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Crystallization and preliminary characterization of a highly thermostable lectin from *Trichosanthes dioica* and comparison with other *Trichosanthes* lectins

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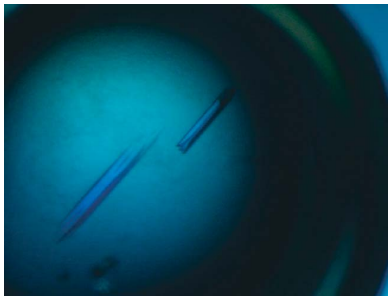
A lectin from *Trichosanthes dioica* seeds has been purified and crystallized using 25%(*w*/*v*) PEG 2K MME, 0.2 *M* ammonium acetate, 0.1 *M* Tris–HCl pH 8.5 and 50 µl 0.5%(*w*/*v*) *n*-octyl β-D-glucopyranoside as thick needles belonging to hexagonal space group $P6_4$. Unit-cell parameters were $a = b = 167.54$, $c = 77.42$ Å. The crystals diffracted to a Bragg spacing of 2.8 Å. Both the structures of abrin-a and *T. kirilowii* lectin could be used as a model in structure determination using the molecular-replacement method; however, *T. kirilowii* lectin coordinates gave better values of reliability and correlation parameters. The thermal, chemical and pH stability of this lectin have also been studied. When heated, its haemagglutination activity remained unaffected up to 363 K. Other stability studies show that 4 *M* guanidinium hydrochloride (Gdn–HCl) initiates unfolding and that the protein is completely unfolded at 6 *M* Gdn–HCl. Treatment with urea resulted in a total loss of activity at higher concentrations of denaturant with no major structural changes. The protein remained stable over a wide pH range, from pH 6 to pH 12, except for partial unfolding at extremely alkaline pH. The role of disulfide bonds in the protein stability was found to be insignificant. Rayleigh light-scattering studies showed no molecular aggregation in any of the extreme treated conditions. The unusual stability of this lectin resembles that of type II ribosome-inactivating proteins (type II RIPs), which is also supported by structure determination. The structural features observed in a preliminary electron-density map were compared with the other two available *Trichosanthes* lectin structures.

1. Introduction

Lectins are carbohydrate-binding proteins that occur ubiquitously in nature and exhibit important biological properties such as blood-group specificity, preferential agglutination of tumour cells and mitogenicity (Lis & Sharon, 1991). In addition, they serve as interesting models for studying the folding and unfolding pathways of oligomeric proteins. The interactions that stabilize the protein structures are electrostatic and hydrophobic interactions, hydrogen bonds and disulfide linkages. Conditions that disturb these stabilizing forces affect the native conformation of the protein, destroying its biological activity. Of the large number of lectins studied, the relationship between structure, conformation and stability has only been established for a few proteins (Ahmed *et al.*, 1998; Reddy *et al.*, 1999; Gaikwad *et al.*, 2002; Gaikwad & Khan, 2003; Sahasrabuddhe *et al.*, 2004).

From our studies, we have found that the two *Trichosanthes* lectins, one from *T. dioica* and the other from *T. anguina*, belonging to the Cucurbitaceae family show unusual stability towards denaturation conditions. *T. anguina* lectin has been independently studied in detail (Komath *et al.*, 1998; Komath & Swamy, 1999; Anuradha & Bhide, 1999; Sultan *et al.*, 2004; Sultan & Swamy, 2005). Both the above lectins are galactose-specific, preferentially binding the β-anomer of galactose, and are heterodimers of two nonidentical subunits joined by disulfide bonds. *T. anguina* lectin has a putative histidine residue (Komath *et al.*, 1998) whilst *T. dioica* lectin has a tyrosine at the sugar-binding site (Sultan *et al.*, 2004). Crystallization of the *T. anguina* lectin has previously been reported and it has been identified as a type II ribosome-binding protein (type II RIP; Manoj *et al.*, 2001). Four other type II RIPs, ricin (Montfort *et al.*, 1987), abrin-a (Tahirov



et al., 1995), mistletoe lectin (Krauspenhaar *et al.*, 1999) and *T. kirilowii* lectin (Li *et al.*, 2001), have been structurally characterized. Type II RIPs are cytotoxic heterodimeric proteins with a lectin-like *B* chain mostly specific towards galactose and an *A* chain showing glycosidase activity (Hartley & Lord, 2004).

In this paper, we describe the crystallization and preliminary characterization of *T. dioica* lectin, the evidence of its highly thermostable nature and also the effect of chemical denaturants and pH on its stability. The structure determination using molecular replacement and the gross structural features in comparison with the known structures of *T. anguina* and *T. kirilowii* lectins are highlighted.

2. Materials and methods

2.1. Materials

Guanidinium hydrochloride (Gdn–HCl) was obtained from Sigma (USA). The lectin from *T. dioica* was purified following a procedure described by Anuradha & Bhide (1999) for *T. anguina* lectin. Buffers used were glycine–HCl for the pH range 2–3, acetate for pH 4, citrate–phosphate for pH 5, phosphate for pH 7, Tris–HCl for pH 8–9 and glycine–NaOH for pH 10–12 (all at 100 m*M* concentration). The stock solutions of 8 *M* Gdn–HCl and 10 *M* urea were prepared in phosphate buffer pH 7 and filtered through a 0.45 µm filter. Crystal Screen solutions were obtained from Hampton Research (USA). The Tris, PEG 2K MME, *n*-octyl *β*-D-glucopyranoside and glycerol used for crystallization experiments were obtained from Sigma (USA). Other chemicals used were obtained locally and were of analytical reagent grade.

2.2. Fluorescence studies

The effect of temperature on stability was studied by incubating the protein samples (1.8 µ*M*) at temperatures in the range 303–368 K for 15 min each. Haemagglutination activity was checked simultaneously by removing aliquots of the samples. The protein samples (1.8 µ*M*) were equilibrated for 4 h at the required denaturant (Gdn–HCl or urea) concentration at 303 K. The intrinsic tryptophan fluorescence emission of the protein was monitored in 1 cm quartz cell in the 300–400 nm range, when excited at 280 nm, in a Perkin–Elmer LS 50B spectrofluorimeter with attached circulating water bath. Excitation and emission band passes of 5 nm each were used. The activity of the sample was measured at the same time. These studies were also carried out with the protein samples in the presence of the reducing agents DTT and *β*-mercaptoethanol (5 m*M*).

2.3. Determination of the lectin activity

Twofold serial dilution of the lectin in saline medium was carried out in a microtitre plate. A 3%(*v*/*v*) erythrocyte suspension was prepared by repeated washing of red blood cells (RBCs) with saline until all proteins were removed and diluting the pellet with saline. 100 µl of this suspension was added to the serially diluted lectin in saline and incubated for 30–60 min at room temperature. Haemagglutination was recorded after 1 h.

2.4. Light-scattering studies

Rayleigh light-scattering experiments were carried out using the spectrofluorimeter to monitor protein aggregation during Gdn–HCl-induced and thermal denaturation. Both excitation and emission wavelengths were set at 400 nm and the time-dependent change in scattering intensity was monitored.

2.5. Renaturation studies

200 µl aliquots were drawn from the samples treated with different concentrations of Gdn–HCl as described above and diluted ten times with 100 m*M* buffer pH 7.0. The fluorescence spectra of the diluted samples were recorded after 24 h and the activity was assayed. A protein sample in the absence of Gdn–HCl, but treated under identical conditions, was used as a control. The protein samples treated at high temperatures were cooled back to 303 K and scans were repeated.

2.6. Crystallization

Initial crystallization trials were carried out using Crystal Screens (Hampton Research, USA). This resulted in forked crystals which grew in condition No. 22 of Crystal Screen I, the composition of which was 0.2 *M* sodium acetate trihydrate, 0.1 *M* Tris–HCl pH 8.5, 30%(*w*/*v*) PEG 4000. The crystal quality was improved by varying the choice and concentrations of salt and PEG. Good crystals were obtained by employing the hanging-drop vapour-diffusion method, in which 1 µl protein solution mixed with 1 µl well solution comprising of 0.2 *M* ammonium acetate, 0.1 *M* Tris–HCl pH 8.5, 25%(*w*/*v*) PEG 2K MME was equilibrated against 1 ml well solution. The well solution also contained 50 µl 0.5%(*w*/*v*) *n*-octyl *β*-D-glucopyranoside as an additive. All crystallization experiments were carried out at 295 K.

2.7. Diffraction data collection, processing and structure determination

X-ray diffraction data were collected on an R-AXIS IV^{++} image plate using Cu *Kα* radiation generated by a Rigaku rotating-anode X-ray generator (RUH-3R) operated at 50 kV and 100 mA. X-rays were focused using a confocal mirror system (Osmic, USA). The crystal was kept frozen at 113 K in a liquid-nitrogen cryostream produced by an X-Stream system (Rigaku–MSI, USA) during data collection, using 30%(*v*/*v*) glycerol in the crystallization solution as cryoprotectant. The crystal-to-detector distance was kept at 200 mm and an oscillation of 0.5° per frame was used. The programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) were used for processing and scaling the data. The structure was determined using the molecular-replacement method implemented in the *AMoRe* (Navaza, 1994) program from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Coordinates of related type II RIPs were used for modelling.

3. Results and discussion

3.1. Effect of temperature

The fluorescence emission spectra of the native lectin showed an emission maximum at 336 nm characteristic of Trp residues in a nonpolar environment. The lectin is active up to 363 K and the fluorescence intensity gradually decreases with increase in temperature. At 363 K the intensity is almost 25% of the original value. On cooling to 303 K the original fluorescence intensity is restored. Although Trp residues are buried, as indicated by the λ_{max} at 336 nm, the microenvironments of these residues seem to be polar. No light scattering was observed under the above conditions, indicating that no aggregation of the protein molecules took place as a consequence of heating. The hydrophobic interactions in the interior of the protein molecules seem to be sufficiently strong to stabilize their respective native structures.

3.2. Effect of Gdn–HCl

On treatment with increasing concentrations of Gdn–HCl, the lectin slowly started losing activity at a 3 *M* concentration of the denaturant. There was no shift in the wavelength corresponding to the emission maximum. At a 4 *M* concentration of Gdn–HCl λ_{max} shifted to 340 nm, indicating a slight increase in the polarity of the Trp environment. A red shift in the λ_{max} to 355 nm observed at 6 *M* must be a consequence of unfolding. This is accompanied by a major activity loss. At concentrations above 3 *M* Gdn–HCl, even the intensity of fluorescence has reduced substantially, indicating a change in the microenvironment of the Trp residues. Renaturation or refolding of the protein was measured by the extent of reappearance of the original spectrum and estimation of the degree of recovery of sugar-binding activity. On diluting the lectin treated with 4 and 5 *M* Gdn–HCl, the activity was partially regained (with a 10–15% increase in the activity) and correspondingly a blue shift of λ_{max} to 340–342 nm was observed after 24 h. This kind of partial renaturation and reactivation was observed also in the case of *Artocarpus hirsuta* lectin when 3–5 *M* Gdn–HCl-treated protein was diluted (Gaikwad *et al.*, 2002).

3.3. Effect of urea

Just as in the case of Gdn–HCl-mediated denaturation, the lectin initially remained stable up to a 3 *M* concentration of urea and then slowly started losing activity when the urea concentration was increased to 4 *M*. A major loss of activity occurred at a urea concentration of 7 *M*, but this was not accompanied by any major structural changes in the protein, as no significant shift in λ_{max} could be detected in the fluorescence. Although no major structural changes in lectin treated with urea could be observed, a comparable loss of activity as in the case of Gdn–HCl-treated lectin was observed. The reason could be that the high concentration of urea is either preventing the access of the lectin-binding site to the RBC surface or causing changes in the geometry of the binding site of the lectin itself.

3.4. Effect of pH

T. dioica lectin remains stable in the pH range 6–12 and is only partially stable at pH 4, while 60% activity is lost at pH 2. The fluorescence intensity is much lower at highly acidic or highly alkaline pHs compared with the neutral pH range. Thus, except for some minor changes in the microenvironment of the Trp residue, no structural transition occurrs over a wide pH range. The decrease in fluorescence intensity may arise from protonated and deprotonated forms of the amino acids on the surface that affect the emission of the Trp residues.

Figure 1
Hexagonal crystals of *T. dioica* lectin, maximum dimension 0.5 mm.

Table 1
Diffraction data statistics for *T. dioica* lectin.

Values in parentheses are for the last shell.

Temperature (K)	295
X-ray source	Rotating anode
Wavelength (Å)	1.5418 (Cu $K\alpha$)
Resolution limits (Å)	40.0–2.80 (2.9–2.8)
No. of measured reflections	148229
No. of unique reflections	30702
Completeness of data (%)	99.8 (99.5)
R_{merge}† (%)	10.9 (38.4)
Average $I/\sigma(I)$	9.9 (3.7)
Mosaicity	0.6
Space group	$P6_4$
Unit-cell parameters (Å)	$a = b = 167.54$, $c = 77.42$
Unit-cell volume (Å^3)	1882020
Matthews coefficient (Å^3 Da^{-1})	2.85
No. of molecules per unit cell (Z)	12
Solvent content (%)	56.8
R factor/R_{free} (after first cycle)	0.38/0.46
Correlation factors	0.69/0.54
Reflections in working set/test set	29807/1544

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

To assess the role of disulfide linkages in the unusual stability of the lectin structure, thermal and Gdn–HCl denaturation studies were carried out in the presence of 5 m*M* DTT as well as 5 m*M* β-mercaptoethanol. In both cases, proteins were found to be stable up to 358 K, ruling out involvement of disulfide linkages in stabilizing the structures of *Trichosanthes* lectins. Previous reports and our own structural investigations indicate that both the lectins contain two non-identical subunits linked by a disulfide bond (Anuradha & Bhide, 1999; Sultan *et al.*, 2004).

3.5. Crystallization and crystal characterization

Needle-shaped crystals of *T. dioica* lectin (Fig. 1) were grown against 1 ml well solution consisting of 0.2 *M* ammonium acetate, 0.1 *M* Tris–HCl pH 8.5, 25%(*w*/*v*) PEG 2K MME along with 50 µl 0.5%(*w*/*v*) *n*-octyl β-D-glucopyranoside as an additive. 30%(*v*/*v*) glycerol introduced into the crystallization solution acted as cryo-protectant. Crystals diffracted to 2.8 Å Bragg spacing. Data-collection parameters and intensity statistics are listed in Table 1.

3.6. Structure determination and comparison

Initially, we could not accurately determine the space group of *T. dioica* crystals because of difficulty in fixing the screw symmetry from systematic absences of reflections. However, on running *AMoRe* for the translation function separately for all possible sixfold rotation axes, inputting the correct rotation-function solutions, the exact screw translation could be identified through higher correlation and lower *R* factors of the solution (Table 2).

On carrying out the molecular-replacement procedure for structure determination, inputting the coordinates of various available type II RIPs, the coordinates of *T. kirilowii* lectin (PDB code 1ggp) gave the best results (Table 3), followed by those of abrin-a (PDB code 1abr). The asymmetric unit contains two heterodimers of the *A* and *B* chains of the input model. The Matthews coefficient (Matthews, 1968) calculated (Table 1) assuming two heterodimers in the asymmetric unit was also within the expected range. Removing the first residues 1–8 of the *B* chain and residues 41–49 of the *A* chain in abrin-a gave slightly improved correlation parameters compared with inputting the whole abrin-a molecule. In fact, in the solution with

Table 2
Calculation of translation function to obtain the correct space group.

The solutions with the correct space group input have a comparatively higher correlation factor and lower *R* factor and are highlighted in bold.

Input space group	Rotation-function solution	Correlation factor	*R* factor (%)
*P*6	1	30.0	54.7
	2	29.8	54.7
$P6_1$	1	32.9	53.5
	2	31.2	54.1
$P6_2$	1	30.0	54.5
	2	29.6	54.7
$P6_3$	1	30.3	54.4
	2	29.5	54.8
***P*6$_4$**	**1**	**37.2**	**52.1**
	2	**34.5**	**53.2**
$P6_5$	1	30.2	54.5
	2	29.5	54.6

Table 3
The molecular-replacement solutions obtained using the *T. kirilowii* lectin structure as model.

The parameters shown are after rigid-body refinement in *AMoRe* using reflections in the resolution range 20–2.8 Å. (CC, correlation coefficient; *R*, *R* factor.)

Solution	α (°)	β (°)	γ (°)	T_x	T_y	T_z	CC	*R*
1	45.30	87.44	146.85	0.7491	0.5007	−0.0002	48.6	48.5
2	22.03	135.53	317.97	0.7153	0.7702	−0.0007		

the whole abrin-a molecule the deleted residues made short contacts with the second molecule of the asymmetric unit. The report of the structure determination of *T. anguina* lectin also mentions the removal of similar residues from the input model (Manoj *et al.*, 2001). The parameters for the second solution were far below those of the first correct solution when ricin coordinates (PDB code 2aai) were used as an input model in the rotation function. Inputting corresponding polyalanine models into *AMoRe* only worsened the solution parameters. Graphic visualization of the crystal packing of correct solutions selected showed no serious short contacts between neighbouring molecules.

In the absence of sequence information, no extensive refinement could be attempted on the initial model. However, an estimated *R* factor of 0.38 and $R_{\rm free}$ of 0.46 were obtained on applying one cycle of refinement (subsequent to rigid-body refinement) prior to electron-density calculation using *REFMAC* (Murshudov *et al.*, 1997), tightly holding the non-crystallographic restraint between the two molecules in the asymmetric unit. The corresponding correlation factors after one cycle of refinement were 0.69 and 0.54 for reflections in the resolution range 25–2.8 Å. By overlapping the initial model on the electron-density map, it could be confirmed that the gross features of the type II RIP structural elements were also conserved in the structure of the *T. dioica* lectin. Out of the five residues, Tyr74, Tyr113, Glu164, Arg167 and Trp198, of the *A* chain of abrin-a identified as important for the ribosome-inactivating mechanism, only the latter four are conserved in the *T. dioica* lectin. There is no sufficient electron density observed in the preliminary electron-density map to accommodate the aromatic side chain of tyrosine for residue 74. This feature makes this lectin closer to both the *T. anguina* and *T. kirilowii* lectins, which also reportedly only have electron density for an Ala residue at this position. All the disulfide bonds found in *T. kirilowii* lectin, including the inter-chain disulfide bond between the *A* and *B* chains, seem to be preserved in this lectin.

One major difference between the present crystal structure and those of the *T. anguina* and *T. kirilowii* lectins is the association of the two heterodimers in the asymmetric unit and the crystal packing. The association of the two molecules of the *T. dioica* lectin in the asymmetric unit takes place exclusively through interactions involving the *A* chains (Fig. 2). This type of dimer formation is not observed in the crystals of other two *Trichosanthes* lectins.

Unfortunately, in the absence of confirmed amino-acid sequence for any of the three *Trichosanthes* lectins, any detailed attempts to correlate stability with structural features at this time may be premature and inconclusive. However, the study reported is highly suggestive of a close relationship between the structural and biochemical characteristics and the type II RIP features common to the three *Trichosanthes* lectins of *T. dioica*, *T. anguina* and *T. kirilowii*.

4. Conclusions

Type II ribosome-inactivating proteins (type II RIPs) are toxic N-glycosidases that depurinate the universally conserved sarcin loop of larger rRNAs. RIPs are widely distributed among different plant genera and are present in a variety of different tissues. Some of them are highly thermostable (Lam & Ng, 2001). It has been pointed out that *Trichosanthes* lectins have a similarity to type II RIPs (Sultan *et al.*, 2004); here, we have shown that like other type II RIPs, the *T. dioica* lectin is also resistant to a wide range of denaturing conditions. We have further substantiated this observation by determining its structure and proving that structurally also it belongs to the class of type II RIPs. However, our data shows that stability is not directly influenced by the presence of structural elements such as disulfide bridges alone. Furthermore, even in the absence of any confirmed amino-acid sequences, we have demonstrated through correlation of gross three-dimensional structural features that the

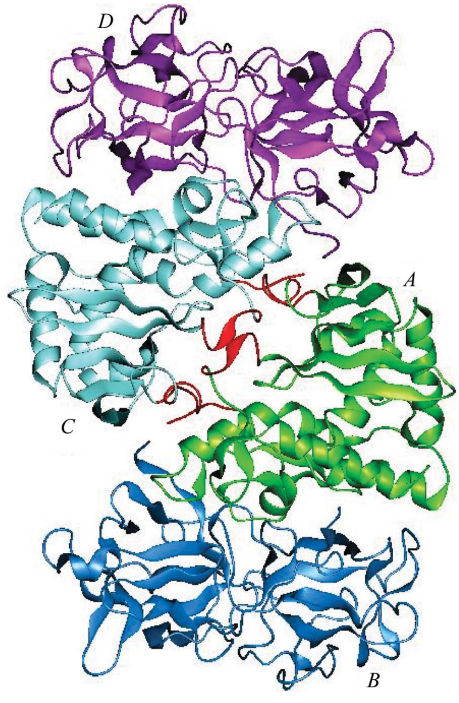


Figure 2
The dimer–dimer association of the two heterodimer molecules of *T. dioica* lectin in its crystal structure. The segments are coloured differently and the subunits are labelled *A*, *B*, *C* and *D*. The two *A* chains (subunits *A* and *C*) come closer in the asymmetric dimer. The parts of the molecules in the interface are shown in red. Strands from the *A* and *C* subunits (residues 45–47 with respect to *T. kirilowii* lectin) form a short β-sheet in the middle of the interface.

three *Trichosanthes* lectins from *T. dioica*, *T. anguina* and *T. kirilowii* are evolutionarily closely related.

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