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1H NMR SPECTRA OF TRYP SIN INHIBITORS FROM SEEDS OF CUCURBITACEAE PLANTS. RESONANCE SIGNALS OF METHYL GROUPS*

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1H NMR spectra (250 MHz) of four trypsin inhibitors isolated from seeds of Cucurbitaceae plants were studied. It was found that structural differences between the inhibitors from the genus Cucurbita and from the genus Cucumis consist, among others, in the presence in the former group of a valine residue strongly shielded from the solvent.

A few years ago Polanowski and Wilusz (Polanowski et al., 1980; Leluk et al., 1983) have detected a new group of polypeptide trypsin inhibitors in seeds of plants of the Cucurbitaceae family. The amino acid sequence of two isoinhibitors from squash (Cucurbita maxima) seeds, designated CMT-I and CMTI-III, was recently determined by Wilusz et al. (1983). On the basis of immunological studies as well as the analysis of 1H NMR spectra of the inhibitors in the range of amide resonances (Szewczuk et al., 1983) it has been concluded that the inhibitors from the particular genera of the Cucurbitaceae family differ in their molecular structure. The inhibitors from the genus Cucurbita (squash, summer squash and zucchini) have a compact molecular structure, as evidenced by the presence in 1H NMR spectra of a group of 9-10 amide protons practically unexchangeable for deuterium. On the other hand, all the amide protons of the inhibitor isolated from cucumber seeds (genus Cucumis) proved exchangeable for deuterium upon dissolution of the sample in 2H2O. Analysis of 1H NMR spectra of the inhibitors over the range of resonance of methyl groups of amino acid side chains indicates that the structural differences between the inhibitors from plants belonging to the Cucurbita and Cucumis genera consist,

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among others, in the presence in the former of a valine residue strongly shielded from the solvent. We are inclined to identify this residue as Val² of the polypeptide chain of CMTI¹. Analysis of ¹H NMR spectra has been performed for the following inhibitors: *Cucurbita maxima* seed trypsin inhibitor I; *Cucurbita pepo* seed trypsin inhibitor II; *Cucurbita pepo var. Gironontia* (zucchini) seed inhibitor I, and *Cucumis sativus* seed trypsin inhibitor IIb.

**MATERIALS AND METHODS**

Virgin forms of the trypsin inhibitors from seeds of squash, summer squash, zucchini and cucumber were isolated under conditions described by Leluk *et al.* (1983); that paper contains also more detailed characteristics of the inhibitors.

¹H NMR spectra (250 MHz) of the inhibitors were recorded on a Bruker WM 250 spectrometer working in pulse Fourier transform mode. Solutions of particular inhibitors, about 1% in 99.75% ²H₂O were used; p₂H of the samples (measured with a Mera-Elwro N 511 instrument) was 5.5, and the temperature of the measurements were placed in 5 mm tubes, with DSS as internal standard.

**RESULTS AND DISCUSSION**

In the present work attention was focused on the range of aliphatic proton resonances in the ¹H NMR spectra of the inhibitors from seeds of the *Cucurbitaceae* plants, in an attempt at identification of at least a part of the resonance signals

<table>
<thead>
<tr>
<th>Residue</th>
<th>CMTI-I</th>
<th>CPTI-II</th>
<th>CPGTI-I</th>
<th>CSTI-IIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ala</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Ile</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Leu</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Val</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Total number of CH₃ groups of Ile, Leu and Val residues</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>18</td>
</tr>
</tbody>
</table>

¹ Abbreviations used: CMTI-I, trypsin inhibitor I from the seeds of squash (*Cucurbita maxima*); CPTI-II, trypsin inhibitor II from the seeds of summer squash (*Cucurbita pepo*); CPGTI-I, trypsin inhibitor I from the seeds of zucchini (*Cucurbita pepo var. Gironontia*); CSTI-IIb, trypsin inhibitor IIb from the seeds of cucumber (*Cucumis sativus*); DSS, 3-(trimethyl-silyl)-1-propanesulphonic acid sodium salt.
of polypeptide methyl groups. Five amino acids which contain methyl groups in their side chains, occur in the molecules of the inhibitors studied: alanine, isoleucine, leucine, valine and methionine. Their content in the particular inhibitors is given in Table 1. $^1$H NMR spectra of CMTI-I, CPTI-II and CPCTI-I were of good quality. In the spectrum of CSTI-IIb the resonance signals were somewhat more broadened which could be due to greater conformational lability of the molecule. The $^1$H NMR spectrum of CPTI-II (aliphatic region) is shown as an example (Fig. 1). In this spectrum, similarly as in those for all other inhibitors studied, a highfield triplet (at 0.62 ppm) assigned to the standard, i.e. DSS, is visible. This signal is, of course, omitted in further analysis.

Fig. 1. A, Aliphatic region of $^1$H NMR spectrum of the summer squash trypsin inhibitor II (CPTI-II); B, highfield fragment of the same spectrum on irradiation of the sample with the resonance frequency of the signal at 0.91 ppm.

Trypsin inhibitor from squash seed (CMTI-I). On the basis of the known amino acid sequence of CMTI-I, the signal at 1.45 ppm (weakly resolved doublet) can be assigned to the Ala$^{18}$ residue, and the singlet at 2.18 ppm to the S-methyl group
of Met$^8$. The position occupied by the signal of the methyl group of Ala$^{18}$ is identical with that of free alanine (1.45 ppm) which could indicate that this residue is fully exposed toward the aqueous phase. The signal of Met$^8$ is slightly shifted (by 0.06 ppm) toward the low field, as compared with that of free methionine.

The resonance signals of methyl groups of leucine, isoleucine, and valine overlap forming a cluster with sharp peaks at 0.90, 0.87 and 0.84 ppm. A highfield, non-symmetrical doublet centered at 0.72 ppm is well separated from this cluster. The ratio of intensity of the 0.72 ppm doublet to the intensity of the remaining methyl signals of leucine, isoleucine and valine is about 1:5. Since a CMTI-I molecule contains a total of 12 methyl residues of these amino acids, it should be concluded that the signal at 0.72 ppm derives from two methyl groups. The shape of the signal seems to exclude its assignment to the isoleucine residue because the highfield $\delta$-CH$_3$ signal of isoleucine should have appeared as a triplet. Thus, the signal analysed could derive from leucine or valine residues. It should be noted that the sequence of methyl group resonances in $^1$H NMR spectra of other proteins which have been extensively examined by means of NMR spectroscopy, e.g. neurotoxin of snake venom or pancreatic trypsin inhibitor (BPTI), was different from that observed in the present work. Neurotoxins were the subject of excellent works of Bystrov (Tsetlin et al., 1979; Bystrov, 1981; Arseniev et al., 1981), and BPTI of extensive studies of Wüthrich (Wüthrich et al., 1978; Brown et al., 1978; Richarz & Wüthrich, 1978; Wüthrich & Wagner, 1981). As demonstrated by Bystrov (cf Arseniev et al., 1981), in the case of neurotoxin II from Naja mossambica mossambica, the $\delta$-CH$_3$ triplet of Ile$^{50}$ residue occurs in higher field than the pair of doublets: $\gamma$-CH$_3$ of Val$^{46}$ and $\delta$-CH$_3$ of Leu$^{52}$. Although in the highest field a signal of one of the two magnetically non-equivalent Leu$^1$-CH$_3$ groups is observed, this could be due to a unique position of this residue in the polypeptide chain. In the case of BPTI (Richarz & Wüthrich, 1978; Wüthrich et al., 1978), a signal of the Thr$^{32}$ methyl groups occurs in the highest field. Then, successively, two triplets of $\delta$-CH$_3$ group of isoleucine appear, and only later the doublet of methyl groups of valine and leucine residues, and of $\gamma$-CH$_3$ groups of isoleucine. The sequence of NMR signals in these proteins is thus the same as could be expected from the positions of the respective signals of free amino acids. In our case the two doublets of methyl groups are distinctly shifted upfield and precede the signals $\delta$-CH$_3$ of isoleucine residues. In our opinion, this could be due to the shielding effect of disulphide bridges. It should be noted that in CMTI-I the Val$^2$, Val$^{21}$, Leu$^{17}$ and Leu$^{23}$ residues are in close vicinity of cysteines. The single isoleucine, Ile$^6$, occupies a position rather remote from that of any cysteine residue. The shielding effect of disulphide bridges has been reported in the literature; for instance, the resonance signals of amide protons of valine residues of 2,7-cystine gramicidin S (Ludescher & Schwyzer, 1971) are shifted upfield by 0.2 ppm as compared with gramicidin S. The authors explain this observation by the shielding effect of 3p-electron pairs of the disulphide sulphur atoms. It seems that a similar interaction could also play a role in our case, causing an upfield shift of the signals of the two methyl groups adjacent to the disulphide bridges. The results obtained by us do not allow to conclude from which of the valine and leucine residues of
CMTI-I these signals originate. A comparison of the results obtained for CMTI-I and those for CPTI-II (see below) permits to suggest that Val\(^2\) is one of these residues.

*Trypsin inhibitor from summer squash seed (CPTI-II).* High similarity in immunological properties, amino acid composition, and \(^1\)H NMR spectra in the range of amide resonances, permits to conclude that the structure of CPTI-II is almost the same as that of CMTI-I. However, at variance with CMTI-I, a molecule of CPTI-II contains but one valine residue, and the intensity of the highfield signal at 0.72 ppm, which is observed in the \(^1\)H NMR spectrum of CPTI-II, corresponds to one methyl group. The resonance signals of the remaining methyl groups of isoleucine, leucine and valine form a cluster, with resolved peaks at 0.82, 0.84, 0.87, 0.91, 0.93, 0.99, 1.00 and 1.01 ppm, respectively. Irradiation of the sample with the second resonance frequency corresponding to that of the peak at 0.91 ppm discloses both component

![Graph](image_url)

**Fig. 2.** Aliphatic region of the \(^1\)H NMR spectrum of CPTI-II recorded on irradiation of the sample with the resonance frequency of the signal at 1.47 ppm.
doublets of the magnetically non-equivalent methyl groups of the residue giving resonance signals (at 0.72 and 0.80 ppm, respectively) at the highest field values (see Fig. 1B).

The resonance signal of CH<sub>3</sub>-Ala occurs, similarly as in the case of CMTI-I, at 1.47 ppm (J<sub>ab</sub> = 6.0 Hz). Irradiation of the sample with the resonance frequency of this group results in distinct changes (see Fig. 2) in not a single one but in two regions of the α-proton resonances, i.e. at 4.0 and 4.45 ppm (indicated in Fig. 2 by asterisks). At the same time, the highfield doublet at 0.72 ppm becomes transformed into a singlet, and a new, well-resolved peak appears at 0.80 ppm. This observation can be explained only by overlapping of the signal of β-proton of valine on the resonance signal of CH<sub>3</sub>-Ala at 1.47 ppm — because, if the highfield pair of doublets at 0.72 and 0.80 ppm were derived from a leucine and not a valine residue, their decoupling could not be accompanied by a simultaneous decoupling of α-hydrogen signal at 4.45 pp. However, it should be noted that in the case of <sup>1</sup>H NMR spectra of proteins (Campbell et al., 1975), the resonance signals of β-protons of valine residues occur at about 2.6 ppm. For free valine they are at 2.27 ppm. The position of the signal analysed (1.47 ppm) corresponds more closely to that of signals of γ-protons of leucine than of β-protons of valine. Thus, according to our interpretation it should be assumed that, in CPTI-II, the signal of β-proton of valine is shifted very strongly upfield. This effect could be explained by the shielding effect of a disulphide bridge. It should be expected that this effect would be much stronger in the case of β-protons than γ-methyl groups of Val (or δ-CH of Leu) because, as it appears from studies on molecular models, the β-methine group of valine can easily enter into close vicinity of sulphur atoms of the bridge. Recent studies of Otlewski (1983) have unequivocally demonstrated that valine in CPTI-II is located in the N-terminal pentapeptide of the inhibitor, thus it corresponds to Val<sup>2</sup>, and not to Val<sup>21</sup> of the CMTI-I molecule. Our results seem to suggest that in CPTI-II and also CMTI-I molecules the residue Val<sup>2</sup> is attached very closely to the disulphide bridge. This suggests also that the N-terminal fragment of the polypeptide chain of CPTI-II has limited mobility.

Out of the two α-hydrogen signals seen at 4.0 and 4.45 ppm, the first belongs to α-proton of alanine. It follows, therefore, that the signal at 4.45 ppm is probably the signal of α-proton of valine. Irradiation of the sample with the resonance frequency of α-proton of alanine (4.0 ppm) does not result in decoupling (see Fig. 3) of any of unexchangeable amide protons. Thus, the amide proton of alanine does not belong to the group of protons hardly exchangeable for deuterium. On the contrary, irradiation of the sample with the resonance frequency of the signal located at 4.45 ppm, results in decoupling of so many as two amide signals. In the initial spectrum they appear as two well-resolved doublets; in the spectrum of the irradiated sample they appear as singlets. These signals are located, respectively, at 8.25 (J<sub>ab</sub> 5.7 Hz) and 9.35 ppm (J<sub>ab</sub> 8.5 Hz). As indicated by these results, the resonance signals of α-proton of valine at 4.45 ppm must be overlapped by the signal of another α-proton belonging to an yet unidentified amino acid residue. The amide proton of
Fig. 3. A, Amide region of the $^1$H NMR spectrum of CPTII, and B, the same part of the spectrum recorded on irradiation of the sample with the resonance frequency of the signal at 4.45 ppm.

This residue is, like the amide proton of valine, strongly shielded from the solvent, thus they both belong to the group of unexchangeable protons. Although at present we are unable to determine which of the two amide signals (at 8.25 or 9.35 ppm) should be assigned to the valine residue, we can in any case suggest that in CMTI-I (and probably in CPT-II) the amide proton of Val$^2$ is buried within the hydrophobic interior of the molecule.

It should be also noted that, on irradiation of the sample with the resonance frequency of the signal located at 1.47 ppm (overlapped β-Ala and β-Val resonances), some changes can be observed in the region of 3.0 ppm and 1.7 ppm (indicated by asterisks in Fig. 2), consisting in changes in relative intensity of the particular resonances. The region of 3.0 ppm corresponds to the range of resonance signals of cystine methylene groups. Thus, our observation could in some measure confirm the spacial proximity of valine residue to one of cysteine residues.

It should be added that the resonance signal of the S-CH$_3$ group of methionine in CPTI-II occurs, like in CMTI-I, at 2.18 ppm.

Trypsin inhibitor from zucchini seeds (CPGTI-I). In the spectrum of this inhibitor, the signals of CH$_3$-Ala (1.47 ppm, $J_{AB}$ 5.5 Hz) and S-CH$_3$Met (2.18 ppm) can be readily identified. Similarly as with CMTI-I and CPTI-II, a rather poorly resolved doublet appears at 0.72 ppm. Its intensity corresponds to that of one methyl group. This indicates that, in the CPGTI-I molecule, like in CPTI-II, a residue shielded by
the disulphide bridge is present; on the basis of previous results we are tempted to identify it as a valine residue.

Trypsin inhibitor from cucumber seed (CSTI-IIb). No signal corresponding to CH$_3$-Ala appeared in the $^1$H NMR spectrum of this inhibitor, which is in agreement with the results of amino acid analysis.

A wide cluster with the maximum at 0.91 ppm, and a weaker broad signal centered at 0.72 ppm are visible in the highfield region. Strong broadening of these signals precludes their more precise assignment.

The two methionine residues present in the CSTI-IIb molecule give two separate signals at 2.11 and 2.18 ppm, respectively. This leads to the conclusion that the microenvironment of two methionine residues of CSTI-IIb is different. Since the resonance signal of the 5-CH$_3$ group of free methionine is also located at 2.11 ppm, this could indicate that the methionine of CSTI-IIb which gives the resonance signal at the same frequency, is more exposed to the solvent than the methionine which gives the signal at 2.18 ppm. As we have reported previously (Szewczuk et al., 1983), the group of signals originating from the amide protons unexchangeable for deuterium, does not appear in the $^1$H NMR spectrum of CSTI-IIb. This indicates that even if, like in other inhibitors, a valine residue is present in position 2 of the polypeptide chain of CSTI-IIb, it nevertheless would be accessible to the solvent molecules. Thus, this result points to the existence, in the region of the N-terminal fragment of the molecules studied, of distinct conformational differences in the spacial structure of the inhibitors of the genus Cucurbita on one hand, and from the genus Cucumis on the other.

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WIDMA $^1$H NMR INHIBITORÓW TRYSYNY Z NASION DYNIOWATYCH.
SYGNAŁY REZONANSOWE GRUP METYLOWYCH

Streszczenie

Zbadano widma $^1$H NMR (250 MHz) czterech inhibitorów trypsyny wyizolowanych z nasion dyniowatych. Stwierdzono, że różnice strukturalne pomiędzy inhibitorami z rodzaju Cucurbita a inhibitorami z rodzaju Cucumis polegają m.in. na obecności w tych pierwszych reszty walinowej silnie ekranowanej od dostępu rozpuszczalnika.

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