
PRELIMINARY CRYSTALLOGRAPHIC REPORTS

Crystallization of a scRIP—Gelonin Isolated From Plant Seeds *Gelonium multiflorum*

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ABSTRACT Single crystals of the protein gelonin isolated from the seeds of *Gelonium multiflorum* have been grown at room temperature by vapor diffusion method. The crystals are monoclinic with $a = 49.4 \text{ \AA}$, $b = 44.9 \text{ \AA}$, $c = 137.4 \text{ \AA}$, and $\beta = 98.3^\circ$. The space group is $P2_1$, with two molecules in the asymmetric unit which are related by a noncrystallographic 2-fold axis along $\psi = 13^\circ$ and $\phi = 88^\circ$. The crystals diffract X-rays to high resolution, making it possible to obtain an accurate structure of this single chain ribosome inactivating protein.

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Key words: protein, ribosome, inactivation, toxin

Two types of ribosome-inactivating proteins (RIPs) have been isolated in large quantities from different parts of plants.^{1,2} The type 1 RIPs (scRIPs), e.g., trichosanthin, gelonin, *Mirabilis* antiviral protein, etc., are single chain polypeptides of molecular weight around 26 kDa, and exhibit an alkaline pI in the range of pH 8 to pH 10.³ The type 2 RIPs, e.g., ricin, modeccin, abrin, etc., consist of two polypeptide chains, A and B, linked together by disulfide bridges. Proteins of both types inhibit protein synthesis in eukaryotes by catalytically cleaving the N-glycosidic bond of a specific adenine residue in rRNA, thereby preventing binding of the elongation factor (EF2) to the 60 S subunit of eukaryotic ribosomes.⁴ The degree of inhibition is species and cell type specific. The catalytic activity of type 2 RIPs is associated with only their A-chain, and the B-chain simply has lectin-like properties. Primary structures of a number of RIPs of both types have been determined, and the sequence homology between type 1 and type 2 RIPs is in the range of 15–30%.¹ The scRIPs are all found to be much more resistant to a variety of physicochemical treatments such as freeze-drying, protease treatment, change of pH, etc., as compared to the A-chains of type 2 RIPs.³ Further, scRIPs are nontoxic to intact cells since they lack cell surface receptor binding ability and

hence cannot gain entry into cells on their own.⁵ Unlike type 2 RIPs, a large number of non-cross-reacting scRIPs have been purified which can be used in succession to escape immune response from host cells to prolonged administration of RIPs. Therefore, scRIPs can be more useful in cancer therapy as “immunotoxins” that can be directed against specific target cells through conjugation with suitable cell recognition molecules such as antibodies. However, detailed three-dimensional structure is needed to understand and fully exploit these properties of scRIPs. Interest in the structure of scRIPs has been further enhanced by recent findings that these proteins have selective toxicity against cells infected with human immunodeficiency virus.⁶ A 3 Å electron density map of α -tricosanthin and crystallization report on *Mirabilis* antiviral protein have been published.^{7,8} We report here production and preliminary X-ray analysis of gelonin crystals that diffract X-rays to 1.8 Å resolution.

Gelonin was isolated from *Gelonium multiflorum* seeds, purchased from United Chemicals and Allied products, Calcutta, India. The extracted protein was purified using ion-exchange CM-cellulose chromatography, as described by Stirpe et al.⁵ Typically 100 mg of pure Gelonin was obtained from 100 g seeds. Its purity and activity were determined, respectively, by SDS-PAGE and the extent to which it inhibited, in vitro, the incorporation of [³⁵S]methionine in protein synthesis using rabbit reticulocyte lysate. The LD₅₀ was found to be 13.68 ng/ml, which is in agreement with that reported by Stirpe et al.⁵

Abbreviations used: RIP, ribosome inactivating protein; scRIP, single chain ribosome inactivating protein; PEG, polyethylene glycol.

Received March 3, 1994; accepted April 19, 1994.

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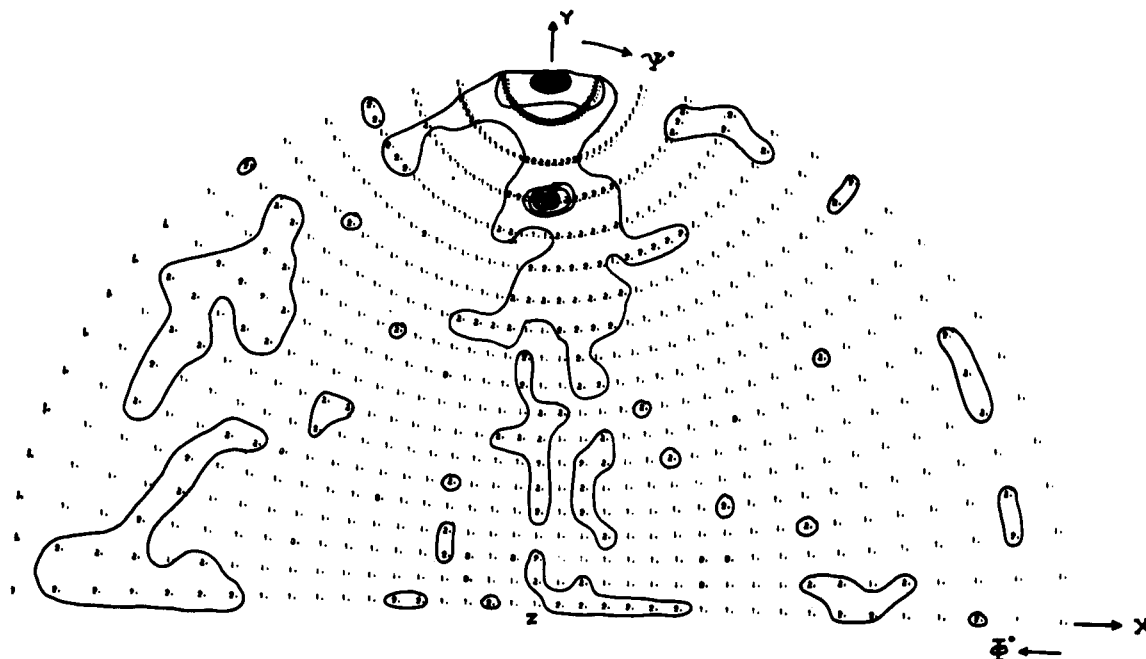


Fig. 1. Stereographic projection of the rotation function map calculated for $\kappa = 180^\circ$, using PROTEIN program package. Diffraction data with d -spacings between 8 and 3.7 Å were included in the calculation. The map has been contoured at intervals of 2σ starting from the average value. Polar angles ψ and ϕ have been varied in steps of 5° .

From chemical modification studies of purified protein, few arginines have been found to be critical for the function of gelonin.^{8,9} The purified protein was extensively dialysed against 10 mM sodium phosphate buffer, pH 7.0, and was then stored frozen at -70°C . Aliquots of this stock solution were thawed and concentrated to 10 mg/ml for crystallization experiments. Protein concentration was estimated photometrically using an absorption coefficient of $E_{280}^{1\%} = 6.7$. Initial conditions for crystallization were determined by the sparse matrix method.¹⁰ These conditions were then refined by systematically varying the pH, ionic strength, and precipitant concentration.

Single crystals suitable for x-ray diffraction studies were obtained by vapor diffusion with PEG 3350 as the precipitant in hanging drops.¹¹ On a siliconized cover slip 5 μl of protein at a concentration of 10 mg/ml in 10 mM sodium phosphate buffer of pH 7.0 was mixed with 5 μl of (23% PEG 3350) reservoir solution B, which contained 0.1 M lithium sulfate, 0.1 M ammonium sulfate, and 0.1 M Tris of pH 8.5. The coverslip was then inverted and sealed on a reservoir of 1.0 ml of solution B. Crystals appeared within a week and grew to a size of $0.2 \times 0.1 \times 0.2 \text{ mm}^3$. Bigger crystals could be grown by the "sitting drop" method. The crystal data and space group symmetry were obtained automatically using Siemen's area detector and the XDS software package.¹² These unit cell dimensions were input while collecting high-resolution data on a RAXIS II

image plate system. The crystals belong to the monoclinic space group $P2_1$ with the following unit cell dimensions: $a = 49.4$, $b = 44.9$, $c = 137.4$ Å, and $\beta = 98.3^\circ$. The crystals are not isomorphous to crystals of other scRIPs reported earlier,^{7,8} and diffract X-rays to 1.8 Å resolution. For a 30-kDa protein, the V_m values are 4.98 or 2.49 Å³/D, depending on whether one or two molecules are present in the asymmetric unit. The latter value is consistent with the range of observed V_m values for protein crystals,¹³ thus suggesting that there are two protein molecules in the asymmetric unit. A total of 105,785 reflections in 180 frames have been measured to 1.8 Å resolution using a RAXIS image plate system mounted on a Rigaku RU300 X-ray generator. The measurements were scaled and merged and the number of unique reflections for which $I/\sigma(I)$ was >1.0 was 38,227. The R_{merge} value was 7.6%.

Self-rotation function¹⁴ maps were calculated in direct space using the software package PROTEIN.¹⁵ Diffraction data with d -spacings between 8 and 3.7 Å were included in Patterson map computation. Patterson vectors lying between spherical shells of different combination of inner and outer radii were chosen for various rotation function calculations. Figure 1 shows a stereographic projection of the self-rotation function map calculated for $\kappa = 180^\circ$. Patterson vectors of length between 15 and 30 Å were used in this calculation. In addition to the crystallographic peak at $\psi = 0^\circ$ (the peak height being 8.7 arbitrary units), there is only one other

strong peak (of height 4.3 units) and $\psi = 13^\circ$ and $\phi = 88^\circ$. The average value of the map is 1.7 units and the standard deviation is 0.1. This peak was present for all choices of Patterson vector lengths. Further, there was no peak of such height, when rotation function maps were calculated for κ values of 60° , 90° , 72° , and 120° . It is, therefore, clear that the two gelonin molecules in the asymmetric unit are related by a noncrystallographic 2-fold axis of symmetry. Further analysis of data to solve the structure at *high resolution* is currently under progress. High resolution structure of gelonin is likely to help in the selection and preparation of conjugates toxic to specific tumor cells or cells infected with HIV.

ACKNOWLEDGMENTS

We are deeply indebted to Dr. Anders Swenssan, Lund University, Lund, Sweden, and Dr. Abelardo Silva, National Cancer Institute, Frederick, Maryland, USA, for their help during the data collection and processing. A short-term fellowship to one of us (K.K.K.) by the Swedish Natural Sciences research foundation is gratefully acknowledged. M.V.H. is grateful to Dr. J.W. Erickson at National Cancer Institute, USA, for the award of a Visiting Scientist position.

REFERENCES

1. Stirpe, F., Barbieri, L., Battelli, M. G., Sonia, M., Lappi, D. A. Ribosome-inactivating proteins from plants: Present status and future prospects. *Biotechnology* 10:405–412, 1992.
2. Eiklid, K., Olsnes, S., Phil, A. Entry of lethal doses of abrin, ricin and modeccin into the crystal of Hela cells *Exp. Cell Res.* 126:321–326, 1980.
3. Barbieri, L., Stirpe, F. Ribosome-inactivating proteins from plants: Properties and uses. *Cancer Surv.* 1:489–520, 1982.
4. Endo, Y., Tsurugi, K. The RNA N-glycosidase activity of ricin-A chain. *J. Biol. Chem.* 263:8735–8739, 1988.
5. Stripe, F., Olsnes, S., Pihl, A. Gelonin, a new inhibitor of protein synthesis, nontoxic to intact cells. *J. Biol. Chem.* 255:6947–6953, 1980.
6. Huang, S.L., Kung, H.F., Huang, P.L., Li, B.Q., Huang, P., Huang, H.L., Chen, H.C. a new class of anti-HIV agents: GAP31, DAPs 30 and 32. *FEBS. Lett.* 291:139–144, 1991.
7. Pan, K., Lin, Y., Fu, Z., Zhou, K., Cai, Z., Chen, Z., Zang, Y., Dong, Y., Wu, S., Ma, X., Wang, Y., Chen, S., Wang, J., Zhang, X., Ni, C., Zhang, Z., Xia, Z., Fan, Z., Tian, G. *Sci. Sin. Ser. B (Chem. Biol. Agric. Med. Earth Sci.)* 30:386–395, 1987.
8. Miyano, M., Appelt, K., Arita, M., Habuka, N., Kataoka, J., Ago, H., Tsuge, H., Noma, M., Ashford, V., Xuong, N-h. Crystallisation and preliminary X-ray crystallographic analysis of Mirabilis antiviral protein. *J. Mol. Biol.* 226: 281–283, 1992.
9. Srinivasan, Y., Ramprasad, M.P., Suroliya, A. Chemical modification studies of gelonin: Involvement of arginine residues in biological activity. *FEBS Lett* 192:113–118, 1985.
10. Jancarik, J., Kim, S.H. Sparse matrix sampling: A screening method for crystallization of proteins. *J. Appl. Cryst.* 24:409–411, 1991.
11. McPherson, A. "Preparation and Analysis of Protein Crystals." New York: John Wiley, 1982.
12. Kabsch, W. Evaluation of single-crystal X-ray diffraction data from a position-sensitive detector. *J. Appl. Cryst.* 21: 916, 1988.
13. Matthews, B.W. Solvent content of protein crystals. *J. Mol. Biol.* 33:491–497, 1968.
14. Rossmann, M.G., ed. "Molecular Replacement Method." New York: Gordon & Breach, 1972.
15. Steigemann, W. Ph.D. thesis, Tectinische Universitat-Munchen, 1974.