

The amino acid sequence of a trypsin inhibitor from the seeds of *Momordica charantia* Linn. Cucurbitaceae

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A trypsin inhibitor (MCI-3) was isolated from the seeds of *Momordica charantia* Linn. Cucurbitaceae in three steps involving the affinity chromatography, CM-Sephadex chromatography and HPLC. It is composed of 62 amino acid residues: Asp₃Thr₃Ser₄Glu₃Pro₄Gly₇Ala₆Val₆Ile₄Leu₁Lys₃Arg₆Trp₁; contained no cysteine; and its M_r was 7443. The amino acid sequence showed significant homology with 'potato inhibitor I family' inhibitors.

Amino acid sequence; Sequence homology; Trypsin inhibitor; (*Momordica charantia*)

1. INTRODUCTION

Trypsin inhibitors are universally found in many plants, especially in leguminous plants [1]. These plant trypsin inhibitors may be classified as a 'Bowman-Birk' and Kunitz inhibitor family [2]. Generally, they contain a high cystine content, the disulfide bridges playing an important role in their inhibitory activities [2]. Recently, we isolated and characterized three trypsin inhibitors (MCI-1, MCI-2 and MCI-3) from the seeds of *Momordica charantia* Linn. (Cucurbitaceae) [3,4]. Of which, MCI-1 (M_r 9000) is composed of 77 amino acid residues and 7 pairs of disulfide bridges, and may belong to the Bowman-Birk family, and MCI-2 is composed of 70 amino acid residues and 2 pairs of disulfide bridges, and seems to belong to the Kunitz inhibitor family. MCI-3 contained no cysteine. In this paper, we report the amino acid se-

quence determination of MCI-3. The amino acid sequence showed that MCI-3 was markedly different from the known trypsin inhibitors, but has significant homology with the 'potato inhibitor I family' inhibitors [5]. This family includes a number of homologous inhibitors from the higher plants [6,7] and one from a lower animal [8]. MCI-3 may also belong to the 'potato inhibitor I family'. This family of inhibitors is as far as we know functionally independent of the presence of disulfide bridges in their structures [9].

2. MATERIALS AND METHODS

2.1. Isolation of MCI-3

MCI-3 was isolated from the seeds of *Momordica charantia* Linn., obtained from Guangzhou Seeds Co., China, by trypsin-Sepharose-4B affinity chromatography followed by CM-Sephadex-C50 ion-exchange chromatography and reverse-phase HPLC on a C₁₈ column as described in [3]. The purity of the protein was identified by SDS-polyacrylamide gel electrophoresis and polyacrylamide gel electrophoresis as described in [3].

2.2. Cleavage with pepsin

MCI-3 (8 mg) was dissolved in 1 ml of 5% formic acid and denatured in boiling water for 5 min, then 250 µg of pepsin (Sigma) was added (enzyme:substrate = 1:30, w/w) and the reaction conducted at 37°C for 1.5 h. The resulting peptides were separated on reverse-phase HPLC.

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Abbreviations: MCI, *Momordica charantia* inhibitor; HPLC, high-performance liquid chromatography; PITC, phenylisocyanate; DABITC, 4-*N,N*-dimethylaminoazobenzene-4-isothiocyanate; DABTH, 4-*N,N*-dimethylaminoazobenzene-4-thiohydantoin

2.3. Cleavage with *Staphylococcus aureus* V8 protease

MCI-3 (12 mg) was dissolved at pH 4.0 in 2.5 ml of 0.10 M acetic acid/ammonium acetate buffer and kept in boiling water for 5 min. Then 300 μ g of *Staphylococcus aureus* V8 protease (Sigma, enzyme/substrate = 1:40, w/w) was added and digested at 37°C for 48 h. The peptides thus obtained were separated on HPLC.

2.4. Cleavage with 0.03 N HCl

MCI-3 (5 mg) and 3 ml of 0.03 N HCl were placed in a hard-glass tube, sealed in vacuum. The reaction was conducted at 110°C for 24 h and the peptides were dried and separated on HPLC.

2.5. Amino acid analysis

The samples were hydrolyzed with 5.7 N HCl containing 0.2% phenol in evacuated sealed tubes at 110°C for 24 h. The amino acid analyses were performed on an LKB 4400 amino acid analyser.

2.6. Amino acid sequence determination

The samples were sequenced by the manual DABITC/PITC double-coupled method [10]. DABTH-amino acids were identified by TLC on polyamide sheets, DABTH-Ile and DABTH-Leu were identified with silica gel plates [10].

2.7. Digestion with carboxypeptidases

MCI-3 was digested with carboxypeptidase A, and B (Sigma), at pH 8.0, in 0.1 M *N*-methylmorpholine buffer and carboxypeptidase Y (Sigma), at pH 5.5, in 0.1 M phosphate buffer, aliquots were withdrawn at suitable time intervals, acidified and applied to the amino acid analyser in a pH 2.2 buffer.

2.8. Separation of peptides by HPLC

The peptide mixture obtained above was separated on a C₁₈ reverse-phase HPLC column eluted using 0.1% trifluoroacetic acid with a linear gradient from 0 to 60% acetonitrile.

3. RESULTS AND DISCUSSION

The amino acid composition of MCI-3 is listed in table 1. The result showed that it contained no cysteine, methionine, tyrosine, phenylalanine and histidine, but a high content of hydrophobic amino acid residues (Val, Gly, Ala and Ile).

The N-terminal residue of the protein was not detected by usual Edman degradation, its N-terminal amino group was blocked, similar to *Vicia* subtilisin inhibitor (SVI) from the broad bean [5] and chymotrypsin inhibitor-2 from barley [12]. Also MCI-3 was not digested by carboxypeptidase A, B and Y under the above described conditions.

The peptic digest was separated into 7 main peaks (P-1 to P-7) on reverse-phase HPLC. P-1 and P-6 were separated once again on HPLC and

Table 1

The amino acid composition of MCI-3

Amino acid	
Asp	3.4 (3) ^a
Thr	4.5 (5)
Ser	4.4 (4)
Glu	5.4 (5)
Pro	3.6 (4)
Gly	7.4 (7)
Ala	7.8 (8)
Val	8.7 (9)
Ile	4.0 (4)
Leu	1.1 (1)
Lys	3.4 (3)
Arg	7.6 (8)
Trp ^b	0.6 (1)
Total	62

^a The value in parentheses was based on amino acid sequence determination

^b Trp as determined by colorimetric analysis [11]

their amino acid compositions were determined. P-2 to P-5 and also P-7 were sequenced by the manual method, the N-terminal amino group of P-1 was blocked, therefore P-1 probably belongs to the N-terminal peptide of MCI-3. The amino acid analysis showed that P-1 consisted of 5 amino acid residues: Thr, Arg, Glx, Gly and Val. To determine the amino acid sequence of MCI-3, it was digested with *Staphylococcus aureus* V8 protease and 0.03 N HCl. The peptides obtained from digestion of *Staphylococcus aureus* V8 protease were separated into 5 peaks (S-1 to S-5) by HPLC, S-1 had a blocked N-terminal and S-2 to S-5 were sequenced. The peptides obtained from 0.03 N HCl hydrolysis were separated into many peaks on HPLC, two of which, A-1 and A-2, had yields high enough to be used directly for sequence analysis. The sequence studies on MCI-3 described above were summarized in fig.1. The total number of amino acid residues was 62.

The amino acid sequence of MCI-3 showed no homology with those of the known trypsin inhibitors, but a significant homology with those of the 'potato inhibitor I family' inhibitors (fig.2). In the comparable part of the sequences (residues 27–88), 18%, 18%, 21%, 25% of the residues in MCI-3 were found in the same positions in LIE, PI-1, VSI and CI-2, respectively. These identical

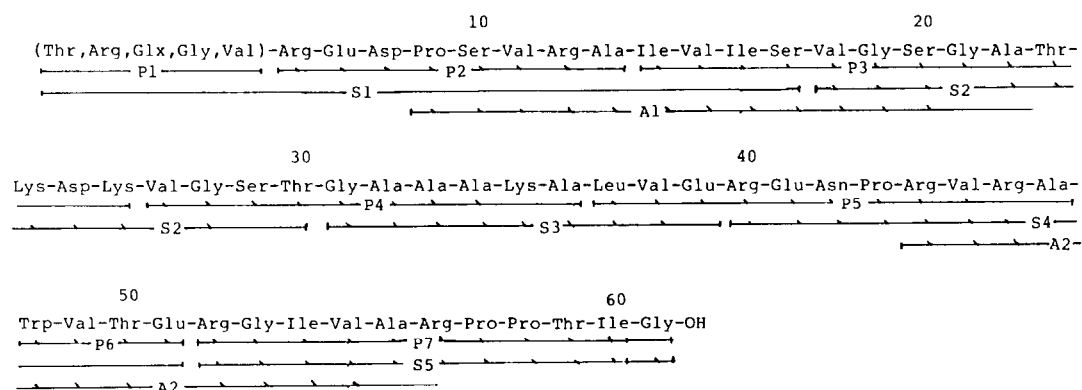


Fig.1. Summary of the sequence studies on MCI-3. P-, S- and A- refer to peptic peptides, *Staphylococcus* proteolytic peptides and acid hydrolysed peptides, respectively. The amino acid sequence was determined by the manual DABITC/PITC double-coupled method [10].

residues mainly appeared in the C-terminal part of proteins. This comparison suggests that MCI-3 may belong to the 'potato inhibitor I family'. The inhibitors of this family contain no cysteine, their inhibitory activities are functionally independent of the disulfide bridges in their structures. The

stabilization of the reactive site may be only dependent on noncovalent forces of the molecules.

The inhibitory specificity of MCI-3 is different from those of LIE, PI-1, VSI and CI-2. It is the first simple protein trypsin inhibitor having no cysteine.

MCI-3	(Thr, Arg
VSI	-Arg-Thr-Ser
CI-2	(Glx, Val, Ser, Ser) Lys-Lys-Pro-Glu-Gly-Val-Asn-Thr-Gly-Ala-Gly-Asp-Arg-His-Asn-Leu-Lys-Thr-Glu
PI-1	Lys-Glu-Phe-Glu-Cys-Asn-Gly-Lys-Leu-Ser
LIE	Thr-Glu-Phe-Gly-Ser-Glu-Leu-Lys-----Ser
MCI-3	Glx-Gly-Val) Arg-Glu-Asp-Pro-Ser-Val-Arg-----Ala-Ile-Val-Ile-Ser-Val-Gly-Ser-Gly-Ala-Thr-Lys
VSI	Trp-Pro-Glu-Leu-Val-Gly-Val-Ser-Ala-Glu-Glu+Ala-----Arg-Gly-Ile-Lys-Glu-----Glu-Lys
CI-2	Trp-Pro-Glu-Leu-Val-Gly-Lys-Ser-Val-Glu-Glu+Ala-----Lys-Lys-Val-Ile-Leu-----Gln-Asn-Lys
PI-1	Trp-Pro-Glu-Leu-Ile-Gly-Val-Pro-Thr-Lys-Leu+Ala-----Lys-Glu-Ile-Ile-Glu-Lys-Gln-Asp-Ser
LIE	Phe-Pro-Glu-Val-Val-Gly-Lys-Thr-Val-Asp-Gln+Ala-----Arg-Glu-Tyr-Phe-THR-----Leu-His-Tyr
MCI-3	---Asp-Lys-Val-Gly-Ser-Thr-Gly-Ala-Ala-Ala-Lys-Ala-Leu-Val-Glu-----Arg-Glu-Asn-Pro+Arg
VSI	Pro-Glu-Ala-Glu-Ile-Gln-Val-Val-Pro-Gln-Gly-Ser-Phe-Val-thr-Ala-Asp-Tyr-Lys-Phe-Gln-----Arg
CI-2	Pro-Glu-Ala-Gln-Ile-Ile-Val-Leu-Pro-Val-Gly-Thr-Ile-Val-Thr-Met-Glu-Tyr-Arg-Ile-Asp-----Arg
PI-1	Leu-Ile-Ser-Asn-Val-Gln-Ile-Leu-Leu-Asn-Gly-Ser-Pro-Val-Thr-Met-Asp-Tyr-Arg-Cys-Asn-----Arg
LIE	Pro-Gln-Tyr-Asn-Val-Tyr-Phe-Leu-Pro-Glu-Gly-Ser-Pro-Val-Thr-Leu-Asp-Leu-Arg-Tyr-Asn-----Arg
MCI-3	Val-Arg+Ala-Trp-Val-Thr-Glu-Arg-Gly-Ile-Val-Ala-Arg-----Pro+Pro+Thr-Ile+Gly.
VSI	Val-Arg+Leu-Tyr-Val-Asp-Glu-Ser-Asn-Lys-Val-Val-Arg---(Ala, Pro, Ala)Ile+Gly.
CI-2	Val-Arg+Leu-Phe-Val-Asp-Lys-Leu-Asp-Asn-Ile-Ala-Glu-----Val+Pro+Arg-Val+Gly.
PI-1	Val-Arg+Leu-Phe-Asp-Asp-Ile-Leu-Gly-Asn-Val-Val-Glu-----Leu+Pro+Arg-Val+Gly.
LIE	Val-Arg+Val-Phe-Tyr-Asn-Pro-Gly-Thr-Asn-Val-Val-Asn-His-Val+Pro+His-Val+Gly.

Fig.2. Comparison of amino acid sequence of MCI-3 with those of the 'potato inhibitor I family' inhibitors. VSI, *Vicia subtilisin* inhibitors from broad bean [5]; CI-2, chymotrypsin inhibitor-2 from barley [9]; LIE, the leech inhibitor eglin [8]; PI-1, potato inhibitor-1 [7]. Invariant amino acid residues are boxed; (---) indicates gaps introduced to maximize homology.

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