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ORIGINAL RESEARCH PAPER

OBTAINING LYSOZYME SPHERULITIC FORMS AT AMBIENT TEMPERATURE USING PYRROLIDINIUM OCTANOAT AS IONIC LIQUID ADDITIVE

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Pyrrolidinium octanoate carboxylate ($Py^+C_nH_{2n+1}COO^-$; PyO in abbreviation) was used as additive for advanced crystallization of Lysozyme protein, to investigate the impact of protic ionic liquid on the protein crystal morphology. The ionic liquid was synthesized by acidic-base Brönsted neutralization, and its purity was checked by HPLC. The protein crystallization was made through the method of vapour diffusion with hanging drops. Crystallization experiments of Lysozyme with the addition of PyO were performed at 0.4 M PyO and respectively 1.6 M. The morphological of spherulitic forms of Lysozyme in aqueous solutions of PyO protic ionic liquid was investigated by optical microscopy after trials were incubated at ambient temperature (18-20°C), in various growth periods (3 days and 1 week). Hanging-drop vapour diffusion crystallization experiments with the addition of 0.4 M of PyO show that Lysozyme crystallized in type I spherulitic form. This is assumed to be a result of heterogeneous nucleation, with thin needles radially growing outward from a more or less spherical particle. Hanging-drop vapour diffusion crystallization experiments revealed that the addition of 1.6 M of PyO led to a second type of spherulitic form of the Lysozyme.

Keywords: protic ionic liquid, lysozyme, crystallization, additive, spherulites

Introduction

Proteins are an essential part of every biological process and understanding biological processes leads to explain the basis of life itself. Protein crystallization has many important applications in two important biotechnological fields: food and pharmaceutical industry.

Proteins frequently precipitate in the form of spherulites, also known as “sea urchin” crystal morphology (Coleman *et al*, 1960; Tanaka *et al.*, 1997; Muschol *et*

al.1997; Heijna *et al.*, 2007; Granasy *et al.*, 2005). Morphologies of the spherulites were observed mainly by optical microscopy. Spherulites start to grow in the direction of crystalline needles, oriented in a radial direction (Coleman *et al.*, 1960; Heijna *et al.*, 2007; Granasy *et al.*, 2005). This explains their almost spherical shape with various forms of densely branched with polycrystalline solidification patterns (Granasy *et al.*, 2005).

According to the nucleation mechanism, spherulites are divided into two morphologic categories (Coleman *et al.*, 1960; Heijna *et al.*, 2007): Type I is the result of heterogeneous nucleation, with thin needles radially growing outward from a more or less spherical particle (Figure 1a), and type II is the result of homogeneous nucleation (Figure 1b).

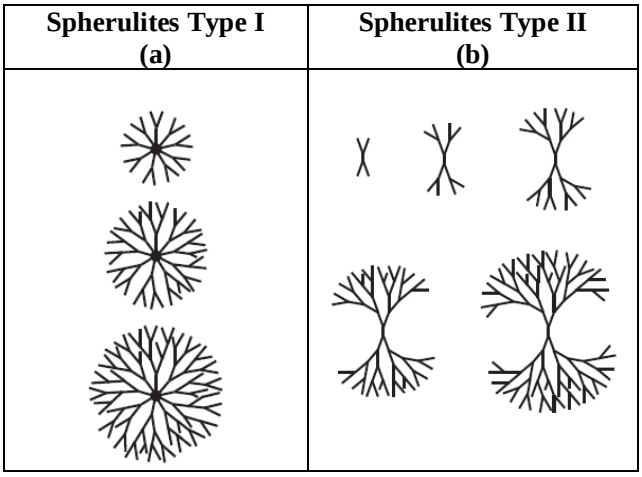


Figure 1. Schematic representation of the formation of spherulites by Coleman *et al.*, 1960 and Heijna *et al.*, 2007: a) Type I spherulites are formed by heterogeneous nucleation on a foreign particle. Crystalline needles radiate outward from this nucleus. b) Type II spherulites forms by the homogeneous nucleation of a single crystalline needle

Heijna *et al.* (2007) show that spherulites frequently coexist with monoclinic crystals, which in most cases appear before the spherulites do. For example, lysozyme spherulites and a monoclinic lysozyme crystal coexist in the same aqueous solution, grown at 18°C from a 15 mg/mL HEWL, 0.6 M $NaNO_3$ and 0.05 M $NaCH_3COO/HCH_3COO$ buffer solution at pH 4.5. When the temperature was raised to 28°C, the spherulites dissolved, while the monoclinic crystals continued to grow (Heijna, *et al.*, 2007).

In protein crystal growth, spherulites are often observed (Echalier *et al.*, 2004; Guilloteau *et al.*, 1996). The mechanisms forming them have not been studied in detail and are shown to be controlled by adding additives (Liu *et al.*, 2002) or by changing supersaturation (Li *et al.*, 2005). In order to prevent spherulitic formation,

a good solution seems to be the increasing of the solute concentration rather than the salt concentration (Heijing *et al.*, 2007).
In this study are presented the possibilities of obtaining the lysozyme spherulites in an aqueous medium with ionic liquid as agent for protein crystallization.

Ionic liquids (ILs) are finding increasing application in the biochemical field in the past decade (Wasserscheid *et al.*, 2003). There are ambient temperature liquids consisting entirely of ions, called ambient molten salts. They are liquids at temperatures below 100°C, exhibiting a great thermal stability, a high solubility power, and extremely low vapour pressure. ILs are good solvents for a wide range of both inorganic and organic materials and are divided into two classes: protic and aprotic ionic liquids. The protic subgroup in the class at ambient temperature molten salts known as ‘‘Protic Ionic Liquids” (PILs) is formed by the transfer of protons between a Brönsted acid and base (Anouti *et al.*, 2008). By the choice of the anion and the cation in their structure, their chemical properties are widely tunable (Wasserscheid *et al.*, 2003) indicating that they have a huge potential for the crystallization of different proteins (Garlitz *et al.*,1999).

In recent years PILs are successfully applied for the crystallization of the insoluble proteins with a low solubility in water (Byrne *et al.*, 2009; Mann *et al.*, 2009).

Materials and methods

Materials for protic ionic liquid synthesis

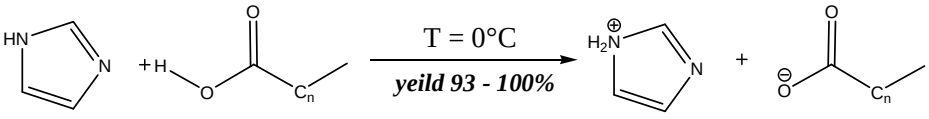
Pyrrolidine (redistilled, 99.5 %) and octanoic acid (98 %) are commercially available from Aldrich and are used without further purification. Lysozyme from chicken egg white BioUltra, lyophilized powder, 98 % (SDS-PAGE), 40,000 units/mg protein are commercially available from SigmaAldrich. Water was purified with a Mili-Q MX (>15 MΩcm) water system.

Materials for protein crystallization

TRIS hydrochloride ($C_4H_{11}NO_3$.HC)l, pH = 7-9 (from SigmaAldrich), purified water (>15 MΩcm), hen egg white Lysozyme, Ly (HEWL from Sigma-Aldrich, 40 000 KDa) and PyO synthesized in our lab.

Methods of preparation of [Pyrr][CnH2n+1COO], n = 7 (pyrrolidinium octanoate protic ionic liquid)

The pyrrolidinium alkyl carboxylates [Pyrr][C_nH_{2n+1}COO](n=7) are synthesized Brönsted acid). The molar ratio of amine/acid is 1:1, according to the procedure described elsewhere (Figure 1) (Anouti *et al.*, 2008).



pyrrolidine n = 7

Figure 1. Neutralization chemical reaction of pyrrolidine (Brönsted base) by the octanoic acid (Brönsted acid) to prepare [Pyrr][C_nH_{2n+1}COO], n=7 (pyrrolidinium octanoate carboxylate)

The carboxylic acid was added slowly to amine while stirring in a tree-necked round-bottom flask immersed in an ice bath and equipped with a dropping funnel and then the stirred to T = 298 K for 4 h. The colourless (pyrrolidinium) ionic liquid was obtained.

Method of preparation of the crystallization solutions

Method of preparation of buffer stock solution

A buffer stock solution of TRIS hydrochloride ($C_4H_{11}NO_3$.HCl, pH = 7-9) was made in deionised water (>15 MΩcm) to result a 0.1 M TRIS hydrochloride solution of pH 7-9.

Preparation of the protein solution

In order to prepare the protein solution, lysozyme was used as source material for crystal growth after dissolving 50 mg in 1 mL buffer TRIS hydrochloride solution.

Preparation of the crystallant solution

A crystallant solution was made in the buffer stock solution of 0.1 M TRIS hydrochloride. 0.2 M ammonium sulfate and 25 % PEG 3450 were used as precipitate agents.

Preparation of the mother liquor

PyO was used as crystallization additive, by using two different concentrations: 0.4 M and respectively 1.6 M in the crystallant solution. Those, two mother liquor were obtained.

Droplets deposition method

The crystallization droplets consisted of 5 µL of protein solution and 5 µL of mother solution containing the PyO additive.

First, the 5 µL droplet of the protein solution is placed on a glass slide and is covered by the second droplet of the mother liquor. The glass slide is placed down to the tank drop containing 0.5 mL mother solution (Figure 2). The assembly is placed at room temperature in a dry place. After 2 days, the trials were analyzed by optical microscopy.

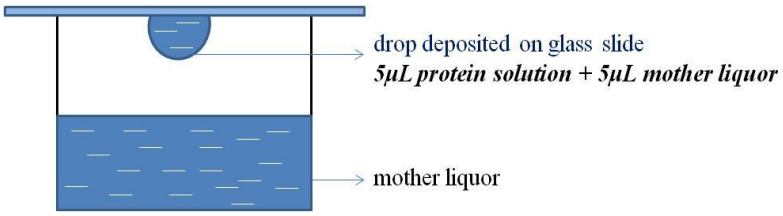


Figure 2. The hanging-drop vapour diffusion method

Coulometric Karl-Fischer titration

Ionic liquid was analyzed for water content using coulometric Karl-Fischer titration prior to any crystallisation experiments. The water content of the final product before crystallisation was 3650 ppm.

HPLC

The synthesis of ILs was checked by HLPC spectrum using a Chromatograf Finnigan Surveyor (Thermo Scientific) equipped with diode detector and autosampler. Analyses were performed using an analytical column BDS

HYPERSIL C18 (150x4.6 mm, 5 µm porosity), and acetonitril 100% as internal standard and as solvent for the samples. The sample was injected in HPLC from the reaction mixture.
Figure 3. shows that the starting materials, pyrrolidine and carboxylic acid, have different retention times in comparison with the new compound, the ionic liquid, and this confirms that the reaction took place.

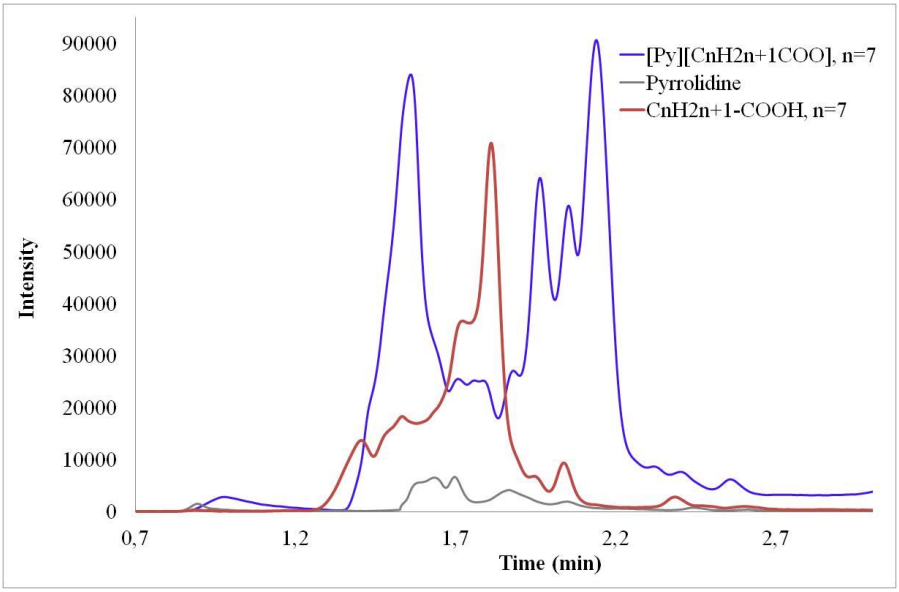


Figure 3. HPLC spectra of PyO (in blue) and their precursors: pyrrolidine (in gray) and octanoic acide $C_nH_{2n+1}COOH$ (n=7) (in red)

Optical microscopy

The formation of crystalline structures in aqueous solutions containing PyO as protic ionic liquid agent was investigated by morphological observation techniques using optical microscopy (Olympus BX41).

Results and discussion

In this study, observations of the spherulites forms grown for Ly in aqueous solutions based on PyO as crystallization additive were made by optical microscopy. There were used two different concentrations of PyO protic ionic liquid: 0.4 M and respectively 1.6 M.

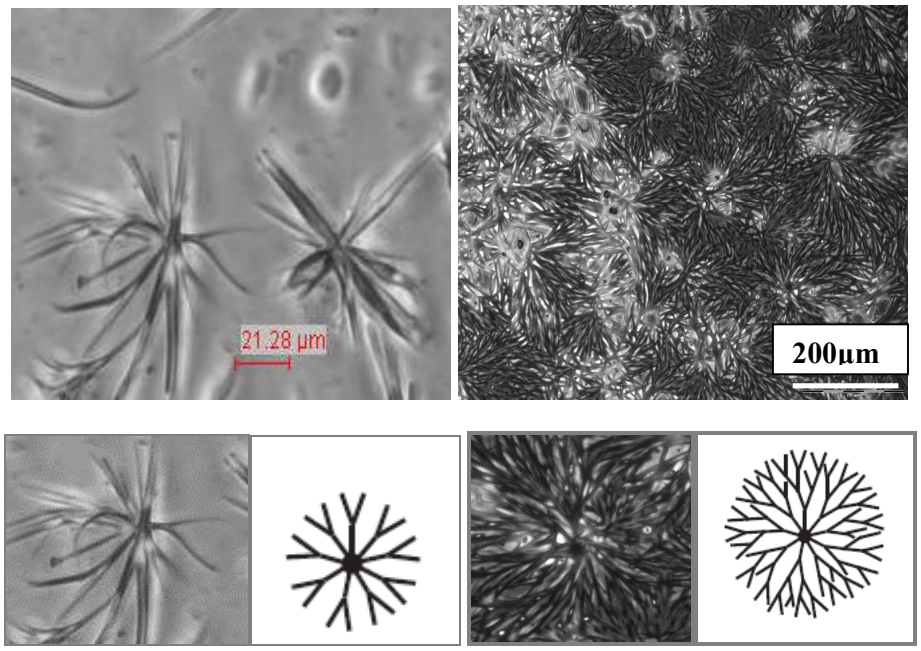


Figure 4. The morphology of two-dimensional Ly spherulites grown at 18-20°C in 0.1 M TRIS hydrochloride solution of pH 7-9 and cristallant agent based on **0.4 M PyO**. The concentration of the protein solution is 50 mg/mL Ly. a) spherulitic growth, 2 days after droplets deposition, b) spherulitic growth, 1 week after droplets deposition

Figure 4. shows the morphology of two-dimensional Ly spherulites grown at 18-20°C in 0.1 M TRIS hydrochloride solution of pH 7-9 based on a cristallant agent with **0.4 M PyO** protic ionic liquid added.

As it can be observed after a 2 days' droplet deposition spherulites forms appear (Figure 4a). One week after droplets deposition, their ramification got enriched (Figure 4b). For both examples, spherulites started to grow in a radial direction which explains their almost spherical shape and confirms the growth described in literature by Heijna *et al.* (2007) for this crystalline species. According to the nucleation mechanism, spherulites shape obtained in this case are type I and are formed by heterogeneous nucleation.

Morphologies of spherulites are in accordance with those found in literature (Coleman *et al*, 1960; Tanaka *et*

As it can be observed, after a 2 days' droplet deposition, the nucleation centers of the spherulites forms appear (Figure 5a). One week after droplets deposition, the spherulites ramifications get enriched in a radial direction, and their final form will be type II (Figure 5b), according to the nucleation mechanism described by Heijna *et al.*, 2007 and Granasy *et al*, 2005. In this case, spherulites are formed by homogeneous nucleation of a single crystalline needle, which subsequently grows and branches off (Coleman *et al*, 1960; Tanaka *et al.*, 1997; Muschol *et al.*, 1997; Heijna *et al.*, 2007; Granasy *et al.*, 2005). Heijna *et al*. (2007) explain that a single

needle can nucleate either homogeneously or heterogeneously, after which the type-II spherulites form.

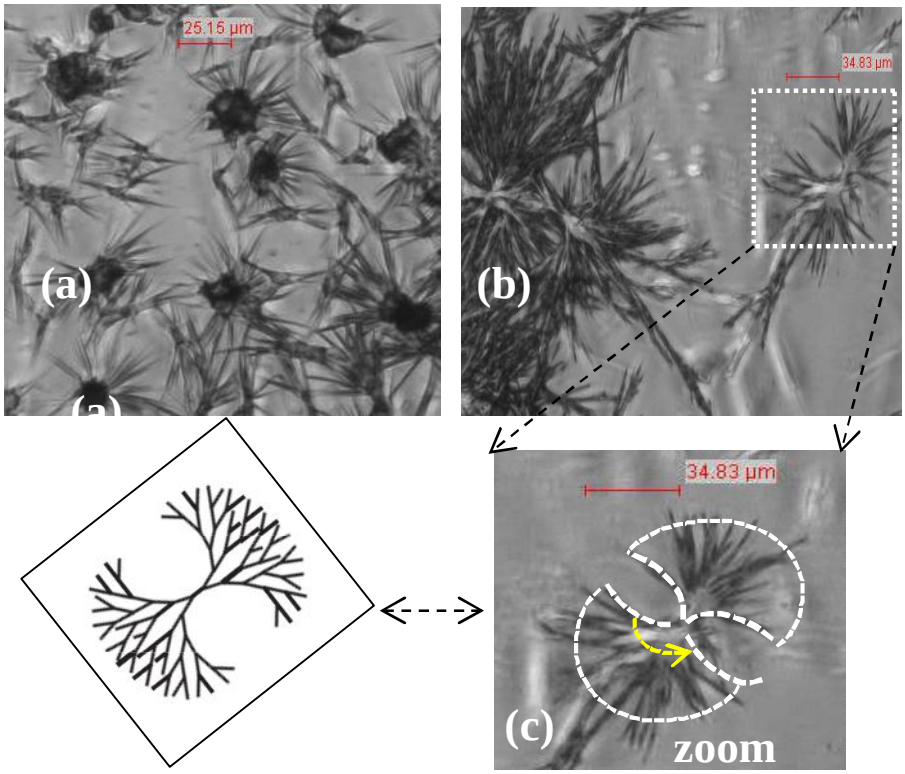


Figure 5. The morphology of two-dimensional Ly spherulites grown at 18-20°C in 0.1 M TRIS hydrochloride solution of pH 7-9 and cristallant agent based on **1.6 M PyO**. The concentration of the protein solution is 50 mg/mL Ly: a) nucleation step, 2 days after droplets deposition, b) spherulitic growth, 1 week after droplets deposition, and c) zoom on the type 2 spherulites form, according to Heijna *et al*. (2007)

Conclusions

There were presented possibilities of obtaining the lysozme spherulites in aqueous medium using a protic ionic liquid as agent for protein crystallization. For this purpose, pyrrolidinium octanoate carboxilate (PyO) has been used as crystallization agent in two different concentrations (0.4 M and 1.6 M). The hanging-drop vapor diffusion method was chosen to perform the crystallization experiments, and the formation of spherulites structures was investigated by morphological observation techniques using optical microscopy. The nucleation and the growth of the spherulites ramifications get enriched in a radial direction in accordance with the term ‘spherulite’ that suggests a nearly spherical shape. Two types of spherulites

were obtained in this way: type I by using of 0.4M PyO (lower concentration) and type II when using a concentration of 1.6 M PyO (higher concentration).

Acknowledgments

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