Trichosanthin, a potent HIV-1 inhibitor, can cleave supercoiled DNA in vitro

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ABSTRACT
Trichosanthin, an abortifacient, immunosuppressive and anti-tumor protein purified from the traditional Chinese herb medicine Tian Hua Fen, is a potent inhibitor against HIV-1 replication. Under normal enzymatic digestion conditions, trichosanthin cleaves the supercoiled double-stranded DNA to produce nicked circular and linear DNA. Trichosanthin has no effect on linear double-stranded DNA. Neither does it convert relaxed circular duplex DNA into a supercoiled form in the presence of ATP. Thus trichosanthin is not a DNA gyrase. However, trichosanthin can cleave the relaxed circular DNA into a linear form, indicating that both the circular as well as the supercoiled forms are essential for trichosanthin recognition. In addition, trichosanthin contains one calcium metal ion per protein molecule, which presumably is related to its endonucleolytic activity.

INTRODUCTION
The discovery that trichosanthin is a potent inhibitor against HIV-1 replication in both acutely infected T-lymphoblastoid cells and chronically infected macrophages (1) has stimulated considerable interest within the scientific community as well as among the general public. Trichosanthin is the principal active ingredient in the Chinese herb medicine Tian Hua Fen (2). It is isolated from the root tuber of the perennial plant Trichosanthes kirilowii Maxim (3–5). Tian Hua Fen has been used for centuries in China for abortion and for the cure and treatment of ectopic pregnancy, choriocarcinoma, hydatidiform moles, and invasive moles (6,7). Recently, the gene that encodes for trichosanthin has been isolated, sequenced (8), and expressed in E. coli (9).

METHODS AND MATERIALS
Trichosanthin was isolated according to the procedures described previously (5). pBluescript II DNA was purchased from Stratagene, φX174 RF DNA and SV40 DNA from Bethesda Research Laboratories.

Elemental analysis of trichosanthin
Trichosanthin solution was placed into a dialysis tubing (M.W. cutoff 2,000, SpectraPor) and dialysed against 1.0 and 10.0 mM EDTA solutions, pH 7.4 at 4°C. The dialysis solution was changed afresh every 10–12 hours four times. After dialysis the trichosanthin solution was diluted to the appropriate concentrations with distilled water and assayed for Ca²⁺, Mg²⁺, Zn²⁺, and Mn²⁺ ions. The measurements were made on a Beckman SpectraSpan VB Direct Current Plasma Emission Spectrometer (DC Plasma/Echelle System) located in the Division of Geological and Planetary Sciences at Caltech. The precision of each measurement is within ±5%. Before and after each measurement was made, the instrument was checked and rechecked with standard solutions, with the last EDTA dialysis

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solution taken as the zero standard. No Mg$^{2+}$, Mn$^{2+}$ and meaningful amount of Zn$^{2+}$ were found in trichosanthin.

**DNase activity assay of trichosanthin**

One microgram supercoiled DNA (φX174 RF, pBluescript II or SV40) was incubated with appropriate amount of trichosanthin in a total volume of 20 μl of 50 mM Tris-HCl, 10 mM MgCl$_2$ (omitted in control experiments), 10 mM CaCl$_2$, 100 mM NaCl, pH 8.0 at room temperature for 1 hour. At the end of the incubation 10 μl Ficoll solution (30% Ficoll, 200 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol FF) was added. The electrophoresis was carried out under non-denaturing conditions in standard TAE buffer (21) in a horizontal fashion in 1% agarose gel at 50V until the tracking dyes moved to the appropriate positions. The gel was stained with ethidium bromide at a final concentration of 0.5 μg/ml. The gel was then washed twice with distilled water before it was placed over a UV illuminator. The photographs were taken with a Polaroid MP4 Land camera.

**Topoisomerase activity assay of trichosanthin**

Seven micrograms of supercoiled φX174 RF DNA was incubated with 15 units topoisomerase I (BRL) in 110 μl 50mM Tris-HCl (pH 7.6), 50mM KCl, 10mM MgCl$_2$, 0.1mM EDTA, 0.5mM dithiothreitol, 30 μg/ml bovine serum albumin for 30 minutes at 37°C. The DNA was phenol extracted and ethanol precipitated(22). The obtained relaxed circular DNA was divided equally into 6 tubes and incubated with 0, 0.2, 0.5, 1, 2, 5μg trichosanthin respectively. The assay was carried out in 20μl 50mM Tris-HCl (pH 7.6), 20mM KCl, 10mM MgCl$_2$, 2mM dithiothreitol, 1.5mM ATP, 5mM spermidine, 50μg/ml BSA for 30 minutes at 37°C. At the end of the incubation, 10μl of 2% sodium dodecyl sulphate, 20% Ficoll, 400μg bromophenol blue/ml was added to the solution to stop the reaction. Subsequent electrophoresis was carried out under the same conditions as described under the DNase assay.

**RESULTS AND DISCUSSION**

**One atom of calcium is associated with trichosanthin**

The data of an elemental analysis are summarized in Table 1. Only calcium and no magnesium, manganese or significant amount of zinc was found in trichosanthin. Although the significance of this finding is not fully understood, it is presumed that calcium may be related to the endonucleolytic activity of trichosanthin. It is noteworthy that even with extensive dialysis against high concentrations of EDTA (10 mM), the calcium is still not depleted. This result strongly implicates calcium as a intrinsic component of trichosanthin. Whether the calcium is structural or has a catalytic function remains to be ascertained. However, it appears that it is not merely a cofactor.

**Trichosanthin cleaves supercoiled double-stranded DNA**

To demonstrate the endonuclease activity of trichosanthin, we tested three supercoiled DNAs pBluescript II, SV40 and φX174 RF. When the supercoiled DNA is incubated with increasing concentrations of trichosanthin, the DNA is first nicked to give a nicked circular form, which moves significantly slower than the supercoiled DNA in an agarose gel (top band in each lane, Fig. 1). When the protein concentration becomes sufficiently high, the linear form DNA is produced (second band from top in lanes 4, 5 and 6, Fig. 1), which moves faster than the nicked circular form but slower than the supercoiled DNA in an agarose gel. Similar effects are observed for pBluescript II and SV40 DNAs (data not shown).

**Trichosanthin has no effect on linear DNA but can cleave covalently closed circular DNA**

Surprisingly, trichosanthin has no effect on the linear double-stranded λ DNA (Fig. 2) and several other linear DNA’s that we have tested. Consistent with this result, once the circular DNA has been converted into a linear one by treatment with a restrictive endonuclease, trichosanthin shows no further effect. This selective activity of trichosanthin on supercoiled DNA has led us to suspect that trichosanthin might be acting as a DNA gyrase, particularly at lower concentrations. Responsible for maintaining the negatively supercoiled state of DNA, a DNA gyrase changes the topological linking number of closed circular DNA in steps of two by transiently creating a double-strand break and passing another DNA segment through the break (23-25). In the absence of ATP, a gyrase removes negative supercoils inefficiently, which is consistent with the results presented in Fig. 1. However, in the presence of ATP, the reaction is reversed, i.e., the gyrase will now introduce negative supercoils into a relaxed covalently closed circular DNA (26,27). If trichosanthin is indeed a DNA gyrase, one would therefore expect that in the presence of ATP trichosanthin will convert a relaxed circular DNA into the supercoiled form, instead of supercoiled DNA, is used as the substrate, trichosanthin cannot convert the relaxed circular DNA into the supercoiled form, indicating clearly that trichosanthin is not a DNA gyrase.

<table>
<thead>
<tr>
<th>Trichosanthin Concentration (μM)</th>
<th>EDTA Concentration (mM)</th>
<th>Ca$^{2+}$ Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.6</td>
<td>(no dialysis)</td>
<td>14.0±0.1</td>
</tr>
<tr>
<td>9.6</td>
<td>1.0</td>
<td>9.4±0.3</td>
</tr>
<tr>
<td>40.1</td>
<td>10.0</td>
<td>40.5±0.1</td>
</tr>
</tbody>
</table>

![Figure 1. The endonucleolytic activity of trichosanthin on φX174 RF DNA. Lanes 1 to 6 represent 1μg φX174 RF DNA incubated with 0, 0.03, 0.05, 0.3, 1, and 5 μg trichosanthin respectively. N, nicked circular; L, linear; S, supercoiled φX174 RF DNA.](image)
Figure 2. The effect of trichosanthin on linear double-stranded λ DNA. Lanes 1 to 8 represent λ DNA incubated with 0, 0.02, 0.5, 1, 2, 4, 8, 16 μg trichosanthin respectively.

The data do show, however, that trichosanthin can cleave the relaxed circular DNA into a linear form, indicating that the supercoiled and circular forms are essential factors for trichosanthin recognition.

It is noteworthy that trichosanthin acts on the three supercoiled DNA’s (pBluescript II, SV40, and φX174 RF) that we have tested in a similar fashion—to produce a nicked circular form at low concentrations and a linear form at sufficiently high concentrations. The question then is whether trichosanthin is a specific endonuclease that recognizes the same sequence in all three of these supercoiled DNA’s. Some interesting observations are obtained when supercoiled DNA is first treated with trichosanthin and then with a known specific endonuclease. In two such joint digestion experiments, one with EcoR I and the other with Pvu I, we have found that trichosanthin seems to have one cleavage site located between T500, the EcoR I cleavage site, and G701, one of the two cleavage sites of Pvu I in pBluescript II DNA. However, this specificity of trichosanthin may be due to the topological features of the conformation of the supercoiled DNA. One possible reason for the low specificity of trichosanthin, especially for covalently closed circular DNA, could be that the active form of the protein is an oligomer, rather than the monomer. Indeed, at the high protein:DNA ratios employed in our experiment, the trichosanthin itself can wrap the DNA, inducing localized torsional stress. Since the linear DNA is not topologically constrained, this would explain the preference for supercoiled doubled-stranded DNA or covalently closed circular DNA. The stronger preference for supercoiled DNA over relaxed covalently closed circular DNA is consistent with this scenario, as well.

The endonucleolytic activity of trichosanthin is not due to the contamination by endonucleases

The activity assays were all conducted under normal enzymatic digestion conditions with Mg$^{2+}$ present in the reaction buffer, since Mg$^{2+}$ is an essential cofactor for all restriction endonucleases. We have addressed the issue of whether Mg$^{2+}$ is really necessary for the novel DNase-like activity of trichosanthin uncovered in this study. If Mg$^{2+}$ is not required, repeating the assay in the absence of Mg$^{2+}$ should prove that the endonucleolytic activity is indeed due to trichosanthin, and not from some contaminating endonucleases. This is an important control experiment in light of the rather low specific activity observed for trichosanthin. The results of such an experiment are presented in Figure 4. The data show that trichosanthin exhibits the endonucleolytic activity in the absence of Mg$^{2+}$.

As has been shown by McGrath and his colleagues, cellular protein synthesis in acutely HIV-1 infected T-lymphoblastoid cells is not affected at concentrations of trichosanthin which result in significant reductions of RNA and protein synthesis in virus (1). Accordingly it is very interesting to note that trichosanthin has no nuclease activity on λ DNA (Fig. 2) as well as several other linear DNAs that we have tested (data not shown). This differential effect of trichosanthin on different forms of DNA substrates may be a significant determinant toward understanding the mechanism of action whereby trichosanthin inhibits HIV replication.

ACKNOWLEDGMENTS

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