ISOLATION OF TWO TRYPsin INHIBITORS FROM RESTING SEEDS
OF THE WHITE BUSH (CUCURBITA PEPO VAR. PATISSONINA)
AND THEIR PROPERTIES *

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Two trypsin inhibitors, CPPTI-I and CPPTI-II of \( M_r \) 3,250 and 7,850,
respectively, were isolated from resting white bush seeds. Both inhibitors are
cysteine-rich proteins. In addition to trypsin, they inhibit a trypsin-like enzyme
isolated from Streptomyces griseus proteinase but they do not act on chymo-
trypsin, kallikrein or subtilopeptidase A. The isolated inhibitors contain a lysine
residue in position \( P_1 \) of the reactive site.

A number of homologous, low-molecular trypsin inhibitors containing
about 30 amino acid residues, have been isolated from resting seeds of the
Cucurbitaceae family plants (Polanowski et al., 1980; Hojima et al.,
1982; Leluk et al., 1983; Otlewski et al., 1984). Some of their properties
(Szewczuk et al., 1983; Siemion et al., 1984a, 1984b) and primary structure
(Wilusz et al., 1983; Joubert, 1984; Wieczorek et al., 1985) have also
been determined. On the basis of the data obtained it proved possible
to synthesize a preparation which showed the activity of one of those
inhibitors (Kupryszewski et al., 1985).

Still, there are several problems to be elucidated of which the most
important is the physiological function of the trypsin inhibitors. According
to Ryan et al. (1974) plant inhibitors of proteinases may be involved
in plant protection against pathogens. However, as found in our experiments,
the inhibitor from zucchini seeds (CPGTI-I) \(^1\) did not inhibit the growth
of, e.g., Fusarium culmorum, Fusarium moniliforme or Pseudomonas lachrymans,

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\(^1\) Abbreviations used: CMTI, Cucurbita maxima trypsin inhibitor; CPTI, Cucurbita pepo
trypsin inhibitor; CPGTI, Cucurbita pepo var. Giromontia trypsin inhibitor; CPPTI, Cucurbita
pepo var. patissonina trypsin inhibitor; BAPNA, \( \alpha \)-N-benzyol-DL-arginine-p-nitroanilide \cdot HCl;
pNPG, \( p \)-nitrophenyl \( p \)'-guanidinobenzoate \cdot HCl.
known phytopathogens of the Cucurbitaceae plants (Leluk, 1983). Attempts to demonstrate in extracts from vegetative parts of zucchini or in extracts from resting or germinating zucchini seeds, the presence of an endogenous proteinase able to bind with the immobilized CPGTI-1 proved unsuccessful (Leluk, 1983). Thus, the physiological role of these inhibitors remains unknown.

Another problem to be elucidated is the heterogeneity of these inhibitors, resulting from differences in their polypeptide chain length (Wieczorek et al., 1985).

In the present work an attempt has been made to establish the reason of this heterogeneity, using as material the seeds of white bush, another plant of the Cucurbitaceae family.

MATERIALS AND METHODS

Resting seeds of white bush (Cucurbita pepo L. var. patissonina) were obtained from Plant Seed Corporation in Wrocław.

The reagents and methods used in the present work, except those described below, were the same as used previously (Leluk et al., 1983).

The activity of subtilopeptidase A (type VII, Sigma Chem. Comp.) and chymotrypsin was determined according to Kunitz (1947) with casein as a substrate. The trypsin-like activity of a Streptomyces griseus proteinase was determined against BAPNA as a substrate by the method of Erlanger et al. (1961).

The presence of inhibitors was assessed by polyacrylamide gel electrophoresis (Hanspal et al., 1983) with the use of the gel co-polymerized with casein.

Preparation of trypsin inhibitor. The meal from ground seeds of white bush was extracted with 5 vol. (w/v) of 0.1 M-acetate buffer, pH 4.5, or with the same volume of 0.15 M-perchloric acid. The suspensions were stirred for 1 h at room temperature, then clarified by centrifugation. To remove perchloric acid, the extract was neutralized with 10 M-KOH, and the potassium perchlorate crystals formed were filtered off. Further steps of the purification procedure, i.e. ammonium sulphate precipitation, chromatography on SP-Sephadex C-25 column, purification on immobilized trypsin and separation of the virgin form of the inhibitor from the modified form (with hydrolysed reactive site peptide bond), were performed as described by Leluk et al. (1983).

RESULTS

Among the seeds of cultivated plants from the Cucurbitaceae family we have so far studied, the white bush seeds were found to be one
of the richest sources of trypsin inhibitors. When the acetate buffer extract was subjected to SP-Sephadex C-25 chromatography, the proteins showing antitrypsin activity were eluted as three distinct peaks (Fig. 1).

![Graph](image_url)

**Fig. 1.** SP-Sephadex C-25 chromatography of the white bush seed trypsin inhibitors. Crude inhibitor preparation (acetate buffer extract) was loaded onto the 3.5 X 22 cm column equilibrated with 0.02 M-citrate buffer, pH 3.2. Fractions of 10 ml were collected at a flow rate of 50 ml/h. —- A280; o, antitrypsin activity; △, NaCl concentration

On polyacrylamide gel electrophoresis at pH 8.6 two of these fractions migrated towards the anode (Plate 1, lane 1) and the third fraction, towards the cathode (not shown). Only the first two inhibitor fractions were further purified (Table 1). The inhibitor present in the first peak, designated CPPTI-I, which on electrophoresis (Plate 1, lane B) showed the presence of a single band with antitrypsin activity, was purified on immobilized trypsin and subjected to rechromatography on SP-Sephadex C-25 column (Fig. 2). Two electrophoretically homogeneous inhibitor preparations were obtained: virgin form, CPPTI-I, and the modified form, CPPTI-I*, with the peptide bond of the reactive site broken during purification on immobilized trypsin (Plate 2, gels A and C). The virgin form became also transformed into the modified form in the presence of catalytic amounts of trypsin at pH 3.5. (Plate 2, gel B).

The inhibitor present in the second chromatographic peak, CPPTI-II, was also subjected to further purification. On polyacrylamide gel electrophoresis two trypsin inhibitors were found (Plate 1, lane C). The slower migrating band corresponded to CPPTI-I. The faster migrating band, CPPTI-II, was further purified on immobilized trypsin and by chromato-
graphy on SP-Sephadex C-25. Two active peaks were obtained (Fig. 3); only the inhibitor of the second peak, differing in electrophoretic mobility from CPPTI-I, was further purified (Table 1) and analysed (Table 2).

Table 1

Preparation of trypsin inhibitors from white bush seeds

The seeds (5 kg) were extracted with 0.1 M-acetate buffer, pH 4.5. Protein, except at the last step, was determined by the microbiuret method (Goa, 1953). Final preparations dried over P₂O₅ were weighed. One unit of the antitrypsin activity was defined as that amount of the inhibitor which reduced by half the activity of 2 mg of trypsin.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Activity</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
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<tr>
<td></td>
<td>total units</td>
<td>specific units/mg protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extract</td>
<td>97,800</td>
<td>2,097</td>
<td>0.02</td>
<td>1</td>
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<tr>
<td>(NH₄)₂SO₄ 0.9 sat.</td>
<td>9,350</td>
<td>920</td>
<td>0.10</td>
<td>5.0</td>
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<tr>
<td>SP-Sephadex C-25</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CPPTI-I</td>
<td>4,510</td>
<td>838</td>
<td>0.18</td>
<td>8.7</td>
</tr>
<tr>
<td>CPPTI-II</td>
<td>930</td>
<td>49</td>
<td>0.05</td>
<td>2.4</td>
</tr>
<tr>
<td>CPPTI-III</td>
<td>810</td>
<td>6</td>
<td>0.01</td>
<td>0.5</td>
</tr>
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<td>Affinity chromatography</td>
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</tr>
<tr>
<td>CPPTI-I</td>
<td>120</td>
<td>782</td>
<td>6.52</td>
<td>304.5</td>
</tr>
<tr>
<td>CPPTI-II</td>
<td>19</td>
<td>42</td>
<td>2.21</td>
<td>103.3</td>
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<td>SP-Sephadex C-25</td>
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<tr>
<td>CPPTI-I</td>
<td>74</td>
<td>531</td>
<td>7.17</td>
<td>335.2</td>
</tr>
<tr>
<td>CPPTI-I*</td>
<td>8</td>
<td>50</td>
<td>6.16</td>
<td>293.2</td>
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<tr>
<td>CPPTI-II</td>
<td>6</td>
<td>19</td>
<td>2.98</td>
<td>139.4</td>
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<tr>
<td>Desalting and freeze-drying</td>
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<td>CPPTI-I</td>
<td>47</td>
<td>349</td>
<td>7.32</td>
<td>342.0</td>
</tr>
<tr>
<td>CPPTI-I*</td>
<td>5</td>
<td>33</td>
<td>6.96</td>
<td>331.3</td>
</tr>
<tr>
<td>CPPTI-II</td>
<td>2</td>
<td>7</td>
<td>3.28</td>
<td>153.5</td>
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</table>

* Modified form of inhibitor.

When trypsin inhibitors were extracted with 0.15 M-perchloric acid instead of acetate buffer, results similar to those presented in Fig. 1. were obtained. The only difference with respect to the acetate buffer extract was the presence of a small amount of the inhibitor emerging from the SP-Sephadex C-25 column before the main peak of activity (not shown). In both cases, over 80% of the antitrypsin activity was located in the main inhibitor peak (CPPTI-I, cf Fig. 1). These main fractions were similar also in electrophoretic mobility (cf Plate 2, gels A and D) as well as in inhibitory action on bovine trypsin (Fig. 4) and the trypsin-like enzyme from St. griseus proteinase (not shown). The CPPTI-I preparations obtained in acetate buffer or perchloric acid extract had the same amino acid
Plate 1: Inhibitory activity against bovine trypsin detected with the use of polyacrylamide gel containing copolymerized substrate casein. A, Crude extract; B and C, fractions CPPTI-I and CPPTI-II, respectively, obtained after the initial SP-Sephadex C-25 chromatography (cf Fig. 1). Electrophoresis was run at pH 8.6

Trân-Chăn Pham et al (facing p. 322)
Plate 2. Polyacrylamide gel electrophoresis of the purified trypsin inhibitors at pH 8.6. A, CPPTI-I, virgin form; B, CPPTI-I after modification with a catalytic amount of trypsin at pH 3.5; C, CPPTI-I*, modified form; D, CPPTI-I, virgin form prepared from the perchloric acid extract; E, CPPTI-II. The preparations A, B, C and E were obtained from the acetate buffer extract.
Fig. 2. SP-Sephadex C-25 rechromatography of CPPTI-I. The inhibitor purified on immobilized trypsin was applied on the 1×12 cm column. Fractions of 5 ml were collected at a flow rate of 40 ml/h. Other conditions of chromatography, and designations were the same as in Fig. 1.

Fig. 3. SP-Sephadex C-25 rechromatography of CPPTI-II. The inhibitor purified on immobilized trypsin was applied on the 1.5×4 cm column. Fractions of 2.5 ml were collected at a flow rate of 15 ml/h. Other conditions of chromatography, and designations were the same as in Fig. 1.
composition (Table 2) and molecular weight (3 200), and both had arginine as the N-terminal residue. The two inhibitor preparations retained full activity after modification of guanidyl residues of arginine with 1,2-cyclohexanediene, whereas acetylation of ε-amino groups of lysine with acetic anhydride led to their inactivation. Neither preparation inhibited bovine chymotrypsin, pancreatic kallikrein or subtilopeptidase A. The results presented point to the identity of the two inhibitor preparations which will be further referred to as CPPTI-I.

**Table 2**

**resting seeds of Cucurbita pepo var. patissonina**

Integral values are given in parentheses. Tryptophan was determined according to Godwin & Morton (1946)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>CPPTI-I*</th>
<th>CPPTI-Ia</th>
<th>CPPTI-IIa</th>
</tr>
</thead>
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<tr>
<td>Lys</td>
<td>3.10 (3)</td>
<td>2.08 (3)</td>
<td>7.10 (7)</td>
</tr>
<tr>
<td>His</td>
<td>1.07 (1)</td>
<td>0.92 (1)</td>
<td>3.69 (4)</td>
</tr>
<tr>
<td>Arg</td>
<td>0.97 (1)</td>
<td>0.87 (1)</td>
<td>2.90 (3)</td>
</tr>
<tr>
<td>CySO₃H</td>
<td>6.02 (6)</td>
<td>5.98 (6)</td>
<td>10.30 (10)</td>
</tr>
<tr>
<td>Asp</td>
<td>2.07 (2)</td>
<td>1.78 (2)</td>
<td>5.30 (5)</td>
</tr>
<tr>
<td>Thr</td>
<td>0.90 (0)</td>
<td>0.00 (0)</td>
<td>0.78 (1)</td>
</tr>
<tr>
<td>Ser</td>
<td>0.88 (1)</td>
<td>1.07 (1)</td>
<td>3.73 (4)</td>
</tr>
<tr>
<td>Glu</td>
<td>3.30 (3)</td>
<td>2.55 (3)</td>
<td>8.13 (8)</td>
</tr>
<tr>
<td>Pro</td>
<td>1.10 (1)</td>
<td>0.95 (1)</td>
<td>2.00 (2)</td>
</tr>
<tr>
<td>Gly</td>
<td>2.10 (2)</td>
<td>2.00 (2)</td>
<td>10.63 (11)</td>
</tr>
<tr>
<td>Ala</td>
<td>1.05 (1)</td>
<td>1.03 (1)</td>
<td>3.25 (3)</td>
</tr>
<tr>
<td>Val</td>
<td>1.05 (1)</td>
<td>0.83 (1)</td>
<td>2.95 (3)</td>
</tr>
<tr>
<td>Met</td>
<td>0.75 (1)</td>
<td>0.85 (1)</td>
<td>0.56 (1)</td>
</tr>
<tr>
<td>Ile</td>
<td>1.94 (2)</td>
<td>1.69 (2)</td>
<td>3.58 (4)</td>
</tr>
<tr>
<td>Leu</td>
<td>3.10 (3)</td>
<td>2.68 (3)</td>
<td>5.52 (6)</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.70 (1)</td>
<td>0.60 (1)</td>
<td>1.70 (2)</td>
</tr>
<tr>
<td>Phe</td>
<td>0.00 (0)</td>
<td>0.00 (0)</td>
<td>0.46 (1)</td>
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<tr>
<td>Trp</td>
<td>0.00 (0)</td>
<td>0.00 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>29</td>
<td>75</td>
</tr>
</tbody>
</table>

*Extraction with acetate buffer; aextraction with perchloric acid; ND, not determined.

The second inhibitor from white bush seeds, CPPTI-II, extracted, with acetate buffer, resembles in several respects CPPTI-I. It has a lysine residue at possition P₁, it inhibits bovine trypsin (Fig. 4) and the trypsin-like enzyme from St. griseus proteinase and, like CPPTI-I, it does not inhibit chymotrypsin, kallikrein or subtilopeptidase A. On polyacrylamide gel electrophoresis at pH 8.6, CPPTI-II migrates faster than CPPTI-I and the modified form CPPTI-I* (Plate 2E), and differs in amino acid composition from CPPTI-I (Table 2) although, like CPPTI-I, it contains a large amount
Fig. 4. Inhibition of bovine trypsin by CPPTI-I (A) prepared from acetate extract (○) or perchloric acid extract (●), and by CPPTI-II (B). The amount of active trypsin was estimated by titration against pNPGB.

Fig. 5. Calibration curve for the determination of $M_r$ of trypsin inhibitors by Sephadex G-50 chromatography. The column (1.8 × 82 cm) was equilibrated with 0.05 M-phosphate buffer, pH 7.5, containing 0.1 M-NaCl. Standards: 1, CMTI-III ($M_r$ 3270); 2, Kazal inhibitor (6150); 3, cytochrome C (12500); 4, myoglobin (17600); 5, chymotrypsinogen A (25000). Inhibitors: A, CPPTI-I and C, CPPTI-II, purified from the acetate buffer extract; B, CPPTI-I purified from the perchloric acid extract.

of cysteine. The $M_r$ values for CPPTI-II determined on the basis of trypsin inhibition (Fig. 4), molecular gel filtration (Fig. 5) and calculated from the amino acid composition were, respectively 7200, 7900 and 7850.
DISCUSSION

Determination of the amino acid sequence of trypsin inhibitors from seeds of the squash family (Cucurbitaceae) plants performed at the Laboratory of Professor M. Laskowski (Wieczorek et al., 1985) permitted to establish that the seeds contain two forms of the inhibitor, differing solely in length of the polypeptide chain at the N-terminal of the molecule: shorter forms, built of 29 amino acid residues (CMTI-III, CPTI-II), and longer ones, with an additional N-terminal sequence His-Glu-Glu (CMTI-IV, CPTI-III).

The occurrence of two forms of the inhibitor in seeds of Cucurbitaceae plants could play an important physiological role. Both forms could arise as products of enzymatic degradation of larger proteins, or the shorter forms could be formed by cleavage of the N-terminal tripeptide His-Glu-Glu from the longer forms. This process could occur either during ripening of the seeds, or during the purification procedure due to the presence of proteolytic enzymes in the extract (Wilimowska-Pelc et al., 1983; Polanowski et al., 1985). To exclude the latter possibility, perchloric acid was applied as a deproteinizing agent for parallel extraction of the inhibitors. Under these conditions, proteinases present in the extract undergo inactivation whereas low-molecular inhibitors remain active. The main inhibitor (CPPT-I) of white bush seeds isolated from the perchloric acid extract, containing 29 amino acid residues, appeared to be identical with the main inhibitor isolated from seeds extracted with acetate buffer. The amounts of the inhibitor obtained from the two extracts were also similar. From these data it follows that the inhibitor composed of 29 amino acid residues does not arise during the purification process.

Both the amino acid composition and properties of CPPT-I from the white the white bush seeds, of the main inhibitor from zucchini seeds, CPPT-I (Leluk et al., 1983) and of the trypsin inhibitor from summer squash seeds, CPTI-II (Otlewski et al., 1984) are identical.

The second inhibitor (CPPT-II) isolated from the white bush seeds is twice as large as CPPT-I. However, the two inhibitors have many properties in common, e.g. reaction with trypsin at equimolar rations, inhibition of the activity of the trypsin-like enzyme from St. griseus proteinase, large content of cysteine, and a lysine residue in position P_1 of the reactive site. Moreover, they do not affect chymotrypsin, kallikrein or subtilopeptidase A. The amino acid composition of the two inhibitors does not exclude the possibility that in vivo the smaller one, CPPT-I, is a product of proteolysis of the larger one, CPPT-II.

On the other hand, neither of the inhibitors isolated from seeds resembles in its amino acid composition and inhibitory properties the main trypsin inhibitor obtained from the white bush fruit. The latter contains only
1-2 cysteine residues and it inhibits, in addition to trypsin, also kallikrein and subtilopeptidase A (Pham et al., 1985). This indicates that the main inhibitor from the white bush fruit cannot serve as precursor of the seed inhibitors.

REFERENCES


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