STRUCTURAL CHARACTERISTICS OF TENECIN 3,
AN INSECT ANTIFUNGAL PROTEIN

Young-Tae Lee1, Daeh-Hee Kim1, Jeong-Yong Suh1, Jae Hoon Chung2, Bok Leul Lee3,
Younghoon Lee1 and Byong-Seok Cho2*

1Department of Chemistry and 2Department of Biological Sciences, Korea Advanced Institute of Science
and Technology, Taejon 305-701, Korea;
3College of Pharmacy, Pusan National University, Pusan 609-735, Korea

Received October 6, 1998
Accepted October 13, 1998

SUMMARY

Tenecin 3, an antifungal protein, previously isolated from the insect Tenebrio molitor,
inhibits growth of the fungus Candida albicans. However, the antifungal mechanism and
functions of tenecin 3 remain unknown. As an initial step to study the mechanism and
functions, physical and structural properties of tenecin 3 were examined by circular
dichroism (CD) analysis and 2D nuclear overhauser effect spectroscopy. These analyses
suggest that tenecin 3 has a propensity of random structure with very loose turn-like
elements. The CD results also indicate that this random structural propensity is not
significantly affected by temperature, pH, and by the presence of organic solvents or
sodium dodecyl sulfate (SDS) micelles. However, the hydrodynamic studies suggest that
tenecin 3 is not in extended form in spite of its random structural feature.

Key words: Antifungal protein, Insect, Protein structure, Tenecin 3

INTRODUCTION

Insects have developed their characteristic host-defense system based on potent
antimicrobial molecules (1-3). Many antibacterial proteins induced by responses of insects
to bacterial challenge or septic injury have been studied extensively (4, 5). In constrast,
relatively a few proteins having antifungal activity have been identified in insects and they
are less studied. Although the small number of antifungal proteins restricts the generality of
classification, we may group them into two categories. (i) Immune-inducible peptides
including drosomycin (6), thanatin (7), and mechnikowin (8): Drosomycin of 44 residues
was found to have cysteine residues engaged in intramolecular disulfide bridges and to
build a cysteine-stabilized αβ motif, which also appeared in insect defensin (9, 10) and
plant defensin (11). Drosomycin shows a specific activity only on fungi, while
methchnikowin exhibits activity against both fungi and Gram-positive bacteria. (ii)
Constitutively expressed antifungal proteins including AFP (antifungal protein) (12),
tenecin 3 (13, 14), and holotricin 3 (15): These proteins show similar biochemical features such as heat stability, no antibacterial activity, and high contents of Gly and His residues.

Among the constitutively expressed antifungal proteins, tenecin 3 of 78 residues, isolated from _Tenebrio molitor_, has distinctive features in its primary structure (13). Gly, His, and Gln constitute 80% of its total residues. It has also a repeated motif Gly-X-X-Gly, where X can be His, Gln or Leu. This motif reiterates 11 times. However, its action mechanism is not yet known. In the present work, we investigated structural properties of tenecin 3 using spectroscopic and hydrodynamic methods as an initial step to understand the action mode of tenecin 3.

**MATERIALS AND METHODS**

**Tenecin 3 expression and purification**

The recombinant tenecin 3 was expressed in _Escherichia coli_ strain BL21(DE3) with plasmid pATF-I which contains the tenecin3 coding sequence under the T7 expression system and purified as previously described (16). The antifungal activity of the purified protein against _Candida albicans_ was confirmed as described (13) prior to structural analysis.

**Spectroscopic methods**

All the CD spectra were obtained by Jasco-720 or Jasco-715 spectropolarimeter. Cellular path length was 1 mm. The concentration of stock solution of protein was determined by amino acid analysis and bicinchoninic acid (BCA) assay (17). The stock solution was diluted to 50-100 µg/ml in appropriate buffers. All the experiments were carried out at 25°C unless any comment was indicated. Scan speed was set with 10 or 20 nm/min. The can was carried out three times and averaged to the mean value. The contents of secondary structure were calculated using the Yang's method (18).

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX-500 spectrometer operating at 500 MHz. The concentration of protein was 1.6 mM and pH was 6.0 in 20 mM sodium phosphate buffer. Nuclear overhauser effect spectroscopy (NOESY) spectra were measured at 295 K. The mixing time of NOESY was 250 ms. The water resonance was suppressed by the pre-irradiation during the relaxation delay of each pulse. Experiments were recorded with 512 t₁ measurements and data points of 1 K was collected for each t₁. The numbers of scan for each t₁ were 80 for NOESY. The time-proportional phase-incrementation (TPPI) was used for the phase sensitive mode detection. All data processing was performed on felix-950. Complex fourier transformation was performed. Apodization was used with sinebell squared window function. Proton exchange with deuterium was measured at 295 K. The spectrum of 0 min was recorded in H₂O/D₂O (9:1) and others were obtained after dissolving the lyophilized protein in D₂O.

**Hydrodynamic method**

HPLC-gel permeation chromatography (GPC) was used to estimate the molecular weight of the protein based on the hydrodynamic property. Standard and sample proteins were
applied to the column (Protein-Pak 125, Waters) with isocratic elution (phosphate 50 mM, pH 6.5, NaCl 100 mM) at 0.5 ml/min. Standard proteins were insulin (5.8 kDa), cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43.5 kDa), BSA (69 kDa). Dipeptide (Gly-L-Leu) and plasmid DNA (>300 kDa) were used to determine the total bed volume and the void volume, respectively. The molecular weight of the protein was obtained by plotting $K_d$, which satisfies the following equation: (elution volume) = (void volume) + $K_d$ (total bed volume), and log(MW) of the standard proteins.

RESULTS AND DISCUSSION

We used the recombinant protein expressed in *Escherichia coli* (16) to examine structural properties of tenecin 3 due to very low availability of the protein from the natural source. First, we examined the presence of secondary structural elements in tenecin 3. Since structural studies in amphiphilic or nonpolar environments may give a picture close to that of the *in vivo* situation, two systems, water/trifluoroethanol (TFE) solution and SDS suspension, were used for examining structural features of tenecin 3. These two systems are the most commonly used systems to provide these environments for proteins (19-21). Fig. 1A shows the CD spectra of tenecin 3 in varying the ratio of TFE/water at 25°C. No prominent structure could be seen at various different concentrations of TFE. All CD spectra showed very similar pattern above 210 nm, but the higher ratio of TFE made the weaker negative band around 200 nm, which is characteristic of random coil. This change at the higher ratio accompanied a slight increase in helix population and the corresponding decrease in random coil population (Table 1). The CD spectra were also collected by varying temperature, pH, and concentration of SDS (Fig. 1B-D). Any significant changes in the CD spectra were not observed under various different solvent conditions. Therefore these CD data suggest that the native structure of tenecin 3 has little structural variations under various conditions including amphiphilic or nonpolar environments. Calculation of the contents of secondary structures according to the Yang's method (18), which was based on the CD results, suggests that tenecin 3 is composed of mainly random coils (39-57%) and turns (28-43%). The CD results are consistent with the structure prediction from the amino acid sequence by both the Chou-Fasman's method (22) and Garnier-Robson's method (23) that any helix or $\beta$-strand does not exist in the entire sequence: The Chou-Fasman's method predicts that most of sequence of tenecin 3 is composed of turns, while the Garnier-Robson's method predicts that tenecin 3 has mainly random coils with a minor portion of turns. To confirm the presence of turns in tenecin 3, we used $^1$H NMR spectroscopy.
Table 1. Estimation of secondary structure contents of tenecin 3 from CD

<table>
<thead>
<tr>
<th>Condition</th>
<th>Alpha (%)</th>
<th>Beta (%)</th>
<th>Turn (%)</th>
<th>Random (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of TFE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>11</td>
<td>35</td>
<td>54</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>10</td>
<td>35</td>
<td>55</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>17</td>
<td>31</td>
<td>53</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>22</td>
<td>31</td>
<td>46</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>21</td>
<td>28</td>
<td>51</td>
</tr>
<tr>
<td>60</td>
<td>2</td>
<td>21</td>
<td>30</td>
<td>46</td>
</tr>
<tr>
<td>70</td>
<td>3</td>
<td>22</td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>80</td>
<td>6</td>
<td>15</td>
<td>37</td>
<td>43</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5°C</td>
<td>0</td>
<td>0</td>
<td>43</td>
<td>57</td>
</tr>
<tr>
<td>15°C</td>
<td>3</td>
<td>10</td>
<td>35</td>
<td>53</td>
</tr>
<tr>
<td>25°C</td>
<td>3</td>
<td>7</td>
<td>36</td>
<td>54</td>
</tr>
<tr>
<td>35°C</td>
<td>0</td>
<td>2</td>
<td>41</td>
<td>57</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>0</td>
<td>17</td>
<td>34</td>
<td>50</td>
</tr>
<tr>
<td>3.7</td>
<td>0</td>
<td>15</td>
<td>35</td>
<td>51</td>
</tr>
<tr>
<td>4.7</td>
<td>0</td>
<td>14</td>
<td>35</td>
<td>52</td>
</tr>
<tr>
<td>5.8</td>
<td>0</td>
<td>12</td>
<td>36</td>
<td>52</td>
</tr>
<tr>
<td>6.7</td>
<td>0</td>
<td>10</td>
<td>36</td>
<td>53</td>
</tr>
<tr>
<td>7.7</td>
<td>0</td>
<td>10</td>
<td>37</td>
<td>54</td>
</tr>
<tr>
<td>Conc. of SDS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1mM</td>
<td>3</td>
<td>19</td>
<td>36</td>
<td>50</td>
</tr>
<tr>
<td>10mM</td>
<td>5</td>
<td>18</td>
<td>37</td>
<td>39</td>
</tr>
<tr>
<td>20mM</td>
<td>4</td>
<td>18</td>
<td>36</td>
<td>41</td>
</tr>
</tbody>
</table>

Fractions of each conformation were calculated by curve-fitting using following constraints: $Y(\lambda) = f_1X_1(\lambda) + f_2X_2(\lambda) + f_3X_3(\lambda) + f_4X_4(\lambda) + f_5X_5(\lambda)$, $\sum f_i = 1$, where $X(\lambda)$ is the mean residue ellipticity at $\lambda$; $f_i$ is the fraction of the $i$th conformation and $X_i(\lambda)$ is the corresponding reference mean residue ellipticity. Reference CD was based on five proteins: myoglobin, lactate dehydrogenase, lysozyme, papain, and ribonuclease A.

Surprisingly, no significant NH-NH crosspeaks were observed in the NOESY spectrum (Fig. 2), suggesting that tenecin 3 does not have any stable turns. Since NH-NH crosspeaks would not be observed if turns are very unstable, tenecin 3 seems to have turns as very loose forms. The hydropathy analysis (Fig. 3) also shows that tenecin 3 is a very hydrophilic protein with no significant hydrophobic segment in the entire sequence. This global hydrophilicity prevents the formation of hydrophobic core for a structured protein. This random structural propensity of tenecin 3 can explain the heat stability of the protein. Taken together, it can be concluded that tenecin 3 is natively unfolded protein which is mainly composed of random coil with very loose turn-like elements.

In order to obtain information about hydrodynamic properties of tenecin 3, we performed a HPLC-GPC experiment and a hydrogen exchange experiment. Assuming that tenecin 3
Figure 1. Far-UV CD spectra of tenecin 3. (A) In varying ratio of TFE/water. The percentage of TFE in solution was varied from 10% to 80% with the increment of 10%. (B) In various temperature in 20mM sodium phosphate (pH 7.0): 5°C (----), 15°C (-- --), 25°C (-- - -) and 35°C (---------). (C) In various pH: 2.0, 3.7, 4.7, 5.8, 6.7 and 7.7. pH of solution was adjusted by 1 mM Na2HPO4/citric acid with 20 mM NaCl. (D) In 1 mM (--- ), 10 mM (-- --) and 20 mM (---------) of SDS (pH 7.0, 20 mM sodium phosphate).

Table 2. Measurement of molecular weight of tenecin 3.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (kDa)</th>
<th>Elution time (min)</th>
<th>Elution volume, Ve (ml)</th>
<th>V_e - V_o</th>
<th>K_d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bed volume, V_i</td>
<td>&lt;1</td>
<td>23.67</td>
<td>11.84</td>
<td>5.92</td>
<td>1</td>
</tr>
<tr>
<td>Insulin</td>
<td>5.8</td>
<td>21.58</td>
<td>10.79</td>
<td>4.87</td>
<td>0.82</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>12.4</td>
<td>18.96</td>
<td>9.48</td>
<td>3.56</td>
<td>0.60</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>29</td>
<td>17.25</td>
<td>8.63</td>
<td>2.71</td>
<td>0.46</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>43.5</td>
<td>15.83</td>
<td>7.92</td>
<td>2.00</td>
<td>0.34</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>69</td>
<td>14.67</td>
<td>7.34</td>
<td>1.42</td>
<td>0.24</td>
</tr>
<tr>
<td>Void volume, V_o</td>
<td>&gt;300</td>
<td>11.83</td>
<td>5.92</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tenecin 3</td>
<td>8.5^</td>
<td>20.33</td>
<td>10.17</td>
<td>4.25</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Dipeptide (Gly-L-Leu) and plasmid DNA (>300kDa) was used to determine the total bed volume and the void volume, respectively. The molecular weight of the protein was obtained by plotting K_d, which satisfies the following equation; (elution volume) = (void volume) + K_d (total bed volume), and log(MW) of the standard proteins.

^ Calculated molecular weight from hydrodynamic results.
would be a globular protein, the HPLC-GPC data estimates its molecular weight to be 8.5 kDa (Table 2). This value is very close to the calculated molecular weight of 7.4 kDa, suggesting that tenecin 3 has a smaller hydrodynamic radius than expected as in a random coiled structure with very loose turn-like elements. Therefore, the shape of protein seems to be a globular-like form rather than an extended form. The hydrogen exchange by $^1$H NMR was examined to see whether hydrogen bonds in tenecin 3 are shielded from solvent molecules. All of amide protons were exchanged with deuterium within 5 minutes (Fig 4), indicating that there is no stable hydrogen bond. Therefore the smaller hydrodynamic radius than expected is not due to the presence of stable hydrogen bonds in tenecin 3. Instead it may arise from the large amount of turn-like elements even though they are very loose.

Figure 2. NH-NH region of NOESY spectrum of tenecin 3. The spectrum was recorded on a Bruker AMX-500 spectrometer operating at 500MHz at 295K. The concentration of protein was 1.6 mM and pH was 6.0 in 20 mM sodium phosphate buffer. The mixing time of NOESY was set at 250 ms. The water resonance was suppressed by the pre-irradiation during the relaxation delay of each pulse.
because turn-like elements can prevent the protein from spreading out.

It would be very intriguing that tenecin 3 with no apparent secondary structural elements has an antifungal activity although how tenecin 3 acts as an antifungal protein remains to be demonstrated. Recently other proteins having natively unfolded structures but carrying out specific functions have been known (24, 25). However, no one knows the basis on the structure-function relationship. Identification of the natively unfolded structure of tenecin 3 provides a model system that can be used to assess the functional significance of the natively unfolded structures.

![Hydrophobic and hydrophilic domains analysis](image)

**Figure 3.** Hydropathy analysis of tenecin 3. The distribution of hydrophobic and hydrophilic domains was analyzed by the method of Kyte and Doolittle (26). This analysis shows that tenecin 3 is a very hydrophilic protein and there is no significant hydrophobic segment in the entire sequence. The number on horizontal line indicates the position of amino acids.

![Proton exchange spectra](image)

**Figure 4.** Spectra of 1D proton exchange experiments. The spectrum of 0 min was recorded in H$_2$O/D$_2$O (9:1) and others were obtained after dissolving the lyophilized protein in D$_2$O.

**ACKNOWLEDGMENT**

This work was supported by a grant (KOSEF 95-0423-09-02-3) from Korea Science and Engineering Foundation.
REFERENCES