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Structural characterization and low-resolution model of BJ-48, a thrombin-like enzyme from *Bothrops jararacussu* venom

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Abstract

Thrombin-like enzymes (TLEs) are important components of snake venoms due to their involvement in coagulopathies occurring on envenoming. Structural characterization of this group of serine proteases is of utmost importance for better understanding their unique properties. However, the high carbohydrate content of some members of this group prevents successful crystallization for structural determination. Circumventing this difficulty, the structure of BJ-48, a highly glycosylated TLE from *Bothrops jararacussu* venom, was studied in solution. At pH 8.0, where the enzyme displays maximum activity, BJ-48 has a radius of gyration (*Rg*) of 37 Å and a maximum dimension (D_{max}) of 130 Å as measured by small-angle X-ray scattering (SAXS) and a Stokes radius (SR) of 50 Å according to dynamic light scattering (DLS) data. At the naturally more acidic pH (6.0) of the *B. jararacussu* venom BJ-48 behaves as a more compact particle as evidenced by SAXS (R_g = 27.9 Å and D_{max} = 82 Å) and DLS (SR = 30 Å) data. In addition, Kratky plot analysis indicates a rigid shape at pH 8.0 and a flexible shape at pH 6.0. On the other hand, the center of mass of intrinsic fluorescence was not changed while varying pH, possibly indicating the absence of fluorescent amino acids in the regions affected by pH variation. Circular dichroism experiments carried out with BJ-48 indicate a substantially random coiled secondary structure that is not affected by pH. Low-resolution model of BJ-48 presented a prolate elongated shape at pH 8.0 and a U-shape at 6.0. BJ-48 tertiary structure at pH 6.0 was maintained on heating up to 52 °C and was completely lost at 75 °C. The possible existence of two pH-induced folding states for BJ-48 and its importance for the biological role and stability of this enzyme was discussed.

Keywords: Snake venom; Serine protease; SVTLE; Solution structure; pH-induced conformational changes; Protein shape

1. Introduction

Venom serine proteases affect prey hemostasis by issuing specific cleavages in factors involved in coagulation, fibrinolysis and in the kallikrein–kinin systems [1,2]. Some of these venom proteases are often referred to as thrombin-like enzymes (SVTLEs) due to their ability to cleave fibrinogen, releasing fibrinopeptide A, fibrinopeptide B or both. SVTLEs show a high level of similarity within them and to a smaller degree with mammalian serine proteases such as trypsin and thrombin [3]. SVTLEs and trypsin-like enzymes have similar primary structures and are believed to share a common ancestor [4]. SVTLEs have been extensively studied, yet there is a lack of three dimensional structural information concerning this group of proteases. Homology models generated for several SVTLEs have fostered the belief that these proteins would display a globular structure similar to other members of the chymotrypsin family, e.g. [3]. However, SVTLEs may show significant differences in the molecular shape mainly due to variations in surface loops length and composition [5] and to distinct carbohydrate content [2].

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Recently, we have biochemically characterized BJ-48, a SVTLE from *Bothrops jararacussu* [6] which is capable of cleaving either the α and β chain of fibrinogen but show preference for the β chain. Its N-terminal sequence is most similar to batroxobin, a TLE isolated from *Bothrops atrox* venom. This enzyme presents the usual pH-activity profile of trypsin-like enzymes, showing highest activity at pH 8.0. Nevertheless, BJ-48 also shows distinct physical–chemical properties from trypsin and thrombin. For instance, it has greater stability to low pH and heating than its mammalian counterparts [7,8]. Indeed, during the purification procedure, BJ-48 can be eluted from the benzamidine–agarose affinity column using acid pH and a rapid increase of pH to prevent loss of enzymatic activity is not necessary as described for thrombin [8].

This stability to acid pH exposure has grabbed our attention because SVTLEs are maintained at pH 6 in the venom glands, where they must be only partially active or inactive. After venom inoculation, SVTLEs are exposed to the higher physiological pH and become active. Hence, such stability to pH changes may have a role in preventing venom degradation while keeping SVTLEs efficiency in the prey's vascular system.

Structural information on BJ-48 is essential to further characterize this protein and better comprehend the relationship between fold, enzymatic activity and its dependency on pH. However, BJ-48 is extensively glycosylated, which poses difficulties to its structural characterization by crystallography. In this context, low-resolution biophysical methods may be specially helpful for understanding the structure and molecular envelope of BJ-48 in solution. In the present work we report the effect of pH on BJ-48 structure by circular dichroism (CD), intrinsic florescence (IF), dynamic light scattering (DLS) and small-angle X-ray scattering (SAXS) studies. *Ab initio* models of BJ-48 were constructed from SAXS data at pH 6.0 and 8.0.

2. Materials and methods

2.1. B. jararacussu venom

B. jararacussu venom was extracted manually from ten specimens kept at the Vital Brazil Institute.

2.2. Purification

BJ-48 was purified as described before [9]. Briefly, the venom (100 mg) was equilibrated in 50 mM Tris–HCl containing 0.5 M NaCl, pH 7.0 and subjected to affinity chromatography on a benzamidine–agarose column. The adsorbed proteins were eluted with 1 mM HCl (pH 3.0), containing 0.5 M NaCl. The eluant was collected on ice and immediately concentrated using P10 centrifugal filters. The concentrated post-benzamidine–agarose preparation was injected in HPLC (Shim-pack Diol-150 column-Shimadzu) previously equilibrated in 50 mM phosphate buffer pH 7.2. The protein was fractionated on an automatic HPLC system (Shimadzu, 6A model) at a flow rate of 1 ml/min, during 28 min at 25 °C. The fraction containing BJ-48 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) using the Laemmli method (1970) in non-reducing and reducing conditions. Gels were stained with Comassie Blue R-250. Phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa) were used as molecular weight/mass standards. The purified BJ-48 sample was dialyzed against 50 mM phosphate buffer pH 6.0 or 50 mM Tris–HCl pH 8.0 and then concentrated to 4 mg/ml using P10 centrifugal filters.

2.3. Dynamic light scattering (DLS)

DLS analysis was performed with DynaPro™ (Protein Solutions™) at room temperature (25 °C). About 0.1 mg of BJ-48 was analyzed for each set of DLS data in 50 mM phosphate buffer, pH 6.0 and pH 8.0. All protein solutions were filtered through a membrane of 0.2 μm porosity to remove any dust prior to adding to the sampling cell.

2.4. X-ray scattering experiments, equipment set up and data analysis

Small angle X-ray scattering (SAXS) experiments were performed at the SAXS beamline of the National Synchrotron Laboratory, Campinas, Brazil [10]. The experimental setup included a temperature-controlled, 1.5 mm diameter capillary tube sample holder and a linear position-sensitive detector. Data acquisition was performed by taking five 600 s frames, using a sample detector distance of 446 mm and 849 mm and X-ray wavelength of 1.488 Å which enabled detection of a q range ($q = (4\pi/\lambda)\sin(\theta)$, λ = wavelength and 2θ = scattering angle) equal to $0.0165\ \text{Å}^{-1} < q < 0.2192\ \text{Å}^{-1}$. All the data treatment of the scattering intensities was performed using the software package TRAT1D [11]. Usual correction for detector homogeneity, incident beam intensity, sample absorption, blank subtraction and intensities averaging were included in this routine. The output of this software provides the corrected experimental intensities and error values. Data analysis and model calculations were performed using the computer programs GNOM [12] and GASBOR [13]. Using Indirect Fourier Transformation the program GNOM gives the pair distance distribution function *p(r)* which was used to calculate the experimental radius of gyration (R_g) and particle maximum dimension (D_{max}). The program GASBOR was used to generate *ab initio* models as described in Section 3.4. The HydroPro software [14] was used to calculate the hydrodynamic parameters, radii of gyration and maximum distances from the *ab initio* models, enabling direct comparison with the experimental values.

2.5. Effect of pH on BJ-48 structure by SAXS

The BJ-48 measurements were performed at a protein concentration of 4 mg/ml in two different buffers: 50 mM Tris–HCl buffer pH 8.0 and 50 mM phosphate buffer pH 6.0 at 37 °C. For comparison, measurements on denatured BJ-48 at pH 6.0 and 7.5 were also performed. For this purpose, BJ-48 aliquots at both pH values were thermally denatured by heating for 1 h at 75 °C rendering samples completely devoid of enzymatic activity. The equivalent molecular weight of BJ-48 protein at pH 8.0 and pH 6.0 was determined using a standard sample of 5 mg/ml of ovoabulmin (42.2 kDa) measured in the same

Table 1
Dimensional data of BJ 48 at pH 6.0 and pH 8.0

Source	R_g	SR	D_{max}
DLS			
BJ-48 pH 6	23.2*	30	60#
BJ-48 pH 8	38.7*	50	100#
SAXS			
BJ-48 pH 6	27.9	36.02*	82
BJ-48 pH 8	37	47.76*	130
Hydropro			
BJ-48 pH 6	29.3	32.3	89.6
BJ-48 pH 8	35.6	41	132.5

* Calculated using $SR=(5/3)^{1/2}\times R_g$.
\# Calculated for spherical molecules using $D_{max}=2\times SR$.

experimental conditions. Molecular masses were obtained from the quotient between their intensity extrapolated to $q=0$ and the $I_{(0)}$ value corresponding to ovabulmin.

2.6. Resistance to thermal denaturation at pH 6 by SAXS

In order to investigate if BJ-48 displays the same resistance to thermal denaturation at pH 6.0 as observed before for pH 8.0, as judged by the preservation of its hydrolytic activity [6], the enzyme sample was heated in small steps from 10 °C to 75 °C (10 °C, 25 °C, 32 °C, 37 °C, 42 °C, 47 °C, 52 °C and 75 °C). After heating, the sample was immediately cooled to 25 °C and a new SAXS data set was collected.

2.7. Circular dichroism analysis

CD measurements were made using a Jasco J-810 spectropolarimeter. BJ-48 was analyzed at 50 mM phosphate buffer pH 6.0 and 10 mM Tris–HCl pH 7.5 and 8.0. The data were collected at a scanning rate of 50 nm/min with a spectral bandwidth of 1 nm using a 0.2 cm path-length cell in far-UV (195–250 nm). Measurements were carried out at room temperature (25 °C) at a final protein concentration of 1 mM. All buffers used were of analytical grade and were filtered before use to avoid light scattering by small particles.

2.8. Intrinsic fluorescence (IF)

IF measurements were followed by exciting the protein samples at 280 nm and measuring the fluorescence emission between 300 and 400 nm. IF spectra were quantified by the center of spectral mass <ν> according to equation below [15]:

$$\langle\nu\rangle = \sum \nu_i \times F_i / \sum F_i$$

where F_i is the fluorescence emitted at wave number ν_i and the summation is carried out over the range cited above. Experiments were performed at 25 °C using 100 mM sodium citrate (pH 5.0 and 5.5), 100 mM phosphate buffer (pH 6.0 and 6.5) and 100 mM Tris–HCl (pH 7.0–9.0). The experiments were performed on JASCO FP-6300.

2.9. Protein determination

Protein content of samples was determined by the DC protein assay (Bio-Rad). Bovine serum albumin was used as standard.

3. Results

3.1. Effect of pH on BJ-48 structure by DLS and SAXS

BJ-48 was purified from *B. jararacussu* venom using two chromatographic steps. In accordance to previously published results [6], the purified BJ-48 sample showed a single band in SDS–PAGE analysis under reducing and non-reducing conditions (data not shown). The apparent radius of BJ-48 was determined at pH 6.0 (natural venom pH) and at pH 8.0 by DLS and SAXS (Table 1). The molecular mass values calculated from BJ-48 SAXS data at pH 8.0 and 6.0 were 50±3 kDa and 55±3 kDa, respectively. In both pH values, BJ-48 was shown to be monomeric by SAXS, DLS and gel filtration chromatography, indicating absence of aggregation in our experiments.

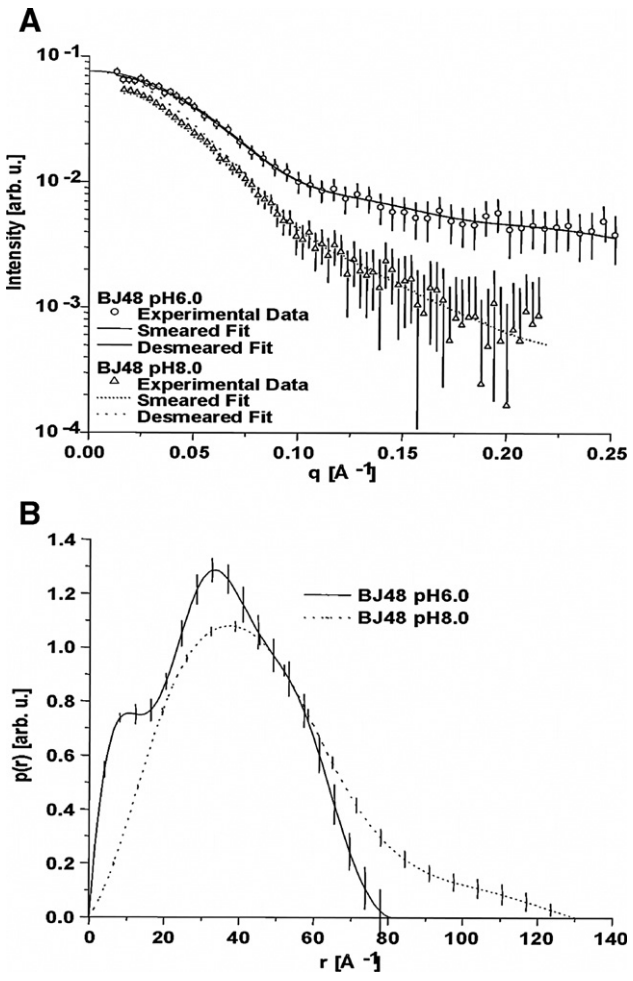

Fig. 1. SAXS analysis of BJ-48 structure at pH 8.0 and 6.0. (A) Small-angle scattering pattern. (B) Pair distribution function [*p(r)*]. The *p(r)* function and fitted curves were calculated from experimental scattering data using the program GNOM [12]. For details, see text.

The superposition of experimental (corrected and normalized) SAXS curves for BJ-48 at pH 8.0 and 6.0 is shown in Fig. 1A. At the more alkaline pH the protein scattering pattern shows a q^{-4} decay that is compatible with a more compact particle. For pH 6.0, a partial q^{-2} contribution to the scattering intensity was evidenced. This indicates that the protein has flexible domains, although it is not a perfect random coil. The SAXS profiles (Fig. 1A) were collected using low concentration solutions and using a 1D gas detector, which does not provide good statistical counting at high angles. For this reason, both curves are noisy in the high q range. A slower decay following q^{-2} at high angles for the flexible protein may be responsible for improved statistics when the log(I) vs. q curve is compared with that of a more compact protein, for which a q^{-4} decay is expected.

The values of R_g and D_{max} were determined from the pair distribution function, $p(r)$: for pH 8.0, it was obtained $R_g = 37 \pm 1$ Å and $D_{max} = 130$ Å, and for pH 6.0 the values derived were $R_g = 27.9 \pm 0.5$ Å and $D_{max} = 82$ Å (Fig. 1B). The R_g and the D_{max} suggest a prolate elongated shape for pH 8.0. At pH 6.0, the pair distribution function indicates a U-shape. Noteworthy, the SR values determined by DLS at pH 6.0 and pH 8.0 were 30 Å and 50 Å, respectively (Table 1). These results are in general agreement with SAXS data, clearly demonstrating two distinct particles and following the same trend (particle dimension at pH 6.0 < pH 8.0).

In accordance to the scattering pattern shown in Fig. 1A, the Kratky plots ($I\,q^2 \times q$) indicate that BJ-48 presents a rigid form at pH 8.0 and possesses higher flexibility at pH 6.0 (Fig. 2). SAXS data for thermally denatured BJ-48 (without enzymatic activity) rendered Kratky plots with ascending curves that undoubtedly characterizes a random coil or non-structured protein independent of the pH value the experiment was performed (Fig. 2). These Kratky plots clearly different from non-denatured BJ-48 at pH 6.0 confirm that the enzyme is structured at this pH.

3.2. Effect of temperature on BJ-48 structure

Our experiments conducted at pH 6.0 showed that at temperatures lower than 52 °C BJ-48 preserves its shape and

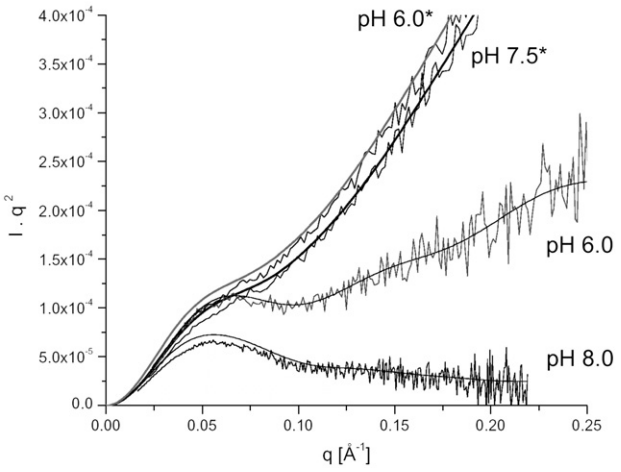

Fig. 2. Effect of pH on BJ-48 flexibility. Kratky plots of native BJ-48 at pH 6.0 and pH 8.0. The curves for thermally denatured BJ-48 (without enzymatic activity) at pH 6.0* and pH 7.5* are plotted for reference.

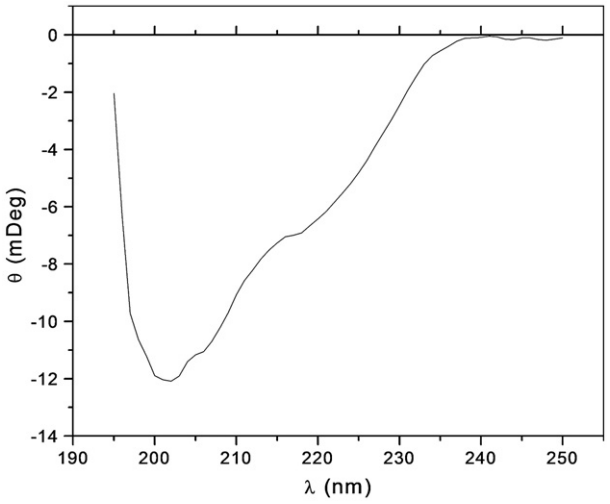

Fig. 3. Circular dichroism analysis. CD spectra of BJ-48 at 1 mg/ml was recorded at pH 7.5 in 50 mM Tris–HCl at 25 °C.

R_g. However, at 75 °C its R_g is increased to 35 ± 3 Å, a value similar to the R_g observed at pH 8.0. The lowering of the temperature back to 25 °C did not result in the recovery of the BJ-48 particle dimensions. In fact, the R_g was further increased to 39 ± 3 Å. The behavior of BJ-48 at pH 6.0 on heating is strictly similar to the resistance of BJ-48 hydrolytic activity at pH 8.0 subjected to analogous thermal treatment [6], indicating that the different conformations of this enzyme present at pH 8.0 and pH 6.0 display similar thermal stability.

3.3. Effect of pH by CD and IF

The CD spectrum of BJ-48 protein in solution buffered at pH 7.5 is shown in Fig. 3. It suggests a higher than expected content of random coil structures for an active enzyme known to belong to the chymotrypsin fold. Besides, comparison of the spectra

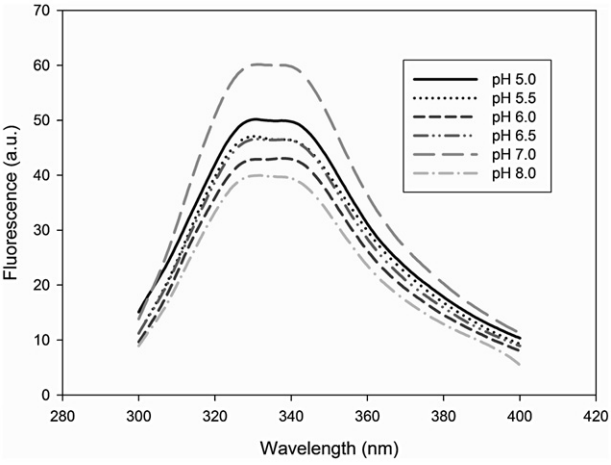

Fig. 4. BJ-48 intrinsic fluorescence spectra on varying pH. Experiments were performed in 100 mM sodium citrate (pH 5.0 and 5.5), 100 mM phosphate buffer (pH 6.0 and 6.5) and 100 mM Tris–HCl (pH 7.0–8.0) with 0.2 mg/ml of BJ-48 at 25 °C. Samples were excited at 280 nm and fluorescence emission was measured between 300 and 400 nm. Fluorescence intensity is reported is arbitrary units (a.u.).

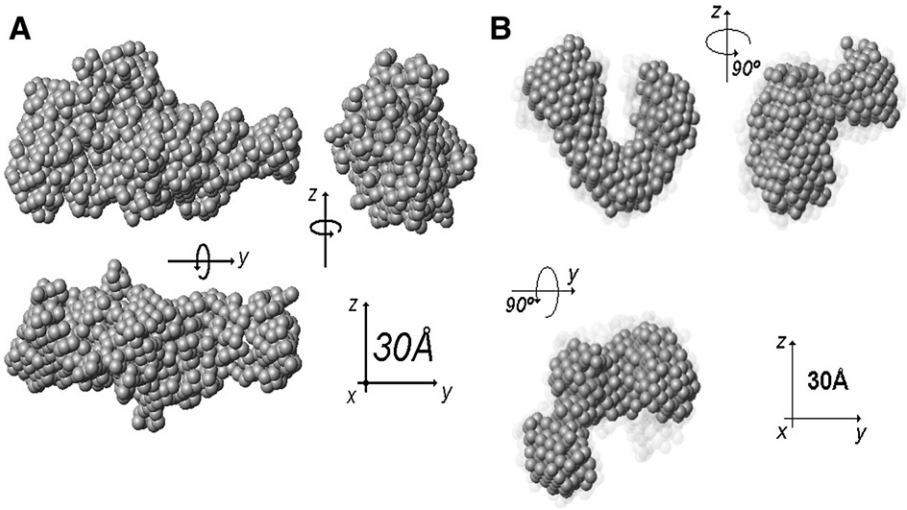

Fig. 5. Molecular envelopes of BJ-48 at pH 8.0 and 6.0. (A) BJ-48 at pH 8.0 and (B) BJ-48 at pH 6.0. *Ab initio* models represent an average of five dummy residue models (DRM) calculations using GASBOR [19]. The light gray spheres in (B) represent the space accessible for the DRM as judged by the degree of conservation of a given dummy residue among the five models.

recorded at different buffer systems reveals that the secondary structure was not affected by pH (result not shown).

The global changes in BJ-48 structure subjected to different pH values were monitored by measuring the IF emission. BJ-48 IF had a maximum located at 331.5 nm, with the centre of mass at 343 nm as shown in Fig. 4. No spectral shift in the centre of mass was observed by pH modification, possibly due to the absence of fluorescent amino acids in the regions affected by pH variation. On the other hand, variation on fluorescence intensities was observed but no simple trend upon pH change could be rationalized. Possibly, differences in the protein flexibility at the distinct pH may be affecting the quantum yields of the fluorophores without changing their exposure to the solvent (as judged by the lack of spectral shift).

3.4. Molecular envelope of BJ-48

The low-resolution molecular models of BJ-48 at pH 8.0 and 6.0 were determined using GASBOR program. Starting from a globular arrangement of interconnected spherical beads (dummy residues) which mimic the protein sequence, the program optimize the structure by rotation and translation of the dummy residues, using a simulated annealing protocol, in order to obtain a three dimensional configuration that gives the best fit of the experimental scattering data. The number of dummy residues used in the calculation is given by the protein size, which is known. Since SAXS is a low-resolution technique it is not possible to obtain one unique solution from this modeling procedure. In order to have a more reliable result we have calculated five independent models for each pH and the average result is shown in Fig. 5. The model average was performed by the program package DAMAVER [16]. In the averaging procedure the calculated models are aligned, compared and as a result the most probable configuration is represented by an average model built using a close packed arrangement of spheres. In agreement with the *p(r)* function, the BJ-48 model at pH 8.0 has a prolate shape (Fig. 5A). For pH 6.0, the generated *ab initio* model revealed that the protein has a U-shape in this condition (Fig. 5B). Since for pH 6.0 it is known that the protein has an intrinsic flexibility, it is necessary to understand this model as a probable average configuration for the protein in this pH.

These results suggest that two different conformational states for BJ-48 are induced by pH variation. The prolate form (Fig. 5A) is most stable in alkaline pH while the U-shape form (Fig. 5B) predominates in acid pH and is associated with a lower activity. Analysis of the models by Hydropro furnished R_g and D_{max} values that were similar to these obtained from the scattering curves (Table 1), giving further support to the analysis of SAXS data.

4. Discussion

The structures of globular proteins can be classified as native, molten globule, premolten globule and unfolded (random coil) conformations (folding states), according to their secondary structure content and overall flexibility [17]. The molten globule state shows the typical structure of native proteins; however the premolten globule and unfolded conformations do not have rigid tertiary structures.

All known crystallographic structures of the chymotrypsin family members are globular (e.g. thrombin [18,19]). As demonstrated in this study, BJ-48 has a non-globular shape, indicating possible significant differences in structural properties for BJ-48, a highly glycosylated serine protease [6], in relation to other members of the chymotrypsin family. Models generated from SAXS data showed that BJ-48 has a prolate shape at pH 8.0 and U-shape at pH 6.0. BJ-48 has a rigid structure at pH 8.0 as supported by its scattering pattern and the analysis of its Kratky plot. Since the native state of most proteins is characterized by more rigid structures when compared to folding intermediates [17], BJ-48 must be in its native

state at pH 8.0. This is in accordance to enzyme activity profile for different pH, which shows highest activity at pH 8.0 [6].

BJ-48 structure at pH 6.0 may suggest that the protein is in an intermediate folded state due to the increase in the protein flexibility when compared with pH 8.0, yet it is not accompanied by an increase in R_g as expected. Moreover, the higher flexibility at pH 6.0 is possibly associated with some loss of tertiary structure as it is accompanied with reduction of proteolytic activity at this pH [6]. Although the protein has intrinsic flexibility it was not possible to fit the SAXS data assuming a simple random coil model (data not shown), which indicates that the protein should have a defined overall shape, as depicted by the *ab initio* model. Unfortunately, it was not possible to use the secondary structure content to characterize the native state since BJ-48 showed a random coil CD spectrum at both pH 6 and 8. Similarly, using fluorescence spectroscopy it was also not feasible to identify an intermediate state, possibly because the tryptophan and tyrosine residues are located in regions not affected by pH variation. Nonetheless, we could confirm that the folding state for BJ-48 at pH 6.0 is completely different from the unfolded state observed in thermally denatured BJ-48.

The detection of two different states induced by pH variation (basic and acid pH) using fluorescent spectroscopy was also observed for acutolysin D, a metallo-protease from *Agkistrodon acutus* venom [20]. Hence, our findings may suggest a general mechanism for regulation of proteolytic activity of venom enzymes.

5. Concluding remarks

The present study demonstrated particular and interesting structural modifications occurring with BJ-48 thrombin-like enzyme on pH change, where a prolate shape is observed in the native state (pH 8) and a U-shape characterizes an intermediate state for the less active enzyme at pH 6. We must state that some of these particularities revealed by SAXS and DLS of BJ-48 can be the result of the extensive *N*-glycosylation on the enzyme molecular surface but experiments with BJ-48 at active pH and at the venom pH have clearly given structural information about novel folding states for the serine proteases in solution.

We propose that such behavior for venom proteases in solution could be the result of a regulatory mechanism that has evolved in snake venom glands to control undesired proteolysis. Possibly, in the slightly acid pH of venom (pH 6.0 as in *B. jararacussu* species) the proteases are partially folded with low activity, which could serve to avoid protein degradation in the snake gland. After snake bite, proteases are inoculated in the prey's blood circulation and exposed to higher pH (i.e., pH 7.4). This would trigger the transition from a less active folding intermediate to the fully catalytically competent native conformation.

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References

[1] Q. Lu, J.M. Clemetson, K.J. Clemetson, Snake venoms and hemostasis, J. Thromb. Haemost. 3 (2005) 1791–1799.
[2] S.M. Serrano, R.C. Maroun, Snake venom serine proteinases: sequence homology vs. substrate specificity, a paradox to be solved, Toxicon 45 (2005) 1115–1132.
[3] F.P. Silva Jr., S.G. De-Simone, S1 subsite in snake venom thrombin-like enzymes: can S1 subsite lipophilicity be used to sort binding affinities of trypsin-like enzymes to small-molecule inhibitors? Bioorg. Med. Chem. 12 (2004) 2571–2587 (Erratum in: Bioorg. Med. Chem. 12 (2004) 3755).
[4] A.M. Lesk, W.D. Fordham, Conservation and variability in the structures of serine proteinases of the chymotrypsin family, J. Mol. Biol. 258 (1996) 501–537.
[5] H.C. Castro, D.M. Silva, C. Craik, R.B. Zingali, Structural features of a snake venom thrombin-like enzyme: thrombin and trypsin on a single catalytic platform? Biochim. Biophys. Acta 1547 (2001) 183–195.
[6] F.P. Silva Jr., H.L.M. Guedes, L.C. Garvey, A.S. Aguiard, S.C. Bourguignon, E. Di Cera, S. Giovanni-De-Simone, BJ-48, a novel thrombin-like enzyme from the *Bothrops jararacussu* venom with high selectivity for Arg over Lys in P1: role of *N*-glycosylation, Toxicon 50 (2007) 18–31.
[7] H. Outzen, I.G. Berglund, A.O. Smalas, N.P. Willassen, Temperature and pH sensitivity of trypsins from atlantic salmon (*Salmo salar*) in comparison with bovine and porcine trypsin, Comp. Biochem. Physiol. 115 (1996) 33–45.
[8] K. Nakamura, T. Suzuki, M. Hasegawa, Y. Kato, H. Sasaki, K. Inouye, Characterization of p-aminobenzamidine-based sorbent and its use for high-performance affinity chromatography of trypsin-like proteases, J. Chromatogr. A 1009 (2003) 133–139.
[9] S.G. De-Simone, C. Correa-Netto, O.A.C. Antunes, R.B. De-Alencastro, F.P. Silva Jr., Biochemical and molecular modeling analysis of the ability of two *p*-aminobenzamidine-based sorbents to selectively purify serine proteases (fibrinogenases) from snake venoms, J. Chromatogr. B 822 (2005) 1–9.
[10] G. Kellermann, F. Vicentin, E. Tamura, M. Rocha, H. Tolentino, A. Barbosa, A. Craievitch, I.L. Torriani, The small-angle X-ray scattering beamline of the Brazilian Synchrotron Light Laboratory, J. Appl. Crystallogr. 30 (1997) 880–883.
[11] C.L.P. Oliveira, D.R. dos Santos, G. Kellermann, T. Plivelic, I.L. Torriani, Technical Note, LNLS, Campinas, Brazil, 1997.
[12] V. Semenyuk, D.I. Svergun, GNOM-a program package for small-angle scattering data processing, J. Appl. Crystallogr. 24 (1991) 537–540.
[13] D.I. Svergun, M.V. Petoukhov, M.H. Koch, Determination of domain structure of proteins from X-ray solution scattering, Biophys. J. 80 (2001) 2946–2953.
[14] J. Garcia De La Torre, M.L. Huertas, B. Carrasco, Calculation of hydrodynamic properties of globular proteins from their atomic-level structure, Biophys. J. 78 (2000) 719–730.
[15] L.M. Lima, R.B. Zingali, D. Foguel, R.Q. Monteiro, New insights into conformational and functional stability of human alpha-thrombin probed by high hydrostatic pressure, Eur. J. Biochem. 271 (2004) 3580–3587.
[16] V.V. Volkov, D.I. Svergun, Uniqueness of *ab initio* shape determination in small-angle scattering, J. Appl. Crystallogr. 36 (2003) 860–864.
[17] V.N. Uversky, Natively unfolded proteins: a point where biology waits for physics, Protein Sci. 11 (2002) 739–756.
[18] V. Bode, V. Mayr, U. Baumann, R. Huber, S.R. Stone, J. Hofsteenge, The refined 1.9 Å crystal structure of human alpha-thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment, EMBO J. 8 (1989) 3467–3475.
[19] W. Bode, P. Schwager, The refined crystal structure of bovine beta-trypsin at 1.8 Å resolution. II. Crystallographic refinement, calcium binding site, benzamidine binding site and active site at pH 7.0, J. Mol. Biol. 98 (1975) 693–717.
[20] X.L. Xu, X.H. Liu, B. Wu, Y. Liu, W.Q. Liu, Y.S. Xie, Q.L. Liu, Metal-ion-and pH-induced conformational changes of acutolysin D from *Agkistrodon acutus* venom probed by fluorescent spectroscopy, Biopolymers 74 (2004) 336–344.