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A dynamic light scattering investigation of the nucleation and growth of thaumatin crystals

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Abstract

The aim of this contribution is to show some important physical–chemical parameters that must be taken into account during the crystallization of proteins. For this purpose, we have calculated the overall free energy, the chemical potential, the entropy, and the enthalpy for a model protein. Dynamic light scattering techniques were used in order to study how far the system was from the equilibrium after obtaining single crystals. Additionally, we show the solubility plot of the model protein in order to explain the plausible crystal growing zone. We have also presented the first experimental approach to infer the equilibrium state by using light scattering techniques. Classical thermodynamic measurements were obtained and compared with our approach. It has also been possible to explain the role of ionic strength and temperature in the crystallization of thaumatin. Finally, we have demonstrated how our crystal quality predictions obtained from dynamic light scattering and X-ray diffraction analyses can be compared with classical predictions. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Obtaining a good quality macromolecular crystal is a critical step within the process of X-ray structural analysis. A knowledge of the 3D molecular structure gives us useful information about the mechanism of action of biomolecules at the atomic level. Such information forms a very important part of biology, as well as medicine, biotechnology, and any field dependent upon new applications of bio-macromolecules.

In order to crystallize a protein, the system has to reach a maximum solubility state (subsaturating zone). From there, it has to be taken far from the equilibrium state to a condition of supersaturation. This can be done by altering one or more physical–chemical parameters of the solution, such as pH, temperature, protein concentration, etc. It is important to note that the action of all these parameters is interdependent, because the solution changes dynamically during the process of nucleation and growth of the crystals [1]. On the way to the supersaturation condition, the solution will cross the metastable zone, where nuclei will appear and compete until a stable nucleus is formed. At this point, the supersaturation zone is reached. In

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the process of forming a macroscopic aggregate, however, the solution gradually returns to the equilibrium state [2]. On the other hand, the difference in the chemical potential which is the driving force for protein crystallization depends in a complicated manner upon the protein concentration, and on the solution conditions (temperature, ionic strength and pH).

Whenever the crystallization of a molecule is studied, we have to face both equilibrium and non-equilibrium conditions. The first condition involves the solubility of a system, the surface energy of the crystal, the osmotic pressure and other thermodynamic parameters. The non-equilibrium condition can involve the flow of a solution around the crystal, as well as the deposition of molecules from saturated solutions [3].

The crystallization can be divided into three stages: nucleation, crystal growth and cessation of growth. We consider the nucleation to be the critical stage in the crystallization process, because at this point the macromolecules in solution aggregate to form either a crystal or an amorphous precipitate depending on the value of the supersaturation achieved. Since nuclei cannot exist in solutions containing protein dissolved at concentrations less than the thermodynamic solubility, we believe that knowledge of the solubility of a system is an essential requirement to control the crystallization and the formation of good quality crystals.

Several authors [4–6] have considered light scattering to be a very useful technique for studying the pre-nucleation and the nucleation stage as well as the solubility and aggregation of proteins under different conditions.

In this work, we have used dynamic light scattering to investigate the solubility and the process of nucleation in thaumatin from *Thaumatococcus daniellii*. This protein was chosen as a model protein, because it possesses substantial range of solubility and stability at high temperatures and at different values of the ionic strength. It is also possible to obtain this protein with a high degree of purity. In addition, to studying the influence of ionic strength and temperature upon the solubility, we have estimated the width of the metastable zone, which sustains the growing of the crystal once a stable nucleus is formed [3]. This

knowledge can be used to improve the quality of crystals by crystallization micro- or macroseeding techniques. Finally, some of the thermodynamic parameters such as Gibbs free energy, enthalpy, entropy and chemical potential are calculated for this protein in an effort to place the crystallization of this protein on a quantitative basis.

2. Materials and methods

As impurities are critical in protein crystallization [7], a SDS-PAGE electrophoresis with silver stain was performed on the thaumatin (T-7638 Sigma Chemical Co). This process revealed no significant contaminants that prevented work with this protein without further purification.

In order to study the influence of ionic strength and temperature on the solubility and aggregation behavior of thaumatin, several solutions were prepared with double distilled water. In these solutions, the final concentration of thaumatin was 20 mg/ml (w/v) at pH 7.0 in $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer (Sigma Chem. Co.). These solutions contained various final concentrations of sodium potassium tartrate (Sigma Chem. Co.) ranging from 7.5 to 20.0%. Samples of these solutions were injected into the DLS equipment Dyna-Pro 801-TC (Protein Solutions). This device is equipped with a laser of 830 nm, a temperature controller, and system for passing the solution through 0.022 μm filters (Wathman Anotop). Light scattering data were collected at 18°C over approximately 4 and 6 h.

To study the influence of the temperature, solutions prepared as explained above, were injected into the DLS equipment. For each concentration of sodium potassium tartrate, data were collected over a period of 30 min at the following temperatures 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C and 45°C.

When the interaction between the precipitating agent and protein was studied by DLS, the protein was in direct contact with the final concentration of the precipitating agent. Therefore the system achieved supersaturation immediately. This is similar to what happens in the batch crystallization method. In order to emulate these DLS

conditions, thaumatin crystals were also grown in batch crystallization using all of the various conditions described above at 4°C and 18°C. From these batches at each condition, we chose crystals of approximately equal size for X-ray diffraction studies. The X-ray diffractometer was a Rigaku with rotating anode generator (100 kV, 50 mA) and an R-AXIS IIC Imaging Plate Detector held at a crystal/detector distance of 100 mm. Subsequently, Wilson plots were obtained for each crystal.

The solubility diagrams of the system were obtained in order to choose the best conditions to crystallize the protein. For this purpose, we obtained the solubility plots at 4°C and 18°C for all sodium potassium tartrate concentrations as follows: a period of one month elapsed after the formation of the crystals. Then, the crystallization containers were opened and the mother solution was extracted under a microscope in order to avoid taking crystals. The solution was placed in Eppendorf tubes and centrifuged in a microcentrifuge (Brinkmann Instruments Inc.) at 12,800 g for 30 s. This was done in order to eliminate any crystals from the solution as well as any non-visible nuclei. Ten microliters of the protein solution were taken and diluted with double distilled water up to 1 ml. The absorption of the solution was measured at 280 nm. It is worth mentioning that the extinction coefficient for the thaumatin was calculated experimentally for our system (Thaumatin from Sigma Chemical Co) with a value of $E_{280\text{nm}}^{1\%} = 11$. This coefficient permitted us to calculate the concentration of each solution.

In order to achieve a better understanding of the solubility of our system from the classical point of view, Green's equation was used to calculate the solubility at 4°C and 18°C. In this equation we included the salting-in (K_i) and salting-out (K_o) constants that are related to the ionic strength of the system. These values were also used to calculate some classical thermodynamic parameters related to the crystallization of the thaumatin. Two kinds of calculations were done, the first was based on classical equations taking the solution as an ideal case, the second approach took into account some of the adjustments required to represent real solutions. We also introduced some new ideas based directly on

nucleation theory, which can be used with dynamic light scattering data to calculate the interfacial energy between a nucleus and the solution.

Green's equation can be written as

$$\text{Log}(S_p/S_0) = K_i(C_{pp})^{0.5} - K_o C_{pp}, \quad (1)$$

where S_p is the current solubility of the protein and S_0 the solubility of the protein in water and C_{pp} is the concentration of the precipitating agent.

The supersaturation, β , is then defined as

$$\beta = C_p/S_p, \quad (2)$$

where C_p is the initial concentration of the protein and S_p is the equilibrium concentration (solubility at specific temperature and precipitating agent concentration).

For processes that happen at constant temperature, the change in the Gibbs free energy (ΔG) is given by the classical thermodynamic equation:

$$\Delta G = \Delta H - T\Delta S, \quad (3)$$

where, ΔH is the change in the enthalpy, ΔS is the change in entropy and T is the absolute temperature.

The enthalpy (H) is a state function equal to the quantity of heat released or absorbed by the system at constant pressure. For the specific case of the dissolution, ΔH is defined by

$$\Delta H_{\text{solution}} = -[R \ln(S_{p2}/S_{p1})]/[1/T_1 - 1/T_2], \quad (4)$$

where R is the universal gas constant ($8.3143 \text{ J K}^{-1} \text{ mol}^{-1}$), S_{p1} and S_{p2} are the solubilities at two different temperatures (T_1 and T_2). This equation applies only to ideal solutions.

By contrast, we can calculate the entropy of solution, ΔS , using the following equation

$$\Delta S = \frac{\Delta\mu_a(\text{pH}, C_p, T_2) - \Delta\mu_a(\text{pH}, C_p, T_1)}{T_2 - T_1}. \quad (5)$$

In Eq. (5), $\Delta\mu_a$ is the difference in the chemical potential of the solute in the supersaturated solution and in the solution in equilibrium with the crystal. Since at equilibrium, the solute must have the same chemical potential as the solute in the crystal, the chemical potential difference in Eq. (7), which is defined by

$$\Delta\mu = k_B T \ln(\gamma C_p/\gamma S_p), \quad (6)$$

represents the driving force for crystallization. It is important to mention that we could multiply Eq. (6) by $k_B T$ in order to obtain the energy per molecule or even multiply by RT obtaining the energy/mol. In Eq. (6), C_p is the solute concentration in the supersaturated solution, γ is the thermodynamic activity coefficient of the solute, and k_B is Boltzmann constant ($1.3806 \times 10^{-23} \text{ J K}^{-1}$). Under ideal solution conditions, the activity coefficients are equal to unity and the driving force can be expressed simply in terms of the nominal supersaturation β ,

$$\Delta\mu = k_B T \ln(\beta). \quad (7)$$

The activity coefficient of the dissolved protein can be expressed in terms of a virial expansion,

$$\ln \gamma = 2B_2 C_p + (3/2)B_3 C_p^2 + \dots \approx 2B_2 C_p, \quad (8)$$

where, B_2 and B_3 are virial coefficients.

In Eq. (8), the standard state is taken so that $\gamma \rightarrow 1$ as $C_p \rightarrow 0$. Combining Eqs. (6)–(8) gives a simple estimate of the thermodynamic driving force for the crystallization:

$$\Delta\mu = k_B T [\beta + 2B_2(C_p - S_p)]. \quad (9)$$

It is apparent from Eq. (9) that $\Delta\mu$ depends not only on the nominal supersaturation, β , but also on the magnitudes C_p and S_p . In particular, the solubility is a sensitive function of the ionic strength and temperature, so $\Delta\mu$ can vary considerably with solution conditions even at constant β .

On the other hand, for comparison with Eq. (8), we can also estimate ΔG by substituting Eqs. (4) and (5) into Eq. (3).

We estimate ΔG at two temperatures (4°C and 18°C) using different concentrations of precipitating agent and constant concentration of

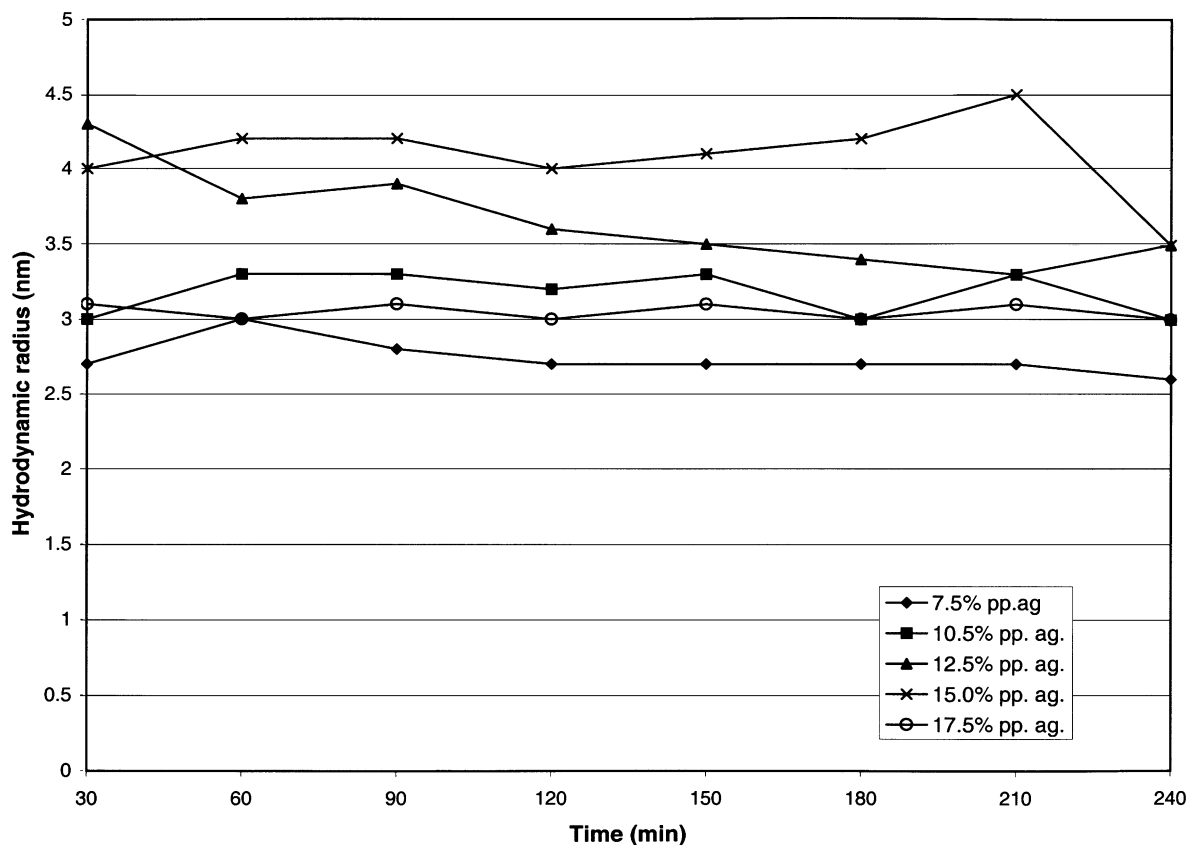


Fig. 1. Study of the hydrodynamic radius as a function of time for the thaumatin at different concentrations of precipitating agent.

protein. In order to understand how far from the equilibrium the system is during crystallization of thaumatin, this thermodynamics estimates our dynamic and static light scattering observations.

3. Results and discussion

DLS is a technique used to measure the particle motion (the translational diffusion coefficient, D_T) of a macromolecule undergoing Brownian motion in solution. The translational diffusion coefficient is related to the hydrodynamic radius through the Stokes–Einstein equation under ideal conditions (a dilute and monodisperse small particle ensemble): $D_T = k_B T / 6\pi\eta R_H$ where k_B is the Boltzmann's constant, T is the absolute temperature, η the viscosity of the protein solution (although strictly this is the viscosity of the solvent) and R_H is the hydrodynamic radius of the cluster.

Using the DYNAMICS program from the Dyna-Pro801 TC equipment from Protein Solutions Co., this method delivers directly the estimated molecular weight as a function of the

temperature and pH. Fig. 1 shows the hydrodynamic radius (R_H) versus time as a function of the increase of the precipitating agent concentration. Some fluctuations were observed at 12.5% (w/v) and 15% (w/v) of Na K tartrate. However, at a higher concentration of the precipitating agent, we observed a decrease in the R_H at 17.5% (w/v) of precipitating agent similar to the value obtained for R_H at 7.5% (w/v). We suggest that the system reaches the supersaturation zone at concentrations of 17.5% (w/v) and higher concentrations, which may be the cause of this effect. At such high concentrations of precipitating agent, high concentration of micro-aggregates (probably retained in the filter) may form. After dissipation of this transient effect, the dimer state reappears, which corresponds to an approximate R_H ranging from 3.0 to 3.1 nm. The value of 20.0% (w/v) of sodium potassium tartrate was not plotted, because it showed a quick linear growth that reached the R_H of 800 nm in a short time, which we believe to be caused by the formation of amorphous precipitate [8].

Fig. 2 shows a photograph of the crystals grown under the same conditions as the light scattering

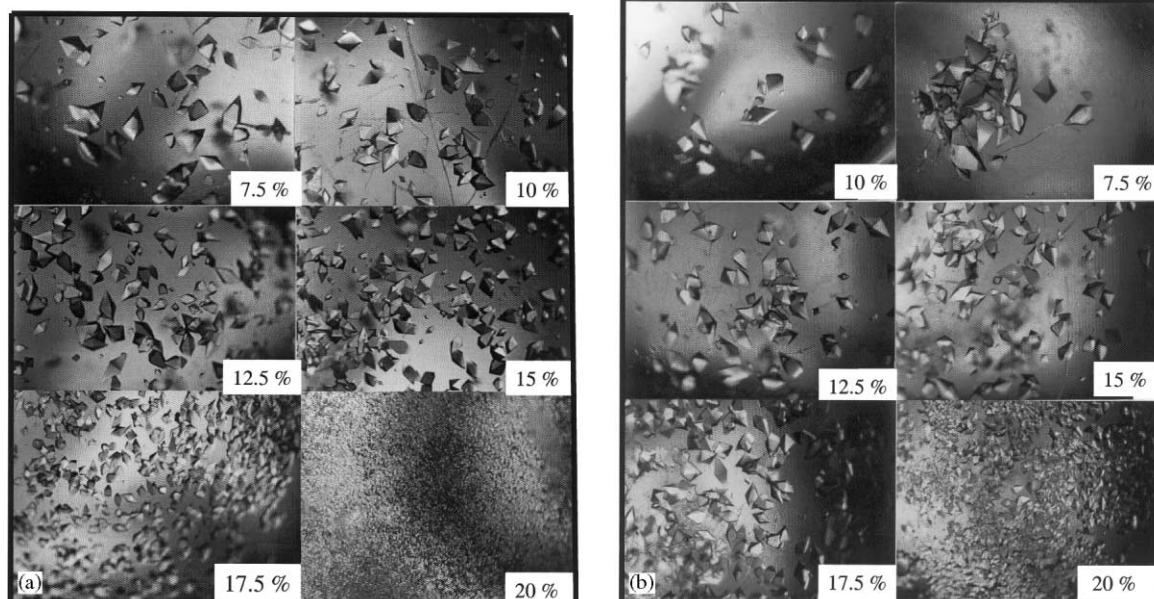


Fig. 2. Recorded photographs of the crystallization of thaumatin with different concentrations of precipitating agent at two temperatures (a) 4°C and (b) 18°C.

experiments. Like the light scattering experiments, these results show clearly that the higher concentrations of precipitate lead to smaller crystals. At 17.5% of the precipitating agent, large quantities of small aggregates are formed in the solution. These crystals cannot grow any bigger, because of the formation of amorphous precipitate, which competes for the available supersaturation. At 20% (w/v) of precipitating agent, we observed dense micro-crystals both covered with and mixed with amorphous precipitate. There was no optical significant difference between the crystals grown at 4°C and the crystals grown at 18°C. In passing, we note that when we tried to grow crystals under the ambient temperature conditions of our laboratory, (data not shown), we also obtained dense micro-crystals mixed with amorphous precipitate.

Our results indicate that the concentration of precipitating agent plays an important role in the

crystallization of thaumatin. In this regard, we agree with Rosenberger, who described the role played by protein solubility and purity in successful crystallizations [9]. To find optimum conditions for crystallization, we have to understand the attractive/repulsive forces of specific groups of the amino acids residues responsible for crystal contacts. To exploit the Debye–Hückel screening of charges, one seeks crystals under conditions of pH far from the isoelectric point. Given that the isoelectric point of thaumatin is at pH 12. Our crystallization carried out at pH 7 satisfied this requirement.

By contrast, when the influence of the temperature was analyzed via DLS, we observed that there is no R_H dependence on temperature (Fig. 3). Although different temperatures were screened, the ionic strength continued being the principal parameter that influenced the crystallization of

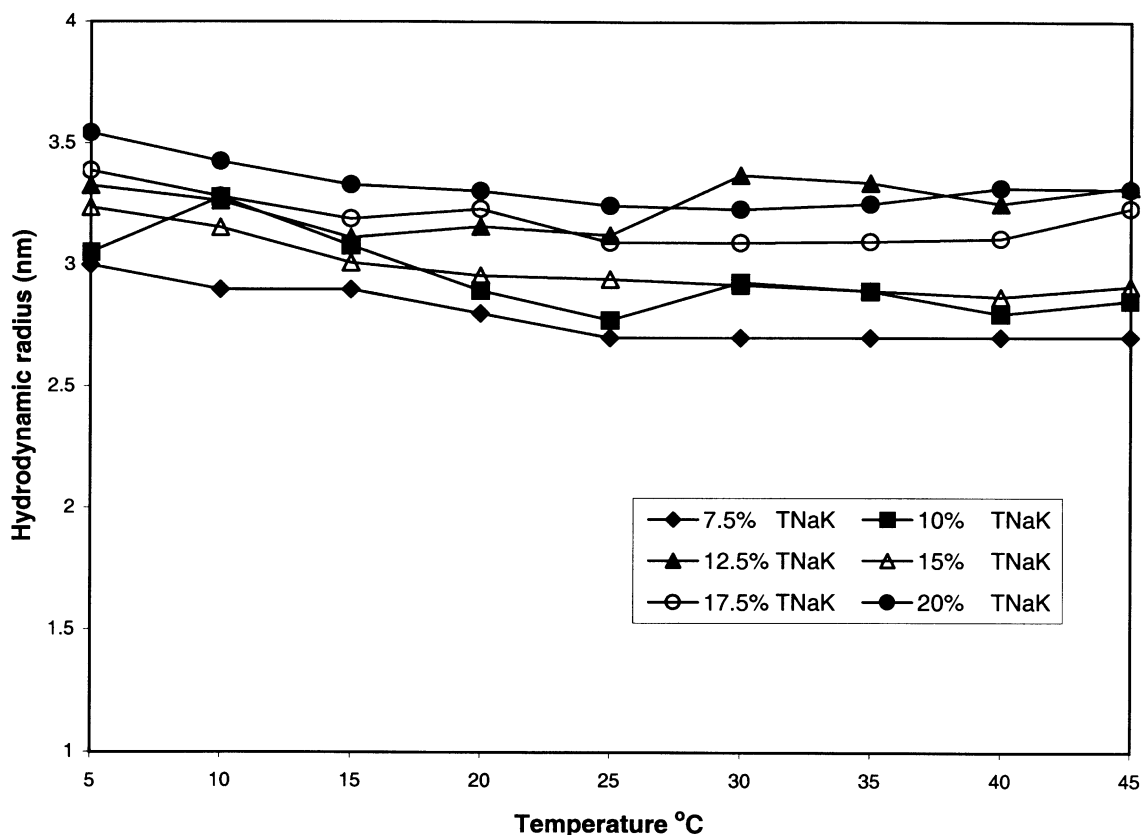


Fig. 3. Study of the hydrodynamic radius as a function of the temperature for different concentrations of precipitating agent.

thaumatin. However, when the crystalline quality was analyzed using the X-ray diffraction techniques (Fig. 4), it was found that the crystals that grew at 4°C had substantially better structural quality than those grown at 18°C. Although the overall resolution ranged from 1.9 to 2.0 Å, the internal average order of these crystals at 7.5% was higher at both temperatures.

However, at higher supersaturation, we may expect both pH and temperature to play a role. This combination generally results in good quality crystals, though of smaller size, if there is not sufficient mass available [10].

The solubility plot (Fig. 5) corroborates the results obtained from the X-ray diffraction analysis. The solubility at 4°C was higher than that at

18°C. It can be observed that the temperature becomes a predominant factor in producing good crystals at lower values of ionic strength. When the concentration of precipitating agent is raised, the ionic strength rather than the temperature is the predominant factor that affects the solubility. Our solubility for thaumatin is similar to the results obtained for the lysozyme published by Howard and co-workers [11]. Both lysozyme and thaumatin have a basic isoelectric point and low molecular weight.

If we compare the crystallization and X-ray results with the solubility plot (Fig. 5), we can also observe that the best crystals correspond to the lowest precipitant concentration in the solubility plot (7.5%). By comparison, in experiments

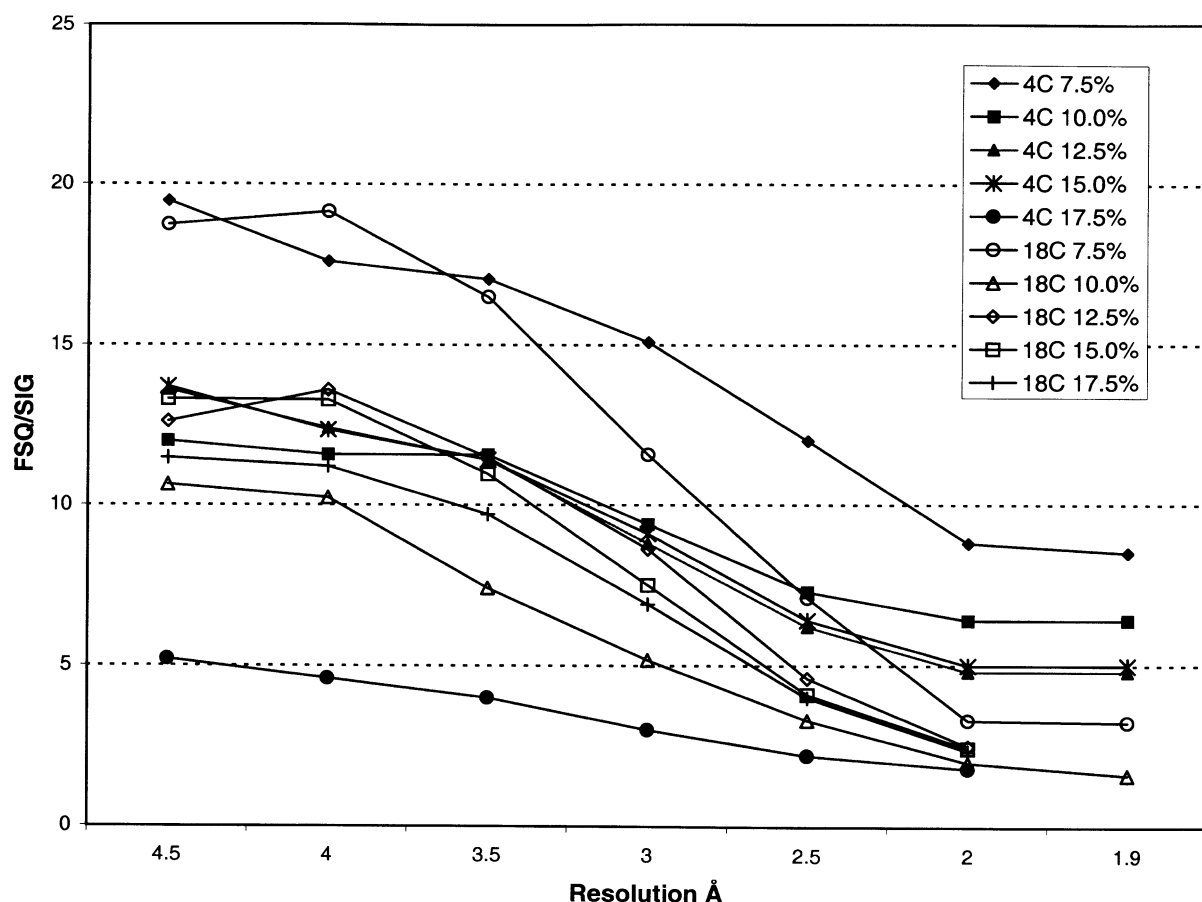


Fig. 4. Wilson's plot for thaumatin crystals grown at different concentrations of precipitating agent at two temperatures: (a) 4°C and (b) 18°C.

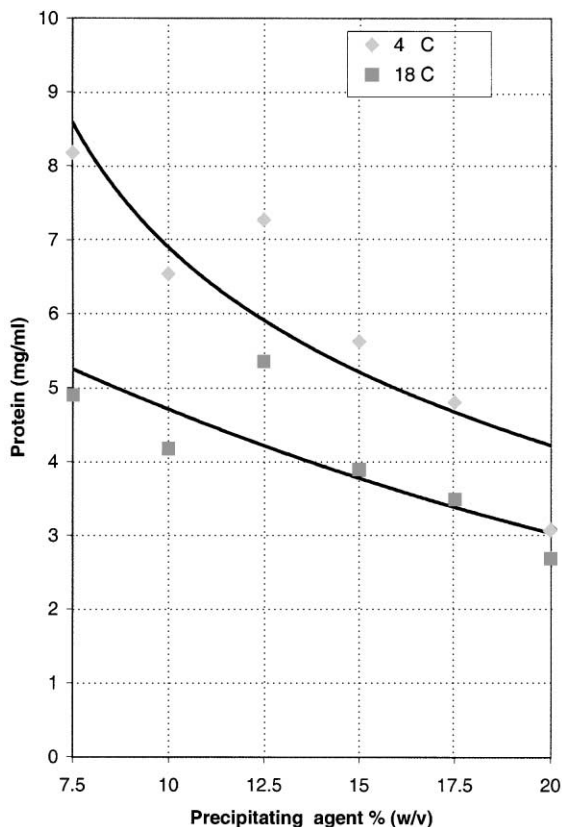


Fig. 5. Solubility plots for thaumatin at 4°C and 18°C.

performed at 0.0%, 1.0% and 3.0% precipitating agent, we did not find any crystals at all. Due to this, better crystals could be obtained above 3.0 and up to 7.5% (w/v). This concentration value presumably corresponds to the lower limit of the metastable zone in the Ostwald–Miers diagram [2], while the value of 17.5% (w/v) corresponds to the higher limit value.

The width of the metastable zone is an important parameter when considering appropriate conditions for growing crystals. This width can be obtained plotting the velocity of the crystal growth against the supersaturation.

Having set the limits of the solubility plot, we decided to analyze the long time solution behavior of thaumatin in the metastable region. During the first few hours at 7.5% (w/v) and 10.0% (w/v) (data not shown) of precipitating agent, the thaumatin molecules probably exist principally as

dimers, since the solution conditions appear not to correspond to the metastable zone. When the ionic strength is increased to 12.5% precipitating agent, the equilibrium presumably shifts toward the formation of crystalline aggregates (Fig. 6). Fluctuations in the R_H can be observed. This process seems to be causal and not statistical regarding the Oswald ripening phenomena [12]. The reported fluctuations probably are due to some effects of the experimental set-up. Under steady state conditions, which occur when a large amount of protein is in solution, aggregated protein in the form of dimers, trimers, and higher oligomers can be expected to exist. In Fig. 7, the peaks representing scattering from objects with different radius would seem to suggest this.

In order to have a better understanding of the crystallization process, we calculated all thermodynamic parameters. The difference in the chemical potential was also estimated using two approaches. The first used Eq. (7) (these results are shown in Table 1) and the second used the Donnan equilibrium taking into account the dynamic light scattering data, for this we used Eqs. (8) and (9) (results shown in Table 2). It is worth mentioning that we used the Kratochvil's equation and static light scattering techniques (total intensity light scattering method) to calculate the second virial coefficient [13,14]. At the same time, the values of these thermodynamic parameters (per molecule) were calculated for the crystal as well (Table 3). The overall Gibbs free energy for the nucleation is positive as it is shown in Table 1 that theoretically speaking, under these conditions the nucleation process would not be spontaneous at all. Nevertheless, it is useful to remember that in a chemical reaction, as well as in precipitation reactions, we have two types of ΔG : the energy of activation, where the kinetic process plays an important role and the ΔG of the equilibrium. Due to the way in which the experiment was performed the overall free energy, ΔG , calculated is the ΔG of the equilibrium, so if the value tends to zero, this indicates that the crystallization has been completed. The nucleation is a process that occurs far from the equilibrium, but tends to it. From the results observed in Tables 1 and 2 the trend of the chemical potential to lower

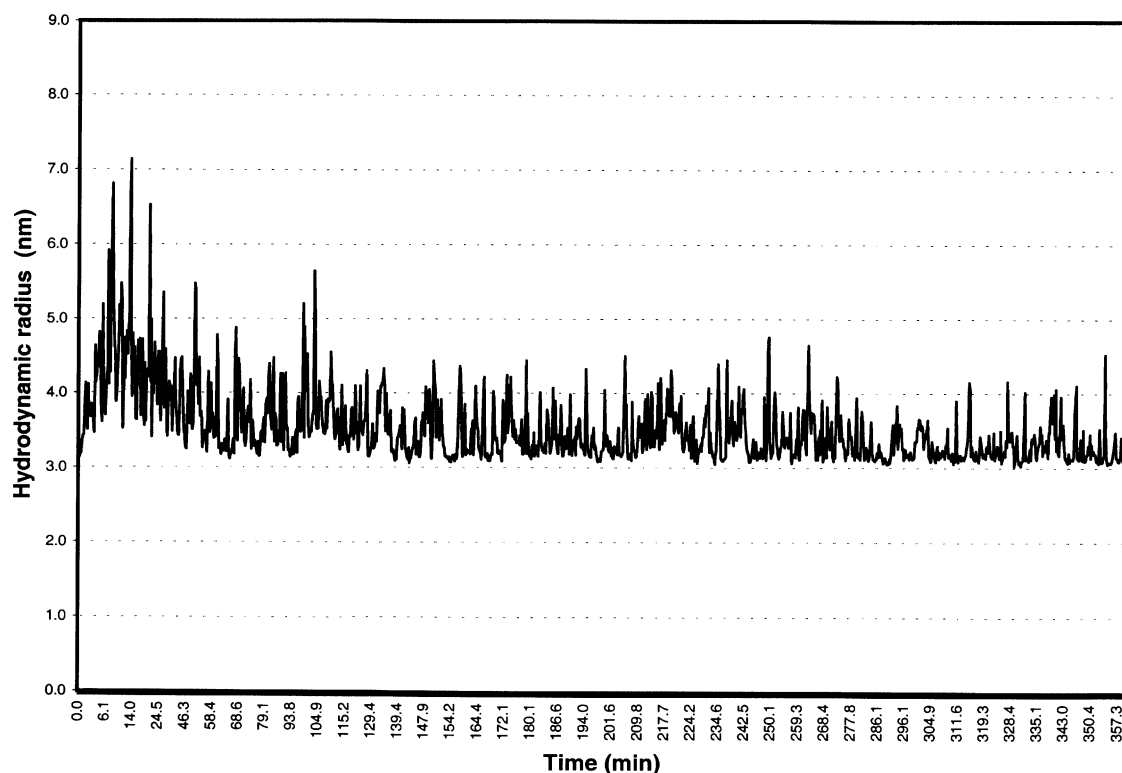


Fig. 6. Study of the behavior in solution of thaumatin by DLS under following conditions: thaumatin 20 mg/ml, phosphate buffer 0.05 M pH 7.0, 18°C and 12.5% (w/v) of sodium potassium tartrate.

value from the higher to the lower concentration of precipitating agent means that the system is closer to the equilibrium at lower concentrations of this precipitating agent. On the other hand, following the proposal published by Grant about the effects of thermodynamic non-ideality in protein crystal growth [14], the Free Gibbs energy for the crystal in a non-ideal case has to be estimated from Eq. (6).

From the plot of $\Delta\mu/kT$ versus $1000/T$ based on Eq. (3) the $\Delta H_{\text{crystal}}$ could be estimated as well. This is the heat released (or absorbed) upon transfer of a molecule A, at pH and concentration C from a fixed point in the solution to a fixed point in the crystal.

From Table 3, using these equations, it is possible to say that the needed energy for transferring a molecule from a fixed point in the solution to a fixed point to the crystal is very low. This means that if the temperature is not well

controlled or even the presence of few impurities the barrier of the potential will be drastically reduced provoking a quick precipitation. These results agree with our observation of thaumatin crystallization done without controlling the temperature, where a precipitation of micro-crystals was obtained. On the other hand, the nucleation theory might provide a better interpretation of these results shown in Table 3 than the classical thermodynamics that we have used. Taking into account this theory, for measuring the interfacial energy on the crystal, some improvements in that regard are described in the next section.

During the editorial review of this manuscript it came to our attention that the hydrodynamic radius of the particles, which form in solutions supersaturated in thaumatin, might be constructed as a measure of the radius of the critical nucleus. Preferably we should have a theory of the distribution of thaumatin monomers, dimers,

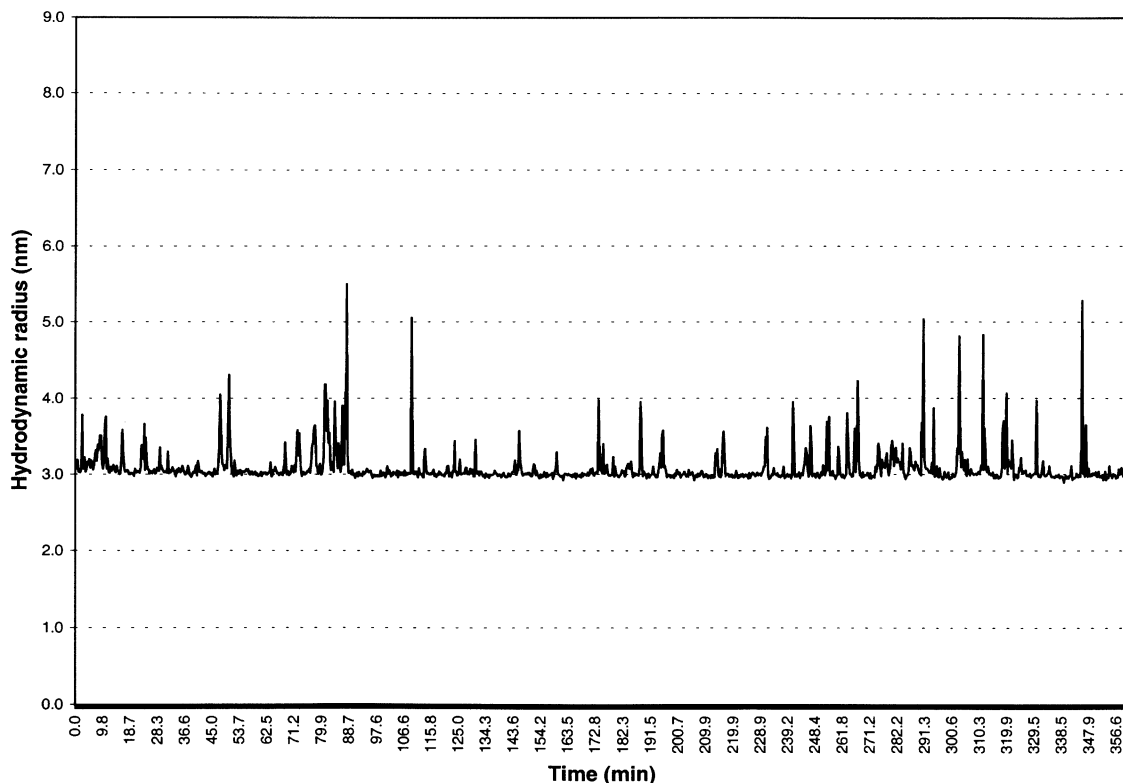


Fig. 7. Study of the behavior in solution of thaumatin by DLS under the following conditions: thaumatin 20 mg/ml, phosphate buffer 0.05 M pH 7.0, 18°C and 17.5% (w/v) of sodium potassium tartrate.

Table 1

Calculated overall thermodynamic parameters (per mol) that affect the crystallization of thaumatin

%NaK tartrate	$\Delta\mu$ 4°C (J)	$\Delta\mu$ 18°C (J)	ΔS (J/K)	ΔH_T (kJ)	ΔG_T 4°C (kJ)	ΔG_T 18°C (kJ)
7.5	2.7×10^{-21}	5.6×10^{-21}	2.0×10^{-22}	24.6	24.6	24.6
10.0	3.8×10^{-21}	5.8×10^{-21}	1.4×10^{-22}	21.3	21.3	21.3
12.5	4.2×10^{-21}	6.2×10^{-21}	9.9×10^{-23}	14.6	14.6	14.6
15.0	4.8×10^{-21}	6.5×10^{-21}	1.1×10^{-22}	17.6	17.6	17.6
17.5	5.44×10^{-21}	6.9×10^{-21}	4.8×10^{-20}	14.7	14.7	14.7
20.0	7.1×10^{-21}	8.7×10^{-21}	1.1×10^{-22}	14.8	14.8	14.8

trimers, etc., up to and including the nucleus of critical size and use this to compute the average radius appropriate for light scattering experiments. In the absence of such a theory, we shall use nucleation thermodynamics to show that knowledge of the apparent hydrodynamic radius can be

used to estimate the interfacial tension between the nucleus and the solution.

Let μ_1^0 be the chemical potential of a protein molecule in the crystal (assumed to be the same as the interior of the nucleus). Let σ be the interfacial tension associated with the contact of the surface

Table 2

Calculated overall thermodynamic parameters (per mol) using non-ideal case

%NaK tartrate	$\Delta\mu$ 4°C (J)	$\Delta\mu$ 18°C (J)	ΔS (J/K)	ΔH (kJ)	ΔG 4°C (kJ)	ΔG 18°C (kJ)
7.5	7.76×10^{-21}	1.74×10^{-21}	6.88×10^{-22}	24.6	24.6	24.6
10.0	1.03×10^{-21}	1.83×10^{-21}	5.71×10^{-22}	21.3	21.3	21.3
12.5	1.15×10^{-21}	2.03×10^{-21}	6.30×10^{-22}	14.6	14.6	14.6
15.0	1.34×10^{-21}	2.20×10^{-21}	6.12×10^{-22}	17.6	17.6	17.6
17.5	1.59×10^{-21}	2.44×10^{-19}	6.08×10^{-20}	14.7	14.7	14.7
20.0	2.45×10^{-21}	3.92×10^{-21}	1.05×10^{-21}	14.8	14.8	14.8

Table 3

Calculated thermodynamic parameters (per molecule) for the crystallization using non-ideal conditions (transferring a molecules from solution to the crystal)

%NaK tartrate	$\Delta G_{\text{crystal}}$ 4°C (J)	$\Delta G_{\text{crystal}}$ 18°C (J)	$\Delta H_{\text{crystal}}$ (J)	$\Delta S_{\text{crystal}}$ 4°C (J/K)	$\Delta S_{\text{crystal}}$ 18°C (J/K)
7.5	-1.840×10^{-20}	-2.138×10^{-20}	4.00×10^{-20}	2.10×10^{-22}	2.10×10^{-22}
10.0	-1.884×10^{-20}	-2.230×10^{-20}	4.96×10^{-20}	2.46×10^{-22}	2.47×10^{-22}
12.5	-1.926×10^{-20}	-2.102×10^{-20}	1.60×10^{-20}	1.27×10^{-22}	1.27×10^{-22}
15.0	-1.982×10^{-20}	-2.268×10^{-20}	3.68×10^{-20}	2.04×10^{-22}	2.04×10^{-22}
17.5	-2.042×10^{-20}	-2.200×10^{-20}	1.08×10^{-20}	1.13×10^{-22}	1.13×10^{-22}
20.0	-2.042×10^{-20}	-2.450×10^{-20}	6.02×10^{-20}	2.90×10^{-22}	2.90×10^{-22}

of the nucleus with the surrounding solution. Consider a spherical nucleus with area A_j containing j molecules. The standard Gibbs energy $G_j^0(n)$ of such a nucleus is ordinarily taken to be

$$G_j^0(n) = j\mu_1^0(c_p) + \sigma A_j. \quad (10)$$

The chemical potential $\mu_1^0(n)$ appropriate to a molecule in this nucleus is

$$\mu_1^0(n) = \partial G_j^0(n)/\partial j = \mu_1^0(c_p) + \sigma[dA_j/dV][dV/dj], \quad (11)$$

where V is the volume. For a sphere, $dA_j/dV = 2/r_j$, where r_j is the radius. Letting $dV/dj = v$, where v is the volume of a thaumatin molecule in the nucleus, Eq. (11) reads

$$\mu_1^0(n) = \mu_1^0(c_p) + [2\sigma v/r_j]. \quad (12)$$

The chemical potential of a thaumatin molecule in the solution is

$$\mu_1(s) = \mu_1^0(s) + k_B T \ln a_1, \quad (13)$$

where $\mu_1^0(s)$ is the solution phase standard chemical potential, a_1 is the thaumatin thermodynamic activity, T is the absolute temperature, and k_B is Boltzmann's constant. At equilibrium, Eqs. (12)

and (13) should be equal, which leads us to

$$\mu_1^0(c) + [2\sigma v/r_j] = \mu_1^0(s) + k_B T \ln a_1. \quad (14)$$

As $r_j \rightarrow \infty$, we approach the thermodynamic solubility, a_1^{sol} , of crystalline thaumatin, so that

$$\mu_1^0(c) = \mu_1^0(s) + k_B T \ln a_1^{\text{sol}}, \quad (15)$$

which serves to evaluate $\mu_1^0(c_p)$. Now use Eq. (15) to replace $\mu_1^0(c_p)$ in Eq. (14). The result is

$$2\sigma v/r_j = k_B T \ln [a_1/a_1^{\text{sol}}]. \quad (16)$$

Noting that

$$a_1 = \gamma_1(c_{p1})(c_{p1}/\tilde{c}), \quad (17)$$

where $\gamma_1(c_{p1})$ is the activity coefficient, c_{p1} is the thaumatin concentration, while \tilde{c} is the concentration in the standard state. Eq. (16) can be rewritten as

$$2\sigma v/r_j = k_B T \ln [(\gamma_1(c_{p1})c_{p1})/(\gamma_1(c_{p1}^{\text{sol}})(c_{p1}^{\text{sol}}))], \quad (18)$$

As it was suggested previously in this manuscript, we should represent $\gamma_1(c_{p1})$ by

$$\ln \gamma_1(c_{p1}) = 2Bc_{p1}, \quad (19)$$

where B is the second virial coefficient. The concept of supersaturation [15] can be defined as

$\beta = c_{p1}/c_{p1}^{\text{sol}}$ and substitute Eq. (19) into Eq. (18). The result is

$$2\sigma v/r_j = k_B T \ln \beta + 2Bk_B T c_{p1}^{\text{sol}}(\beta - 1). \quad (20)$$

It is proposed that we have to identify r_j with the hydrodynamic radius and use Eq. (20) to determine σ . If we know the mass density, d , of solid thaumatin, then $v = M/N_A d$, where M is the molecular weight, and N_A is Avogadro's number. If not, a specific volume of $0.73 \text{ cm}^3/\text{g}$, which is appropriate to most proteins in solution, can be used to estimate v . From your measurements of r_j as a function of S , we should be able to determine σ .

It is not rare if σ depends upon pH and ionic strength. In writing Eq. (10), we have conveniently represented all the surface effects by the term σA_j . If thaumatin is like lysozyme, small crystals (and perhaps also nuclei) carry surface charges, which depend upon pH [16]. The surface densities of these adsorbed ions are in equilibrium with their bulk concentrations in the solution. These equilibria are expected to contribute some terms proportional to A_j to the right hand side of Eq. (10). The coefficients of A_j in terms, which depend upon pH and ionic strength, can be lumped together with σ to make an "effective" interfacial tension.

The "effective" interfacial tension is expected to be temperature dependent. Using the general relation, $s = -\partial\mu/\partial T$, between chemical potential per molecule, μ , and entropy per molecule, s , we can differentiate Eq. (12) to obtain

$$s_1^0(n) = s_1^0(c_p) + s_1^0(\text{surface}), \quad (21)$$

where

$$s_1^0(\text{surface}) = -\partial/\partial T(2\sigma v/r_j). \quad (22)$$

In Eq. (21), $s_1^0(n)$ is the entropy per molecule for formation of a nucleus of radius r_j , $s_1^0(c_p)$ is the molecular entropy of formation of the crystal (this is probably not known), while $s_1^0(\text{surface})$ is the entropy required to establish a molecule in the surface. If v has the units $\text{cm}^3/\text{molecule}$, then the entropies are on a molecular basis. Since $2\sigma v/r_j$ is free energy, one can go on to calculate the enthalpy per molecule in the surface, $h_1^0(\text{surface})$, using

$$2\sigma v/r_j = h_1^0(\text{surface}) - T s_1^0(\text{surface}). \quad (23)$$

The reference state for both $h_1^0(\text{surface})$ and $s_1^0(\text{surface})$ is an infinite crystal with a flat surface.

4. Conclusions

The overall values of ΔG , ΔH reported in this contribution have shown how far from the equilibrium the system is after crystallization. This expressed to some extent that after obtaining the crystals, we have to wait for a long time to be sure that the quasi-equilibrium is reached. Additionally, the use of dynamic light scattering could help us to estimate not only certain important thermodynamic parameters in the crystallization process, but also to estimate the width of the metastable zone. This is particularly useful in order to grow bigger crystals for X-ray analyses.

This work showed a novel approximation to the study of the metastable zone and crystallization behavior of thaumatin by DLS combining these data for predicting some thermodynamic parameters and a novel approach using the nucleation theory for calculating in detail the surface energy for any molecule for a growing crystal. More experiments must be done with other proteins in order to see how physical–chemical factors (such as pH, temperature, etc.) affect in different ways the crystallization process. For thaumatin, we have demonstrated that the ionic strength is crucial to be taken into account at certain temperatures. Although, at the beginning in our experiments the controlled temperature did not have a remarkable influence (by simple observation of crystal morphology) on the crystal obtained, this was important where the quality of crystals was studied by X-ray diffraction.

Finally, additional experiments need to be designed in order to know which is the minimum number of molecules required to reach the critical nucleus; this would be necessary to control the nucleation (inside the metastable zone) from the experimental view point. A kinetic study of the nucleation process will be done in order to know the value of the activation energy of the crystallization. Future investigations focused on analytical biochemistry will help us to be sure if our

system could be in a total equilibrium after obtaining crystals or never will be.

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