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Thaumatin crystallization in hanging drop and in thin layer by vapour diffusion method

D.S. Tsekova

Department of Organic Chemistry, University of Chemical Technology and Metallurgy, 1756 Sofia

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Crystallization of the protein Thaumatin in a drop and in a thin layer at hanging drop set up has been studied. Two main orientations of its tetragonal crystal were observed for crystals formed and grown on a flat foreign surface. They were well shaped inside the drop and show habitus changes as well as depression formations when growing in a thin layer. Crystals nucleated and grown at the end of the layer, often in aglomerates, also show specific morfology.

Keywords: Protein crystallization, Thaumatin, hanging drop method, solution layer, morphological (in)stability

INTRODUCTION

Crystallization of biological macromolecules is important for the X-ray diffraction studies, but it is also intensively exploited topic because of the efforts to understand the crystallization process. Biological macromolecules crystallize usually from complex aqua solutions with specific parameters like type and pH of the buffer, ionic strength, inorganic and organic additives and concentrations of all of the ingredients [1,2]. Developments in crystallization methodologies, protocols, and reagents are also facilitating crystallization efforts. The most often applied approach used for obtaining protein crystals is the vapour diffusion one, particularly as a hanging drop method. There are many publications relating to the application of this method and very few considerations of the processes that happen in such systems [3,4]. In this set-up several areas with different influence on the protein solution behaviour exist: bulk, contact of protein solution with the cover slide from one side and with the air – from the other side, as well as simultaneous contact of the protein solution with both solid surface and air. Studies of the influence of the cover slide surface, in some cases additionally treated to obtain new templates, as well as other solid foreign substrates, on the crystallization behaviour of proteins are published by many research groups [5]. It is well known that heterogeneous nucleation is easier than homogenous, so in case of attraction between a foreign surface and protein molecules, crystallization is facilitated. Kaishev developed a model, to show that heterogeneous surface nucleation is easier than in the bulk, and also that the nucleation in a concave corner is easier than the surface one [6].

Here, crystallization of the protein Thaumatin in a hanging drop and in a solution layer again in the hanging drop set-up is presented. Solution layer, instead of hanging drop is a modification which is not recorded up to now in the literature. Results obtained concern microscope observations of the grown at these conditions crystals. Thaumatin is a sweet protein with molecular weight (Mw) of 22 kDa, 207 amino acids and isoelectric point of 12 [7]. Even it was crystallized in several different singonies, the most often used conditions for crystallization are from buffers with pH around 7 and the presence of tartaric acid or its sodium/potassium salts. Crystals grown and examined in this report were obtained also following this protocol and belong to the tetragonal space group P41212.

MATERIALS AND METHODS

Materials

Thaumatin from Thaumatococcus daniellii (T-7638.) was purchased from Sigma-Aldrich:. Salts, buffers components, all used solvents were analytical-grade reagents (Sigma, Merck). Cover Glass 18x18 mm – Borosilicate glass, VWR (Cat No 631–0120).

Crystallization experiments were performed using 48 wells Linbro tissue boxes for vapour diffusion crystallization in hanging drop. Crystals

* To whom all correspondence should be sent:
E-mail: d_tsekova@uctm.edu

were observed by optical microscope Olympus BX51M equipped with a CCD video camera.

Cover slides cleaning

Glass slides were cleaned with a hot mixture of concentrated nitric and sulphuric acids (3:1 concentrated H_2SO_4:HNO_3) at 50–60 ºC and then rinsed in deionized water until removing the acidic mixture. The washed slides were dried in an oven at 120 ºC and after that cooled to room temperature.

Solutions used for protein crystallization

Thaumatin crystallization solutions were in 50 mM PIPES, pH=6.8, sodium potassium tartrate in the interval 0.34M to 1 M and Thaumatin from 16 to 75 mg/ml.

All solutions of protein and salts were prepared in appropriate buffer concentrations and were filtered prior to mixing for crystallization through 0.22 µm porous filters.

Concentrations are defined according balance data for the solute and data for final solution volume presented in mg/ml, percent [%] or molar [M] units.

Hanging drop crystallization

The hanging drop method was performed in two variants – classical – protein solution is in the shape of a drop [1,2] and in a thin layer (see Figure 1).

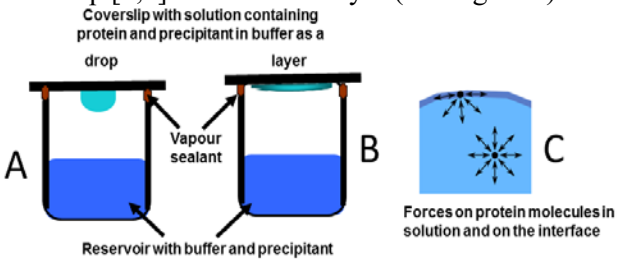

Fig. 1. Hanging drop crystallization set-up: **A.** in the classical (drop), and **B.** new modified variant (layer). **C.** Protein molecules in the bulk and on the interface. Intermolecular forces that arise between two equal molecules are demonstrated by arrows.

The set-up used consisted of 2-5 µl protein containing solution placed on a clean or a salinized glass cover slide and stabilised over the reservoir solution of 200-500 ml. The reservoirs did not contain any protein. Crystallization occurs only in the protein solution on the cover slide. Normally, the initial relation of precipitant concentration in the protein solution (C_A) versus that in the reservoir ($2C_A$) is 1:2, which during the equilibrium leads to the protein solution volume shrinking twice (because of water evaporation) and adequate increasing of its supersaturation. In the classical variant the protein solution is just dropped on the cover slide, which immediately is reversed and sealed over the reservoir.

A modification has been applied in the shape and the disposal of the protein solution for this study. Dropped by means of automatic pipette protein solution was purposely spared on the cover glass surface. In this procedure, the real spreading depended also on the hydrophobicity of the surface. The diameter of the layers in the described here experiments was in the range 5 – 8 mm and adequate layer thickness: 50–250 µm.

In this set-up several important regions can be recognized (see Figure 1 C) – first - in the bulk, where the supersaturation is the main factor and second - on the interface, where solution contact either the surface of the cover slide or the air, and interactions with the foreign surface can have contribution to the process of crystal formation [5]. Besides these two cases of bulk and „solution-foreign surface“ interactions, we can distinguish a third possible location of protein molecules taking part in a nucleus formation – „solution-glass surface-air“, which is the circus line around the layer, laying on the cover slide. Interactions of the protein molecules situated exactly on this line differ from above commented cases – in the bulk and on smooth substrates. It is well known and also visible in the Figure 1 A and B that areas of contact of protein solution: „cover slide surface-solution“, „air-solution“ and „solution-glass surface-air“, in the solution layer variant are several times more extended than in the drop and it is on the account of the bulk interactions in the solution.

RESULTS AND DISCUSSIONS

Bulk crystallization of Thaumatin.

Crystals nucleated and grown at the conditions used and presented above, belong to the P41212 tetragonal space group. Typical habitus observed for such formed crystals is presented in the Figure 2. Bulk nucleated and grown crystals develop full number of faces typical for this space group, like it is presented in the Figure 2A. Of course, when crystal growth is hampered by different obstacles this shape is usually changed in various ways – faces smoothness violation, change in the number of appeared faces and so on.

Inside the droplet or the layer contacting with a flat foreign surface

The influence of solid surfaces, having different chemical composition and surface properties on the

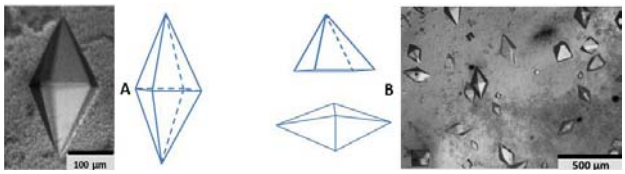

Fig. 2. Tetragonal Thaumatin crystals formed in a hanging drop. **A.** Bulk nucleated and grown crystal – photo and depicted crystal morphology. **B.** The most often revealed morphologies, containing reduced number of faces – depiction and photo. Crystals grown from 50 mM PIPES, pH=6.8, sodium potassium tartrate 0.5 M and Thaumatin 26 mg/ml.

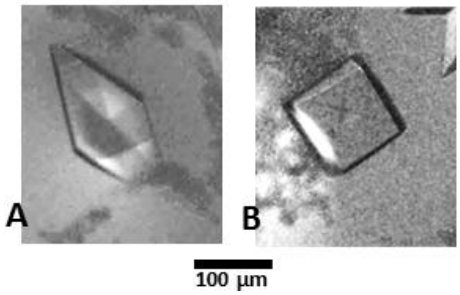

Fig. 3. Thaumatine crystals grown in a thin layer vapor diffusion. Two different orientations are shown, corresdponding to these given in the Figure 2B. Crystals grown from Thaumatin of 16 mg/ml and sodium potassium tartrate 0.34 M in 50 mM PIPES, pH=6.8, solution layer on a glass slide covered by phenyl groups (see [8]).

crystallization of Thaumatin in consideration of number and sizes of crystals, was published as a different study [8]. Here attention is paid on the morphology of the crystals formed on an alien surface. Obviously the heterogeneously formed and grown on flat surfaces crystals develop reduced number or particularly developed faces like these ones presented in the Figure 2B. The same phenomena – growth of different orientations of crystals formed and grown on foreign surfaces was found for lysozyme and ferritin [9].

When the growth happens in a thin layer the lack of space reflects on the shape of the growing crystal. First of all, as it is visible from the Figure 3, apexes and edges necessary to have pyramidal constructions, like these presented in the Figure 2 miss here, instead new „faces“ parallel to the substrate tried to appear. Another important element in these crystals is the lack of smoothness of these „new faces“ and depression formations. This phenomena called „morphological instability“ was noticed before for other two proteins - lysozyme and ferritin crystals growing in a very thin (40 µm) glass cell [10] reaching during their growth a phase boundary. Actually papers concerning this polyhedral instability in case of

Fig. 4. Steps generated from the apexes that move towards the central part of a facet. The stability (flatness) of this face is determined by the step advance velocity and the step generation rate at the corners.

protein crystallization are limited in number [10,11]. More reports have been published for low molecular systems, where various attempts have been made to understand and predict morphological stability of growth of polyhedrons from solution [12].

When a polyhedral crystal grows from solution, the solute concentration around it is no uniform distributed, but the highest is around the edges and the lowest is in the central parts of the faces. That is why it is believed that 2D nucleation process of new layers is realized at the edges and continues propagation as new crystal layers towards the center of the face (see Figure 4). In the Figure 4 it is indicated how the steps generated from the two neighbouring corners are moving in opposite directions. When two steps meet, step annihilation occurs and a new facet is formed.

Chernov's theory for polyhedral stability [12a] is based on the idea, that the concentration inhomogeneity is compensated by the change of the kinetic coefficient in the center of the crystal face. He suggested: $\sigma_{corner}k_{corner} = \sigma_{center}k_{center}$, where σ is the supersaturation, given as a difference between real concentration in the corner (C_{corner}) or center (C_{center}) and equilibrium one (C_e); ($C_{corner} - C_e$ or $C_{center} - C_e$), and k is the kinetic coefficient. This compensation would work when the difference $C_{corner} - C_{center}$ is in a reasonable range, suggested to be lower than 10-20% [11]. If the solution becomes sufficiently thin, solute transfer along the growing face will be hampered and this difference will become higher, then the spreading speed of the steps will be low. As the steps are continuously generated from the corner of the crystal then the step density will be much higher around the corners decreasing toward the central part, so the face will lose the flatness and will develop depression in its center.

Depressions formed in these crystals, as it is well visible from the photos in the Figure 3, are polygonysed and do not look as rough morphological defects, which is most probably a result from the slow kinetics of protein crystal growth.

Crystals nucleated and grown on the contact area „solid surface-protein solution-air“

When a solution layer is used, often crystals formed on this border line (solid surface-protein solution-air) are many and sometimes agglomerate during their growth if they are very close each to other (see Figure 5). Reasons for that are more than one. According the theory, nucleation of crystals in

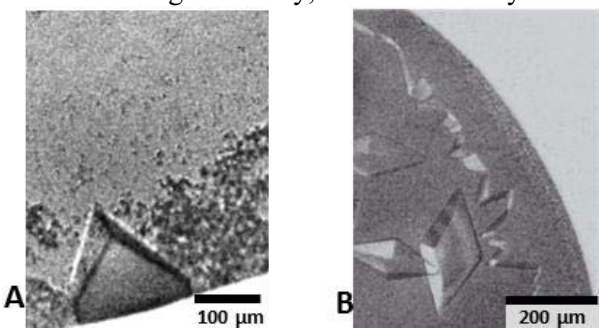

Fig. 5. Crystals nucleated and grown at the end of the solution layer. **A.** A monocrystal. Besides the reduced number of faces, the developed morphology also much differ than typical habitus of tetragonal Thaumatin crystals. **B.** Agglomerations surrounding the solution layer. Growing crystals merge and there is no visible boundary between different crystals.

a concave corner is even easier than on a smooth foreign surface [6] so many crystals are expected to be nucleated at this boundary. Although air cannot be considered as solid substrate, the forces acting on the interface solution-air favour increasing the concentration of molecules with hydrophobic regions on their surfaces [13,5h]. So to some extent the effect of the interphase can be expected to be similar to the hydrophobic surface. Therefore, the nuclei formed and grown on the border line – „substrate-solution-air“ most probably represent examples of crystals that nucleate in the concave corner [6], which are even smaller and have less number of faces than these formed on the surface. Also, right there it is expected the increasing in the local concentration (supersaturation) to be most pronounced because of the bevelling the layer, which causes additionally significant change in the morphology of growing there crystals.

CONCLUSIONS

The results obtained show that the crystallization of proteins is highly dependent on the presence of phase boundaries in the solution, e.g. solid substrates, air bubbles, solid impurities like dust, synthetic or natural fibres and so on. They can be used both to facilitate nucleation and to track the growth of crystals, which is important for understanding the fundamental process of crystallization of proteins.

Depression formation on the surface of crystals growing at a phase boundary where the diffusion supply is hampered confirms that growth of the crystal face occurs by 2D nucleation and propagation of separate monolayers.

Results obtained reveal also that the equilibrium shape of crystals grown depends on where the nucleus was formed, as it was considered and derived by Kaischew [6]. Indeed, recent studies have shown that crystallization of some proteins and other low molecular compounds passes through two phases - liquid dense clusters and then solid nucleus inside the liquid phase [14], however, the observed phenomena clearly show that the surfaces have an impact on both the process of nucleation [5] and the shape of crystals further developed.

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КРИСТАЛИЗАЦИЯ НА ТАУМАТИН ВЪВ ВИСЯЩА КАПКА И В ТЪНЪК СЛОЙ ЧРЕЗ ПРИЛАГАНЕ НА ПАРНО ДИФУЗИОННИЯ МЕТОД

Д.С. Цекова

Катедра по органична химия, Химикотехнологичен и металургичен университет, София 1756

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(Резюме)

Изследвана е кристализацията на белтъка Тауматин в капка и в тънък слой по метода на висящата капка. Две основни ориентации на неговите кристали от тетрагоналната пространствена група са наблюдавани, типични за случаите на образуване и растеж върху плоска подложка. Кристалите са добре оформени, когато са нараствани в капка и с променен хабитус, често с образуване на вдлъбнатини, когато са нараствани в тънък слой. Образуваните и нарастнати в края на слоя кристали, нерядко групирани в агломерати, също проявяват специфична морфология.