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Trypsin inhibitors from the garden four o'clock (*Mirabilis jalapa*) and spinach (*Spinacia oleracea*) seeds: Isolation, characterization and chemical synthesis

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Abstract

Five serine proteinase inhibitors (*Mirabilis jalapa* trypsin inhibitors, MJTI I and II and *Spinacia oleracea* trypsin inhibitors, SOTI I, II, and III) from the garden four-o'clock (*M. jalapa*) and spinach (*S. oleracea*) seeds were isolated. The purification procedures included affinity chromatography on immobilized methylchymotrypsin in the presence of 5 M NaCl, ion exchange chromatography and/or preparative electrophoresis, and finally RP–HPLC on a C-18 column. The inhibitors, crosslinked by three disulfide bridges, are built of 35 to 37 amino-acid residues. Their primary structures differ from those of known trypsin inhibitors, but showed significant similarity to the antimicrobial peptides isolated from the seeds of *M. jalapa* (MJ-AMP1, MJ-AMP2), *Mesembryanthemum crystallinum* (AMP1), and *Phytolacca americana* (AMP-2 and PAFP-S) and from the hemolymph of *Acrocinus longimanus* (Alo-1, 2 and 3). The association equilibrium constants (K_a) with bovine β-trypsin for the inhibitors from *M. jalapa* (MJTI I and II) and *S. oleracea* (SOTI I–III) were found to be about 10^7 M^{-1}. Fully active MJTI I and SOTI I were obtained by solid-phase peptide synthesis. The disulfide bridge pattern in both inhibitors (Cys1–Cys4, Cys2–Cys5 and Cys3–Cys6) was established after their digestion with thermolysin and proteinase K followed by the MALDI-TOF analysis.

Keywords: *Mirabilis jalapa*; *Spinacia oleracea*; Purification; Chemical synthesis; Primary structure; Disulfide bonds topology; *Mirabilis* serine proteinase inhibitor family; Trypsin inhibitors

1. Introduction

Proteolytic enzymes are involved in numerous biochemical and physiological processes. They are responsible not only for cellular protein digestion, but also for intracellular protein turnover associated with defense mechanisms, elimination of misfolded proteins, signal propagation, the activation of proenzyme, regulatory proteins, and receptors, the release of hormone and biologically active peptide, assembling processes, cellular differentiation and ageing, seed development, mobilization of storage protein during seed germination or seedling growth, pathogen suppression, and pest proteinases (Ehrmann and Clausen, 2004; Antão and Malcata, 2005). All of these processes are under precise control, amongst which naturally occurring proteinase inhibitors play a very important function.

Plant seeds and storage organs have proven to be a very rich source of protein proteinase inhibitors, particularly the inhibitors of cysteine and serine proteinases. The last ones,

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on the basis of amino-acid sequences, disulfide bridge topology, localization of the reactive sites, and mechanism of action, were grouped into at least seven well-characterized families: Kunitz, Bowman–Birk, Potato I, Potato II, Squash, Cereal, and Mustard (Laskowski and Kato, 1980; Birk, 2003; De Leo et al., 2002). Some of these inhibitors, especially the very small ones, such the inhibitors belonging to the squash family, built of barely 30 amino-acid residues (Kowalska et al., 2006), are of particular interest as therapeutics because they inhibit trypsin, plasmin, kallikrein, cathepsin G, and the blood clotting factors X_a and XII_a (Otlewski and Krowarsch, 1996). In addition, such small proteins are very attractive models for the design of molecules with simplified structure (Rolka et al., 1992) with selective activity against chymotrypsin, human leukocyte elastase (Rolka et al., 1991), or even against carboxypeptidase A (Le-Nguyen et al., 1989).

In this paper, we report the purification and characterization of five novel trypsin inhibitors: two from *Mirabilis jalapa* (MJTI I and II) and three other from *Spinacia oleracea* (SOTI I–III) seeds. For all of them besides MJTI II, their complete amino-acid sequences, and for MJTI I and SOTI I the disulfide bond topology were established. The isolated inhibitors showed no homology to any other inhibitor; instead they are homologous to a class of antimicrobial peptides from *M. jalapa* seeds (Cammue et al., 1992). Two of the inhibitors (MJTI I and SOTI I) were obtained by chemical synthesis.

2. Results and discussion

2.1. Purification of MJTIs

Two trypsin inhibitors (MJTI I and II) were isolated from the acidic extract (pH 4.0) of garden four-o'clock seed meal by precipitation with cold ethanol (96%) and affinity chromatography (in batch procedure) on immobilized methylchymotrypsin in the presence of 5 M NaCl. The adsorbed inhibitors were eluted with water. In this step of purification we used catalytically inactive enzyme, with His57 converted to 3-methylhistidine, although still capable of forming complexes with inhibitors (Ryan and Feeney, 1975). Employing affinity chromatography on this support, we isolated not only chymotrypsin-specific inhibitors (Wojtaszek et al., 2006) but also trypsin inhibitors when the procedure was carried out in a presence of 5 M NaCl (Polanowski et al., 2003). This procedure provides facilities for isolating the inhibitors in their virgin forms (reactive site peptide bond unsplit).

The next step of the purification procedure was preparative native-PAGE at pH 9.4 (Fig. 1). The inhibitory fractions with similar electrophoretic mobility were pooled, lyophilized, and then purified on a Nucleosil-100 C-18 column (Fig. 2). After rechromatography (not shown), two inhibitors (MJTI I and II) were obtained. From 1 kg of garden four-o'clock seeds, about 20 mg of both inhibitors were obtained.

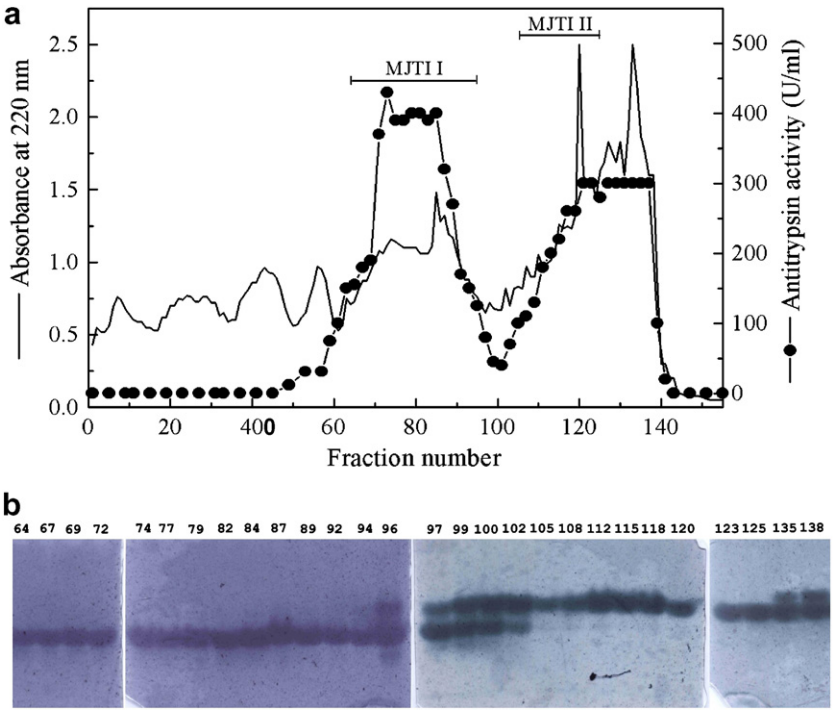

Fig. 1. Preparative electrophoresis at pH 9.4 under native conditions (a) and electrophoretic mobility of the obtained fractions (b). (a) Proteins (127.2 mg) were applied on 7.5% polyacryloamide gel. Electrophoresis was performed in a discontinuous system at a constant voltage of 450 V at 4 °C. Ten-ml fractions at a flow rate of 1 ml/min of water were collected. Fractions marked by "I–I" were pooled. (b) Inhibitors were separated in 7.5% gel copolymerized with 0.1% edestin.

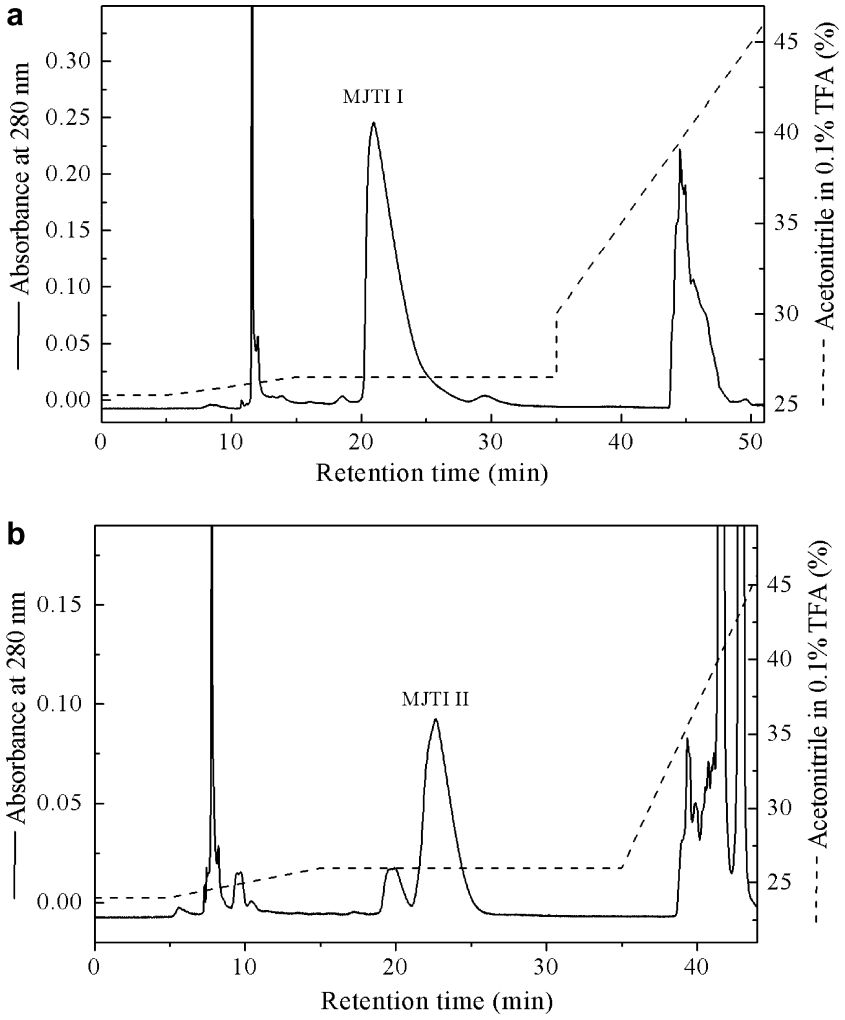

Fig. 2. RP–HPLC chromatography of MJTI I (a) and MJTI II (b). Proteins were applied on Nucleosil-100 C-18 column (10 μm, 250 × 8.0 mm) and then eluted with acetonitrile in 0.1% TFA at a 1 ml/min flow rate.

2.2. Purification of SOTIs

Three trypsin inhibitors (SOTI I–III) were isolated from the acidic extract (pH 4.5) of spinach seed meal by salting out the proteins with ammonium sulfate (0.9 saturation) and affinity chromatography on immobilized methylchymotrypsin in the presence of 5 M NaCl. All trypsin inhibitory activity was adsorbed on the column and then was eluted from the column with water (not shown). In the next step of purification, chromatography on DEAE-Sephadex A-25 at pH 8.6 was used (Fig. 3). About 65% of the antitrypsin activity did not bind on the ion exchanger (SOTI I) or is clearly retarded (SOTI II) and thus the later one was eluted isocratically (Fig. 3); each of them was additionally purified on a SP-Sephadex C-25 column at pH 2.8 (Fig. 4). The inhibitor adsorbed on the DEAE-Sephadex A-25 was eluted at ca. 0.15 M NaCl concentration (Fig. 3). Finally, all three inhibitory preparations were purified by RP–HPLC on a C-18 column, displaying single major, symmetrical peaks. As judged by SDS–gel electrophoresis and mass spectrometry analysis (not shown), the use of RP–HPLC allowed obtaining three homogenous trypsin inhibitors: SOTI I (not adsorbed on the DEAE-Sephadex A-25), SOTI II (eluted isocratically) and SOTI III (adsorbed on the ion exchanger). From 1 kg of spinach seeds, 680 μg, 630 μg, and 810 μg of SOTI I, SOTI II, and SOTI III, respectively, were obtained.

2.3. Amino-acid composition and primary structure of MJTIs and SOTIs

The amino-acid compositions of the inhibitors isolated from the garden four-o'clock and spinach seeds were very similar within the group of inhibitors isolated from the same material (Table 1). The molecular masses of the MJTIs and SOTIs assayed by mass spectrometry were about 3.5–4 kDa and coincided with the molecular masses calculated from their amino-acid sequences (Table 1).

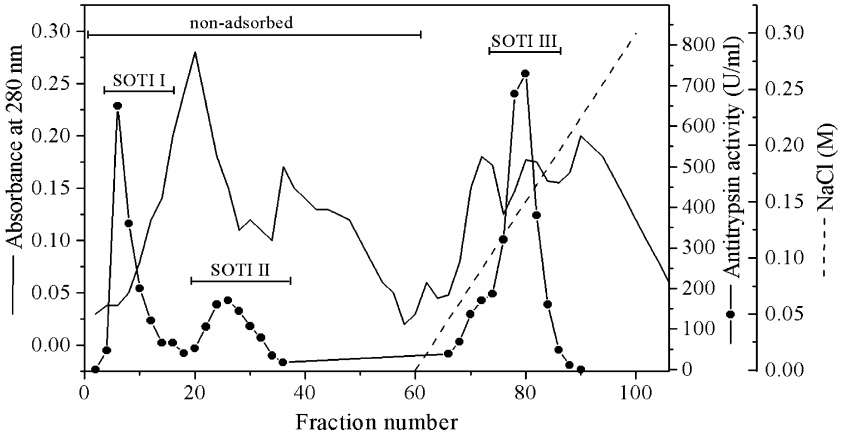

Fig. 3. DEAE-Sephadex A-25 chromatography of SOTI inhibitors. Proteins were applied on DEAE-Sephadex A-25 column (220 × 15 mm) equilibrated with 0.05 M Tris–HCl buffer, pH 8.6. The adsorbed proteins were eluted with linear gradient of NaCl 0–0.5 M at a 1 ml/min flow rate. Fractions 4–16 (SOTI I), 20–38 (SOTI II), and 74–86 (SOTI III) were collected separately.

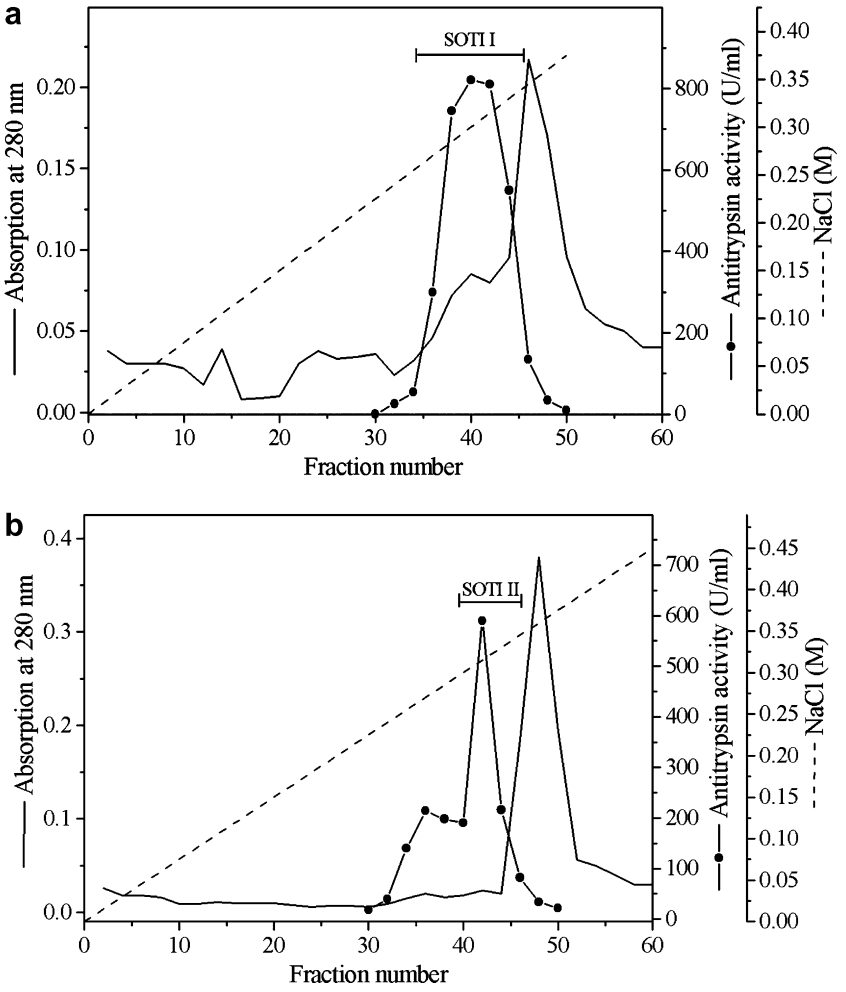

Fig. 4. SP-Sephadex C-25 chromatography of SOTI I (a) and SOTI II (b). Proteins were applied on SP-Sephadex C-25 column (130 × 15 mm) equilibrated with 0.05 M citric acid/sodium phosphate buffer, pH 2.8. The proteins were eluted with linear NaCl gradient.

The primary structure of the reduced and alkylated MJTI I was determined. In the case of SOTIs, the amino-acid sequences were carried out on non-reduced forms of inhibitors; therefore no signal was detected at cysteine residues. Based on the absence of the signal, amino-acid compositions, molecular masses, and homology with the

Table 1
Amino-acid compositions and molecular masses of MJTIs and SOTIs

Amino-acid	MJTI I	MJTI II	SOTI I	SOTI II	SOTI III
Asp/Asn	3.98 (4)	4.19	0.00 (0)	0.97 (1)	0.91 (1)
Ser	1.94 (2)	1.94	3.23 (4)	3.35 (4)	4.59 (4)
Glu/Gln	4.00 (4)	4.11	3.12 (3)	3.15 (3)	4.26 (4)
Gly	1.47 (1)	1.10	3.76 (4)	3.82 (4)	5.16 (4)
His	0.95 (1)	1.08	1.10 (1)	1.10 (1)	1.49 (1)
Arg	2.01 (2)	2.07	1.03 (1)	1.03 (1)	1.28 (1)
Thr	0.99 (1)	0.93	0.00 (0)	0.00 (0)	0.49 (0)
Ala	2.15 (3)	2.53	1.87 (2)	1.85 (2)	2.05 (2)
Pro	4.37 (5)	5.26	4.32 (5)	4.40 (5)	5.44 (5)
Tyr	1.45 (1)	1.86	0.75 (0)	0.73 (0)	0.51 (0)
Val	1.99 (2)	2.02	1.95 (2)	2.01 (2)	2.47 (2)
Met	0.00 (0)	0.00	0.00 (0)	0.00 (0)	0.00 (0)
Lys	0.96 (1)	0.76	1.04 (1)	1.07 (1)	1.08 (1)
Ile	1.79 (2)	1.94	2.70 (3)	2.78 (3)	3.33 (3)
Leu	1.10 (1)	1.08	1.16 (1)	1.03 (1)	1.32 (1)
Phe	0.91 (1)	1.05	1.95 (2)	2.00 (2)	2.77 (2)
Cys	5.72 (6)	5.58	ND (6)	ND (6)	ND (6)
Total residues	37		35	36	37
M_w (Da)					
ESI-MS	4060.0	4044.1	3586.5	3709.2	3838.4
Amino-acid sequence	4061.6		3587.3	3702.3	3831.5

The numbers in parentheses represent the value from sequence analysis. ND, not determined.

trypsin inhibitor from *M. jalapa*, the position of the cysteine residues in SOTIs was established (Fig. 5). The amino-acid sequences of MJTI I and SOTI I–III have been deposited in the SWISS PROT protein data bank under following accession numbers: MJTI I-P84778, SOTI I-P84779, SOTI II-P84780, SOTI III-P84781.

MJTI I and II have almost identical amino-acid compositions (Table 1). Unfortunately, amino-acid sequence of MJTI II was not determined by Edman degradation. Probably, this peptide has pyroglutamic acid residue at N-terminus.

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MJTI I    EDEECAKTDQICPPNA-PNYCCSGSCVPHPRLRIFVCA
SOTI I       KCSPSGAICSGFGPPEQCCSGACVPHPILRIFVCQ
SOTI II    DKCSPSGAICSGFGPPEQCCSGACVPHPILRIFVCQ
SOTI III  EDKCSPSGAICSGFGPPEQCCSGACVPHPILRIFVCQ
MJ-AMP1     <ECIGNGGRCNENVGPPYCCSGFCLRQPGQGYGYCKNR
MJ-AMP2       CIGNGGRCNENVGPPYCCSGFCLRQPNQGYGVCRNR
PAFP-S      AGCIKNGGRCNASAGPPYCCSSYCFQIAGQSYGVCKNR
AMP-2        ACIKNGGRCVASGGPPYCCSNYCLQIAGQSYGVCKKH
AMP1        AKCIKNGKGCREDQGPPFCCSGFCYRQVGWARGYCKNR
Alo-1         CIKNGNGCQPDGSQGNCCSRYCHKEPGWVAGYCR
Alo-2         CIANRNGCQPDGSQGNCCSGYCHKEPGWVAGYCR
Alo-3         CIKNGNGCQPNGSQGNCCSGYCHKQPGWVAGYCRRK
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Fig. 5. Sequence alignment of sequences of MJTI I and SOTIs with homological proteins. MJ-AMP1and MJ-AMP2 – antimicrobial peptide 1 and Antimicrobial peptide 2 from *Mirabilis jalapa* seeds (Cammue et al., 1992); PAFP-S – anti-fungal protein 1 from *Phytolacca americana* seeds (Shao et al., 1999); AMP-2 – antimicrobial seed protein from *Phytolacca americana* seeds (Liu et al., 2000); AMP1 – antimicrobial peptide 1 from *Mesembryanthemum crystallinum* seeds (Michalowski and Bohnert, 1998); Alo-1, Alo-2 and Alo-3 – antifungal peptide from hemolymph of *Acrocinus longimanus* (Barbault et al., 2003). Position of reactive site peptide bond in the inhibitors is indicated by an arrowhead; "–" indicates a gap inserted into the sequence for optimal alignment of inhibitors.

MJTI and SOTIs revealed homology to the antimicrobial peptides isolated from the *M. jalapa* seeds and from *Mesembryanthemum crystalinum*, to antifungal protein and antimicrobial protein from the seeds of *Phytolacca americana*, and to antifungal peptide from the hemolymph of *Acrocinus longimanus* (Fig. 5).

2.4. Activity of MJTIs and SOTIs

Chemical modification of guanidyl groups of arginine residues in SOTI I with 1,2-cyclohexanedione (CHD) led to the complete loss of activity of all inhibitors (not shown). The antitryptic activity was not affected by acetylation of free amino groups with acetic anhydride. Taking into account that all the inhibitors isolated from spinach seeds contain only one arginine residue in the C-terminal part of molecules (Fig. 5), we can conclude that the Arg–Ile peptide bond forms the reactive site in these inhibitors. The position of reactive site in MJTI molecules was established on the basis of primary structure homology with SOTIs.

The equilibrium association constants (K_a) of MJTI I and SOTI I with bovine β-trypsin are 5.36×10^7 and 1.85×10^7 M^{-1}, respectively.

2.5. Solid-phase synthesis of MJTI I and SOTI I

The synthesized peptides showed a correct molecular mass assayed by mass spectrometry using MALDI-TOF (Table 2). HPLC analysis confirmed that the synthetic inhibitors are identical to those isolated from plants. The synthetic inhibitors coinjected with the corresponding

Table 2
Chemical characteristic of MJTI I and SOTI I

Peptide	Retention time (min)	Molecular weight (Da)	
		Calculated	Determined MALDI-TOF
MJTI I	15.82	4061.6	4061.9
SOTI I	20.52	3587.3	3588.4

Table 3
Fragmentation of digested MJTI I by MALDI-TOF

Proposed disulfide bridge connectives	Calculated M_w with corresponding fragment	Ion mass obtained	Proposed fragment
Cys1–Cys4	408	407.8	CS BCA
Cys2–Cys5	440	439.2	SC IC
Cys3–Cys6	474	475.4	CA YC

standards isolated from the natural source displayed single major peaks at HPLC (Fig. 6). This indicates that the disulfide bridge topology in synthetic and wild-type inhibitors is the same. Both synthesized peptides displayed trypsin inhibitory activity similar to inhibitors isolated from natural sources.

2.6. Disulfide bridge topology

Inhibitors were digested by thermolysin and proteinase K and then analyzed by MALDI-TOF. The assignment of peaks corresponding to the peptide fragments are summarized in Tables 3 and 4. Based on these results we are able to confirm that disulfide bridge pattern in both peptides fits well in the knottin disulfide pattern that is: Cys1–Cys4, Cys2–Cys5, Cys3–Cys6.

3. Conclusions

Since MJTI I and II and SOTI I, II, and III shown unique amino-acid sequences with no homology to any other proteinase inhibitors, we propose to designate them

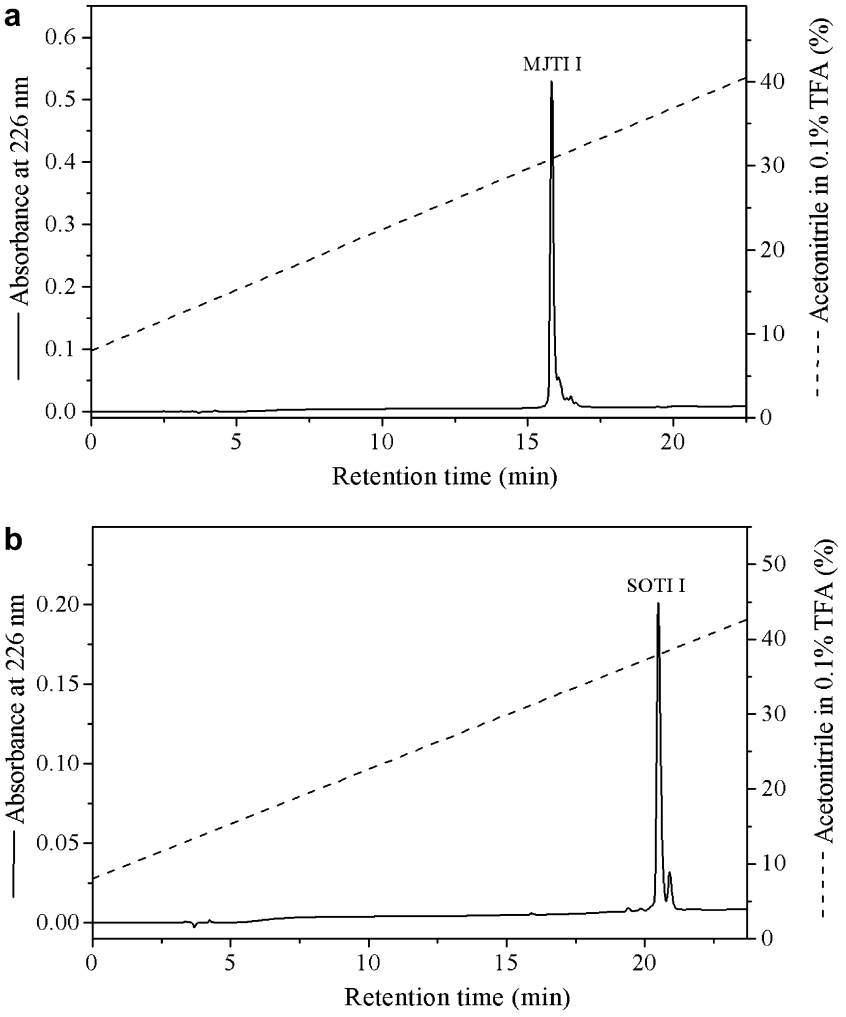

Fig. 6. Coinjection of synthetic and native peptides: MJTI I (a) and SOTI I (b). Proteins were applied on Kromasil-100 C-8 column (5 μm, 250 × 8.0 mm) and then eluted with acetonitrile in 0.1% TFA at a 1 ml/min flow rate.

Table 4
Fragmentation of digested SOTI I by MALDI-TOF

Proposed disulfide bridge connectives	Calculated M_w with corresponding fragment	Ion mass obtained	Proposed fragment
Cys1–Cys4	609	609.2	KCSP CSG
Cys2–Cys5	835	835.2	AICS GACVP
Cys3–Cys6	984	983.3	EQC IFVCCQ

as a new *Mirabilis* serine proteinase inhibitor family. The disulfide bridge topology displayed by these miniproteins permits recognizing them as cysteine knottin family inhibitors.

4. Experimental

4.1. Materials

The study was carried out on garden four o'clock (*M. jalapa* L.) and spinach (*S. oleracea* L.) seeds supplied by M. Legutko Breeding and Seed Company, Smolice (Poland). Bovine trypsin (EC 3.4.21.4) was prepared according to Wilimowska-Pelc and Mejbaum-Katzenellenbogen (1978) and bovine β-trypsin was isolated from this preparation as described by Liepniecks and Light (1974). Edestin was prepared as described by St. Angelo et al. (1968). Bovine α-chymotrypsin (EC 3.4.21.1), thermolysin (EC 3.4.24.27), proteinase K (EC 3.4.21.64), bovine serum albumin (BSA), *N*α-benzoyl-DL-Arg-*p*-nitroanilide (BAPNA), *N*-benzoyl-Val-Gly-Arg-*p*-nitroanilide, *N*-(*p*-tosyl)-Gly-Pro-Arg-nitroanilide, dimethyl sulfoxide (DMSO), Triton X-100, tris(hydroxymethyl)aminomethane (Tris), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), β-alanine, divinyl sulfone (DVS), phenylmethanesulfonyl fluoride (PMSF), acrylamide, molecular weight markers for SDS–PAGE, diisopropylcarbodiimide (DIPCDI), piperidine, *N*,*N*-dimethylformamide (DMF), *N*-methyl-2-pyrrolidone (NMP), and bicinchoninic acid kit for protein determination were from Sigma (St. Louis, MO, USA). *p*-Nitrophenyl *p*-guanidinobenzoate × HCl (NPGB) was from Merck (Darmstadt, Germany). Sodium veronal was from LOBA Feinchemie (Fischamend, Austria). Methyl *p*-nitrobenzenesulfonate, *N*,*N*′-methylenebisacrylamide, phenol, trifluoroacetic acid (TFA), and sodium chloride were from from Fluka (Buchs, Switzerland). *N*,*N*,*N*′,*N*′-tetramethylethylenodiamine (TEMED) was from Serva Feinbiochemica (Heidelberg, Germany). 1,2-Cyclohexanedione (CHD) and Amido Black were purchased from ICN Biomedicals (Aurora, OH, USA). Methylchymotrypsin was prepared by the method of Nakagawa and Bender (1970) and traces of unmodified enzyme were inactivated with PMSF. Sepharose 4B, DEAE-Sephadex A-25, SP-Sephadex C-25 were from Pharmacia Biotech (Uppsala, Sweden). The RP–HPLC Nucleosil-100 C-18 column (10 μm, 250 × 8.0 mm) and Kromasil-100 C-8 column (5 μm, 250 × 8.0 mm) were from Knauer (Berlin, Germany). Acetonitrile was from J.T. Baker (Deventer, Netherlands). The AccQTag reagent kit, AccQTag Eluent A, and AccQTag C-18 column (4 μm, 150 × 3.9 mm) were purchased from Waters (Milford, MA, USA). Amino-acid standards and 6 N hydrochloric acid were from Pierce (Rockford, IL, USA). YM 1 membrane filters were from Amicon (Denvers, MA, USA). Ethanol was from Kompania Spirytusowa "Wratislavia" Polmos Wrocław (Wrocław, Polska). Tenta Gel S AC-Gln(Trt)-Fmoc and Tenta Gel S AC-Ala-Fmoc were from Rapp Polymere (Tübingen, Germany). Hydroxybenzotriazole (HOBt) and 2-(7-azabenzotriazol-1-yloxy)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were from GL Biochem (Shanghai) Ltd. (Shanghai, China). All other reagents were of analytical or HPLC-grade purity and were purchased from Polskie Odczynniki Chemiczne (POCH) (Gliwice, Poland).

4.2. Purification of inhibitors

Ground seeds of garden four-o'clock and spinach defatted with acetone were extracted for 1.5 h at room temperature under mechanical stirring with 3–5 volumes (w/v) of 0.05 M acetate buffer, pH 4.0 or 4.5, respectively. After centrifugation for 30 min at 900*g*, the supernatant was decanted and the sediment reextracted with two volumes of the same buffer. Both extracts were pooled. Proteins extracted from the garden four-o'clock seeds were precipitated with 10 vol. of cold ethanol (96%), whereas proteins from the spinach seeds were salted out with ammonium sulfate at 0.9 saturation. After 20 h the precipitated proteins were collected by centrifugation (30 min at 9000*g*), and the sediment was solubilized in water and dialyzed for 24 h against water using a membrane with a molecular weight cut-off 1 kDa.

The dialyzed solutions were clarified by centrifugation and the resulting supernatants were brought to pH 8.0 with 1 M Tris and then NaCl was added to a final concentration of 5 M. Proteins from spinach were applied to an immobilized methylchymotrypsin-Sepharose 4B column (170 × 40 mm), pre-equilibrated with 0.05 M Tris–HCl buffer, pH 8.0, containing 5 M NaCl. Proteins from garden four-o'clock seeds were allowed to react with methylchymotrypsin-Sepharose 4B in a batch procedure. The unbound proteins were washed out with starting buffer. The adsorbed proteins were eluted first with water and then with 0.01 N HCl. The inhibitory peak fractions eluted with water were concentrated and desalted on a YM 1 membrane filter. The inhibitors from the garden four-o'clock seeds were then purified by means of preparative PAGE under native conditions at pH 9.4. The inhibitors from spinach seeds were first separated by ion-exchange chromatography on a DEAE-Sephadex A-25 column (220 × 15 mm) equilibrated with 0.05 M Tris–HCl buffer, pH 8.6, and unadsorbed fractions were additionally purified on SP-Sephadex C-25 column (130 × 15 mm) equilibrated with citric-phosphate buffer, pH 2.8. Finally, all inhibitors were purified on a Nucleosil-100 C-18 column (10 μm, 250 × 8.0 mm).

4.3. Determination of protein concentration of protein

Protein was estimated with bicinchoninic acid (Smith et al., 1985) using bovine albumin as a standard or by spectrophotometric measurement of the absorbance at 215 and 225 nm and calculating the concentration using the equation: C_{protein} (μg/ml) = $(A_{215} - A_{225}) \times 144$ (Wolf, 1983).

4.4. Trypsin and trypsin inhibitory assay

Trypsin and antitrypsin activities were measured using BAPNA as substrate (Erlanger et al., 1961). One unit of inhibitory activity was defined as the amount of protein required to reduce the activity of 2 μg of trypsin to 50% of the original value.

4.5. Immobilization of methylchymotrypsin

Methylchymotrypsin (2 g) was immobilized on cyanogen bromide activated Sepharose 4B (140 ml) according to March et al. (1974).

4.6. Polyacrylamide slab gel electrophoresis

SDS–PAGE was done according to a discontinous procedure on 16.5% (w/v) polyacrylamide gels slabs according to Schägger and von Jagov (1987).

Trypsin inhibitors were examined by native PAGE according Davis method (1964) in gels containing a copolymerized 0.1% edestin dissolved in 0.05 M acetic acid (Polanowski et al., 1992). After electrophoresis the gels were incubated in trypsin solution (20 μg/ml) in 0.1 M Tris–HCl buffer, pH 8.0. The undigested edestin was stained with 0.1% Amido Black in acetic acid.

4.7. Preparative PAGE

Preparative polyacrylamide gel electrophoresis in native condition was performed using the Model 491 Prep Cell (Bio-Rad Laboratories; Hercules, CA, USA). Proteins were dissolved in 1 ml of 40% glycerol containing 0.01% bromophenol blue and were then loaded on 7.5% polyacrylamide gel (65 × 28 mm). Trypsin inhibitors were purified in a continuous system using Tris-CAPS buffer, pH 9.4 (McLellan, 1982). Electrophoresis was carried out at 450 V in a cold room. Elution was performed with water at a flow rate of 1.0 ml/min and 10-ml fractions were collected. The elution of the proteins was monitored by absorbance at 220 nm and the antitrypsin activity was assayed in each fraction.

4.8. Analysis of amino-acid composition

Samples containing 1–3 μg of protein were hydrolyzed in a protein hydrolyzer (Knauer, Germany) by vapor-phase HCl hydrolysis at 120 °C for 22 h. Amino-acid derivatization was performed with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Cohen and Michaud, 1993). The derivatized amino acids were separated by HPLC on an AccQ-Tag column (150 × 3.9 mm, Waters, USA). Cysteine was determined after oxidation of inhibitors with performic acid (Carne, 1988).

4.9. Amino-acid sequence determination

Sequence analyses were performed on a gas-phase sequencer (Model 491, Perkin–Elmer/Applied Biosystems, Foster City, CA, USA) at the BioCenter of the Jagiellonian University, Kraków, Poland. The PTH-derivatives were analyzed by online gradient HPLC on a SPHERI-5 PTH C-18 column (5 μm, 220 × 2.0 mm) using a Model 140C microgradient delivery system equipped with a Model 785A programmable absorbance detector.

4.10. Modification of arginine and lysine residues

Guanidyl groups of arginine residues were modified with CHD (Patthy and Smith, 1975). Free amino groups of lysine residues were acetylated with acetic anhydride (Riordan and Vallee, 1967).

4.11. Mass spectrometry analysis

Mass spectra were recorded on a Finnigan MAT TSQ 700 triple-stage quadrupole mass spectrometer equipped with an electrospray ion source (ESI-MS). Samples were dissolved in methanol/water/acetic acid (50:45:5, v/v/v) and introduced into the electrospray needle by mechanical infusion through a microsyringe at a flow rate of 2 μl/min. A potential difference of 4.5 kV was applied between the electrospray needles. Nitrogen gas was used to evaporate the solvent from the charged droplets. At least 20 scans were averaged to obtain each spectrum. Transformation of the resulting spectra were performed with the BioWorks software package (Finningan MAT).

Matrix-assisted laser-desorption ionization (MALDI) mass spectrometry was run on a Biflex III MALDI-TOF mass spectrometer (Bruker, Germany) using α-cyano-4-hydroxycinnamic acid as the matrix. The spectrometer was operated at an accelerating voltage of 20 kV with pulsed extraction optimized for ions of masses of about 3000 Da.

4.12. Standardization of enzyme and inhibitor solutions

The concentration of bovine β-trypsin was determined by spectrophotometric titration of the active center with NPGB (Chase and Show, 1970) and standardized trypsin was in turn used to titrate trypsin inhibitors.

4.13. Determination of equilibrium association constants (K_a)

The equilibrium association constants were determined according to Empie and Laskowski (1982). The concentra-

tion of enzyme $[E_0]$ conformed the equation: $2 < [E_0] \times K_a < 50$. The inhibitor concentration $[I_0]$ ranged from 0 to $2 \times [E_0]$ and the final substrate (Bz-Val-Gly-Arg-*p*-nitroanilide or Tos-Gly-Pro-Arg-nitroanilide) concentration did not exceed $0.2 \times K_M$. Kinetic measurements were made at 410 nm, and residual enzyme concentration was calculated from the initial slopes. The experimental data were fit to the equation:

$$[E] = \frac{1}{2} \cdot \left([E_0] - F \cdot [I_0] - K_a^{-1} + \sqrt{\left([E_0] + F \cdot [I_0] + K_a^{-1} \right)^2 - 4 \cdot [E_0] \cdot F \cdot [I_0]} \right)$$

where $[E_0]$ and $[I_0]$ are total enzyme and inhibitor concentrations, respectively, $[E]$ is the residual enzyme concentration, and F is the enzyme-inhibitor equimolarity factor.

4.14. Peptide synthesis

MJTI I and SOTI I were synthesized by the solid-phase method using Fmoc-chemistry. Tenta Gel S AC-Gln(Trt)-Fmoc and Tenta Gel S AC-Ala-Fmoc (substitution of amino acid 0.2 meq/g resine; Rapp Polymere, Germany) were used as a support. The syntheses were carried out manually. Deprotections of Fmoc-groups were performed with 20% piperidine in DMF/NMP (1:1, v/v). Couplings were achieved using mixtures of DIPCDI/HOBt or HATU/HOBt. The peptides were removed from the solid support together with side-chain protections in a one-step procedure using a TFA/phenol/triisopropylsilane/water (88:5:2:5) mixture (Sole and Barany, 1992). The reaction was carried out in room temperature for 150 min. After completing the synthesis, the crude peptides were dissolved in 5% acetic acid, pH was adjusted to 7.5 using 1 M ammonium acetate, and the peptide solutions were stirred at room temperature. The progress of oxidation was monitored by HPLC. The process was completed in approximately 48 h.

4.15. Determination of the disulfide bridge connectivity

To determine the disulfide bridge topology in MJTI I and SOTI I, the method described by Stachowiak et al. (1990) was applied. Each protein was dissolved in 250 µl of 100 mM ammonium acetate buffer, pH 6.5, containing 2 mM $CaCl_2$ to achieve the inhibitor concentration of 50 mM. Then 10 µg of thermolysin were added and the mixture was incubated at 49 °C for 66 h. Afterwards the pH was adjusted to 4.5 with acetic acid, the temperature was reduced to 45 °C, and 5 µg of proteinase K were added. The mixture was incubated for an additional 24 h at 49 °C. The digested peptide fragments were separated by RP–HPLC on a Kromasil-100 C-8 column (5 µm, 250 × 8.0 mm). The peptides were eluted with a linear gradient of acetonitrile in 0.1% TFA. The collected fractions were evaporated and analyzed by MALDI-TOF spectrometry.

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