STUDIES ON CRYSTALLINE ABRIN: X-RAY DIFFRACTION DATA, MOLECULAR WEIGHT, CARBOHYDRATE CONTENT AND SUBUNIT STRUCTURE

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1. Introduction

Abrin is a protein present in the seeds of the legume *Abrus precatorius* having an extremely high toxicity toward animals, its LD_{50} being 0.02 mg/kg body weight in rats [1]. Like ricin from castor beans and concanavalin A from Jack beans, it possesses the ability to agglutinate blood cells [2] and is, therefore, classified as a lectin [3]. In addition to the agglutinating property, abrin is a potent inhibitor of protein synthesis both *in vitro* [4] and *in vivo* [5]. Olsnes and Pihl [6] have shown that abrin may prevent peptide chain elongation and suggest that abrin may function in a catalytic manner. It appears similar to diphtheria toxin in this respect although unlike the bacteria toxin, it does not stimulate incorporation of NAD into the elongation factor [4]. Abrin has also been shown to inhibit DNA synthesis after an appreciable time lag [5] though the mechanism of this function is not known.

Abrin administered at sub-lethal doses has been shown to be an effective inhibitor of several types of tumors in rats and mice [1, 7], producing complete remission under some conditions [1]. Examination of tumor cells treated with abrin shows drastic cytoplasmic abnormalities and observable chromosomal aberrations [7].

Abrin binds galactose with considerable specificity which probably forms the basis of its agglutinating function. One can take advantage of this property in the purification procedure by employing affinity chromatography on Sepharose, a galactose containing polymer [8]. Sephadex gel filtration indicates a total mol. wt. of 260 000. SDS-polyacrylamide gel electrophoresis demonstrates that abrin is a tetramer composed of four identical or very similar subunits each of which is comprised of two polypeptide chains of molecular weights, 35 000 and 30 000, which may be joined by disulfide bridges. Each polypeptide has carbohydrate linked to it which does not dissociate upon denaturation.

X-ray diffraction analysis of single crystals of abrin reveals space group symmetry $P2_12_12_1$ with a = 138Å, b = 142Å, c = 178Å and one entire tetramer as the asymmetric unit. The diffraction patterns which extend to high resolution exhibit a degree of pseudo symmetry indicating the presence of at least one exact or near exact 2-fold axis relating pairs of subunits.

2. Materials and methods

2.1. *Materials*

Abrus precatorius seeds were collected on the farms of Robert and Alexander McPherson in Stuart, Florida. Human erythrocytes were obtained from the M.I.T. clinic. Sepharose and Sephadex was purchased from Pharmacia, polyacrylamide from Bio-Rad, galactose from Sigma and Dulbecco's phosphate buffered saline (DPBS) from Grand Island Biologicals.

2.2. *Agglutination tests*

Agglutination tests were conducted on human erythrocytes, washed five times and suspended in DPBS at a concentration of about 10^5 cells/ml as determined with a hemacytometer. Results were evaluated visually without magnification on a 0.1 ml sample.

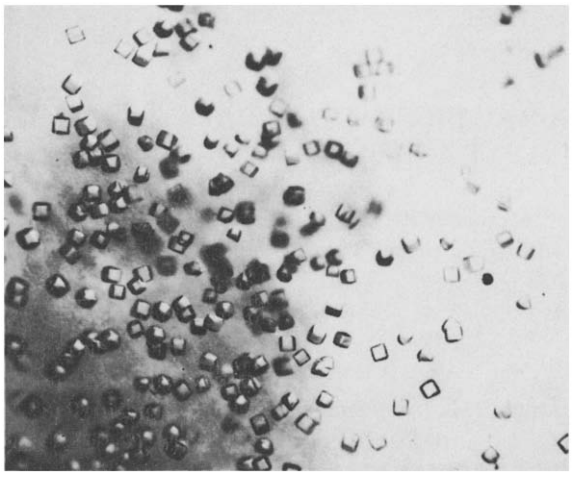

Fig. 1. Photomicrograph of crystals of abrin grown from ammonium sulfate in the presence of glutathione.

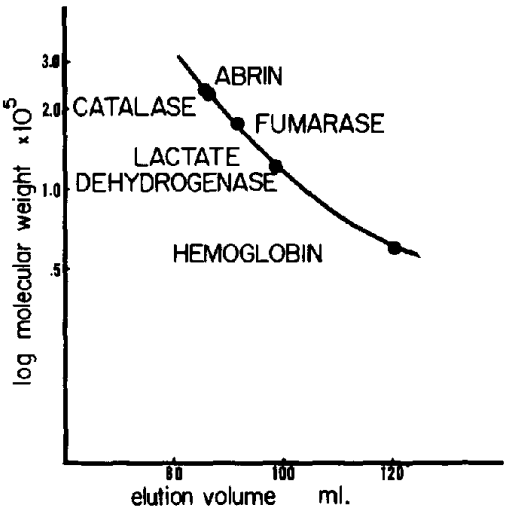

Fig. 2. Plot of the elution volumes from a Sephadex G-200 gel filtration column for abrin and four proteins of known molecular weight versus their log molecular weight. Flow rates were approximately 10 ml/hr. Dextran blue appeared with an elution volume of 64 ml.

2.3. *Purification*

Purification of abrin was by a modification of the procedure described by Tomita et al. [8]. Seeds were ground and extracted with DPBS over a period of two days and cleared of residue by centrifugation. The fraction soluble between 30% and 65% saturation with ammonium sulfate was dialyzed 24 hr versus DPBS and applied to a Sepharose 4B column equilibrated with the same. The column was washed with DPBS until no more protein was observed and then eluted with 0.2 M galactose. The fractions containing agglutinating activity were combined, dialyzed against distilled water for 24 hr to remove sugar and lyophilized. From 50 g of seeds, approximately 60 mg of purified abrin was obtained.

2.4. *Electrophoresis*

Electrophoresis of the purified abrin was conducted according to the description of Laemmli [9] on SDS-polyacrylamide gels (10% acrylamide, 1% SDS) in the presence of mercaptoethanol and using a Tris–glycine discontinuous buffer system. Staining for protein was with Coomassie blue and molecular weights of polypeptide chains were determined by the method of Weber and Osborn [10]. To test for the presence of carbohydrate in the abrin molecule, electrophoresis was performed as described and the gels stained by the PAS procedure [11]. The gels were scanned on a modified Gilford spectrophotometer at 560 nm, stained with Coomassie blue, and rescanned for protein at 550 nm. The two scans were then superimposed to determine which polypeptide chains were glycopeptides.

2.5. *Crystallization*

Crystallization of the purified abrin was by the technique of vapor equilibration [12] of 30 μl droplets of the protein solution against 25 ml reservoirs of 42% saturated ammonium sulfate. The crystallization droplet was 3 mg/ml in abrin, 25 mM in glutathione and 30% saturated with ammonium sulfate. Crystallization occurred within 3–10 days at 4°C. The crystals varied in size from microcrystals up to nearly 1 mm on a side. Some typical examples are shown in fig. 1.

2.6. *X-ray examination*

X-ray examination was conducted on crystals mounted by the conventional means in quartz capillaries. Diffraction photographs were recorded on a Buerger precession camera using nickel filtered CuKα radiation generated by an Elliot rotating anode source run at 40 kV and 40 mA. All X-ray work was carried out at 14°C.

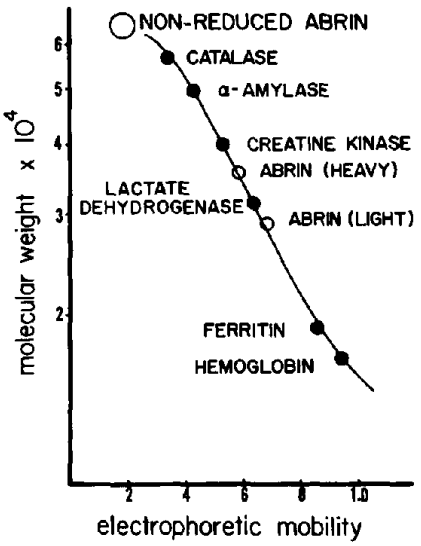

Fig. 3. Plot of electrophoretic mobility versus log molecular weight for abrin and several standard proteins of known molecular weight. In the presence of mercaptoethanol the indicated light and heavy chains are obtained while in its absence a broad diffuse band is obtained extending from 60–70 000 mol. wt. suggesting mixed aggregates of the two constituent chains.

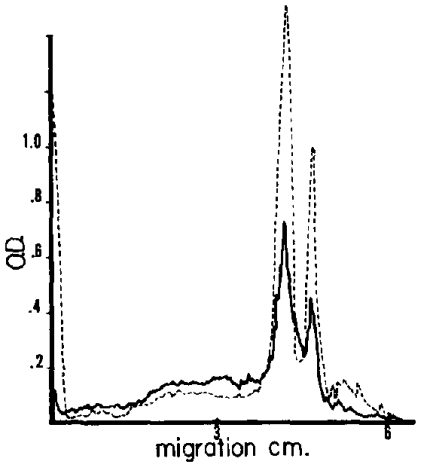

Fig. 4. Superimposed scans of an SDS-polyacrylamide gel containing reduced abrin measured first at 560 nm for carbohydrate (——) after staining by the PAS procedure [11] and then at 550 nm for protein (-----) after restaining with Coomassie blue. The two protein peaks have electrophoretic mobility corresponding to 35 000 and 30 000, and both are glycopeptides.

2.7. *Gel filtration*

Gel filtration of abrin was carried out on a Sephadex G-200 column of dimensions 2.6 cm X 45 cm equilibrated with 0.05 M Tris–HCl buffer at pH 7.8. The molecular weight of abrin was estimated by comparing its elution volume with that of several standard proteins of known molecular weight.

3. Results

3.1. *Molecular weight*

Fig. 2 shows a plot of the elution volumes of abrin compared with those of hemoglobin, lactate dehydrogenase, fumarase, and catalase versus their log molecular weights. Based on this data the molecular weight of abrin is judged to be approximately 260 000.

3.2. *Subunit structure*

Fig. 3 shows a plot of log molecular weights of several standard proteins on SDS-polyacrylamide gels along with the position of abrin both in the presence and absence of mercaptoethanol. With no reducing agent present, abrin yields a diffuse band in the molecular weight range of 60–70 000. With mercaptoethanol present, two distinct bands are observed corresponding to molecular weights of 30 000 and 35 000. These results agree well with those reported by Olsnes and Pihl [6]. From the combined Sephadex gel filtration and SDS-polyacrylamide gel results it appears that abrin is a tetrameric protein of about 260 000 composed of four identical or at least extremely similar subunits of 65 000 mol. wt. and that each of these subunits is in turn composed of two distinct polypeptide chains of mol. wt 35 000 and 30 000.

3.3. *Carbohydrate determination*

Fig. 4 shows the results of the sequential PAS and Coomassie blue staining procedures applied to the polyacrylamide gels. When stained with PAS, two bands were observed indicating carbohydrate. When stained with Coomassie blue, two intense protein bands corresponding to 35 000 and 30 000 mol. wt. appeared and superimposed precisely on the carbohydrate positions. The extent of staining for both polysaccharide and protein was not equal between the

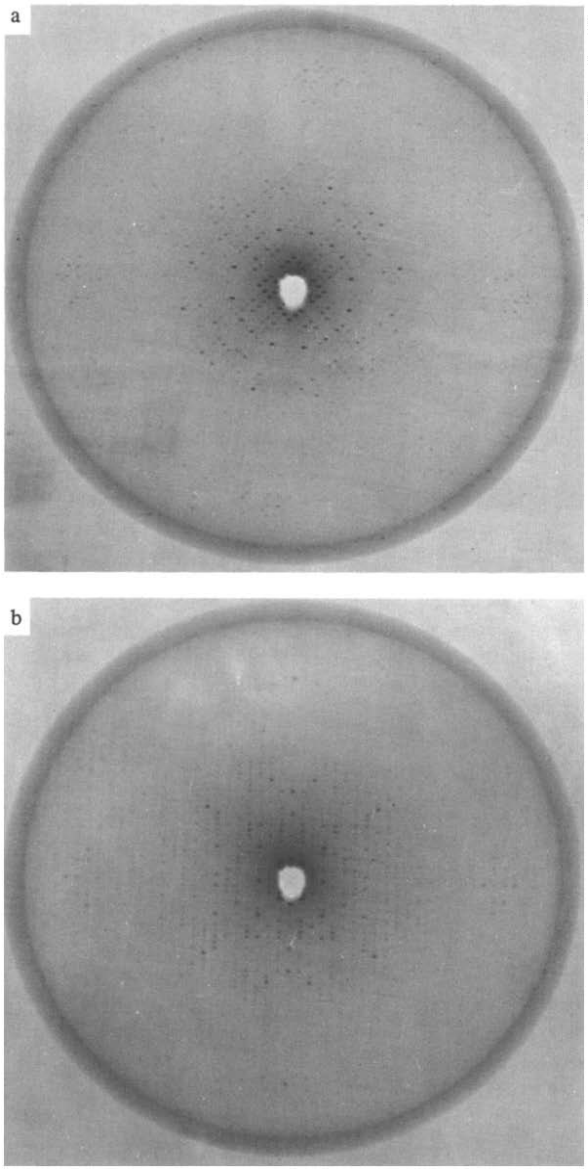

Fig. 5. 12° X-ray diffraction photographs of (a) the hkO and (b) the hhl reciprocal lattice sections of orthorhombic abrin crystals. Note the presence of pseudo 4-fold symmetry in the hkO zone and the apparent absence or weakness of $00l \neq 4n$ reflections in the hhl zone. Crystal to film distance was 10 cm and exposure times were about 30 hr.

two bands in either case, but was stronger for the heavier polypeptide in both cases. This was particularly true for the PAS procedure.

3.4. *X-ray data*

Precession X-ray photographs of the hkO and hhl reciprocal lattice sections are shown in fig. 5. Both exhibit mm symmetry with all odd ordered axial reflections not equal to $2n$ systematically absent. There are no additional systematic absences with respect to upper levels. Since proteins are composed only of L-amino acids, all inversion symmetry is eliminated and the space group must be $P2_12_12_1$. The cell dimensions are a = 138Å, b = 142Å and c = 178Å. The volume of the unit cell is $v = 3.44 \times 10^6 Å^3$.

The density, ρ, of the abrin crystals was measured on a bromobenzene–xylene gradient saturated with water and found to be 1.15 g/cm^3. The molecular weight, M', of the asymmetric unit, *protein plus solvent*, can be calculated from the formula

$$M' = \frac{(0.6023)V\rho}{n}$$

where: ρ is the density of the crystals (g/cm^3); V is the unit cell (A^3) and n is the number of asymmetric units per unit cell. For space group $P2_12_12_1$, $n = 4$ and $M' = 621\,600$. This is best satisfied by assigning a single tetrameric molecule plus its associated solvent as the asymmetric unit of the crystal. In this case, the amount of protein in the crystal is

$$\% \text{ protein} = \frac{260\,000}{621\,600} = 42\%.$$

The value for Vm, the ratio of asymmetric unit volume to protein weight is 3.31 which is high, but within the range of crystalline proteins compiled by Matthews [13].

The crystals diffract well to a resolution of at least 3.0 Å resolution and show no significant degradation until about 50 hr of X-ray exposure has elapsed at which time they begin decaying at a very rapid rate. The crystals seem quite suitable for a high resolution X-ray analysis.

It should be noted, that the hkO diffraction pattern shown in fig. 5 exhibits a strong tendency toward 4-fold symmetry at low resolution. In addition, the 001 line, visible in the hhl diffraction photo also shown in fig. 5, has only $001 = 4n$ reflections present for about the first twenty orders. It appears, therefore, that at low resolution, the symmetry is indicative of the tetragonal space group $P4_12_12$ which has eight

asymmetric units per unit cell. The full implications of the pseudo symmetry to the packing arrangement of the molecules in the unit cell will be developed elsewhere, but one consequence is that the tetrameric molecule must possess at least one exact or near exact 2-fold axis relating pairs of monomers within the molecule. This does not exclude the possibility that additional exact or near exact 2-fold axes may also be present in the molecule, but are not expressed in the crystallographic symmetry.

4. Conclusions

The results presented above indicate that abrin is a protein of mol. wt. 260 000 composed of four identical or very similar subunits of mol. wt. 65 000. Each subunit is comprised of two polypeptide chains of mol. wt. 35 000 and 30 000 each of which is associated with carbohydrate moieties which are covalently attached and not dissociated upon denaturation. X-ray results indicate that the molecule contains at least one exact or near exact 2-fold axis relating pairs of monomers in the molecule. It further shows that the abrin crystals provide a suitable subject for a high resolution structure analysis.

Acknowledgements

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