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PURIFICATION AND PROPERTIES OF THE PROTEINASE INHIBITORS FROM *ACACIA SIEBERANA* (PAPERBARK ACACIA) SEED

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Key Word Index—*Acacia sieberana*, paperbark acacia, Leguminosae, proteinase inhibitors, inhibitor activities, MWs, two polypeptide chains, *N*-terminal sequences

Abstract—Two proteinase inhibitors were purified from *A sieberana* seed by gel filtration on Sephadex G-50 followed by ion exchange chromatography on DEAE-cellulose They comprise 168–169 amino acids including four half-cystine residues The two inhibitors, with MWs of *ca* 19 000, contain two polypeptides chains linked by a disulphide bond The *N*-terminal primary structures of the chains showed homology with those of Kunitz-type inhibitors Both DE-1 and DE-2 from the seed of *A sieberana* strongly inhibit porcine trypsin as well as bovine α-chymotrypsin Titration data show that the inhibitors stoichiometrically bind trypsin and chymotrypsin in a molar ratio of *ca* 1 1

INTRODUCTION

The genus *Acacia*, a legume of the subfamily Mimosoideae, occurs on various continents and is indigenous to South Africa and many other parts of the African continent [1, 2] In addition over the years many species of *Acacia* have been introduced to southern Africa, frequently for commercial reasons, and among some of the most important are the wattles [1] Practically all these exotics can be separated from the indigenous species by their lack of thorns and spines [2] In South Africa many species of *Acacia* are important sources of timber, tannins, gums and animal feed [1]

Until recently nothing was known about the proteinase inhibitors of the Mimosoideae Odani *et al* [3] described the trypsin and chymotrypsin inhibitors from the seed of *Albizzia julibrissin* (silk tree) and Jermyn *et al* [4] reported the presence of trypsin inhibitors in the seeds of various *Acacia* species In the present communication the occurrence of proteinase inhibitors in the seeds of several southern African species is established, and the purification and characterization of two proteinase inhibitors from *Acacia sieberana* DC (paperbark acacia) are described While this work was in progress, Kortt and Jermyn [5] reported on the purification and properties of the trypsin inhibitors from the seed of *Acacia elata*

RESULTS

Figure 1 shows the elution profile obtained for the crude extract of the seeds of *A sieberana* on Sephadex G-50 in 0 2 M ammonium hydrogen carbonate solution Several peaks were evident, of which only the S_3 peak exhibited trypsin and chymotrypsin inhibitor activities

The yields and the specific trypsin and chymotrypsin inhibitor activities of crude preparations and S_3 peaks of the seeds of four species of *Acacia*, are summarized in Table 1 The crude preparation and S_3 peaks exhibited trypsin as well as chymotrypsin inhibitor activities, but the yields and almost all of the inhibitor activites of seed of *Acacia sieberana* were the highest It was, therefore, decided to do further work on the seed of this species 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 Tris–HCl at pH 8 This gave two major proteinase inhibitor peaks (Fig 2) Peaks C_1 and C_2 were rechromatographed, using similar conditions as in the first separation The chromatograms each showed a major proteinase inhibitor peak (DE-1 and DE-2) The major peaks represented *ca* 80% of the total concentration The purification of the proteinase inhibitors is summarized in Table 2

Inhibition of porcine trypsin and bovine α-chymotrypsin at pH 8 by increasing levels of DE-1 and DE-2 was obtained Some of the properties of the inhibitors are given in Table 3 The two inhibitors were essentially pure as judged by disc electrophoresis at pH 8 9 Analysis by dodecyl sulphate gel electrophoresis at pH 7 2 without 2-mercaptoethanol revealed single bands with an apparent MW of *ca* 18 000 In the presence of a reducing agent, DE-1 and DE-2 yielded two bands The relative mobilities of the bands indicated MWs *ca* 14 000 and 5000 Therefore the proteinase inhibitors of *A sieberana* seed contain two polypeptide chains linked by a disulphide bond

An attempt was made to separate the two chains of reduced and *S*-carboxymethylated DE-1 and DE-2 by gel filtration on Sephadex G-75 The elution profile for DE-2 gave a major peak (S_1), which eluted at the void volume of the column, and two minor peaks (S_2 and S_3) Peak S_1 represented the larger chain (a chain) and peaks S_2 and S_3 the smaller chains (b and b′ chains) Similar results were obtained for reduced and *S*-carboxymethylated DE-1 The amino acid composition of intact DE-1 and DE-2 and their a, b and b′ chains together with that of the Kunitz soybean trypsin inhibitor is shown in Table 4

DISCUSSION

Odani *et al* [3] purified four Kunitz-type proteinase inhibitors from the seed of *Albizzia julibrissin* (silk tree)

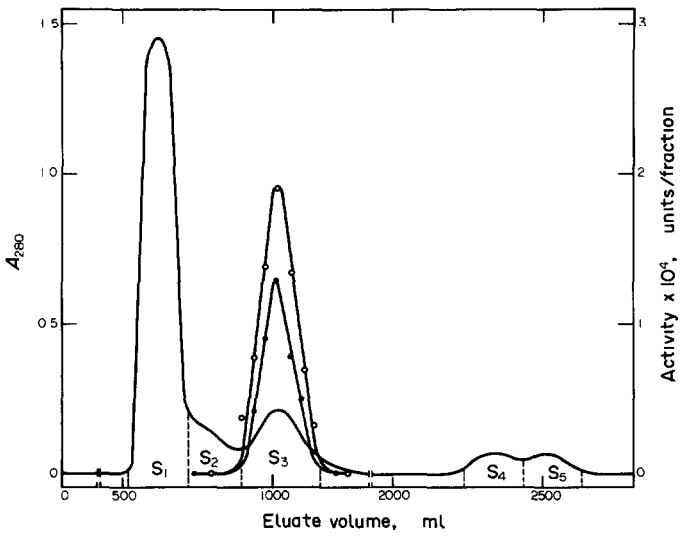

Fig 1 Gel filtration of the crude extract of the seeds of *A sieberana* Crude extract (2 g) was loaded on a Sephadex G-50 column (3 8 × 150 cm) and eluted with 0 2 M ammonium hydrogen carbonate solution at a flow rate of 50 ml/hr The column temperature was 20° and the eluate was monitored at 280 nm Absorbance at 280 nm ———, inhibitor activities against trypsin ●———● and chymotrypsin O———O

Table 1 Proteinase inhibitor activities of various *Acacia* species

Species	Yield		Specific inhibitor activity*			
	Crude preparation (g/100 g seed)	Sephadex S_3 (mg/g crude preparation)	Crude preparation		Sephadex S_3	
			T	C	T	C
Acacia albida (Anatree)	2 8	67	609	471	6190	2910
Acacia karroo (Sweet thorn)	7 4	67	502	495	3620	3190
Acacia sieberana var *woodii* (paperbark acacia)	8 2	80	736	559	5620	4190
Acacia tortilis subspecies *heteracantha* (umbrella thorn)	8 3	53	318	332	3710	3580

*In units/mg T, Trypsin inhibitor, C, chymotrypsin inhibitor

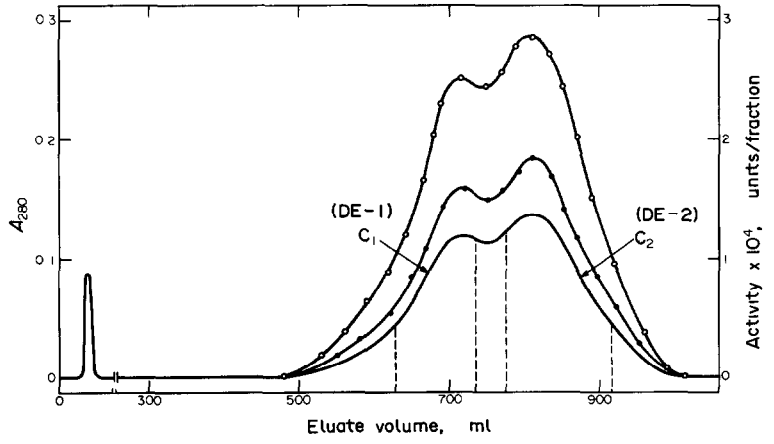

Fig 2 Chromatography of peak S_3 on DEAE-cellulose Peak S_3 (0 25 g) was loaded on a DEAE-cellulose column (0 9 × 15 cm) and eluted by a linear sodium chloride gradient (0–0 4 M over 21) in 0 05 M Tris–HCl at pH 8 at a flow rate of 50 ml/hr The column temperature was 20° and the eluate was monitored at 280 nm Absorbance at 280 nm ———, inhibitor activities against trypsin ●———● and chymotrypsin O———O

Table 2 Summary of the purification of proteinase inhibitor DE-1 and DE-2 from *A sieberana* seeds

Step	Protein (mg)	Total inhibitor activity* (units)	Specific inhibitor activity (units/mg)	Yield (%)
Crude preparation	2000	T 14 72 × 10^5	736	100
		C 11 18 × 10^5	559	100
Sephadex G-50	160	T 8 99 × 10^5	5620	61 1
		C 6 70 × 10^5	4190	59 9
DEAE-cellulose	25	T 2 33 × 10^5	9310	15 8
DE-1		C 1 37 × 10^5	5490	12 3
DE-2	42	T 3 87 × 10^5	9220	26 3
		C 2 36 × 10^5	5610	21 1

*T, trypsin inhibitor, C, chymotrypsin inhibitor

Table 3 Summary of the properties of proteinase inhibitors DE-1 and DE-2

Property	DE-1	DE-2
Disc electrophoresis	One band	One band
SDS*-gel electrophoresis†	One band	One band
SDS-gel electrophoresis‡	Two bands	Two bands
MW by		
(i) gel filtration§	18 500	18 700
(ii) SDS-gel†	18 100	18 500
(iii) SDS-gel‡	14 000 and 4 800	14 000 and 5100
Inhibitor activities	Trypsin	Trypsin
	Chymotrypsin	Chymotrypsin
Free SH	None found	None found

*Sodium dodecyl sulphate
†Without 2-mercaptoethanol
‡In the presence of 2-mercaptoethanol
§Markers used were soybean trypsin inhibitor (20 100), myoglobin (17 800), ribonuclease (13 700) and *Naja nivea* toxin α (7900) In 0 05 M Tris–HCl pH 8 + 0 2 M NaCl

and they found that the inhibitors contained two polypeptide chains linked by a disulphide bond Recently, Kortt and Jermyn [5] isolated two trypsin inhibitors from the seed of *Acacia elata* The two inhibitors are homologous with the soybean trypsin inhibitor (Kunitz) but they were composed of two polypeptide chains linked by a disulphide bond In contrast the Kunitz-type proteinase inhibitors from soybean [6–8], the beans of *Psophocarpus tetragonolobus* [11, 12] the seeds of *Peltophorum africanum* [13] and various species of *Erythrina* [14, 15] are single polypeptide chains

The MW of the proteinase inhibitors (DE-1 and DE-2) from *A sieberana* seed by gel filtration and dodecyl sulphate gel electrophoresis, in the absence of a reducing agent, was *ca* 18 000 Hence the inhibitors comprise 168–169 amino acids including four half-cystine residues No sulphydryl groups could be demonstrated in the intact inhibitors and, therefore, they are cross-linked by two intramolecular disulphide bonds The MWs and the low disulphide content of the *A sieberana* inhibitors resemble the Kunitz-type proteinase inhibitors In the presence of a reducing agent DE-1 and DE-2 yielded each a long a chain and two short b and b′ chains Furthermore, the amino-terminal sequence of the a and b chains are, respectively, homologous to the amino- and carboxy-terminal regions of the Kunitz trypsin inhibitor and to the a and b chains of the Kunitz-type inhibitors from *Albizzia julibrissin* and *Acacia elata* seeds (Fig 3) The amino acid composition of the b′ chains of DE-1 and DE-2 resemble those of the b chains The sequence of the b′ chain of DE-2 was, however, not very homologous to that of the b chain In spite of the apparent homogeneity by gel electrophoretic criteria of DE-1 and DE-2, the above revealed micro-heterogeneity particularly in the carboxyl-terminal regions of the molecules

A sieberana seed contains two major Kunitz-type proteinase inhibitors (DE-1 and DE-2), which not only strongly bind trypsin but they also strongly inhibit α-chymotrypsin The titration curves were similar and they show that the inhibitors stoichiometrically inhibited trypsin and α-chymotrypsin in a molar ratio of *ca* 1 1

The inhibitory characteristics and the number of peptide chains of the Kunitz-type proteinase inhibitors from various seeds and beans are summarized in Table 5 A few inhibitors are specific for chymotrypsin and do not inhibit trypsin Some Kunitz-type inhibitors are potent inhibitors for trypsin, but also inhibit α-chymotrypsin to varying degrees The inhibitor activities of proteinase inhibitors DE-1 and DE-2 from *A sieberana*, in general, resemble those of the Kunitz-type inhibitors from other legume seeds and beans

EXPERIMENTAL

Materials Acacia sieberana DC seed were collected from a large tree in Pretoria The source of trypsin, α-chymotrypsin and chemical reagents have been described previously [15]

Methods The physicochemical methods, the reduction and *S*-carboxymethylation of the proteinase inhibitors and the *N*-terminal sequence determination with the Beckman sequencer have been detailed in a previous communication [15]

The esterolytic activities of trypsin and α-chymotrypsin were measured spectrophotometrically according to the method of ref [17] as described earlier [15] 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 nm and 237 nm, respectively The

Table 4 Amino acid composition of proteinase inhibitors DE-1 and DE-2 and the a, b and b′ chains given as mols of residue per mol of inhibitor or peptide chain

Amino acid	DE-1				DE-2				Kunitz soybean trypsin inhibitor [6–8]
	Whole inhibitor	a chain	b chain	b′ chain	Whole inhibitor	a chain	b chain	b′ chain	
Aspartic acid	17 4(17)	10 2(10)	6 3(6)	4 3(4)	18 0(18)	9 9(10)	6 7(7)	4 3(4)	26
Threonine	5 3(5)	5 2(5)	0 2(0)	1 1(1)	5 6(6)	5 1(5)	0 1(0)	1 2(1)	7
Serine	15 1(15)	10 5(11)	2 9(3)	3 2(3)	14 1(14)	11 6(12)	3 0(3)	3 2(3)	11
Glutamic acid	19 9(20)	17 4(17)	3 2(3)	4 6(5)	21 4(21)	17 8(18)	2 9(3)	4 6(5)	18
Proline	11 3(11)	9 0(9)	1 2(1)	2 3(2)	10 3(10)	8 8(9)	1 1(1)	2 3(2)	10
Glycine	17 4(17)	12 7(13)	3 9(4)	2 8(3)	17 2(17)	12 8(13)	3 8(4)	3 9(4)	16
Alanine	6 9(7)	5 4(5)	2 0(2)	1 8(2)	8 2(8)	5 6(6)	2 4(2)	1 3(1)	8
Half-cystine	3 5(4)*	2 6(3)†	0 9(1)†	0 7(1)†	3 6(4)*	2 7(3)†	1 0(1)†	0 8(1)†	4
Valine	9 6(10)	6 6(7)	3 4(3)	3 6(3)	10 1(10)	7 2(7)	3 4(3)	2 7(3)	14
Methionine	0 1(0)	0	0	0	0	0	0	0	2
Isoleucine	6 3(6)	5 3(5)	1 0(1)	1 5(2)	6 3(6)	5 3(5)	1 0(1)	1 4(1)	14
Leucine	20 4(20)	16 4(16)	3 9(4)	4 7(5)	20 1(20)	16 3(16)	3 9(4)	4 6(5)	15
Tyrosine	6 8(7)	5 3(5)	1 0(1)	1 6(2)	6 4(6)	5 4(5)	1 0(1)	1 4(1)	4
Phenylalanine	6 0(6)	5 1(5)	1 1(1)	1 6(2)	5 1(5)	5 4(5)	1 1(1)	1 4(1)	9
Lysine	7 8(8)	4 6(5)	3 0(3)	2 0(2)	8 0(8)	4 8(5)	2 8(3)	1 8(2)	10
Histidine	2 0(2)	2 0(2)	0 4(0)	0 8(1)	3 2(3)	2 9(3)	0 4(0)	0 8(1)	2
Arginine	12 8(13)	7 7(8)	4 7(5)	3 0(3)	12 0(12)	7 4(7)	4 4(4)	2 9(3)	9
Tryptophan‡	0	0	0	0	0 7(1)	0 8(1)	0	0	2
Total	168	126	38	41	169	130	39	38	181

*Determined as cysteic acid by the method of ref [9]
†Determined as *S*-carboxymethylcysteine
‡Determined by the method described by Liu and Chang [10]

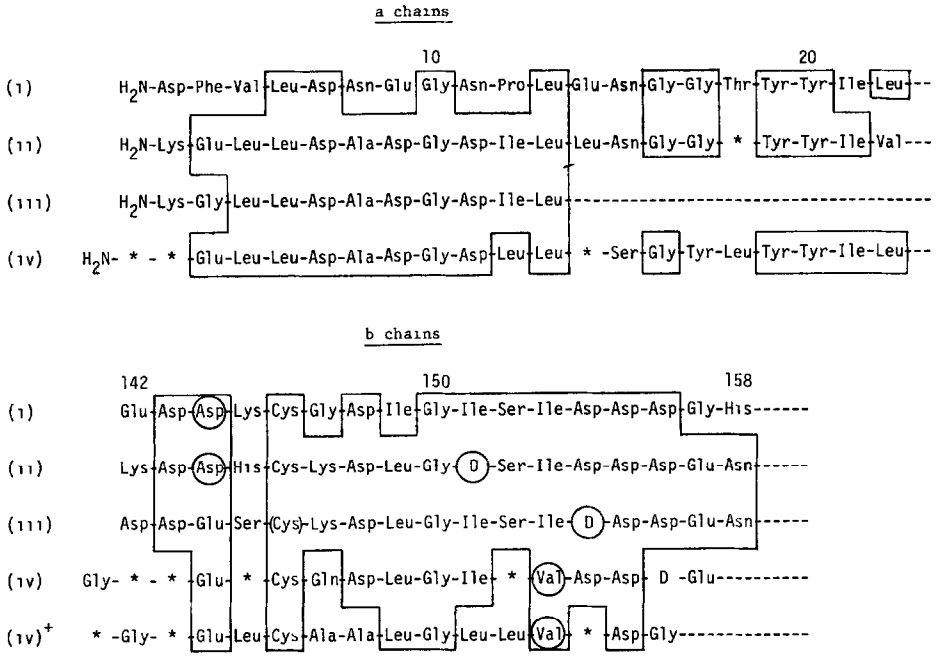

Fig 3 Comparison of the *N*-terminal primary structures of the a and b chains of Kunitz-type proteinase inhibitors from various sources (i) Kunitz soybean trypsin inhibitor [6–8], (ii) *Albizzia julibrissin* A-II [3], (iii) *Acacia elata* [5], and (iv) *Acacia sieberana* DE-2 (this paper) *, Residue not determined, D, deletion, +, sequence of the b^1 chain of DE-2 from *A sieberana* The circles in a boxed region indicate variant amino acids

Table 5 The inhibitory characteristics and number of polypeptide chains of Kunitz-type proteinase inhibitors from various legume seeds and beans

Legume seed or bean	Inhibitor	No of peptide chains	Inhibitor activities		Reference
			Trypsin	Chymotrypsin	
Soybean		1	Strong	Weak	[16]
Erythrina latissima	DE-1	1	Weak	Strong	[14]
	DE-3	1	Strong	Weak	[14]
Erythrina lysistemon	DE-1	1	None	Strong	[15]
	DE-2	1	Strong	Weak	[15]
	DE-3	1	Strong	Strong	[15]
	DE-4	1	Strong	Weak	[15]
Peltophorum africanum	DE-1	1	Strong	Strong	[13]
Psophocarpus	2	1	Strong	Weak	[11]
tetragonolobus	3	1	Strong	Weak	[11]
	B	1	None	Strong	[12]
Albizzia julibrissin	AII	2	Strong	Strong	[3]
	AIII	2	Strong	Strong	[3]
	BI	2	None	Strong	[3]
	BII	2	None	Strong	[3]
Acacia elata	2	2	Strong	Strong*	[5]
Acacia sieberana	DE-1	2	Strong	Strong	†
	DE-2	2	Strong	Strong	†

*The binding of the enzyme by the inhibitor was weak
†This paper

inhibitors' activities were estimated from the residual enzymatic activities as described previously [15] The concn of the enzymes was corrected for inactive materials as determined by active-site titrations [18]

1 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° 1 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

Preparation of the crude proteinase inhibitor Ground *A sieberana* seeds (100 g) were extracted with 10% NaCl soln (1 l) overnight at 10° The suspension was then macerated for 5 min in a Waring blendor The extract was clarified by centrifugation at 16000 *g* brought to 75% satn with $(NH_4)_2SO_4$ and the ppt recovered by centrifugation The ppt was redissolved in 10% NaCl soln, dialysed against H_2O and lyophilized The yield of the extract was 8 2 g

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