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PROTEIN INHIBITORS OF TRYPSIN FROM THE SEEDS OF *CUCURBITACEAE* PLANTS*

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Seven trypsin inhibitors were isolated from the seeds of *Cucurbitaceae* plants: two from cucumber (*Cucumis sativus*) and red bryony (*Bryonia diotica*) and one from figleaf gourd (*Cucurbita ficifolia*), spaghetti squash (*Cucurbita pepo* var. (*vegetable spaghetti*) and water melon (*Citrullus vulgaris*). The inhibitors were purified by fractionation with ammonium sulphate, followed by ion-exchange chromatography and affinity chromatography using immobilized trypsin or anhydro-trypsin.

The homogeneous inhibitors from cucumber and water melon are made up of 32 and 30 amino acid residues, respectively, whereas the remaining ones of 29 residues. All inhibitors contain three disulphide bridges and are free of threonine, phenylalanine and tryptophan. Inhibitors from spaghetti squash and CSTI IIb from cucumber are inactivated by acetylation of free amino groups whereas the remaining ones are inactivated by modification of arginine with 1,2-cyclohexanedione. Thus the P_1 residues of the reactive sites of the inhibitors are lysine and arginine, respectively.

Within the classification of serine proteinase inhibitors proposed by Laskowski Jr. & Kato [1] trypsin inhibitors from *Cucurbitaceae* constitute a new family [2]. These low molecular weight polypeptides composed of approximately 30 amino acids and containing three disulphide bridges have been the subject of our research since the late seventies [3 - 6]. Also Hojima *et al.* [7] extracted from the seeds of squash a low molecular weight

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inhibitor (29 amino acid residues) which inhibits both trypsin and the activated Hageman factor (XIIa).

Some physico-chemical, immunological and structural properties of these inhibitors have already been investigated [8, 9, 10]. The amino acid sequences of seven inhibitors isolated at our Institute [11, 12] and of two isolated by Joubert [13] are known. The heterogeneity of the primary structure of these proteins is due to genetic and post-translational modifications. These inhibitors are, to some extent, homologous with agglutinin domains from wheat germ [14]. On the basis of the known amino acid sequence two biologically active iso-inhibitors from squash seeds were chemically synthesized [15, 16].

Isolation and analysis of the new trypsin inhibitors may contribute to a better understanding of the enzyme-inhibitor interaction and, at the same time, allow for the design of chemical synthesis of mutated inhibitors of desired properties.

In the present work we describe the isolation and purification of trypsin inhibitors from cucumber seeds (*Cucumis sativus*), red bryony (*Bryonia diotica*), Water melon (*Citrullus vulgaris*), figleaf gourd (*Cucurbita ficifolia*) and spaghetti squash (*Cucurbita pepo* var. *vegetable spaghetti*).

The primary structure of two inhibitors from cucumber seeds was described previously [12] and that of the inhibitors from the water melon and red bryony will be published elsewhere.

MATERIALS AND METHODS

Material. Cucumber (*Cucumis sativus* Winsconsin), figleaf gourd (*Cucurbita ficifolia*), spaghetti squash (*Cucurbita pepo* var. *vegetable spaghetti*) and water melon (*Citrullus vulgaris*) were obtained from Plant Seed Corporation (Wrocław, Poland). The seeds of red bryony were extracted from ripe fruits picked at Wrocław Botanical Garden and stored for two months at −20°C.

Reagents. Sepharose 4B, SP-Sephadex C-25, DEAE-Sephadex A-25 and QAE-Sephadex A-25 were from Pharmacia Fine Chemicals (Uppsala, Sweden); Bio-Gel P-2 from Bio-Rad Labs. (Richmond, Calif., U.S.A.); BAPNA, PMSF were from Sigma Chem. Co. (St. Louis, MO., U.S.A.); TLCK and

[1] Abbreviations used: BAPNA, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide·HCl; pNPGB, *p*-nitrophenyl p'-guanidinebenzoate·HCl; PMSF, phenylmethylsulphonyl fluoride; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone; CMTI, *Cucurbita maxima* trypsin inhibitor; CPGTI, *Cucurbita pepo* var. *Giromontia* trypsin inhibitor; CPTI, *Cucurbita pepo* trypsin inhibitor; CSTI, *Cucumis sativus* trypsin inhibitor; BDTI, *Bryonia dioica* trypsin inhibitor; CFTI, *Cucurbita ficifolia* trypsin inhibitor; CVTI, *Citrullus vulgaris* trypsin inhibitor; CPSTI, *Cucurbita pepo* var. *vegetable spaghetti* trypsin inhibitor; CPPTI, *Cucurbita pepo* var. *patissonina* trypsin inhibitor.

pNPGB from Merck (Darmstadt, F.R.G.); 1,2-cyclohexanedione from Koch-Light Labs. Ltd. (Colnbrook, Bucks., England); guanidine hydrochloride and reagents for polyacrylamide gel electrophoresis were from Fluka A.G. (Buchs S.G., Switzerland). Bovine trypsin was prepared according to Wilimowska-Pelc & Mejbaum-Katzenellenbogen [17], and soybean trypsin inhibitor according to Polanowski & Olichwier [18]. Anhydro-trypsin, prepared as described by Ako *et al.* [19], was purified on immobilized soybean inhibitor and traces of trypsin activity were inactivated with TLCK. The remaining reagents were from POCh (Gliwice, Poland).

Methods. Protein was determined by the microbiuret method of Goa [20]. Trypsin concentration was determined by titration of the enzyme active centers with pNPGB according to Chase & Shaw [21]. Trypsin and trypsin inhibitor activities were assayed under conditions described by Erlanger *et al.* [22] against BAPNA as a substrate. One unit of the antitrypsin activity was defined as the amount of the inhibitor which reduced by 50% the activity of 1 mg of trypsin.

Free amino groups were acetylated with acetic acid anhydride under conditions described by Riordan & Vallee [23]. Guanidyl groups of arginine were modified with cyclohexanedione according to Patty & Smith [24]. *N*-Terminal amino acids were assayed by the dansyl procedure of Hartley [25]. Trypsin, anhydro-trypsin and soybean inhibitor were immobilized on Sepharose 4B according to March *et al.* [26].

Electrophoresis was performed on 7.5% polyacrylamide gel at pH 8.6 according to Davies [27]. Protein was revealed by staining with 1% Amido Black in 7% acetic acid. Amino acid composition was determined after 24, 48 and 72 h hydrolysis in 5.7 M HCl at 110°C with an automatic amino acid analyser. Cysteine was assayed following its oxidation with performic acid to cysteic acid [28].

Preparation of the inhibitors. Inhibitors were isolated and purified according to modified methods described previously by Leluk *et al.* [4] and Pham *et al.* [6]. All steps, unless indicated otherwise, were carried out at room temperature.

Step 1. Extraction. Ground seeds were extracted with 5 vol. (w/v) of 0.05 M acetate buffer, pH 4.7, with mechanical stirring for 1 h, and subsequently clarified by centrifugation.

Step 2. Ammonium sulphate precipitation. Protein was precipitated with ammonium sulphate at 0.9 saturation. After 24 h the precipitate was collected by centrifugation, dissolved in 5 vol. of water, adjusted to pH 3.1 with 2 M HCl, heated to 80°C for 15 min and then stored overnight at room temperature. The precipitate was collected by centrifugation or filtration. The supernatant was diluted with water to conductivity corresponding to that of 0.05 M NaCl.

Step 3. SP-Sephadex C-25 chromatography. The supernatant from step 2 was applied to a SP-Sephadex C-25 column equilibrated with 0.02 M citrate buffer, pH 3.1. Bound proteins were eluted with a NaCl concentration gradient in the starting buffer.

Step 4. Affinity chromatography on immobilized trypsin or anhydro-trypsin. Active fractions from step 3 were concentrated, adjusted to pH 7.5 with 2 M NaOH and applied onto a column (3 cm × 15 cm) with immobilized trypsin or anhydrotrypsin equilibrated with 0.05 M Tris buffer, pH 7.5. Inactive proteins were washed out with the starting buffer, containing 0.5 M NaCl followed by water. The bound inhibitor was subsequently eluted with 0.01 M HCl.

Step 5. SP-Sephadex C-25 chromatography. Inhibitors from step 4 were rechromatographed on SP-Sephadex C-25 column (2 cm × 12 cm) as in step 3. Inactive fractions were removed and the virgin forms of the inhibitor were separated from the trypsin modified ones, i.e. with a hydrolyzed peptide bond at the reactive site.

Step 6. Concentration of inhibitors. The inhibitor solutions were diluted three-fold with distilled water and applied on to a SP-Sephadex C-25 column (0.8 cm × 4 cm) equilibrated with 0.02 M citrate buffer, pH 3.1. The column was washed with diluted acetic acid, pH 4.0 and the bound inhibitor eluted with a small volume of aqueous ammonia, pH 10.5.

Step 7. Removal of non-proteinous contaminations. The inhibitor solutions from step 6, adjusted to pH 8.5 with 1 M acetic acid were adsorbed on a DEAE-Sephadex A-25 column (2 cm × 15 cm) equilibrated with 0.05 M Tris buffer, pH 8.5. The inactive material formed a brown band close to the top. Under these conditions the inhibitors from red bryony were not adsorbed whereas the inhibitors from figleaf gourd, spaghetti squash and water melon were adsorbed and eluted with 0.3 M NaCl. Inhibitor CSTI IIb from cucumbers was separated from other inhibitors by elution with NaCl concentration gradient in 0.05 M Tris/HCl buffer, pH 7.5. Eluted proteins were lyophilized, dissolved in 4 M guanidine hydrochloride, desalted on Bio-Gel P-2 column equilibrated with 0.5 M acetic acid and lyophilized.

RESULTS AND DISCUSSION

Isolation and purification of trypsin inhibitors

As shown in Table 1 there are significant differences in *Cucurbitaceae* seeds. *Cucurbita ficifolia* was found to be the richest source of trypsin inhibitors their level being about ten-fold higher than in seeds of squash, zucchini and cucumber.

The ammonium sulphate treatment gives preparations of inhibitors with 2- to 5-fold purification and yield of at least 80%. Heating of these preparations did not increase the specific activity but clarified the solutions and inactivated proteinases. This step is necessary, as we have observed

Table 1

The level of antitrypsin activity in the seeds of some Cucurbitaceae plants

Species	Total activity units/100 g
Cucurbita maxima	36.0
Cucurbita pepo var. *Giromontia*	31.0
Cucurbita pepo var. *patissonina*	41.9
Cucurbita pepo var. *vegetable spaghetti*	44.0
Cucurbita pepo	60.5
Cucurbita ficifolia	339.5
Cucumis sativus	32.8
Cucumis melo	38.5
Citrullus vulgaris	60.7
Bryonia dioica	80.6

that aspartyl proteinase extracted from squash and cucumber seeds [30, 31] inactivates endogeneous trypsin inhibitors through selective proteolysis of one peptide bond (in preparation).

Proteins exhibiting antitrypsin activity were initially fractionated into the individual inhibitors on a SP-Sephadex C-25 column. Elution profiles of the inhibitors from particular species are presented in Fig. 1A - E. The yield of trypsin inhibitors at this step range from 35% (figleaf gourd) to 67% (red bryony). Significant purification was achieved by affinity chromatography. Initially immobilized trypsin was tested but, even under conditions of kinetically controlled dissociation [32] the column gave a mixture of both the virgin forms and the modified ones with a hydrolyzed peptide bond in the reactive site. The virgin form of the inhibitor was in this case separated from the trypsin-modified one by SP-Sephadex C-25 chromatography, at pH 3.1 (Fig. 2A). To avoid proteolysis the immobilized anhydro-trypsin was used henceforth. This catalytically inactie trypsin derivative, in which the active Ser^{195} was replaced by dehydroalanine, still is able to form complexes with the inhibitors. It avoids modification of the inhibitors and permits separation by affinity chromatography of only the virgin forms of the inhibitors (Fig. 2B) with a yield no less than 75%.

Inhibitor preparations obtained thus far still contained from 5 to 25% of inactive material which was removed effectively by SP-Sephadex C-25, DEAE or QAE-Sephadex A-25 chromatographies. Trace contaminations

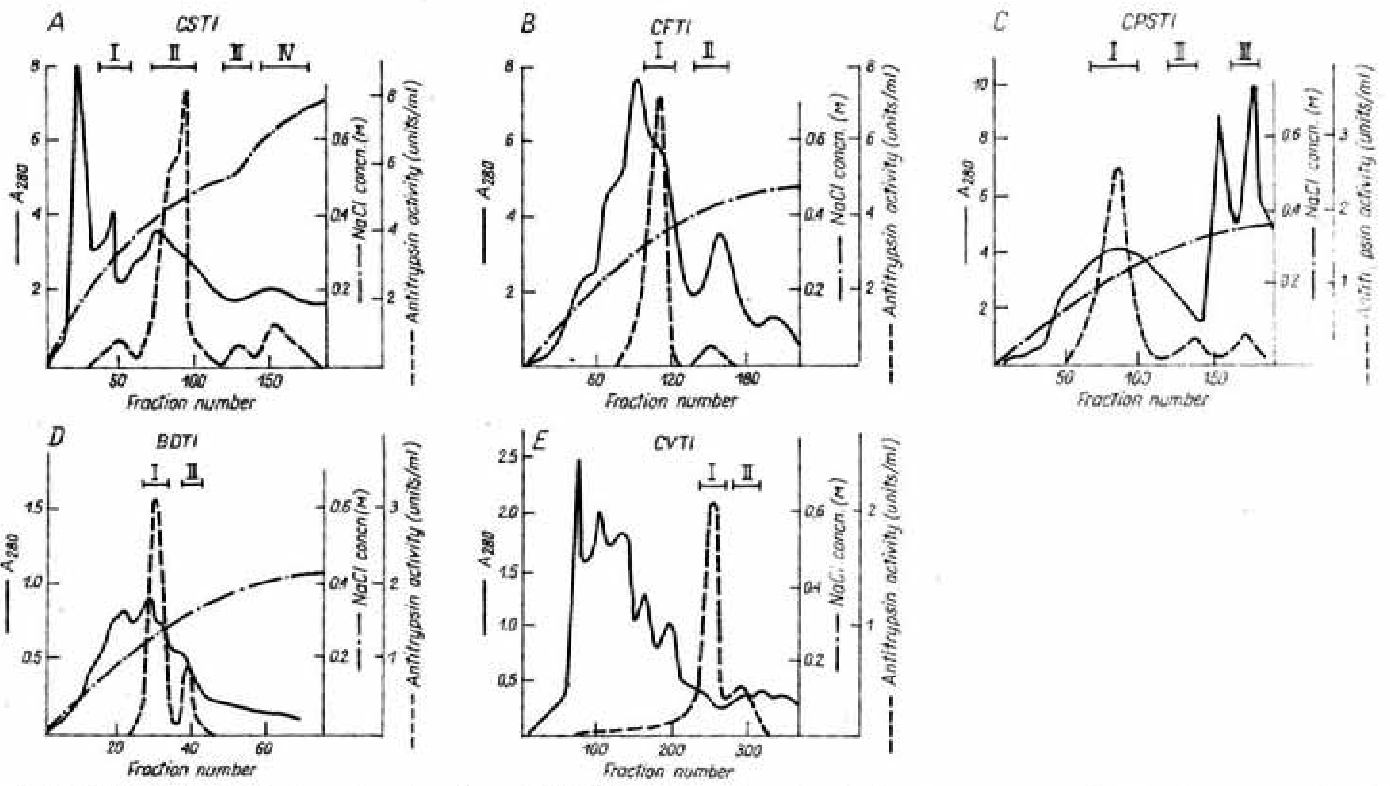

Fig. 1. SP-Sephadex C-25 chromatography of trypsin inhibitors from the seeds of: A. *Cucumis sativus* (5 kg); B. *Cucurbita ficifolia* (5 kg); C. *Cucurbita pepo* var. *vegetable spaghetti* (3 kg); D. *Bryonia diotica* (0.3 kg); E. *Citrullus vulgaris* (1.8 kg). Each inhibitor is denoted by the first letter of the Latin name of the species and the Roman numbers stand for the order in which the inhibitors were eluted from the column. For details see Methods

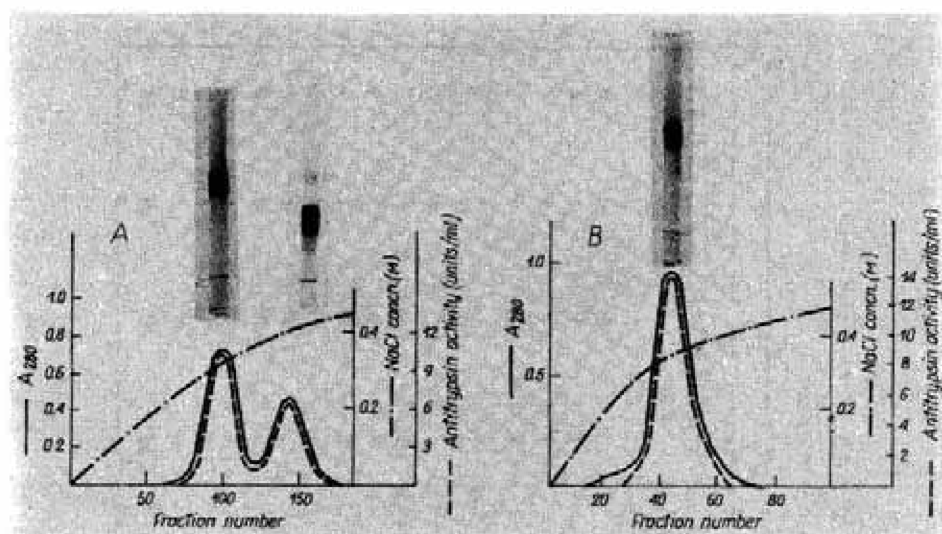

Fig. 2. SP-Sephadex C-25 chromatography of trypsin inhibitor CFTI I from *Cucurbita ficifolia* seeds following purification by affinity chromatography on: A, immobilized trypsin; B, immobilized anhydro-trypsin. Insert: gel electrophoresis of the preparations at pH 8.6

remaining in some preparations were finally removed on Bio-Gel P-2 columns.

Purification of inhibitors from cucumber seeds requires an additional comment. Inhibitors from these seeds were separated into four fractions. As seen in Fig. 1A, the nonsymmetrical second protein peak CSTI II contained the bulk of antitrypsin activity. This inhibitor purified on immobilized trypsin and rechromatograped on SP-Sephadex C-25 was still heterogeneous on polyacrylamide gel electrophoresis. Homogeneity of the inhibitor CSTI IIb (Fig. 3) was obtained only after DEAE-Sephadex A-25 chromatography.

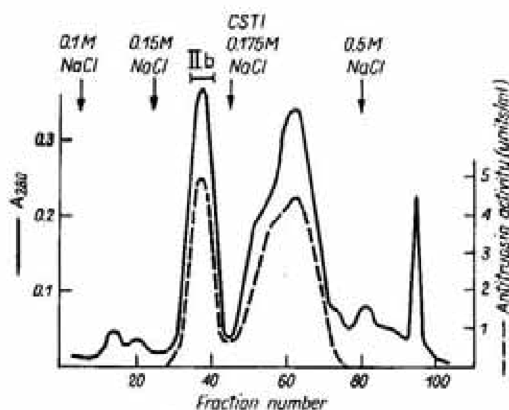

Fig. 3. DEAE-Sephadex A-25 chromatography of trypsin inhibitor CSTI II from *Cucumis sativus* seeds

Table 2

Amino acid composition of trypsin inhibitors from the seeds of Cucurbitaceae

Numbers in parentheses are the nearest integers

Amino acid	Residues/mol									
	CFTI I	CFTI II	CPSTI I	BDTI I	BDTI II	CVTI I	CSTI* IIb	CSTI* IV	CPGTI* I CPTI* II	CMTI* I
Asx	1.80(2)	1.95(2)	2.10(2)	2.85(3)	3	3	4	2	2	2
Thr	0.00(0)	0.03(0)	0.10(0)	0.05(0)	0	0	0	0	0	0
Ser	0.80(1)	0.85(1)	0.91(1)	0.90(1)	1	0	2	1	1	1
Glx	3.00(3)	2.82(3)	2.99(3)	2.07(2)	1	2	1	2	3	3
Pro	0.90(1)	0.93(1)	1.03(1)	0.92(1)	1	1	1	2	1	1
Gly	2.04(2)	2.00(2)	2.00(2)	3.85(4)	4	3	2	2	2	2
Ala	1.33(1)	1.02(1)	1.06(1)	1.09(1)	1	2	0	0	1	1
1/2 cystine	5.70(6)	5.50(6)	5.80(6)	5.57(6)	6	6	6	6	6	6
Val	1.90(2)	1.87(2)	0.96(1)	1.06(1)	1	1	3	1	1	2
Met	0.98(1)	0.83(1)	0.90(1)	0.86(1)	1	1	2	3	1	1
Ile	1.10(1)	1.02(1)	1.78(2)	0.85(1)	1	2	2	2	2	1
Leu	2.87(3)	2.97(3)	3.07(3)	1.93(2)	2	2	4	3	3	3
Tyr	0.81(1)	0.70(1)	0.88(1)	0.74(1)	1	1	1	1	1	1
Phe	0.07(0)	0.02(0)	0.00(0)	0.05(0)	0	0	0	0	0	0
His	1.00(1)	0.83(1)	0.95(1)	0.08(0)	0	1	1	2	1	1
Lys	2.15(2)	1.75(2)	2.77(3)	2.10(2)	2	1	3	2	3	2
Arg	2.06(2)	2.30(2)	1.08(1)	4.10(4)	4	4	0	1	1	2
Total	29	29	29	30	29	30	32	30	29	29

* values from amino acid sequences according to Wieczorek *et al.* [12]

By the above procedures the following homogeneous inhibitors were obtained: CSTI IIb from cucumbers; CFTI and CFTI II from figleaf gourd; BDTI I and BDTI II from red bryony; CVTI I from water melon and CPSTI I from spaghetti squash. However, inhibitor CSTI IV obtained from cucumbers showed in electrophoresis two closely migrating bands. When their amino acid sequence was established, it turned out that they differed only in one *N*-terminal methionine residue [12].

Characteristics and some properties of the inhibitors

The inhibitors described in this paper are built of 29 - 32 amino acids (including 6 cysteine residues) and like the previously described inhibitors from *Cucurbitaceae* plants they lack threonine, phenylalanine and tryptophan (Table 2).

The molecular mass values calculated from amino acid composition, are given in Table 3. All of the inhibitors inhibited linearly the activity of bovine trypsin at the enzyme:inhibitor weight ratio of 1:~0.14. Chemical

Table 3

Trypsin inhibitors from the seeds of plants of the Cucurbitaceae family

Origin	Inhibitor	Yield of inhibitors mg/kg	Specific activity units/mg	Molecular mass	P_1 residue of the reactive site
Cucumis sativus	CSTI IIb	3.5	6.8	3529	Lys
	CSTI IV	0.7	7.0	3424	Arg
Bryonia dioica	BDTI I	48.0	7.2	3332	Arg
	BDTI II	8.0	7.4	3203	Arg
Citrullus vulgaris	CVTI I	9.0	6.6	3638	Arg
Cucurbita ficifolia	CPTI I	105.0	7.3	3269	Arg
	CPTI II	6.0	7.3	3269	Arg
Cucurbita pepo var. *vegetable spaghetti*	CPSTI I	19.0	7.3	3255	Lys

modification of free amino groups led to 90% inactivation of the inhibitors from cucumber seeds (CSTI IIb) and figleaf squash (CPSTI I). The remaining inhibitors (CSTI IV, BDTI I and II, CVTI I, CFTI I and II) were inactivated on modification of the guanidyl groups of arginine with 1,2-cyclohexanedione. These results indicate that lysine is present at the P_1 position of the reactive site of CSTI IIb and CPSTI I and arginine at this position in all other inhibitors.

As mentioned previously the seeds of figleaf gourd constitute the richest source of trypsin inhibitors among the studied seeds of *Cucurbitaceae* plants. Moreover, these seeds contain almost exclusively one inhibitor. Although the antitrypsin activity of the initial protein extract subjected to SP-Sephadex chromatography was recovered in two peaks, upon further purification it turned out that their amino acid composition and electrophoretic mobility were identical. They both contained arginine residue at the *N*-terminal and P_1 position of the reactive site. Similar behaviour on ion-exchange chromatography was observed during purification of an inhibitor from summer squash.

Inhibitor from figleaf seems to be identical with the previously described CMTI I inhibitor from the seeds of squash. Both have the same amino acid composition, similar electrophoretic mobility on polyacrylamide gel, arginine at the *N*-terminal and P_1 position and isoleucine at the P'_1 position of the reactive site. Similarly on the basis of amino acid composition, electrophoretic mobility, the presence of arginine at the *N*-terminal position, lysine at the P_1 position and isoleucine at the P'_1 position of the reactive site, one may assume that the inhibitor from spaghetti squash is identical with the previously described inhibitors CPTI II and CPGTI I.

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