SAXS study of crotapotin at low pH

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ABSTRACT The structure of crotapotin, a protein extracted from the venom of the *Crotalus durissus terrificus,* in solution at pH = 1.5, was studied by SAXS. The experimental results yield structural parameter values of the molecular radius of gyration R_g = 13.6 Å, volume v = 16.2 × 10^3 Å^3 and maximal dimension D_{max} = 46 Å. The distance distribution function deduced from the scattering measurements is consistent with an overall molecular shape of an oblate ellipsoid of revolution with assymetry parameter ν = 0.45.

1. INTRODUCTION

Crotapotin is an acidic subunit of the crotoxin complex extracted from the venom of the *Crotalus durissus terrificus.* Such a protein is highly important to enhance the toxicity of crotalus phospholipase A. It is a protein with molecular weight of 9,000 D and isoelectric point of PK_i 3.4. It consists of three polypeptide chains interconnected by sulfide bridges. The chains A, B, and C, consist of 40, 34, and 14 aminoacid residues, with molecular weights of 4,300, 3,700 and 1,600 D, respectively (Breithaupt et al., 1974).

This paper aims at the study of the structure of crotapotin in acidic solutions, at pH = 1.5, by the small-angle x-ray scattering (SAXS) technique. A low value for the pH was used in order to compare the present results, concerning the crotapotin, with those corresponding to the crotoxin molecule which is not soluble at medium and high pH.

2. MATERIALS AND METHODS

2.1. Samples

Purified crotapotin samples were prepared as follows: ~400 mg of crude venom of the *crotalus durissus terrificus* were dissolved in 3.0 ml of ammonium formate 0.05 M, pH 3.5, and then filtered through a 4 × 45 cm Sephadex G-75 column, buffered at a flow rate of 0.8 ml/min (Laure, 1975). Fig. 1 shows the chromatographic profile of filtering of the crude venom. The five fractions A, B, C, D, and E are 5, 12, 49, 25, and 3% (wt/wt), respectively. Fraction C, which corresponds to ~50% of the whole venom, was identified as being the crotoxin. Three pools of crotoxin were refiltered under the same conditions. These pools correspond to the dashed line in Fig. 1. The horizontal bar indicates the mixed fraction of crotoxin corresponding to peak C.

Fig. 2 represents the chromatographic profile obtained by fractioning the crotoxin in crotapotin and phospholipase A in ionic exchange resins SP-Sephadex C-25 in the presence of guanidine chloride 1 M, pH 4.3. Fractions I, II, and III correspond to crotapotin, to a protein with phospholipatic activity and to phospholipase A, respectively. These three fractions were eluted using 1.0, 2.0, and 3.0 M, respectively, of guanidine chloride at pH = 3.0, a flux of 30 ml/h and fractions of 5.0 ml/tube in a discontinuous gradient. Fraction I was rechromatized under the same conditions, desalinated, dialyzed, and lyophilized. Electrophoresis of the purified crotapotin in a polyacrilamide gel, containing 12% acrilamide, leads to the same polypeptide as that described by Breithaulp et al. (1974).

2.2. SAXS measurements

Protein solutions of several concentrations were placed in Lindemann glass capillary tubes of 1.0 mm diameter (Marck-Korchen, Berlin) for SAXS measurements. The solvent was a solution of 30% formic acid in water, at pH 1.5. First, a 60 mg/ml stock solution of 3.0 mg of protein in 50 × 10^{-6} ml solvent was prepared. The remaining solutions of different concentrations (50, 40, 30, and 20 mg/ml) were obtained by increasing dilution steps.

The SAXS experiments were performed using a Rigaku small-angle goniometer and a classic X-ray generator with a Cu tube at 40 Kv and 30 mA. The scattered x-ray intensity was measured by means of a Tennelec PSD-1000 position-sensitive detector located at 500 mm from the sample and a Tracor Northern TN-1710 multichannel analyzer in a 256 channel mode. The x-ray radiation from the Cu tube was monochromatized using a Ni filter. Three vertical collimation slits were employed. Two of them (0.5 × 10 mm and 0.3 × 10 mm) define the incoming beam having a linear cross-section. The third set was located close to the sample, reducing the parasitic scattering from the collimating slits. A vacuum beam path was placed between the sample and the detector in order to reduce scattering by the air. A linear 2.8 mm wide beam-stopper was inserted close to the detector inside the vacuum path tube.

Typical counting times of 5 h were used for recording the scattering curves corresponding to each concentration. The maximum total number of photons was 3 × 10^4/channel at the lower angles for the most concentrated solution. Lower photon numbers were counted at higher angles and lower concentrations.

The experimental SAXS intensity for each concentration was determined as a function of the modulus of the scattering vector, h, defined as $h = 4\pi \sin\theta/\lambda$, where θ is half the scattering angle and λ the x-ray wavelength used in the experiments (λ = 1.54 Å). The scattering intensity was measured in the h-range between 2.0 × 10^{-2} Å^{-1} and 30 × 10^{-2} Å^{-1}.

The parasitic scattering produced by the solvent, capillary tube, air, and slits was subtracted from the total scattering curves corresponding to all the concentrations. The resulting scattering curves, were plotted in Fig. 3*a*, were corrected to normalize them equivalent sample absorption, concentration and thickness. Appropriate extrapolations for each scattering angle, as described by I. Piltz (1982), on the set of four SAXS

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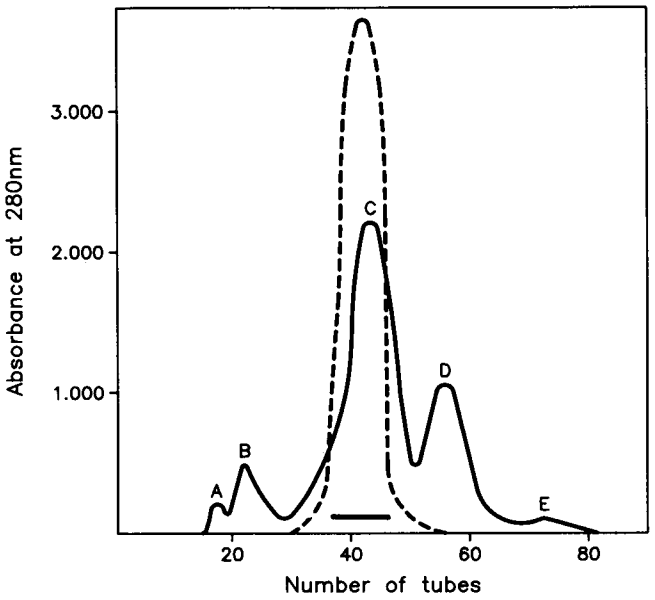

FIGURE 1 Chromatographic peaks obtained from the crude venom of the Brazilian snake (*crotalus durissus terrificus*) when filtered through a 4 × 45 cm, Sephadex G-75 column. Peak C corresponds to the crotoxin fraction and the dashed peak to refiltered crotoxin pools.

experimental curves corresponding to different concentrations, yielded the $I_N(h)$ curves at infinite dilution (or zero concentration), which is shown in Fig. 3 *b*. This extrapolation procedure allows the SAXS intensity function to be obtained free from intermolecular interference effects.

The scattering curves, corresponding to the different crotapotin concentrations, were analyzed after a mathematical desmearing process to eliminate the geometric effects due to the finite linear cross-section using the ITP program (Glatter, 1982). The described theory was applied to the desmeared and smoothed scattering curves for the determination of the structural parameters of the studied molecule.

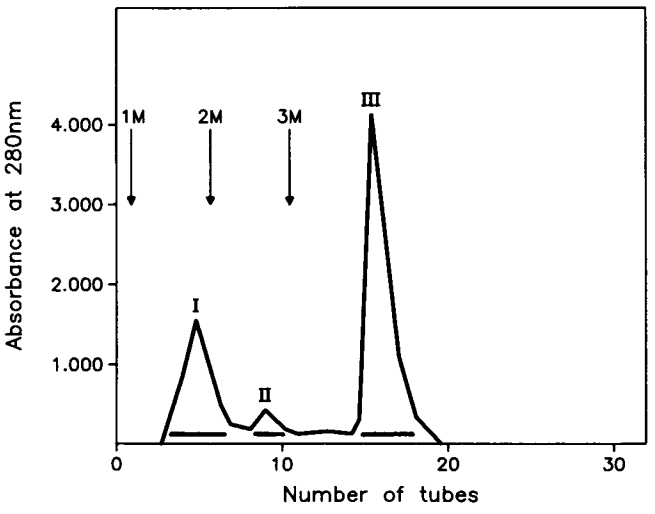

FIGURE 2 Chromatographic peaks obtained from crotoxin when filtered through a SP-Sephadex C-25 (2.5 × 40 cm) column. Peaks I and III correspond to crotapotin and phospholipase A, respectively.

TABLE 1 **Structural parameters of crotapotin**

R_g (Å)	$V(10^3 \times Å^3)$	D_{max} (Å)
13.6 ± 0.4	16.2 ± 0.4	46 ± 2

3. RESULTS AND DISCUSSION

3.1. Scattering curves and molecular parameters

The desmeared and smoothed scattering curve corresponding to zero concentration is plotted in Fig. 4 *a*. The radius of gyration, R_g, of the protein was determined using Guinier's law from the slope γ of the linear region of the log $I(h)$ versus h^2 curve at low h (Guinier and Fournet, 1955):

$$I(h) = I(0)e^{-1/3R_g^2h^2} \quad \text{and}$$
$$R_g(Å) = 2.628\sqrt{\gamma\,(Å^2)}. \tag{1}$$

In order to determine other structural parameters, the protein solution was assumed to be a two-electronic-density system composed of monodisperse particles, i.e., protein molecules of space-constant electron density, and of equal size, immersed in a homogeneous solvent. Under this assumption, the SAXS intensity was expected to have an assymptotic behavior described by Porod's law (Guinier and Fournet, 1955):

$$I(h) = \alpha/h^4, \tag{2}$$

where α is a constant. For real systems, Eq. 2 is not generally obeyed because of a usually weak contribution to SAXS from short range electronic density fluctuations in the protein molecule. This provides a constant contribution β to the SAXS intensity. Under this condition, the expected assymptotic behavior of the scattered intensity by two-phase systems, including electronic short range density fluctuations, can be written as follows:

$$I_N(h)h^4 = \alpha + \beta h^4 \quad (h \to \infty). \tag{3}$$

This behavior was observed experimentally as shown in Fig. 4 *b*. In order to determine the intensity function $I(h)$ associated with crotapotin and its solvent, described as a two-density system, the constant contribution from the density fluctuations was subtracted:

$$I(h) = I_N(h) - \beta, \tag{4}$$

β being deduced from the plot shown in Fig. 4 *b*.

The volume of the molecule was determined by using the following equation (Guinier and Fournet, 1955):

$$V = \frac{2\pi^2 I(0)}{Q}, \tag{5}$$

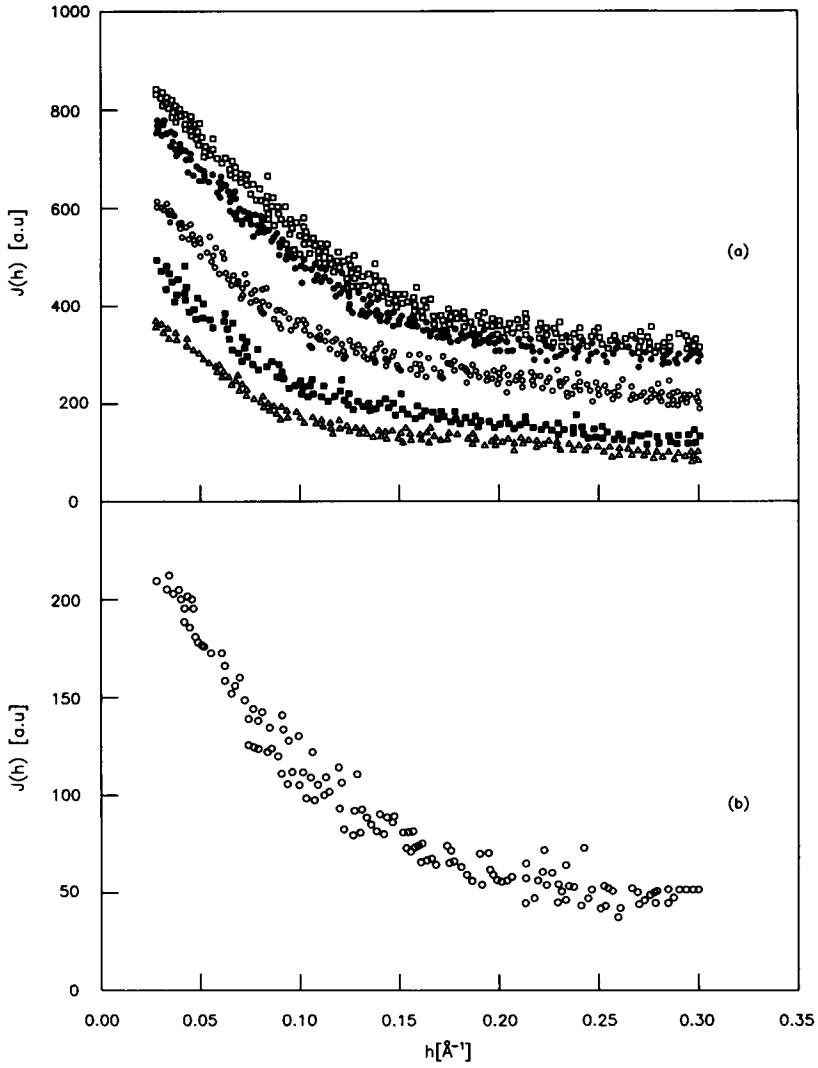

FIGURE 3 (*a*) Experimental SAXS intensities before normalization and correction for collimation effects. Protein concentrations are (△) 20, (■) 30, (○) 40, (●) 50, and (□) 60 mg/ml. (*b*) SAXS intensities extrapolated to zero concentration. The intensities corresponding to fixed h values were extrapolated after normalization of each scattering curve to equivalent concentration and absorption.

where $I(0)$ is the value of the extrapolated scattered intensity at $h = 0$ and Q is the "invariant" parameter given by:

$$Q = \int_0^\infty I(h)h^2 \, dh. \tag{6}$$

The value of Q was calculated numerically from a minimum value of h, h_m, up to the maximum value, h_M, for which the SAXS intensity was measured. For $h < h_m$ and $h > h_M$, Guinier and Porod behaviors of the scattering function (Eqs. 1 and 2, respectively) were assumed.

The distance distribution function of the molecule, $P(r)$, was determined from the experimental SAXS intensity as follows (Glatter and Kratky, 1982):

$$P(r) = \frac{1}{2\pi^2} \int_0^\infty I(h)hr \sin(hr) \, dh. \tag{7}$$

$P(r)$ depends on the molecular shape and defines an additional structure parameter, the maximal molecular dimension D_{max}. The experimental distance distribution function, was derived from the scattering intensity $I_N(h)$ by applying Eq. 7 and using the ITP81 program (Glatter, 1982). The analysis of the SAXS experimental results led to the structural parameters for the crotapotin molecule listed in Table 1. R_g and V were determined from Eqs. 1 and 5, respectively, and D_{max} from the $P(r)$ function (Eq. 7). The following step was to establish the molecular shape consistent with the parameter values shown in Table 1. We have disregarded the simplest assumption of a spherical shape because the volume of a

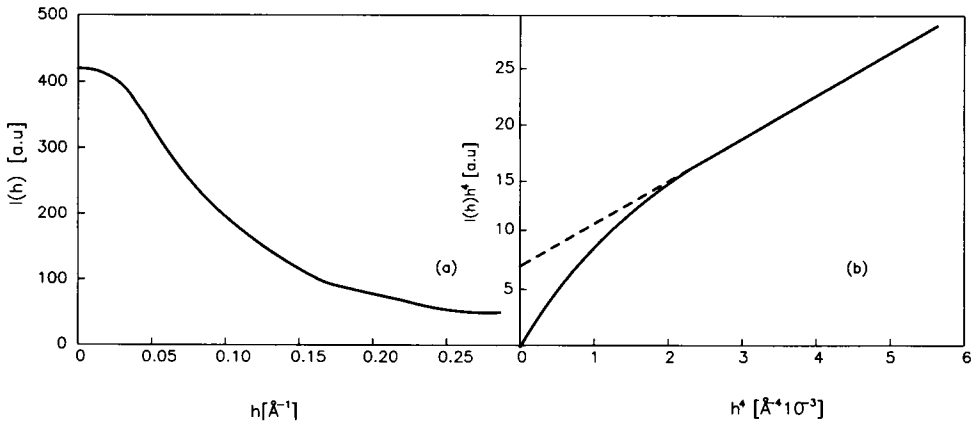

FIGURE 4 (*a*) SAXS curve after desmearing, smoothing and extrapolation down to $h = 0$ by using the ITP program (Glatter, 1982). (*b*) Porod plot of the curve of Fig. 4 *a*.

sphere calculated from the gyration radius R_g = 13.6 Å, $V_{sphere} = 22.6 \times 10^3$ Å^3, is higher than the equal to the experimental volume, $V_{exp} = 16.2 \times 10^3$ Å^3.

The subsequent assumed molecular shape was that represented by an ellipsoid of revolution, with semi-axes a, $b = a$, and c, and assymetry parameter $\nu = c/a$. In Fig. 5 the volume associated to an ellipsoid of revolution with a radius of gyration of 12.6 Å versus the ratio of the semi-axis ν was plotted. The intercepts of this curve with the horizontal line corresponding to the experimental molecular volume, $V_{exp} = 16.2 \times 10^3$ Å^3 yield the semi-axes ratio $\nu_0 = 0.45$ and $\nu_\rho = 1.95$, which are related to oblate and prolate ellipsoids, respectively. Both shapes are then consistent with the experimental R_g and V values.

In order to decide between the prolate and oblate shapes for crotapotin, a comparison of the experimental and theoretical distance distribution function $P(r)$ was tried. The experimental $P_E(r)$ distance distribution function calculated using Eq. 7 is plotted in Fig. 6. The theoretical distance distribution functions $P_T(r)$, which were calculated using the Multibody program (Glatter, 1982) for ellipsoidal molecules assuming R_g = 13.6 Å, and ν = 0.45 and 1.95, for oblate and prolate ellipsoids, respectively, are also plotted in Fig. 6. It is apparent that the theoretical function associated with an oblate ellipsoid with semi-axes $a = b$ = 22 Å and c = 10 Å agrees with the experimental function better than that of a prolate ellipsoid. This implies that the presented experimental SAXS results are consistent with a molecular shape of an oblate ellipsoid of revolution.

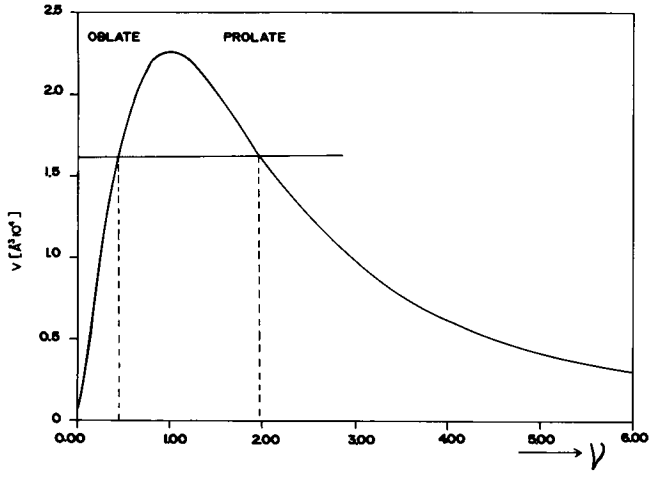

FIGURE 5 Volume associated with an ellipsoid of revolution with R_g = 13.6 Å as a function of the assymetry factor. The horizontal line indicates the volume $V_0 = 16.2 \times 10^3$ Å^3.

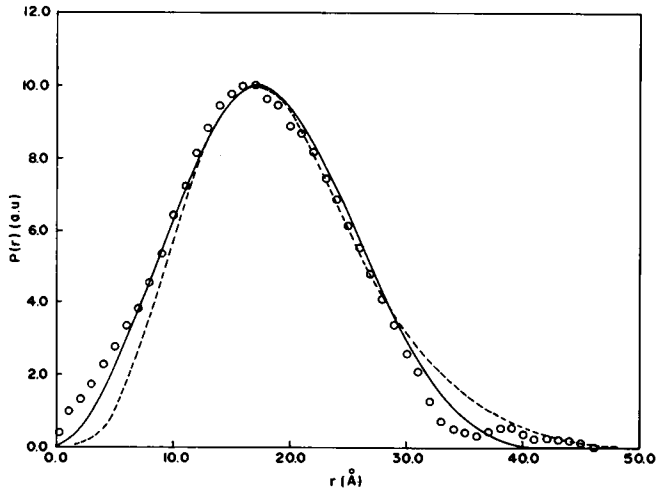

FIGURE 6 Comparison between the experimental (O) and theoretical distance distribution functions $P(r)$ for two structural models of crotapotin. The continuous and dashed lines correspond to oblate and prolate ellipsoids, respectively.

CONCLUSION

The presented experimental SAXS results indicate that the overall shape of crotapotin in solution at pH = 1.5 corresponds to an oblate ellipsoid of revolution with semi-axes $a = b = 22$ Å and $c = 10$ Å.

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