins of different *Erythrina* species exhibit many similarities in molecular size, amino acid composition, carbohydrate content, sugar specificity and N-terminal amino acid sequence [9, 10]. Recently, Joubert and co-workers confirmed that seeds from Southern African species of *Erythrina*, namely, *E. acanthocarpa* [11], *E. caffra* [12], *E. humeana* [13], *E. latissima* [14], *E. lysistemon* [15] and *E. zeyheri* [16] contain large concentrations of trypsin and chymotrypsin inhibitors. Besides the purification of several inhibitors, some of the properties of the inhibitors were studied. They contain 164–166 amino acids (M_r 18 000) including four half-cystine residues and resemble the Kunitz-type inhibitors. The N-terminal amino acid sequences of the inhibitors show a high degree of homology. For a number of inhibitors no free N-terminal was found. The N-terminal amino acid could be blocked with an acetyl group or a pyroglutamyl residue.

In continuation of the study on proteinase inhibitors from the seeds from Southern African species of *Erythrina*, the present communication describes the purification and some of properties of proteinase inhibitors from the seeds of *E. corallodendron* and *E. cristagalli*. These species are, respectively, native of the West Indies and Brazil and their properties, viz. the leaves, flowers, colour of bark of the trees and the seeds are different from the Southern African species.

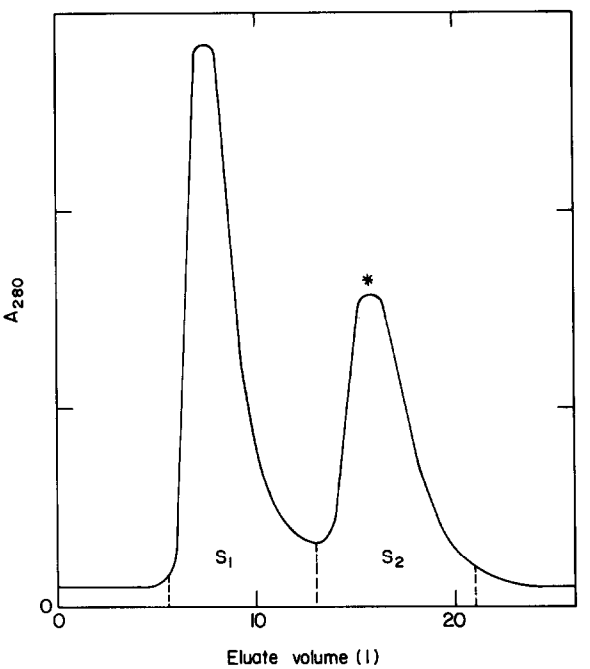

Fig. 1. Gel filtration of the crude extract of the seeds from *E. corallodendron*. Crude extract (2 g) was loaded on a Sephadex G-50 column (3.8 × 150 cm) and eluted with 0.2 M ammonium hydrogen carbonate solution at a flow rate of 50 ml/hr. The column temperature was 20° and the eluate was monitored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

RESULTS

Figure 1 shows the elution profile obtained for the crude extract of *E. corallodendron* seed on Sephadex G-50 in 0.2 M ammonium hydrogen carbonate solution. Two major peaks were evident with only peak S_2 exhibiting trypsin as well as chymotrypsin inhibitor activities. An almost identical elution profile was obtained for the crude extract of *E. cristagalli* seed. Each of the S_2 peaks was lyophilized and further fractionated on DEAE-cellulose using as eluant a linear sodium chloride gradient (0.2 M over 2 l.) in 0.05 M Tris–HCl at pH 8. This revealed a number of major and minor proteinase inhibitor peaks for both seeds (Figs 2 and 3). The C-peaks were each rechromatographed on DEAE-sepharose columns on a linear sodium chloride gradient (0–0.2 M over 1 l.) in 0.05 M Tris–HCl at pH 8 (Figs 4 and 5). The C-peaks afforded DE-1 to DE-8 from the seed *E. corallodendron* and DE-1 to DE-8 from the seed of *E. cristagalli*. The purification of the proteinase inhibitors is summarized in Tables 1 and 2 and some of the properties of the inhibitors are shown in Tables 3 and 4. Disc electrophoresis both in the absence and presence of dodecyl sulphate showed that

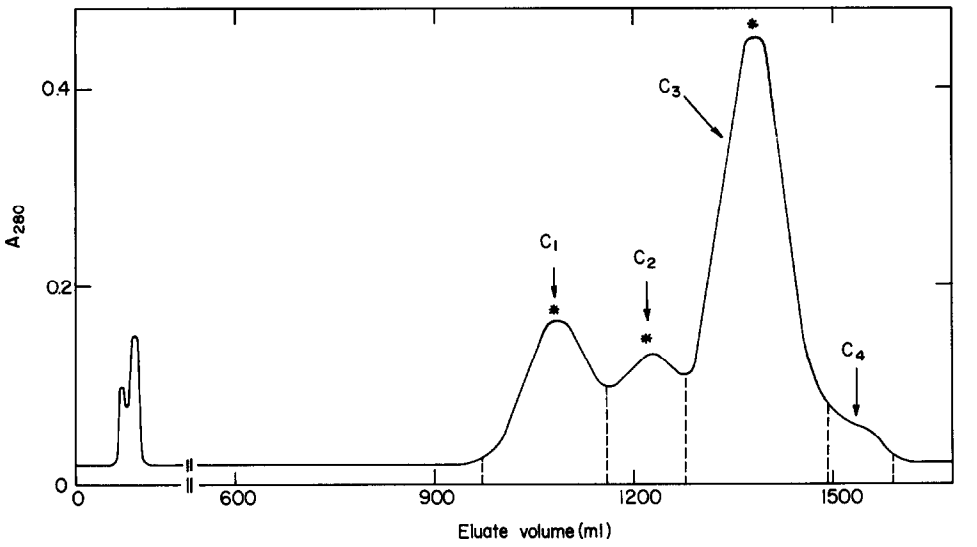

Fig. 2. Chromatography of peak S_2 from *E. corallodendron* seed on DEAE-cellulose. Peak S_2 (0.25 g) was loaded on a DEAE-cellulose (0.9 × 15 cm) column and eluted by a linear sodium chloride gradient (0–0.2 M over 2 l.) in 0.05 M Tris–HCl, pH 8, at a flow rate of 50 ml/hr. The column temperature was 20° and the eluate was monitored at 280 nm. The asterisks denote peak containing trypsin and α-chymotrypsin inhibitor activities.

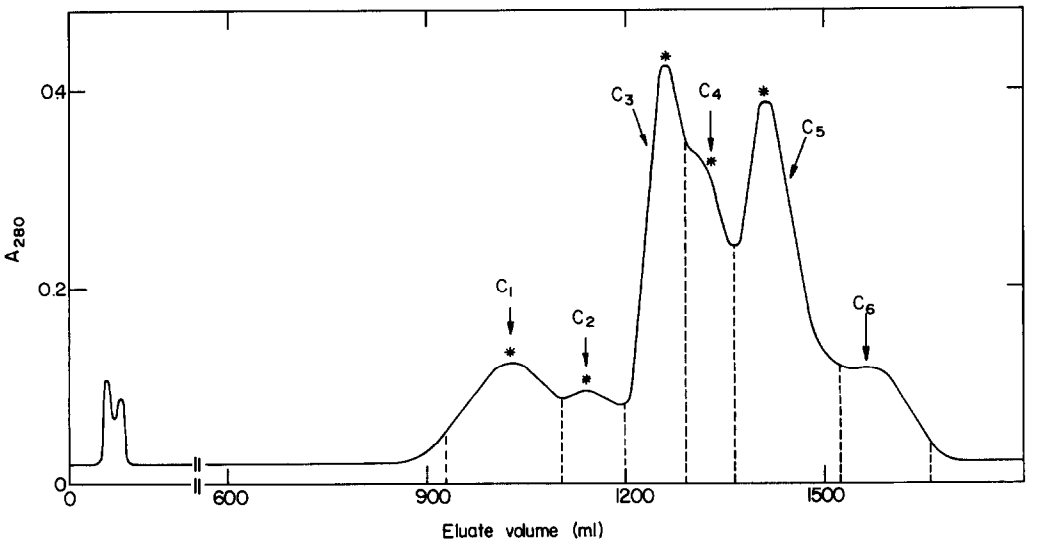

Fig. 3. Chromatography of peak S_2 from *E. cristagalli* seed on DEAE-cellulose. The experimental conditions were the same as in Fig. 2. The asterisks denote peak containing trypsin and α-chymotrypsin inhibitor activities.

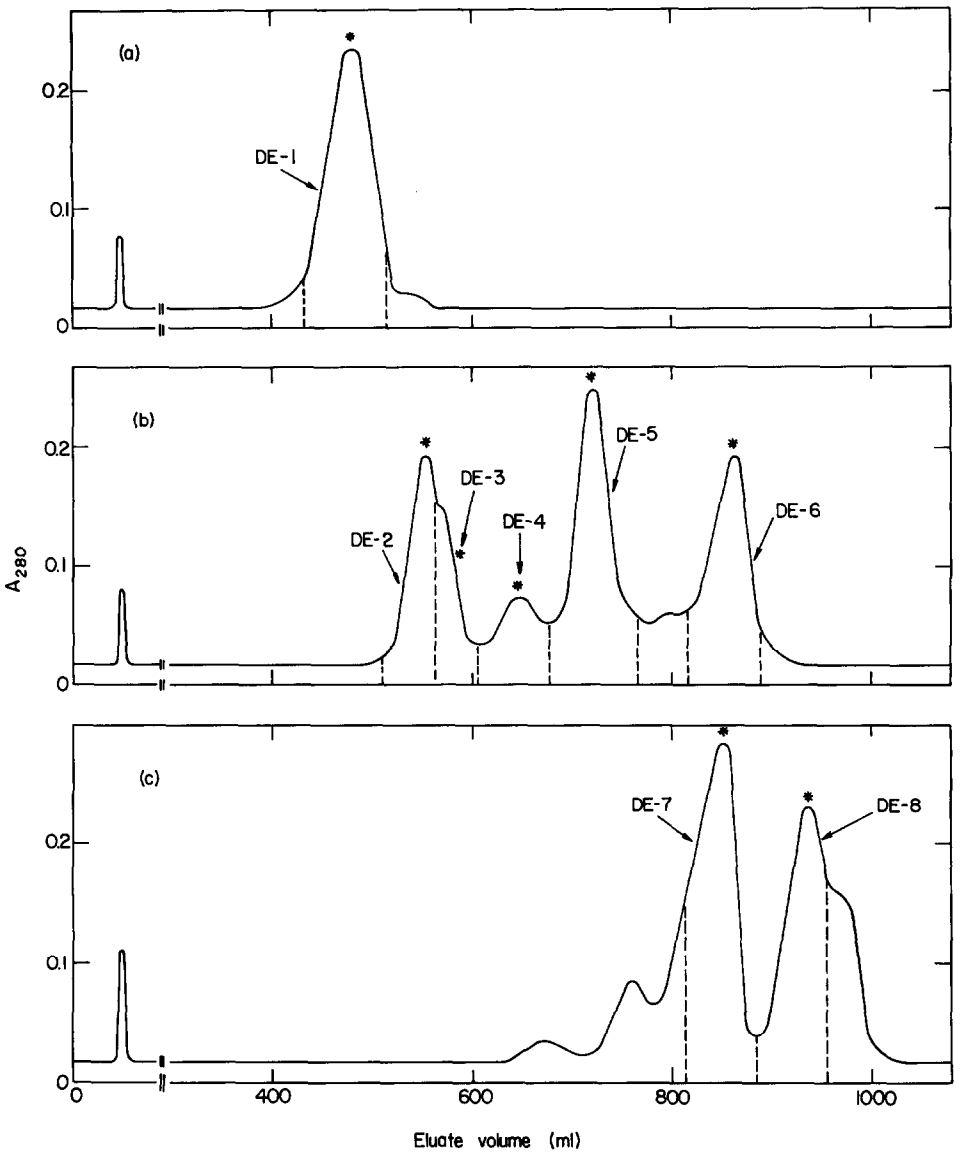

Fig. 4. Rechromatography of peaks C_1, C_2 and C_3 from *E. corallodendron* seed on DEAE-sepharose. Each of the peaks (50 mg) was loaded on a DEAE-sepharose column (0.9 × 50 cm) and eluted by a linear sodium chloride gradient (0–0.2 M over 2 l.) in 0.05 M Tris–HCl, pH 8, at a flow rate of 12 ml/hr. The column temperature was 20° and the eluate was monitored at 280 nm. (a) Peak C_1, (b) peak C_2 and (c) peak C_3. The asterisks denote peaks containing trypsin and α-chymotrypsin inhibitor activities.

the proteinase inhibitors from the seed of *E. corallodendron* and DE-1 to DE-4 and DE-8 from the seed of *E. cristagalli* were homogeneous but disc electrophoresis revealed two major bands for DE-5, -6 and -7 from the latter seed. Further purification of DE-5, -6 and -7 was attempted but it was not successful. The amino acid composition of the pure proteinase inhibitors is given in Tables 5 and 6. The N-terminal primary sequence of reduced and S-carboxymethylated trypsin inhibitor DE-7 from *E. corallodendron* seed, determined on the Beckman sequencer, is shown in Fig. 6(f). However, Edman degradation with the sequencer failed to yield any N-terminal for the other proteinase inhibitors (see Table 7).

Inhibition of porcine trypsin and bovine α-chymotrypsin at pH 8 by increasing levels of the various proteinase inhibitors is shown in Figs 7 and 8.

DISCUSSION

Leguminosae seeds are rich sources of proteinase inhibitors and a number of inhibitors have been isolated and extensively studied. One class of inhibitors (Kunitz-type) have M_rs of approximately 20 000 and a low cystine content usually (two disulphides) [17]. A second class of inhibitors (Bowman–Birk-type) have M_rs of 8000 to 10 000 and high cystine content (usually 7 disulphides) [18]. So far, the proteinase inhibitors from various species of *Erythrina* resemble the Kunitz-type inhibitors. The

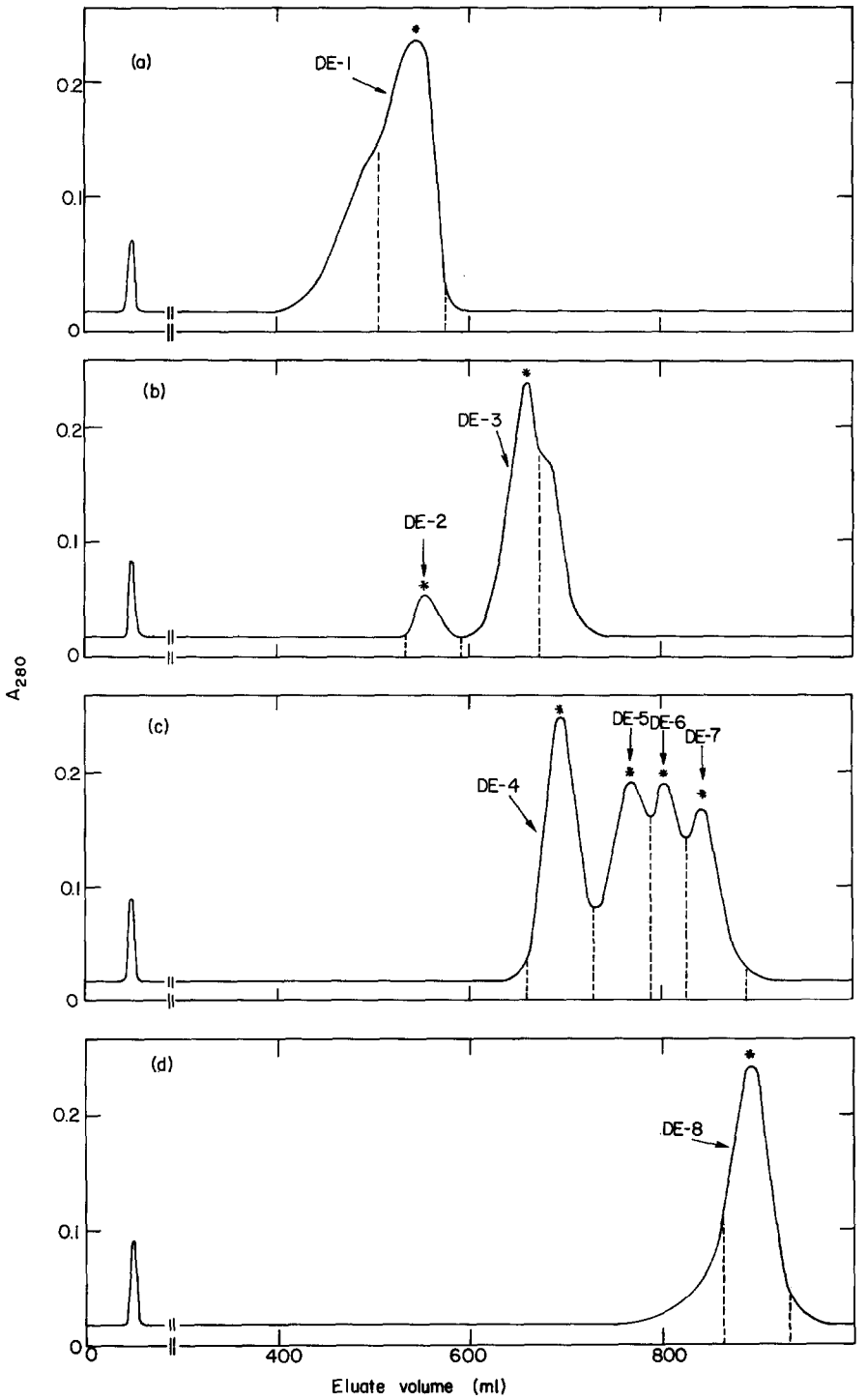

Fig. 5. Rechromatography of peaks C_1, C_2, C_3 and C_4 from *E. cristagalli* seed on DEAE-sepharose. The experimental conditions were the same as in Fig. 4. (a) Peak C_1, (b) peak C_3, (c) peak C_4 and (d) peak C_5. The asterisks denote peaks containing trypsin and α-chymotrypsin inhibitor activities.

amino composition of the Kunitz-type inhibitors from different *Erythrina* species is characterized by a high content of dicarboxylic and hydroxy amino acids (over one-third of the total) and a low content of the aromatic amino acids and cystine.

The proteinase inhibitors isolated from the seeds of *E. corallodendron* and *E. cristagalli* have M_rs of the order of 18 000 and comprise 161–163 amino acids including four half-cystine residues. Since no sulphydryl groups could be detected in the intact inhibitors, they are cross-linked by

Table 1. Summary of the purification of proteinase inhibitors DE-1 to DE-8 from *E. corallodendron* seed

Step	Protein (mg)	Total inhibitor activity (units × 10^3)	Specific inhibitor activity (units/mg protein)	Yield (%)
Crude preparation	2000	T 3220	1610	100
		C 1900	950	100
Sephadex G-50	470	T 2230	4740	69.3
		C 1430	3040	75.3
DEAE-cellulose and DEAE-sepharose				
DE-1	25	T 0	0	0
		C 228	9100	12.0
DE-2	6	T 0	0	0
		C 39	6530	2.1
DE-3	8	T 0	0	0
		C 57	7070	3.0
DE-4	16	T 0	0	0
		C 126	7900	6.6
DE-5	18	T 26	1470	0.8
		C 122	6800	6.4
DE-6	15	T 127	8440	3.9
		C 32	2140	1.7
DE-7	94	T 744	7920	23.1
		C 452	4810	23.7
DE-8	47	T 415	8830	12.8
		C 36	780	1.9

T = trypsin inhibitor; C = α-chymotrypsin inhibitor.

Table 2. Summary of the purification of proteinase inhibitors DE-1 to DE-8 from *E. cristagalli* seed

Step	Protein (mg)	Total inhibitor activity (units × 10^3)	Specific inhibitor activity (units/mg protein)	Yield (%)
Crude preparation	2000	T 1880	940	100
		C 1780	890	100
Sephadex G-50	350	T 1310	3740	69.7
		C 1410	4040	79.2
DEAE-cellulose and DEAE-sepharose				
DE-1	24	T 205	8550	10.9
		C 78	3230	4.4
DE-2	6	T 0	0	0
		C 56	9330	3.1
DE-3	52	T 0	0	0
		C 511	9820	28.7
DE-4	19	T 0	0	0
		C 185	9730	10.4
DE-5	14	T 95	6760	5.0
		C 53	3800	3.0
DE-6	13	T 44	3360	2.3
		C 74	5720	4.2
DE-7	12	T 81	6720	4.3
		C 34	2840	1.9
DE-8	62	T 534	8620	28.4
		C 71	1140	4.0

T = trypsin inhibitor; C = α-chymotrypsin inhibitor.

Table 3. Some of the properties of proteinase inhibitors DE-1 to DE-8 from *E. corallodendron* seed*

Property	DE-1	DE-2	DE-3	DE-4	DE-5	DE-6	DE-7	DE-8
Disc electrophoresis	One band	One band	One band	One band	One band	One band	One band	One band
SDS†-gel electrophoresis	One band	One band	One band	One band	One band	One band	One band	One band
Molecular weight								
(i) Gel filtration‡	17000	17000	17000	ND	18000	16000	17000	17000
(ii) SDS-gel	19000	20000	20000	ND	19000	20000	20000	19000
Inhibitor activity	—	—	—	—	Trypsin§	Trypsin	Trypsin	Trypsin
	Chymotrypsin	Chymotrypsin	Chymotrypsin	Chymotrypsin	Chymotrypsin	Chymotrypsin§	Chymotrypsin§	Chymotrypsin§
N-Terminal amino acid	None	None	None	None	None	None	Valine	None

*The inhibitors contain no free SH-groups.
†Sodium dodecyl sulphate.
‡In 0.05 M Tris–HCl, pH 8, + 0.2 M NaCl.
§Weak activity.

Table 4. Some of the properties of proteinase inhibitors DE-1, DE-2, DE-3, DE-4 and DE-8 from *E. cristagalli* seed*

Property	DE-1	DE-2	DE-3	DE-4	DE-8
Disc electrophoresis	One band	One band	One band	One band	One band
SDS†-gel electrophoresis	One band	One band	One band	One band	One band
M_r by					
(i) Gel filtration‡	16000	ND	17000	17000	16000
(ii) SDS-gel	19000	ND	20000	19000	20000
Inhibitor activities	Trypsin	—	—	—	Trypsin
	Chymotrypsin§	Chymotrypsin	Chymotrypsin	Chymotrypsin	Chymotrypsin§
N-Terminal amino acid	None	None	None	None	None

*The inhibitors contain no free SH-groups.
†Sodium dodecyl sulfate.
‡In 0.05 M Tris–HCl pH 8 + 0.2 M NaCl.
§Weak activity.

Table 5. Amino acid composition of proteinase inhibitors DE-1 to DE-8 from *E. corallodendron* seed

Amino acid	DE-1	DE-2	DE-3	DE-4	DE-5	DE-6	DE-7	DE-8
Asp	17.2 (17)	15.8 (16)	16.8 (17)	16.1 (16)	17.3 (17)	12.8 (13)	14.5 (15)	14.3 (14)
Thr	10.3 (10)	11.3 (11)	10.6 (11)	10.3 (10)	9.2 (9)	8.5 (9)	8.3 (8)	6.9 (7)
Ser	12.1 (12)	12.4 (12)	12.7 (13)	12.2 (12)	13.0 (13)	14.5 (15)	13.7 (14)	17.4 (17)
Glu	19.2 (19)	16.9 (17)	18.1 (18)	19.2 (19)	20.3 (20)	16.6 (17)	20.3 (20)	20.2 (20)
Pro	10.8 (11)	12.9 (13)	11.0 (11)	12.2 (12)	10.6 (11)	11.9 (12)	13.4 (13)	10.8 (11)
Gly	12.0 (12)	13.1 (13)	12.4 (12)	12.5 (13)	12.3 (12)	15.6 (16)	14.1 (14)	15.4 (15)
Ala	11.5 (12)	12.3 (12)	11.8 (12)	11.5 (12)	10.8 (11)	4.1 (4)	7.6 (8)	3.9 (4)
½Cyst	3.5 (4)	3.6 (4)	3.7 (4)	3.5 (4)	3.6 (4)	3.7 (4)	3.4 (3)	3.6 (4)
Val	11.3 (11)	10.8 (11)	11.5 (12)	11.3 (11)	11.5 (12)	10.5 (11)	11.2 (11)	12.5 (13)
Met	0.8 (1)	0.2 (0)	0.2 (0)	0.9 (1)	1.0 (1)	1.3 (1)	0.6 (1)	1.3 (1)
Ile	6.1 (6)	6.2 (6)	6.2 (6)	6.1 (6)	6.3 (6)	9.2 (9)	6.2 (6)	8.6 (9)
Leu	16.0 (16)	15.4 (15)	15.8 (16)	15.4 (15)	15.7 (16)	12.7 (13)	15.3 (15)	12.6 (13)
Tyr	6.0 (6)	6.9 (7)	6.2 (6)	6.5 (7)	6.0 (6)	10.2 (10)	8.0 (8)	8.7 (9)
Phe	5.3 (5)	5.5 (6)	5.4 (5)	5.2 (5)	5.2 (5)	5.9 (6)	4.8 (5)	5.3 (5)
Lys	8.7 (9)	7.5 (8)	7.8 (8)	8.3 (8)	8.5 (9)	8.5 (9)	9.9 (10)	10.7 (11)
His	2.2 (2)	2.0 (2)	2.1 (2)	2.1 (2)	1.7 (2)	2.2 (2)	2.2 (2)	1.4 (2)
Arg	7.3 (7)	7.6 (8)	7.5 (8)	7.1 (7)	7.3 (7)	7.5 (8)	7.2 (7)	6.5 (7)
Try	1.6 (2)	1.7 (2)	1.7 (2)	1.7 (2)	1.6 (2)	1.6 (2)	1.5 (2)	1.7 (2)
Total	162	163	163	162	163	161	163	163

Values are given as mol of residue/mol inhibitor on the basis of M_r of 18000.

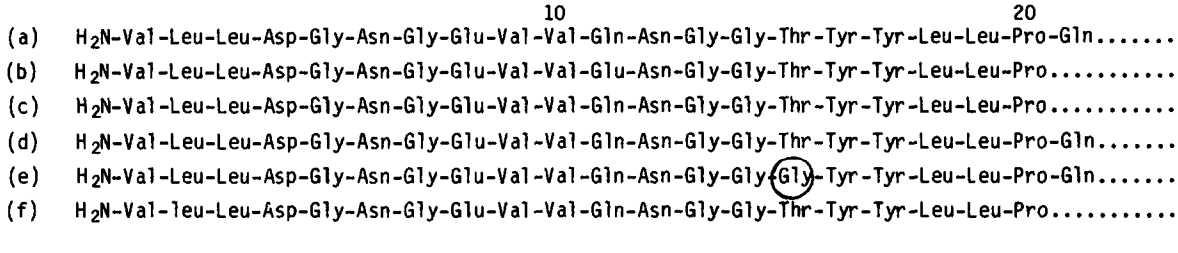

Fig. 6. Comparison of the N-terminal primary structures of Kunitz-type proteinase inhibitors from various *Erythrina* seeds. (a) *E. acanthocarpa* DE-1 [11]; (b) *E. caffra* DE-3 [12]; (c) *E. humeana* DE-3 [13]; (d) *E. latissima* DE-3 [14]; (e) *E. lysistemon* DE-3 [15] and (f) *E. corallodendron* DE-7 (this paper). The circle shows the only difference.

Table 6. Amino acid composition of proteinase inhibitors DE-1, -2, -3, -4 and -8 from *E. cristagalli* seed

Amino acid	DE-1	DE-2	DE-3	DE-4	DE-8
Asp	14.3 (14)	16.6 (17)	17.3 (17)	15.8 (16)	16.3 (16)
Thr	7.4 (7)	9.4 (9)	9.4 (9)	10.4 (10)	7.0 (7)
Ser	17.0 (17)	11.8 (12)	12.1 (12)	12.0 (12)	15.3 (15)
Glu	19.5 (20)	20.6 (21)	19.1 (19)	17.7 (18)	20.2 (20)
Pro	10.1 (10)	12.7 (13)	12.7 (13)	13.3 (13)	10.9 (11)
Gly	15.3 (15)	12.8 (13)	12.7 (13)	13.4 (13)	14.7 (15)
Ala	3.2 (3)	10.4 (10)	11.4 (11)	11.4 (11)	4.2 (4)
Cys	3.6 (4)	3.7 (4)	3.5 (4)	3.4 (3)	3.6 (4)
Val	13.5 (14)	11.9 (12)	12.2 (12)	10.6 (11)	14.2 (14)
Met	1.0 (1)	0.9 (1)	0.1 (0)	0.7 (1)	3.1 (3)
Iso	9.0 (9)	6.4 (6)	6.8 (7)	7.1 (7)	8.7 (9)
Leu	12.4 (12)	15.0 (15)	16.0 (16)	15.6 (16)	9.9 (10)
Tyr	9.8 (10)	5.9 (6)	5.5 (6)	6.0 (6)	8.5 (9)
Phe	5.4 (5)	6.0 (6)	5.4 (5)	5.4 (5)	5.3 (5)
Lys	10.2 (10)	7.3 (7)	6.5 (7)	6.8 (7)	9.7 (10)
His	1.1 (1)	2.3 (2)	2.4 (2)	2.8 (3)	2.1 (2)
Arg	8.0 (8)	7.0 (7)	7.3 (7)	7.6 (8)	7.0 (7)
Try	1.6 (2)	1.5 (2)	1.7 (2)	1.5 (2)	1.6 (2)
Total	162	163	161	163	163

Values are given as mol of residue/mol inhibitor on the basis of M_r of 18000.

Table 7. The inhibitor characteristics and the alanine content (given as mol of alanine/mol inhibitor) of proteinase inhibitors isolated from various *Erythrina* seeds

Seed	Inhibitor	N-Terminal amino acid	Inhibitor activities (units/mg protein)		Alanine content (mol/mol)*	Ref.
			Trypsin	Chymotrypsin		
E. acanthocarpa	DE-1	Val	strong	weak	6	11
	DE-2	none	very weak	strong	12	11
E. caffra	DE-1	none	strong	strong	12	12
	DE-2	none	very weak	strong	12	12
	DE-3	Val	strong	weak	7	12
	DE-4	none	strong	very weak	3	12
E. corallodendron	DE-1	none	none	strong	12	This paper
	DE-2	none	none	strong	12	This paper
	DE-3	none	none	strong	12	This paper
	DE-4	none	none	strong	12	This paper
	DE-5	none	weak	strong	11	This paper
	DE-6	none	strong	weak	4	This paper
	DE-7	Val	strong	weak	8	This paper
	DE-8	none	strong	very weak	4	This paper
E. cristagalli	DE-1	none	strong	weak	3	This paper
	DE-2	none	none	strong	10	This paper
	DE-3	none	none	strong	11	This paper
	DE-4	none	none	strong	11	This paper
	DE-8	none	strong	weak	4	This paper
E. humeana	DE-3	Val	strong	weak	7	13
E. latissima	DE-1	none	very weak	strong	13	14
	DE-3	Val	strong	very weak	7	14
E. lysistemon	DE-1	none	none	strong	12	15
	DE-2	none	strong	weak	4	15
	DE-3	Val	strong	weak	6	15
	DE-4	none	strong	weak	4	15
E. seyheri	DE-1	Val	strong	weak	7	16
	DE-2	none	none	strong	12	16
	DE-3	none	strong	weak	4	16
	DE-4	none	none	strong	11	16
	DE-5	none	strong	weak	4	16

*From the amino acid composition of the inhibitors.

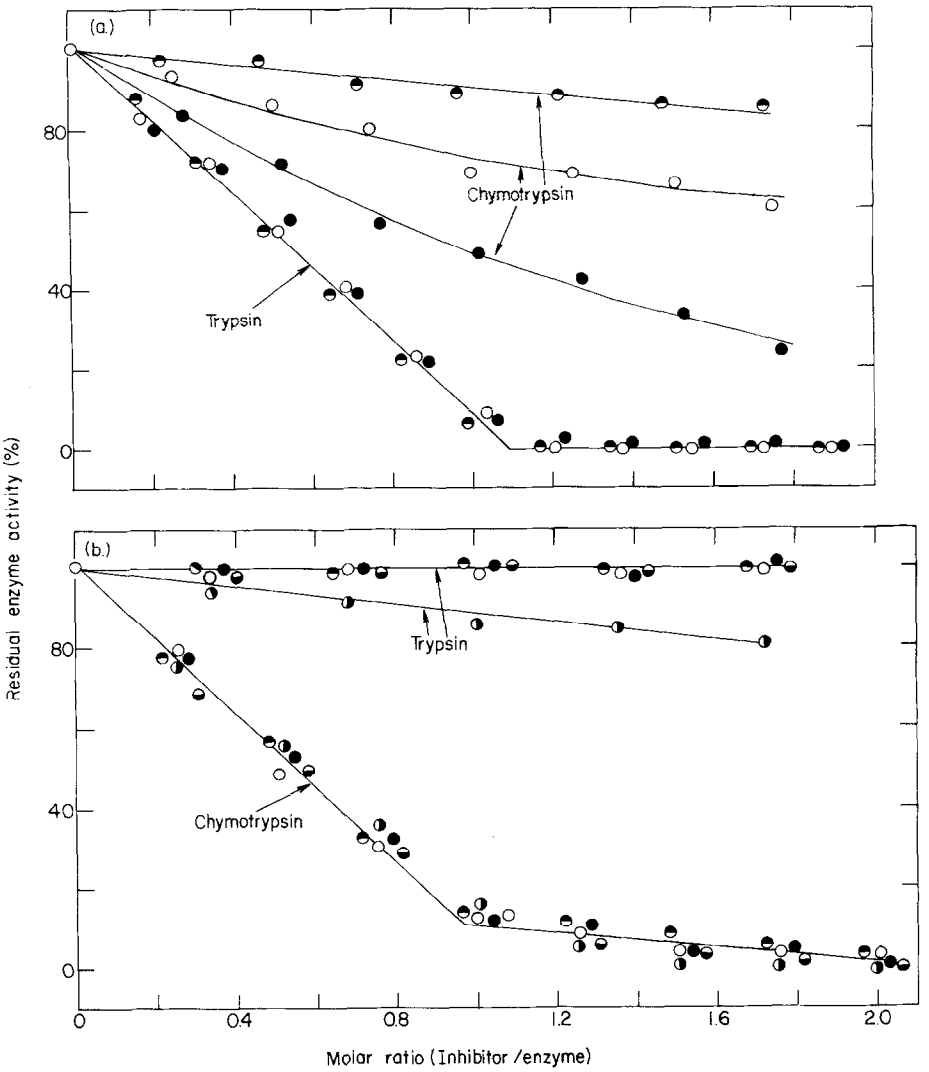

Fig. 7. Inhibition of porcine trypsin and bovine α-chymotrypsin by increasing amounts of Kunitz-type proteinase inhibitors from *E. corallodendron* seed. (a) DE-6 (O——O), DE-7 ●——● and DE-8 ◓——◓. (b) DE-1 O——O, DE-2 ●——●, DE-3 ◓——◓, DE-4 ◒——◒ and DE-5 ◑——◑.

two intramolecular disulphide bridges. The M_rs and low disulphide contents show that the inhibitors belong to the Kunitz-type inhibitors. Furthermore, when the N-terminal primary structure of trypsin inhibitor DE-7 from *E. corallodendron* seed is compared to those of other *Erythrina* species (Kunitz-type inhibitor) (Fig. 6) a high degree of homology is quite obvious.

The inhibitor characteristics of the Kunitz-type proteinase inhibitors from the *E. corallodendron* and *E. cristagalli* seed were varied and different. DE-1, -2, -3, -4 and -5 from *E. corallodendron* seed contain very potent inhibitors for α-chymotrypsin but the inhibition of trypsin is none or small. The titration data (Fig. 7b) shows that inhibitors DE-1 to DE-5 each stoichiometrically inhibit chymotrypsin in a almost molar ratio of 1 : 1. DE-6, -7 and -8 from *E. corallodendron* seed inhibit trypsin strongly and also inhibit α-chymotrypsin weakly to varying degrees. DE-6, -7 and -8 stoichiometrically inhibit trypsin in a molar ratio of 1 : 1 (Fig. 7a).

DE-1 and DE-8 from *E. cristagalli* seed contain very potent inhibitors for trypsin and they also inhibit α-chymotrypsin weakly. In contrast, DE-2, -3 and -4 contain very strong inhibitors for α-chymotrypsin and they have practically no effect on trypsin. The titration data (Fig. 8a and b) show that inhibitors DE-1, -2, -3, -4, -8 each stoichiometrically inhibit trypsin or α-chymotrypsin in a molar ratio of nearly 1 : 1.

The inhibitor characteristics, the free N-terminal amino acids and the alanine content of the Kunitz-type proteinase inhibitors from the seeds of various species of *Erythrina* are summarized in Table 7. A number of inhibitors are specific for chymotrypsin and do not inhibit trypsin. Some inhibitors are potent inhibitors for trypsin, but also inhibit α-chymotrypsin to varying degrees. The inhibitor activities of proteinase inhibitors from *E. corallodendron* and *E. cristagalli*, in general, resemble those of the inhibitors from other species of *Erythrina*. All the chymotrypsin and some of the trypsin inhibitors have no

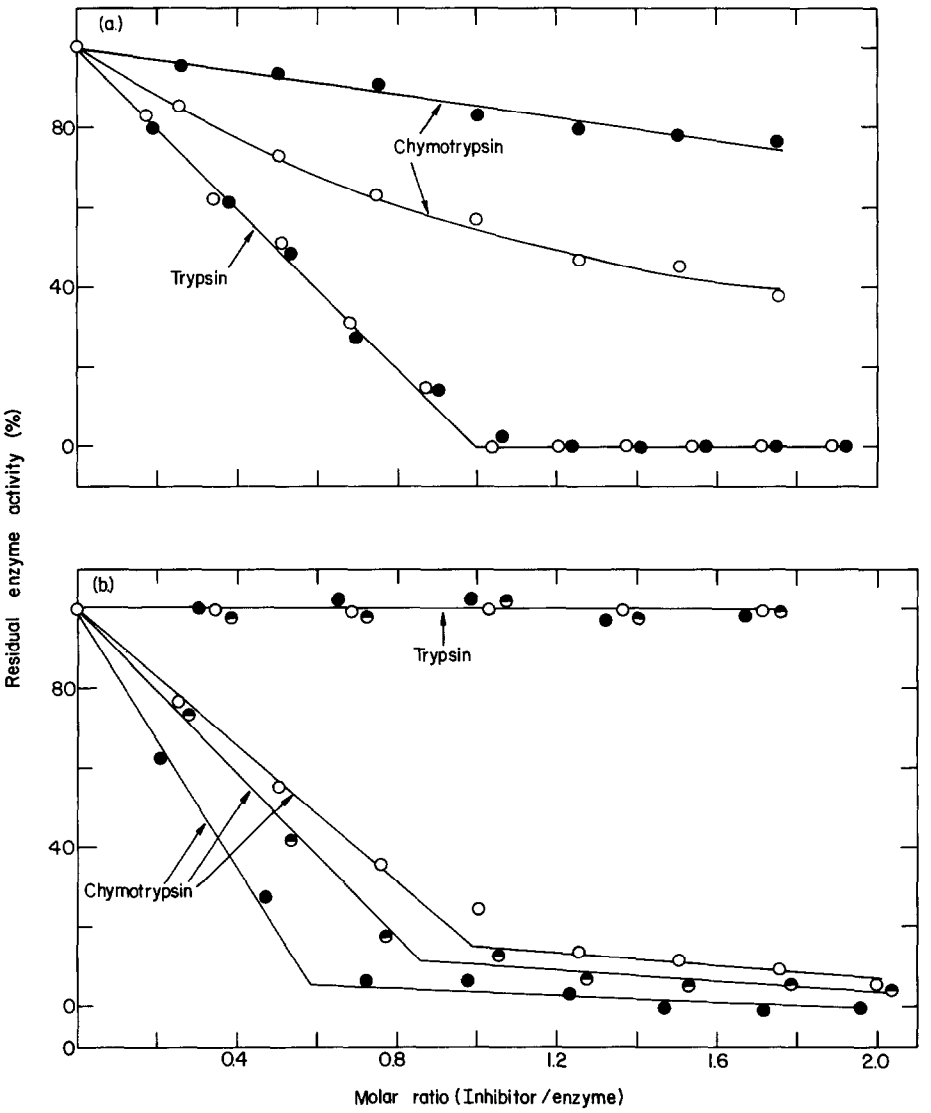

Fig. 8. Inhibition of porcine trypsin and bovine α-chymotrypsin by increasing amount of Kunitz-type proteinase inhibitors from *E. cristagalli* seed. (a) DE-1 O——O and DE-8 ●——● (b) DE-2 O——O, DE-3 ●——● and DE-4 ⊖——⊖.

free N-terminal amino acids. The N-terminal amino acid could be blocked with an acetyl group or a pyroglutamyl residue. The amino acid composition of the trypsin and chymotrypsin inhibitors from different *Erythrina* species is characterized by major differences in the contents of alanine, threonine, isoleucine, tyrosine and lysine. It is interesting to note that there is only a relationship between the alanine content of the inhibitors and their activities. The inhibitors containing a high alanine content (11–13 mol/mol) are associated with potent α-chymotrypsin activities while the inhibitors of low alanine content (3–8 mol/mol) inhibit trypsin strongly.

EXPERIMENTAL

Materials. Erythrina corallodendron L. seeds were collected in Israel and *E. cristagalli* L. seeds were collected by Mr. Jose L. Iglesias in Uruguay. Procine trypsin (3 times crystallized) was supplied by Miles Laboratories (Pty) Ltd., Cape Town. Bovine α-chymotrypsin was obtained from Worthington. N-α-Benzoyl-L-arginine ethyl ester hydrochloride (BzArgOEt) and N-acetyl-L-tyrosine ethyl ester (AcTyrOEt) was obtained from BDH Chemicals and Merck, respectively. 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 on M_rs by gel filtration was carried out as described in ref. [19] on 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 was

performed with a 15% gel according to the method of ref. [20]. SDS gel electrophoresis at pH 7.2 was carried out with a 10% gel as described in ref. [21].

Proteinase inhibitor assays. Assays used were based on the method developed in ref. [22]. 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 absorption at 253 nm and 247 nm, respectively.

Both enzymes were kept as stock soln (3 mg/ml in 1 mM HCl). Each substrate was used at 1 mM concentration 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 and thereafter assaying for enzyme activity remaining. The conc of the enzymes was corrected for inactive materials as determined by active-site titrations [23]. 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 analyser. Samples were hydrolysed with 6 M HCl acid for 24 hr in sealed evacuated tubes; phenol was added to prevent destruction of tyrosine [24]. Half-cystine was determined as cysteic by the method of ref. [25]. For the determination of tryptophan the samples were hydrolysed with 3 M *p*-toluene sulphonic acid as described in ref. [26]. Free sulphydryl groups were assayed in intact proteinase inhibitor samples in 6 M guanidinium chloride according to ref. [27].

N-Terminal amino acid sequence. The N-terminal sequence of reduced and S-carboxymethalated proteinase inhibitor samples was determined with Beckman sequencer as described [28].

Preparation of the crude proteinase inhibitor. Ground defatted seeds (100 g) were extracted with 0.5 M 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 10000 rpm and brought to 70% satn with $(NH_4)_2SO_4$, and the ppt was recovered by centrifugation. The ppt was redissolved in 0.05 M NaCl soln, dialysed against distilled H_2O and lyophilized. The yield of the extracts were, respectively, 16.8 and 12.8 g from the seeds of *E. corallodendron* and *E. cristagalli*.

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