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Jack bean urease (EC 3.5.1.5) aggregation monitored by dynamic and static light scattering

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Abstract

Aggregation of jack bean urease (JBU) is involved in many alterations of its biological properties, notably the ureolytic and entomotoxic activities. In order to investigate this phenomenon, protein aggregates were characterized by dynamic (DLS) and static light scattering (SLS) spectroscopies through determination of apparent hydrodynamic radii, the average molecular masses, radii of gyration and second virial coefficients. No effect of disulfide reducing agents on protein association was observed contrasting with previous reports implicating their function in the prevention of JBU aggregation. The influence of freeze–thawing cycles on protein aggregation was also investigated. Our results showed that after freeze–thawing cycles the native form of JBU with apparent hydrodynamic radius of 7 nm and radius of gyration of 12 nm is replaced by high-order oligomers and this aggregation is not reverted neither by dithiothreitol (DTT) treatment nor by high concentration of salts. Altogether the data help to understand the complex behavior of JBU in solution and may correlate with the diversity of biological properties of this enzyme.

Keywords: Urease; Oligomerization; Freeze–thawing; Hydrodynamic radius; Radius of gyration

1. Introduction

Urease (EC 3.5.1.5; urea amidohydrolase) is a nickel dependent enzyme [1] that catalyzes the hydrolysis of urea to form ammonia and carbon dioxide. In 1926, jack bean urease (JBU) was crystallized by Sumner [2] and these first crystals of a characterized enzyme demonstrated the proteinaceous nature of the enzymes. JBU exists as monomers, trimers and hexamers of identical 91 kDa chains, each containing two nickel ions per subunit [3]. Although JBU was the first protein ever crystallized, its structure has yet to be determined. Heterogeneity, insolubility, molecular flexibility or polydispersity of JBU in solution could be related to difficulties in obtaining crystals suitable for X-rays diffraction studies [4].

Many works have investigated the oligomerization/aggregation behavior of JBU and how this relates to its catalytic properties [5–7]. It has been shown that different jack bean seeds contain distinct molecular forms of JBU and the multiplicity of urease isoenzymes, in conjunction with their interconvertibility, may underlie many catalytic complexities of this enzyme [8].

Recent studies of our group have shown that JBU displays other biological activities, which are unrelated to its ureolytic property, such as activation of blood platelets, interaction with glycoconjugates and entomotoxic activity, this latter suggesting that urease(s) may be involved in plant defense [9–11]. Interestingly, it was observed that many of these biological activities are modified under storage conditions in which protein oligomerization takes place (unpublished). Moreover, aggregation of JBU is believed to impact the shelf life of diagnostic kits based on this enzyme [12]. Aggregation is also a major obstacle to useable crystal growth [13] and may be the reason of the difficulties encountered for growing crystals of JBU capable of diffracting X-rays at low angles [14,15].

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Urease aggregation and precipitation usually follows when the protein solution is stored in some conditions such as high protein concentrations, high temperature, absence of reducing agents, low pH or presence of salts [13]. The mechanisms underlying protein aggregation are not well understood, but it is believed that partial or transient exposure of hydrophobic clusters at the protein surface is responsible for this phenomenon [13]. Furthermore, the oligomeric states of urease are not yet identified.

Using dynamic light scattering (DLS) and static light scattering (SLS) spectroscopies, two non-perturbative and highly sensitive techniques, we have investigated in this work the influence of protein concentration, buffer salts, reducing agent and freeze–thawing cycles on the behavior of JBU in solution.

2. Experimental

2.1. Urease purification

The jack bean enzyme was purified from jack bean meal following the method of Blakeley et al. [5] with modifications. Briefly, dry seeds (Casa Agrodora, São Paulo, Brazil) were powdered and 25 g of defatted meal was extracted with buffer A (20 mM sodium phosphate, pH 7.5, 1 mM EDTA, 2 mM 2-mercaptoethanol) for 1 h at 4 °C. The meal was removed by centrifugation (30,000 × *g*, 20 min, 4 °C), and 28% (v/v) ice-cold acetone (final concentration) was added to the supernatant. The suspension was kept at 4 °C overnight and the precipitated proteins were removed by centrifugation (30,000 × *g*, 20 min, 4 °C). The concentration of acetone in the supernatant was then increased to 31.6% (v/v) and, after stirring at room temperature for 10 min, the precipitate was removed by centrifugation (30,000 × *g*, 20 min, 4 °C) and the supernatant was dialysed against buffer B (20 mM sodium phosphate, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoetanol). The resulting material was mixed with 25 ml of Q-Sepharose resin (Amersham-Biotech Pharmacia) equilibrated in buffer B, and after stirring in a beaker for 30 min in an ice bath, the mixture was filtered and the resin washed with 100 mM NaCl in buffer B to remove the non-retained proteins. Elution of urease-enriched fractions was achieved by adding 300 mM NaCl to buffer B. Active fractions were pooled and concentrated by using a CentriPrep cartridge (Millipore). The urease-enriched material was then applied into a Superose 6 HR 10/30 gel filtration column (Amersham-Biotech. Pharmacia) equilibrated in 20 mM sodium phosphate, pH 7.5, 1 mM EDTA, mounted in an FPLC system. The peak fraction containing active urease was concentrated on a CentriPrep cartridge (Millipore) and the protein solution (2 mg/ml) was stored at 10 °C for 20 days, in 20 mM sodium phosphate buffer, pH 7.5, in the presence of 1 mM EDTA, before the light scattering measurements. The purified protein used in all experiments described here was analysed by capillary electrophoresis (Fig. 1).

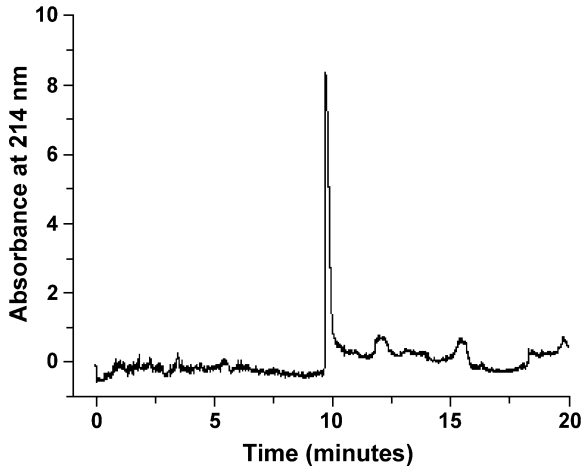

Fig. 1. Analysis of JBU by capillary electrophoresis. Capillary electrophoresis was performed at the Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, SP, Brazil, in an HP3DCE model apparatus (Agilent) equipped with a diode array UV–Vis detector. JBU sample in 20 mM sodium phosphate, pH 7.5, 1 mM EDTA at 37 °C, was injected at a pressure of 50 mbar and − 15 kV voltage. The signs observed in the region after 11 min are due to other components in the solution, such as EDTA.

2.2. Protein determination

The protein content of samples was determined by their absorbance at 280 nm. Alternatively, the methods of Ref. [16] or [17] were used.

2.3. Capillary electrophoresis

Capillary electrophoresis was performed on an HP3DCE model apparatus (Agilent, Waldborn, Germany) equipped with a diode array for UV–visible detection, at the Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, SP, Brazil. Urease samples in 20 mM sodium phosphate, pH 7.5, 5 mM 2-mercaptoethanol, 1 mM EDTA, at 37 °C, were injected under a pressure of 50 mbar, − 15 kV, during 6 s. The capillary (PVA) dimensions were 60 cm (51.7 cm to detector) long and 50 μm internal diameter. The proteins were monitored by the absorbance at 200, 214 and 254 μm.

2.4. Sample preparation

Urease solutions used for DLS and SLS measurements were prepared by dilution of the stored urease solution (2 mg/ml, in 20 mM sodium phosphate, pH 7.5, 1 mM EDTA), to give final concentrations of 0.1, 0.2, 0.4, 0.8, 1.4, and 2.0 mg/ml. The solutions were then centrifuged (14,000 × *g*, 20 min, 4 °C) and the supernatant was filtered through 0.2 μm pore diameter membranes (Millipore) into dust-free cells. This procedure was performed 2 days before the light scattering analysis. In order to avoid dust in the system, the cells were previously cleaned with distilled acetone in a closed system and the samples were always handled in a laminar flow box.

In order to evaluate the effect of the reducing agents, we investigated the influence of 2-mercaptoethanol or dithiothreitol (DTT) on the aggregation of JBU. Reducing agents were added to urease samples to give a final concentration of 5 mM and then DLS measurements were carried out after 1, 6, 18 and 72 h. For analysis of the effect of Tris-buffer, the same urease samples from the previous experiment were dialysed at 4 °C against 25 mM Tris–HCl, pH 7.5, 5 mM 2-mercaptoethanol, 1 mM EDTA. The freeze–thawing cycles were performed freezing the urease samples at −20 °C or −196 °C in presence of 20 mM sodium phosphate, pH 7.5, 1 mM EDTA and then thawing at room temperature (20 °C). We also analysed the effect of freeze–thawing cycles on JBU in the presence of 5 mM DTT or 100 mM NaCl.

2.5. Light scattering

2.5.1. Theory

The theory of light scattering has been reviewed in several monographs [18–20] and only a brief introduction to the method will be given in this work [21]. In a typical light-scattering experiment, a laser beam hits a solution and the scattering light is recorded by a photomultiplier. The spacial resolution of the experiment is defined by the scattering vector q, whose magnitude is given in Eq. (1):

$$|q| = (4\pi n/\lambda)\sin(\theta/2) \tag{1}$$

where λ denotes the wavelength of the incident light, n the refractive index of the solution, and θ the scattering angle.

In a DLS experiment, the fluctuations of the scattering light due to the Brownian motion of the particles are analysed in terms of an autocorrelation function (ACF), which contains the distribution of relaxation times, τ, and scattering amplitudes of the examined components [21], Eq. (2):

$$G^{(1)}(\tau) \propto \int_{R\min}^{R\max} N(R)M^2(R)P(q)S(q)\exp(-mR^{-1}q^2\tau_{\mathrm{rel}})\mathrm{d}R \tag{2}$$

where m is a proportionality constant, $N(R)$ and $M(R)$ denote number and mass of particles with radius R between the integration limits $R_{\min}$ and $R_{\max}$. $P(q)$ is the "intraparticle scattering factor" and it arises from intraparticle interference effects. $S(q)$ is defined as the "interparticle scattering factor" because it describes the interference effects arising between particles. In liquids, $S(q)$ is related to the radial distribution function, and in crystalline solids, it provides information on the unit cell.

Real systems may contain unimodal particle populations (characterized by a mean size and distribution of sizes around that mean) or, more often, two or more populations may coexist in the solution. The Brownian motion of each particle population results in superimposition of fluctuation patterns of different intensities in the light-scattering detector [18,19]. If the particles are small compared to the employed wavelength, the z-average translational diffusion coefficient D_z, can be determined through Laplace inversion of the autocorrelation function. From D, the hydrodynamic radii, R_h, of the particles are calculated according to the Stokes–Einstein equation (Eq. (3)):

$$R_h = k_B T/6\pi\eta D_0 \tag{3}$$

where k_B denotes the Boltzmann constant, T the absolute temperature, and η the viscosity of the solvent in the same temperature.

In a SLS experiment, the time average intensity of light scattering by the sample $\langle I\rangle$ is recorded as a function of the scattering vector $\boldsymbol{q}$. In SLS, the data are obtained according to the simplified Zimm procedure [22], in which the intensity of the scattering light is related to the average molecular weight, M_w, the second virial coefficient, A_2, and the radius of gyration, R_g, through Eq. (4) [23]:

$$Kc/R(\theta,c) = [1/M_w][1 + (R_g q)^2/3] + 2A_2 c \tag{4}$$

where K is an optical parameter called optical contrast factor [23] and $R(\theta, c)$ is the excess absolute time-average light scattering intensity (excess Rayleigh ratio). In order to obtain R, the solvent scattering intensity is subtracted from the protein solution intensity. In this work, the excess Rayleigh ratio of buffer A was taken as reference. To extract the macromolecular parameters, measurements were extrapolated to infinitive dilution ($c \to 0$) and zero scattering angle ($q \to 0$). The magnitude of R_g gives important information about particle shape. Particles of different shape with identical M_w values have different R_g values. The precise shape of a particle can be determined according to mathematical models that consider values for R_h and R_g combined with other available data, such as results from DLS [18,19].

2.6. Experimental setup and data analysis

Light scattering experiments (DLS and SLS) were performed on a Brookhaven Instruments standard setup (BI-200 M goniometer, BI-9000 AT digital correlator) with an He–Ne laser ($\lambda = 632.8$ nm) as light source. The scattering volume was minimized using a 0.4 mm aperture and an interference filter before detecting the signal on the photomultiplier. The time correlation functions were measured in the multi-τ modo using 224 channels. The sample cell was placed in the index matching liquid decahydronaphthalene (decalin, Aldrich).

The measurements in DLS were performed in the angular range 35–140° in steps of 10° or 15°. To avoid excessive information, we only show here the results for an angle of 45° which are representatives of all light scattering angles analysed. The ACFs were first analysed by the Cumulants Method [24] and then the ACF spectra were Laplace-inverted by CONTIN [25,26]. Due to the large amount of recorded spectra, only the "best" solutions found by CON-

TIN (i.e., those having a probability of rejection close to 0.5) were considered.

The SLS measurements were carried out in the angular range of 35–140° in steps of 10° or 15° and the molecular parameters M_w, A_2 and R_g, were obtained according to the simplified Zimm procedure [23].

3. Results and discussion

Since its isolation by Sumner [2], many questions regarding the behavior of JBU in solution have arisen. It had been previously established by many researchers that the native form of JBU is a hexamer (540 kDa) and in some conditions, a trimer (270 kDa). Although other studies have addressed the oligomerization/aggregation behavior of JBU and how this affects its catalytic properties [7,8,27–29], few works were able to correlate the behavior of JBU in solution with properties of medium. Elucidation of these correlations is a crucial step towards understanding JBU biological properties, not only the catalytic activity but also its other recently described pharmacological [9] and insecticidal properties [10,11]. Crystal growth [30] is also greatly influenced by JBU aggregation. In the present work, the oligomerization/aggregation of JBU has been approached experimentally using dynamic and static light scattering.

3.1. Effect of reducing agents on JBU aggregation

Highly purified JBU (Fig. 1) in sodium phosphate buffer at different concentrations was stored at 10 °C for 20 days in absence of any reducing agents and then the particle size distribution was determined by DLS. No changes were observed in the pattern of light scattering by JBU in phosphate buffer, pH 7.5, at a range of 0.4 to 2.0 mg/ml and following long-term storage. The analysis of the correlation functions for this system revealed a single form with apparent hydrodynamic radius of ~ 7 nm (Fig. 2A, inset), and so it can be concluded that under these conditions only the hexamer, the native form of urease, is present in the solution. It has been reported that reducing agents could dissociate high order oligomeric states of JBU into its native form and stabilize the enzyme by preventing its aggregation[5,7,31]. Here we show (Fig. 2A) the correlation functions obtained by DLS measurements for urease solutions at concentrations of 0.8 and 1.4 mg/ml, in absence or presence of 5 mM 2-mercaptoethanol. No effect of the reducing agent was observed and a single form of urease is present in all the solutions (Fig. 2A, inset; Fig. 4), even at higher protein concentrations (up to 2 mg/ml; data not shown). Besides, no changes were observed from 1 up to 72 h after the addition of 2-mercaptoethanol or using dithiothreitol as reducing agent. To find no changes in the particle size in the presence of reducing agent was a surprise since significant effects of these compounds on urease stability have been reported, supposedly due to protection against aggregation of the protein [7]. In our study, protein concentrations, incubation times, pH, disulfite reducing agents concentration and phosphate buffer were similar to those described in other works [5,7,31]. Since the effects of reducing agents upon JBU catalytic properties [5–8] are not due to modifications in its quaternary structure (this work), this fact might be related to some intramolecular aspect of the protein, most probably the maintenance of reduced cysteine(s) near the catalytic site of JBU.

Static light scattering measurements were performed in order to characterize the oligomers of JBU. Fig. 3 shows the

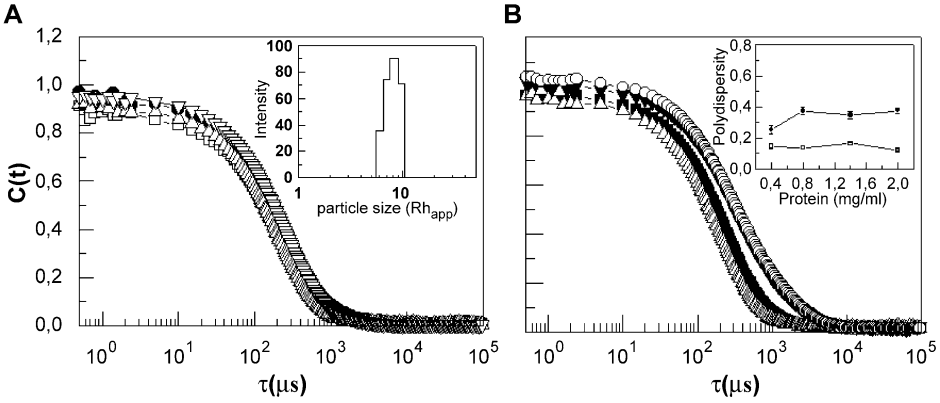

Fig. 2. Effect of 2-mercaptoethanol (2-ME) and the buffer on the behavior of urease in solution, as monitored by DLS. (A) Correlation functions of DLS measurements obtained for JBU in phosphate buffer in the presence and absence of 2-ME. Samples: urease 0.8 mg/ml with (up triangle) and without 2-ME (square); urease 1.4 mg/ml with (down triangle) and without 2-ME (solid circle). (Inset) Particle size distribution obtained by CONTIN analysis (intensity distribution) of DLS data for JBU in sodium phosphate buffer, 1 mM EDTA, pH 7.5, 5 mM 2-mercaptoethanol, obtained by the inverse Laplace transformation of the correlation functions. (B) Effect of the buffer on the behavior of urease in solution. Samples: urease 0.8 mg/ml in 20 mM sodium phosphate buffer (up triangle) and in 25 mM Tris–HCl buffer (solid down triangle); urease 1.4 mg/ml in 20 mM sodium phosphate buffer (solid square) and in 25 mM Tris–HCl buffer (circle). (Inset) Polydispersity measured by DLS of JBU solutions at different concentrations, in 20 mM sodium phosphate, 1 mM EDTA, pH 7.5 (square) and 25 mM Tris–HCl, 1 mM EDTA, pH 7.5 (solid circle). Both buffer solutions contained 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5. The DLS measurements were performed at 45°.

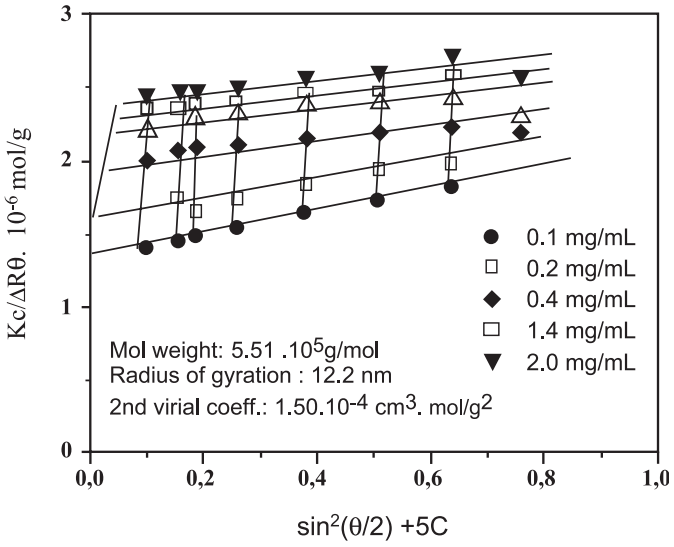

Fig. 3. Zimm plot of JBU, in 20 mM sodium phosphate, 5 mM 2-mercaptothanol, 1 mM EDTA. The Zimm plot was prepared using the Zimm Plot Program 4.3. In the determination of molecular weight, the extrapolation for a concentration of zero gives a value of 5.51×10^5 g/mol ($\pm$ 7.8%) and for an angle of zero, 5.51×10^5 g/mol (20.2%). The relative difference in the extrapolations for the molecular weight is of 1.85×10^{-3}%, indicating a good fitting of Zimm plot.

Zimm plot for JBU in 20 mM sodium phosphate, 5 mM 2-mercaptoethanol, pH 7.5. Under our conditions, a single form of JBU was seen with $R_{h\ (app)}$ of $\sim$ 7 nm, R_g of $\sim$ 12 nm, second virial coefficient of 1.5×10^{-4} cm^3 mol/g^2 and an average molecular weight of 5.51×10^5 g/mol, consistent with the hexameric form of the enzyme. The relative difference of extrapolation ($c \rightarrow 0$ and $\theta \rightarrow 0$) for the molecular weight was 1.85×10^{-3}%, indicating a good fit of the Zimm plot. The ratio R_g/R_h (denotes as ratio ρ) of 1.7 suggests an elongated form of the JBU hexamer (polymers displaying a spherical shape have a ratio of R_g/R_h near 1, while linear polymers have values >1).

3.2. Effect of the buffer on JBU aggregation

The oligomerization of JBU was studied by DLS as a function of protein concentration in two routinely used solutions buffered at pH 7.5: 20 mM sodium phosphate and 25 mM Tris–HCl. Fig. 2B shows the modification in the correlation functions and the polydispersity of the system (Fig. 2B, inset) of JBU in Tris-buffered or in phosphate buffered solutions. The changes in the correlation functions indicated that JBU easily aggregates in presence of Tris–HCl, but not in phosphate buffered solutions. Fig. 4 shows a clear conversion of the urease with $R_{h\ (app)} \sim 7$ nm into a form with $\sim$ 70 nm which accounted for 55–65% of total urease. However, in concentrated solutions (2 mg/ml) the particle with $R_{h\ (app)} \sim 70$ nm was not observed, but instead, two new forms with $R_{h\ (app)} \sim 137$ nm and $\sim$ 21 nm, which together corresponded to 70% of the total protein, were seen. Little change was observed in size distribution with the course of time (data not show).

Cesareo and Langton [32] investigated the influence of different buffers on the enzymatic activity of JBU. The most significant finding was a decrease of $\sim$ 60% in the enzymatic activity when urease was stored in the presence of 25 mM Tris–HCl, as compared to the protein kept in phosphate buffer. Taken together with our observation that JBU aggregates spontaneously in a Tris-buffered medium, with only about 30% of the initial form with $R_{h\ (app)} \sim 7$ nm remaining in solution, these results indicates that the 70 nm form of JBU has either no enzymatic activity or only a residual activity, when compared to the 7 nm native species. Thus it can be concluded that: (i) in Tris-buffered solutions, JBU forms few and discrete oligomer states; (ii) the oligomeric states of JBU depend on the protein concentration and the salts present in the medium.

3.3. Effect of freeze–thawing on JBU aggregation

To investigate the gradual loss in the enzymatic activity and toxic properties of urease upon storage at -20 °C, we studied the influence of cycles of freeze–thawing on protein aggregation. Figs. 4 and 5 show the particle size distribution of JBU in phosphate-buffered solution after freeze–thawing cycles. The particle with $\sim$ 7 nm disappeared and a second form, which seems to be two hexamers associated ($R_{h\ (app)}$ 14 nm), took place after a single cycle. After two cycles of freeze–thawing, the particle with $R_{h\ (app)} \sim 14$ nm practically disappeared (residual 5% of total protein) and forms with hydrodynamic radii of 100 to 600 nm are seen (Fig. 4). These results may explain the multiplicity of JBU isoenzymes described so far and throw some light on how the different molecular forms of urease may have arisen. We

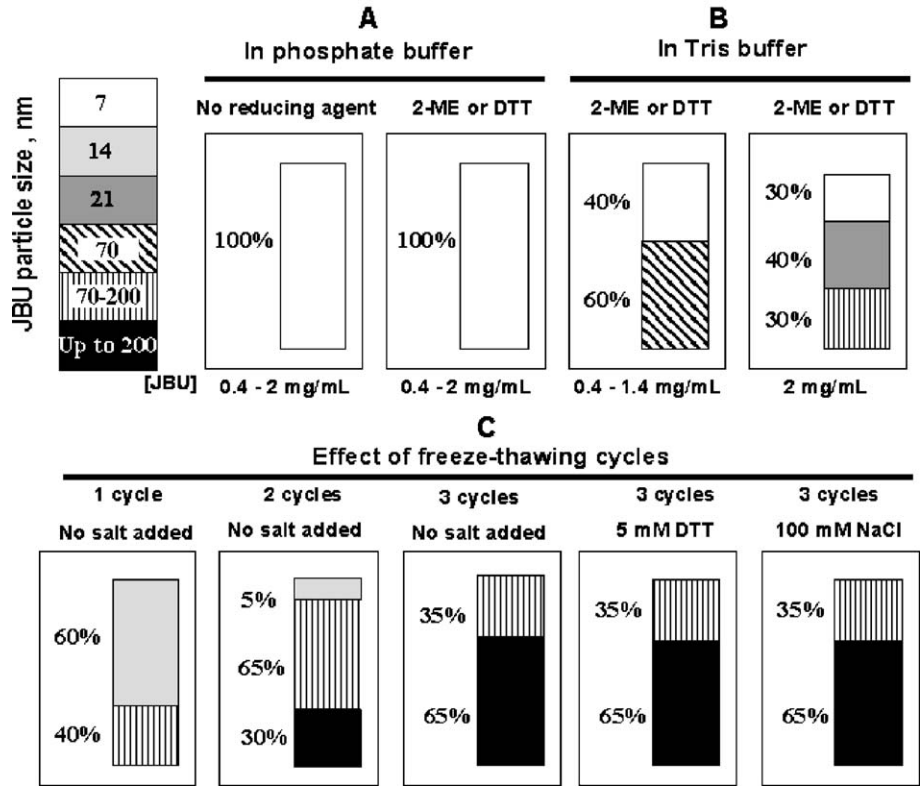

Fig. 4. Relative intensity distributions of JBU oligomers produced under different buffers and salt conditions, and after freeze–thawing cycles, as determined by DLS. (A) JBU (0.4–2 mg/ml) samples in 20 mM sodium phosphate, pH 7.5, 1 mM EDTA in the presence and absence of 5 mM 2-mercaptoethanol (2-ME) or 5 mM DTT. (B) JBU (0.4–2 mg/ml) in 25 mM Tris–HCl, pH 7.5, 5 mM 2-mercaptoethanol, 1 mM EDTA. (C) JBU (0.4–2 mg/ml) in 20 mM sodium phosphate, pH 7.5, 1 mM EDTA submitted to one, two and three cycles of freeze–thawing and the effect of 5 mM DTT and 100 mM NaCl on the aggregates of JBU.

also analysed the effect of freeze–thawing cycles on JBU aggregation in presence of 5 mM DTT or 100 mM NaCl. No changes were observed in the pattern of aggregation of JBU in presence of DTT or NaCl in course of the time, indicating that neither reducing agent nor higher ionic strength are able to prevent or revert the oligomeric process.

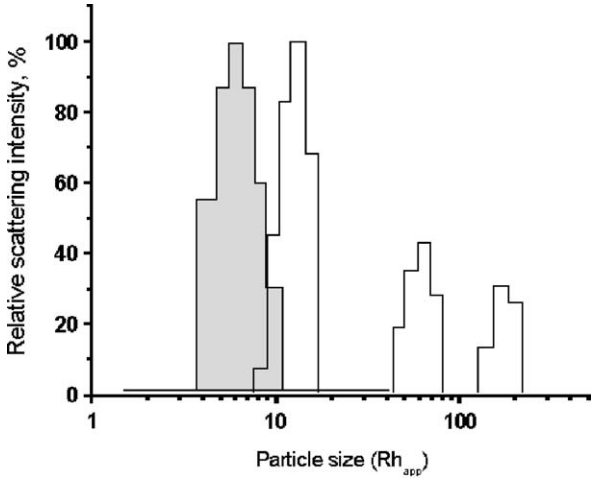

Fig. 5. Distribution of particle sizes obtained by CONTIN analysis (intensity distribution) of DLS data for urease oligomers submitted to a single cycle of freeze–thawing. JBU (0.4 to 2.0 mg/ml) in 20 mM sodium phosphate, 1 mM EDTA, pH 7.5, (gray) was freeze–thawed one time, and the hydrodynamic radii of oligomers obtained after this procedure (transparent) were determined. The DLS measurements were performed at 45°.

After two cycles of freezing and thawing of JBU in phosphate buffer, the Zimm Plot showed a strong angular dependence of the intensity of the light scattering (Fig. 6). From the Zimm Plot for this system, the existence of two populations of particles can be deduced, one with R_g of 68.2 nm and M_w to 1.73×10^6 g/mol and another with R_g of 29.7 nm and M_w of 2.62×10^6 g/mol, corresponding to low and high angles of the plot, respectively (Fig. 6). Moreover, the negative second virial coefficients after freeze–thawing calculated from these two distinct regions, -2.80×10^{-5} and -1.87×10^{-5} cm^3mol/g^2, agree with the formation of aggregates.

3.4. Acid-catalyzed model for the oligomerization of JBU

A pertinent question is why JBU aggregates when submitted to freeze–thawing cycles. The eutectic freezing and thawing process of an aqueous solution provides an explanation for the formation of JBU aggregates (Fig. 7). As a solution decreases in temperature, an equilibrium between the solid and liquid phases of salt and water is maintained. Therefore, at any given temperature, the ratio of solutes in the liquid phase of this solution depends on their relative solubilities and is called the "eutectic composition" [33].

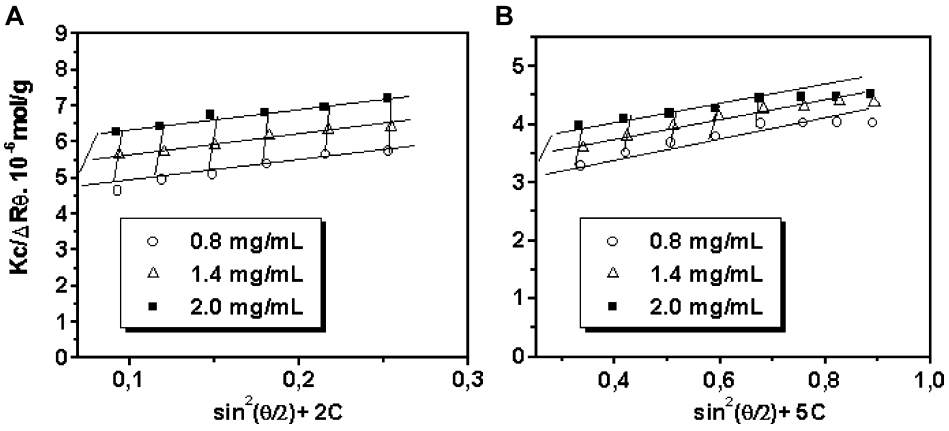

Fig. 6. Zimm plot of JBU, in 20 mM sodium phosphate, pH 7.5, 1 mM EDTA, after two cycles of freeze–thawing. From the Zimm Plot for this system, the existence of two populations of particles can be deduced, one with R_g of 68.2 nm and M_w of 1.73×10^6 g/mol and another with R_g of 29.7 nm and M_w of 2.62×10^6 g/mol, corresponding to low (A) and high (B) angles of the plot, respectively. The negative second virial coefficients after freeze–thawing calculated from these two distinct regions, -2.80×10^{-5} and -1.87×10^{-5} cm^3mol/g^2, agree with the formation of aggregates.

As the solution cools down, ice crystals begin to form and the solutes become highly concentrated as the volume of liquid water available decreases. The dibasic component of sodium phosphate solidifies, leaving the more soluble monobasic form in the liquid phase to acidify the solution and neutralizing the protein's carboxylates by protonation [34]. It should be noted that the initial pH of a phosphate buffer solution has no effect on the pH of the solution at the eutectic freezing point, which is always 3.6 [34]. As JBU is an acidic protein with isoeletric point ~ 4.6, the acidification of medium, which leads to acidic residues protonation, can decrease the electrostatic repulsion of the negative charges and thus could lead to aggregation of the protein molecules. Upon warming, these steps are reversed leaving a neutral solution rich in JBU aggregates. Our data show that neither NaCl (100 mM) nor DTT (5 mM) have any interference in this process and that aggregates of JBU formed are stable up to 2 weeks. If a salt-mediated pathway is involved [35], the addition of high concentrations of NaCl in the medium should lead to shielding of the charged protein residues, disrupting ionic interactions. As no effect of added NaCl was observed, we can conclude that the neutralization of the protein's carboxylates by protonation may be enough to induce JBU aggregation.

The rate of freezing and thawing seems to be an important feature and affects both the production of JBU

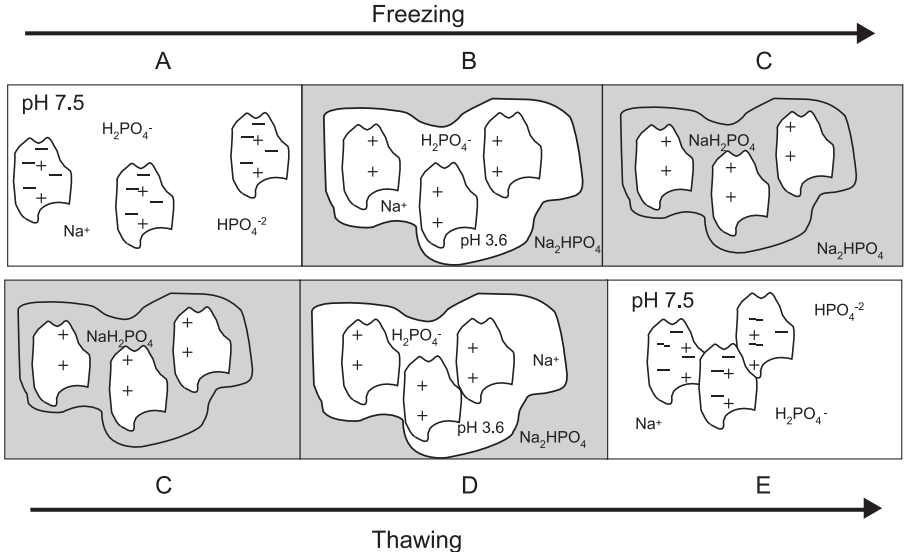

Fig. 7. Hypothetic pathway for the formation of JBU oligomers by freezing and thawing. As the solution of JBU in 20 mM sodium phosphate buffer, pH 7.5 (A) cools down, ice crystals begin to form and the solutes become highly concentrated as the volume of liquid water available to them decreases. The dibasic component of sodium phosphate solidifies, leaving the more soluble monobasic form in the liquid phase to acidify the solution (pH 3.6) and neutralizing the protein's carboxylates by protonation (B) and (C). Upon warming, these steps are reversed, first protonating the concentrated JBU solutions (D), which stabilizes the closed interfaces in newly formed oligomers. Then the ice melts, leaving a neutral solution rich in JBU aggregates (E).

aggregates and the distribution of their size. So in the fast freezing of the JBU solution at −196 °C, no significant aggregation was observed (data not shown) contrasting with the freezing at −20 °C. In general, the slower these process are, the greater the degree of oligomerization of JBU. The formation of crystalline ice is required for the oligomerization to occur [36]. The role of freezing in the reaction is to create high concentrations of the protein in the spaces between regions of growing ice crystals, where the acidic conditions induced by cooling phosphate buffer lead to protonation of acidic residues of the protein, which then oligomerize as the solution thaws. The high concentration of the protein in these temporary spaces promotes oligomerization by lowering the entropic barrier to aggregation [36]. Taken together, these observations explain why the biological activities of JBU are affected when a solution of this protein is stored at −20 °C, being this due to protein aggregation. Biochemical properties of JBU after freeze–thawing in phosphate buffer could be a model to study the behavior of the protein in acid pH and it might be important in the investigations of the role of microbial ureases in the pathogenesis of some diseases, such as gastric ulcers caused by *Helicobacter pylori*, which survives in acidic pH.

In conclusion, using dynamic light scattering and static light scattering spectroscopies, we were able to establish the influence of protein concentration, buffer salts, reducing agent and freeze–thawing cycles on the oligomerization behavior of jack bean urease in solution. The results showed that JBU forms few and discrete oligomeric states depending on the protein concentration and the salts present in the medium and it is not influenced by disulfide reducing agents. After freeze–thawing cycles, the initial form of JBU with apparent hydrodynamic radius of 7 nm is converted into high-order oligomers, which are not reverted by DTT or sodium chloride. Altogether the data help in understanding the complex behavior of jack bean urease in solution and may correlate with the diversity of biological properties displayed by this enzyme.

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