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TRYPSIN ISOINHIBITORS FROM *MOMORDICA REPENS* SEEDS

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Key Word Index—*Momordica repens*, Cucurbitaceae, trypsin isoinhibitors, inhibitor activities, sequences

Abstract—Four trypsin isoinhibitors (CM-1 to CM-4) were purified from *Momordica repens* seeds by gel filtration on Sephadex G-50 followed by ion exchange chromatography on CM-cellulose They comprise 29 amino acids including six half-cystine residues The amino acid sequences of CM-1 and CM-3 have been elucidated The sequences resemble those of the isoinhibitors from squash seed For CM-4 no free N-terminal amino acid was found Inhibitors CM-1, CM-2 and CM-3 inhibit trypsin strongly and they have practically no action on α-chymotrypsin The trypsin isoinhibitors which occur in squash and *Momordica repens* seeds are probably the smallest inhibitors of serine proteinases described so far

INTRODUCTION

In a survey of plant materials for serine proteinase inhibitors Hojima *et al* [1, 2] found that numerous plants are good sources of inhibitors with a variety of specificities These pumpkin seed extracts are remarkable, since they contain a low MW trypsin inhibitor that strongly inhibits Hageman factor fragment but does not inhibit plasma kallikrein Hojima *et al* [3] reported the purification and characterization of this interesting inhibitor from *Cucurbita maxima* (pumpkin) seed, which belongs to the plant family Cucurbitaceae Earlier, Polanowski *et al* [4] described the purification of three trypsin inhibitors form *Cucurbita maxima* (squash) seed Subsequently, the Polish and American workers [5] published the amino acid sequences of two isoinhibitors from squash seed Both isoinhibitors contain 29 amino acids including six half-cystine Their sequences differ only in one position and arginine in position 5 is present at the reactive site of both isoinhibitors

The present communication describes the purification and some of the properties of five low MW trypsin isoinhibitors from *Momordica repens* seeds, also from the plant family Cucurbitaceae

RESULTS

Purification and properties of the isoinhibitors

Figure 1 shows the elution profile obtained for the crude extract on Sephadex G-50 in 0 2 M ammonium hydrogen carbonate solution Several peaks were evident, of which only the S_5 peak exhibited trypsin inhibitor activity Peak S_5 was lyophilized and further fractionated on CM-cellulose using a linear gradient of ammonium acetate–acetic acid buffer pH 5 (Fig 2) The chromatogram showed various peaks, all of which, apart from C_1 and C_7, possessed trypsin inhibitor activities The active peaks were rechromatographed using similar conditions as in the first separation The chromatograms each showed a major trypsin inhibitor peak which afforded CM-1, CM-2, CM-3, CM-4 and CM-5 The purification of the inhibitors is summarized in Table 1 The specific activity of CM-5 was small in comparison with those found for CM-1 to CM-4 Therefore, no further work was done on CM-5 Some of the properties of the inhibitors are summarized in Table 2 Disc electrophoresis revealed a single band for CM-1 to CM-4 Their amino acid composition is given in Table 3 The inhibitors were devoid of free sulphydryl groups Inhibition of porcine trypsin and bovine α-chymotrypsin by increasing levels of inhibitors CM-1, CM-2 and CM-3 is shown in Fig 3

Amino acid sequences of reduced and S-carboxymethylated CM-1, CM-3 and CM-4

The tryptic digest of reduced and S-carboxymethylated inhibitor CM-1 was fractionated on a column of DEAE-cellulose and the amino acid composition of pure peptides is shown in Table 4 The amino acid sequence of CM-1 is shown in Fig 4(a) The amino-terminal sequence of reduced and S-carboxymethylated CM-1 was determined using a Beckman sequencer The known N-terminal sequence directly established the alignment of peptides T-1, T-2, T-3, T-4 and T-5 The only tryptic peptide which was not positioned is T-6 This peptide contained no arginine or lysine and had to be derived from the C-terminus of CM-1 The sequence of T-6 was determined by the manual Edman procedure

The N-terminal sequence of reduced and S-carboxymethylated CM-3 is shown in Fig 4(b) Limited hydrolysis of CM-3 with trypsin at pH 3, and subsequent reduction, S-carboxymethylation and gel filtration yielded one peak (S_1) Such limited hydrolysis occurs at the reactive site of the inhibitors [6–8] Table 3 shows that S_1 comprises 25 amino acids and its N-terminal sequence is revealed in Fig 4(c) The N-terminal sequence of CM-3 and S_1 were aligned [see Fig 4(b) and (c)] and it was obvious that of the 29 residues of CM-3, 24 have been sequenced The C-terminal peptide of CM-3 was isolated from a tryptic digest as described above and its sequence determined This sequence revealed that inhibitor CM-3

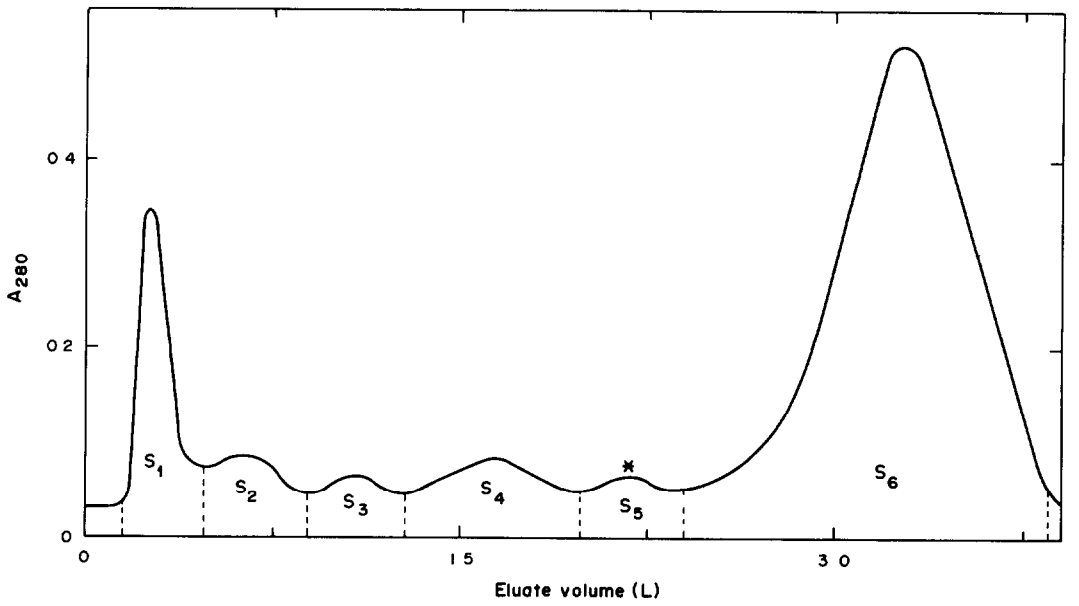

Fig 1 Gel filtration of the crude extract of the seeds of *Momordica repens* Crude extract (2 g) was loaded on Sephadex G-50 column (3 8 x 150 cm) and eluted with 0 2 M ammonium hydrogen carbonate solution at a flow rate of 50 ml/hr The column temperature was 20° and the eluate was monitored at 280 nm The asterisk indicates trypsin inhibitor activity

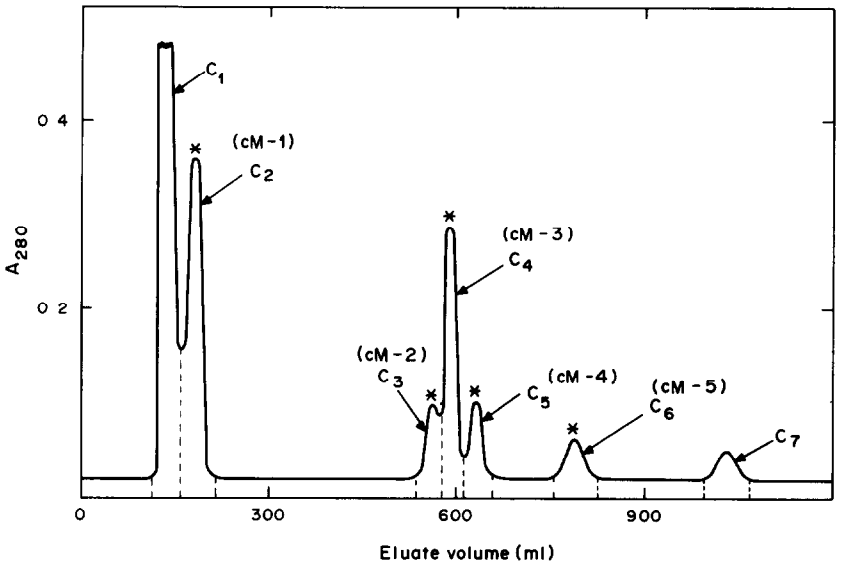

Fig 2 Chromatography of peak S_5 on CM-cellulose Peak S_5 (0 2 g) was loaded on CM-cellulose column (0 9 x 15 cm) and eluted by a linear gradient of 0 05–0 6 M ammonium acetate–acetic acid buffer of pH 5 over 2 l at a flow rate of 50 ml/hr The column temperature was 20° and the eluate was monitored at 280 nm The asterisks indicate trypsin inhibitor activities

contains two or more isoinhibitors It was obvious that limited hydrolysis by trypsin at pH 3 cleaved CM-3 at its trypsin reactive site, Arg^5-Ile^6

Edman degradation with the sequencer failed to yield any N-terminal sequence for CM-4 The N-terminal sequence could be blocked with an acetyl group or pyroglutamyl residue [9]

DISCUSSION

The properties of the four trypsin inhibitors (CM-1 to CM-4) from *Momordica repens* seeds are presumably very similar Their trypsin inhibitor activities were alike, their amino acid compositions were almost identical and the sequences of CM-1 and the isoinhibitors of CM-3 were

Table 1 Summary of the purification of the trypsin isoinhibitors

Steps	Protein (mg)	Total inhibitor activity (units, $\times 10^{-5}$)	Specific inhibitor activity (units/mg, $\times 10^{-3}$)	Yield (%)
Crude	5000	47 00	0 94	100
Sephadex G-50	200	32 12	16 06	68 3
CM-cellulose				
CM-1	16	5 44	34 02	11 6
CM-2	8	2 68	33 61	5 7
CM-3	31	10 23	33 01	21 8
CM-4	18	3 77	20 96	8 0
CM-5	10	0 35	3 48	0 7

Table 2 Summary of the properties of trypsin isoinhibitors CM-1 to CM-4

Properties	CM-1	CM-2	CM-3	CM-4
Disc electrophoresis	One band	One band	One band	One band
SDS* gel electrophoresis	One band	One band	One band	One band
Molecular weight				
(1) Gel filtration†	4400	4500	4400	4000
(2) SDS gel electrophoresis	‡	‡	‡	‡
Inhibitor activities	Trypsin	Trypsin	Trypsin	Trypsin
Free SH	None	None	None	None
N-terminal amino acids	Glycine	ND	Alanine	None

*Sodium dodecyl sulphate
†In 0 05 M Tris–HCl pH 8 plus 0 2 M NaCl
‡Migrated very close to tracker dye (bromophenol blue)

Table 3 Amino acid composition of protease isoinhibitors CM-1, CM-2, CM-3, CM-4 and fragment S_1 given as mols of residue per mol of isoinhibitor

Amino acid	CM-1	CM-2	CM-3	CM-4	Fragments S_1†
Asp	2 2 (2)	2 2 (2)	2 4 (2)	2 4 (2)	2 4 (2)
Thr	(0)	(0)	(0)	(0)	(0)
Ser	1 4 (1)	1 4 (1)	2 4 (2)	2 4 (2)	2 2 (2)
Glu	3 4 (3)	3 1 (3)	2 4 (2)	2 4 (2)	2 0 (2)
Pro	1 3 (1)	1 4 (1)	1 8 (2)	1 8 (2)	1 0 (1)
Gly	2 9 (3)	2 6 (3)	3 3 (3)	4 0 (4)	2 9 (3)
Ala	1 2 (1)	0 7 (1)	1 7 (2)	1 8 (2)	1 3 (1)
Half-Cys*	5 6 (6)	5 5 (6)	5 4 (6)	5 7 (6)	4 7 (5)
Val	0 9 (1)	0 7 (1)	0 2 (0)	0 4 (0)‡	0 4 (0)‡
Met	0 8 (1)	0 7 (1)	0 2 (0)	(0)	0 (0)
Ile	1 5 (2)	1 7 (2)	1 6 (2)	1 7 (2)	1 4 (1)
Leu	1 7 (2)	1 7 (2)	1 3 (1)	0 9 (1)	1 0 (1)
Tyr	0 9 (1)	0 9 (1)	1 0 (1)	0 9 (1)	0 8 (1)
Phe	(0)	0 2 (0)	(0)	0 1 (0)	0 1 (0)
Lys	1 9 (2)	2 1 (2)	1 9 (2)	1 0 (1)	1 5 (2)
His	(0)	0 1 (0)	0 1 (0)	(0)	(0)
Arg	2 9 (3)	3 4 (3)	3 6 (4)	4 1 (4)	2 7 (3)
Total	29	29	29	29	24

*Determined as cysteic acid by the method of Hirs [16]
†Obtained by limited hydrolysis of CM-3 with trypsin at pH 3
‡Obtained after hydrolysis for 72 hr

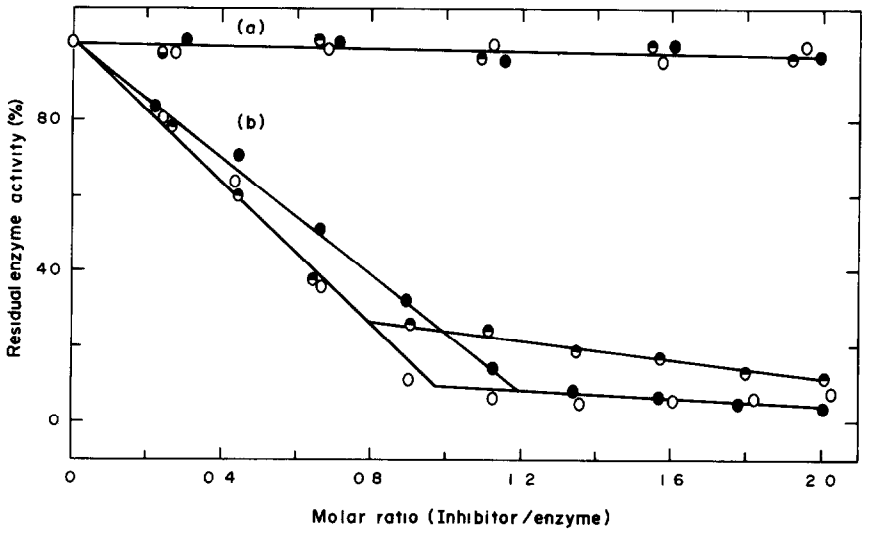

Fig 3 Inhibition of (a) porcine trypsin and (b) of bovine α-chymotrypsin by increasing amounts of trypsin isoinhibitors from *Momordica repens* seed Inhibitors CM-1 ○—○, CM-2 ●—● and CM-3 ◔—◔

Table 4 Amino acid composition of tryptic peptides of reduced and S-carboxymethylated CM-1 (1 μmol) given as mols of residue per mol of peptide

Amino acid	T-1	T-2	T-3	T-4	T-5	T-6	Sequence	Analysis
CMC*	1 0(1)	1 0(1)		3 0(3)		0 8(1)	6	5 6
Asp				2 0(2)			2	2 2
Thr							0	0
Ser				1 0(1)			1	1 4
Ala		1 1(1)		1 1(1)		1 0(1)	3	3 4
Pro	0 9(1)						1	1 3
Gly	0 7(1)					2 0(2)	3	2 9
Ala				1 1(1)			1	1 2
Val				1 1(1)			1	0 9
Met		0 9(1)					1	0 8
Ile	1 0(1)	0 9(1)					2	1 5
Leu		0 9(1)		1 2(1)			2	1 7
Tyr						1 0(1)	1	0 9
Phe							0	0
Lys		1 1(1)		1 2(1)			2	1 9
His							0	0
Arg	1 2(1)		1		1		3	2 9
Total	5	6	1	11	1	5	29	

*S-carboxymethylcysteine

very similar The inhibitors each contain 29 amino acids including six half-cystine residues Since no sulphydryl groups could be detected in the intact inhibitors, they are cross-linked by three disulphide bridges The sequences of trypsin inhibitors CM-1 and CM-3 from *Momordica repens* seed are highly homologous with those of the two trypsin isoinhibitors from squash seed (Fig 5)

The inhibitors from squash and *Momordica repens* of MW *ca* 3300 are probably the smallest inhibitor of serine proteases described so far Previously the sequences of carboxypeptidase inhibitors from tomato fruit and potatoes were reported [10] These inhibitors contain 36–37 amino acids including six half-cystine residues The sequences of the carboxypeptidase inhibitors were, however, not homologous to those of the isoinhibitor from the Cucurbitaceae seeds

Momordica repens seeds contain various potent isoinhibitors for porcine trypsin The titration data of Fig. 3 revealed that inhibitors CM-1, CM-2 and CM-3 each inhibited trypsin in a molar ratio of nearly 1 1 and the enzyme was almost completely inhibited These inhibitors have practically no action on α-chymotrypsin

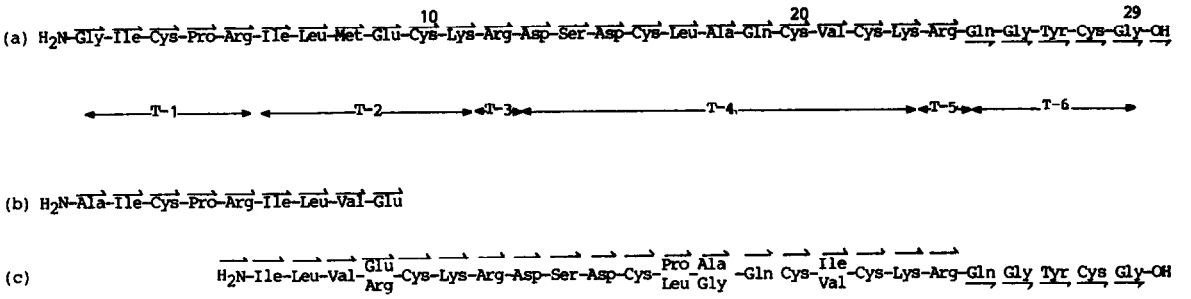

Fig 4 The amino acid sequences of the isoinhibitors from *Momordica repens* seed (a) Complete sequence of CM-1, (b) N-terminal sequence of CM-3 and (c) the complete sequence of fragment S_1 obtained by limited hydrolysis of CM-3 with trypsin at pH 3 The reactive site amino acid residues are indicated by the asterisks The upper half-arrows indicate the residues identified by the sequencer and the lower half-arrows the residues identified by the manual Edman procedure

(a)	R	V	C	P	R*	I*	L	M	E	C	K	K	D	S	D	C	L	A	E	C	V	C	L	E	H	G	Y	C	G
(b)	R	V	C	P	R*	I*	L	M	K	C	K	K	D	S	D	C	L	A	E	C	V	C	L	E	H	G	Y	C	G
(c)	G	I	C	P	R*	I*	L	M	E	C	K	R	D	S	D	C	L	A	Q	C	V	C	K	R	Q	G	Y	C	G
(d)	A	I	C	P	R*	I*	L	V	E/R	C	K	R	D	S	D	C	P/L	A	Q	C	I/V	C	K	R	Q	G	Y	C	G

Fig 5 Comparison of the sequences of the trypsin isoinhibitor from squash seed with those from *Momordica repens* seed (a) squash, ITD I [5], (b) squash, ITD III [5], (c) *Momordica repens*, CM-1, (d) *Momordica repens*, CM-3 The IUPAC one-letter notation for amino acids is used (*Eur J Biochem* **5**, 151, 1968)

EXPERIMENTAL

Materials *Momordica repens* seeds were supplied by Major A K Smith, Sabie, Eastern Transvaal The sources of trypsin, α-chymotrypsin and chemical reagents have been described previously [11]

Methods The physicochemical methods, the digestion of the inhibitors with trypsin and the purification of the peptides, the sequence determination of the reduced and S-carboxymethylated trypsin isoinhibitors and of the fragment obtained by limited hydrolysis with trypsin with the Beckman sequencer have been detailed previously [11, 12]

The esterolytic activities of trypsin and α-chymotrypsin were measured spectrophotometrically according to the method of ref [13] as described earlier [11] 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 inhibitor's activities were estimated from the residual enzymatic activities as described previously [11] The concs of the enzymes were corrected for inactive materials as determined by active-site titrations [14]

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 the inhibitor which inhibited 1 unit of enzyme activity Specific inhibitor activity was expressed as inhibitor units per mg inhibitor

Limited hydrolysis with trypsin The procedure used for limited hydrolysis, at pH 3 of the inhibitor with trypsin, was the same as that described in ref [15]

Preparation of the crude inhibitor Ground defatted *Momordica repens* seeds (100 g) were extracted with 0 2 M ammonium hydrogen carbonate 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 16 000 *g* and lyophilized The yield of the extract was 24 g

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