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ARTICLE

Crystallization Control of Thermal Stability and Morphology of Hen Egg White Lysozyme Crystals by Ionic Liquids

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Ionic liquids (ILs) exhibit a variety of properties that make them attractive additives for biomaterials. 1-Butyl-3-methylimidazolium tetrafluoroborate ([C_4mim]BF_4), 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl), 1-butyl-3-methylimidazolium bromide ([C_4mim]Br), and 1,3-dimethylimidazolium iodine-([bmim]I), as additives during lysozyme crystallization, were tested for their effects on the thermal stability and morphology of lysozyme crystals obtained. [C_4mim]Cl was chosen to evaluate the effect of IL addition concentration on the thermal stability of lysozyme. It is shown that the characteristic peak temperature and endothermic enthalpy values (ΔH) for denaturation increase with increasing addition concentration. As for the degradation, peak temperatures decrease, whereas endothermic enthalpy values markedly increase with the rise of [C_4mim]Cl addition concentration. In the case of adding [C_4mim]BF_4, [C_4mim]Br, and [bmim]I, similar thermal behaviors of lysozyme crystals were observed. The effect of ILs on thermal behaviors of lysozyme can be attributed to enhancing crystal contacts, changing conformational stability, or interaction among molecules, as evidenced by difference in crystal growth morphology. This study is especially helpful in controlling the thermal stability of lysozyme crystals and in gaining initial insight into potential crystallization conditions for prescreening ILs that stabilize the protein and other macromolecule crystals.

KEYWORDS: Crystallization; thermal stability; morphology; lysozyme; ionic liquids

INTRODUCTION

Crystallization of macromolecules is receiving much attention because of its industrial and scientific importance in areas of modern molecular biology and biotechnology, particularly in the genetic engineering of proteins and rational drug design (*1*). The wide screening space of crystallization conditions is continually expanded by new methodologies and techniques of growing crystals for the determination of the three-dimensional structure of proteins (*2*). Ionic strength, temperature, pH, type of salt, and additives are just a few of the factors that might influence the outcome of the crystallization experiment (*2*−*5*).

Recently, ionic liquids (ILs) have been very attractive in both academia and industry due to their unique physical and chemical properties with low melting points that can exhibit intrinsically useful characteristics such as a wide liquid range, a negligible vapor pressure, thermal and chemical stability, and chemical tunabilities and recyclability (*6*−*8*). One advantage to the use of ILs is the wide range of possible structures. The applications of ILs related to protein crystallization are being reported because of the potential for productive interaction between ILs and protein. Pusey et al. (*9*) have used ethyl ammonium nitrate as a precipitating agent for the crystallization of lysozyme, and good diffraction crystals were provided. Judge et al. (*10*) investigated the use of ILs as precipitating agents and additives for protein crystallization for six model proteins. The results indicated that ILs produced changes in crystal morphology and significant increases in crystal size in some cases. Lange et al. (*11*), working with imidazolium-based ILs, suggested that ILs that are excluded from the protein surface tend to stabilize protein and promote salting out. Thus, the presence of ILs can also change the intermolecular interactions and crystalline disorder and hence influence the free energy, thermodynamic activity, thermal stability, and bioavailability. Particularly interesting is the use of ILs in biocatalysis (*12*, *13*) to preserve enzyme stability (*14*, *15*).

Imidazolium-based ILs have also been used to enhance protein folding and suppress aggregation (*11*). Given these applications with biomaterials and their ability to participate in ionic, hydrophobic, and hydrogen bond interactions, ILs are potential additives for use in protein crystallization. Some probable mechanisms of the effect of ILs on protein crystallization were proposed as the ability of ILs to influence the interactions between protein molecules and to alter the surface energy of the crystal such that those formed are more defined and a more ordered or higher dimensional solid state is attained (*4*, *9*). Some literature (*9*, *10*) has shown that protein stability can be affected by changes in intermolecular interaction and that thermal stability of a protein can be significantly enhanced with specific ILs. However, to the best of our knowledge, the effect of ILs on the thermal behavior of protein crystals has not been documented yet.

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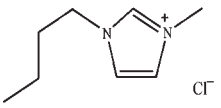
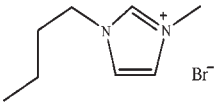
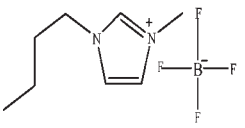
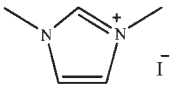
Table 1. Structures and Molecular Weights of Ionic Liquids Used in This Study

ILs	Abbreviation	Structure	Molecular weight
1-butyl-3-methylimidazolium chloride	$[C_4mim]Cl$		174.7
1-butyl-3-methylimidazolium bromide	$[C_4mim]Br$		219.1
1-butyl-3-methylimidazolium tetrafluoroborate	$[C_4mim]BF_4$		226.0
1,3-dimethylimidazolium iodine	[bmim]I		223.9

In the present study, crystallization trials of hen egg white lysozyme were performed and crystals were obtained in the presence of four soluble ILs, 1-butyl-3-methylimidazolium tetrafluoroborate ($[C_4mim]BF_4$), 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$), 1-butyl-3-methylimidazolium bromide ($[C_4mim]Br$), and 1,3-dimethylimidazolium iodine([bmim]I). Differential scanning calorimetry (DSC) was used to analyze the thermal stability of lysozyme crystals obtained in the presence of ILs, aiming to gain some insights into the effect of ILs on the thermal behavior of lysozyme crystals. An additional purpose of this study was to look for a link between the thermal stability of a protein and the performance of ILs and to present ILs as potentially useful additives for crystallization studies and thermal stability improvement of other important proteins.

EXPERIMENTAL PROCEDURES

Reagents. Hen egg white lysozyme (>99% mass fraction purity, according to supplier) was purchased from Genview, USA (Houston, TX). Proteins were used without further purification. Distilled deionized water was used in all of the experiments. The analytical grade anhydrous sodium acetate, glacial acetic acid, and sodium chloride were purchased from Tianjin Kewei Co. of China. The ionic liquids (>99% mass fraction purity) were purchased from Shanghai Cheng Jie Chemical Co. Ltd. of China. The structures of the ionic liquids are shown in **Table 1**.

Batch Crystallization Trials of Model Protein Using ILs as Additives. The batch method was chosen to perform crystallization experiments using known crystallization conditions. The crystallization solution for lysozyme was as follows: 5% (w/w) NaCl, 0.1 M sodium acetate of pH 4.5. The starting material was made up of equal weights of protein solution and precipitant NaCl solution. The known weight ILs were injected into the protein solution. Supersaturated solutions were obtained by mixing protein stock solutions with precipitant solutions. The stirring bar ensured proper mixing and rapid temperature equilibration of the protein solution in the crystallizer. The supersaturated solutions were then left at 4 °C to produce crystals. All conditions of crystallization trials are listed in **Table 2**.

Differential Scanning Calorimetry. A differential scanning calorimeter (Mettler 823e) equipped with a data station (TA analysis) was employed to determine the thermal behaviors of the lysozyme crystals. The temperature axis and the cell constant of the DSC cell were calibrated with indium. A sample of about 4 mg in a punctured aluminum pan was heated at 10 °C/min under a nitrogen purge at 45 mL/min.

Polarizing Microscope. Final samples were observed with a polarizing microscope (Olympus BX51) with an attached CCD video camera, and images were taken to analyze effect of ILs on crystal morphology.

Table 2. Summary of the Effects of Added ILs on Lysozyme Crystallization Trials

IL	NaCl content (% w/w)	pH	IL content (wt %)
$[C_4mim]Cl$	5	4.5	1, 3, and 5
$[C_4mim]Br$	5	4.5	1
$[C_4mim]BF_4$	5	4.5	1
[bmim]I	5	4.5	1

RESULTS AND DISCUSSION

ILs have the potential to serve as bases or acids on the basis of the presence of different cations and anions. Although ions are fully dissociated in aqueous solution, the pH change caused is very small at the low range of IL concentration. Therefore, it is believed that lysozyme crystallizes at constant pH condition in this work. As crystallization additives, there is the possibility that IL concentration influences the strength of specific intermolecular interactions among lysozymes or IL and individual lysozymes. IL $[C_4mim]Cl$, as a representative case, was used to investigate IL addition concentration during crystallization on the thermal behavior of lysozyme crystals. The DSC curves of lysozyme crystals obtained in the presence of IL $[C_4mim]Cl$ at different addition concentrations, are illustrated in **Figure 1**, where two thermal events are observed in each curve. The DSC trace yielded two characteristic endothermic peaks, which were related mainly to thermal denaturation and degradation. The first, large endothermic peak is attributed to thermal denaturation of the lysozyme crystals, and the second, relatively small one corresponds to the degradation of lysozyme. The characteristic peak and endothermic enthalpy values for denaturation and degradation from DSC are listed in **Table 3** and shown in **Figure 2**. From **Figure 2**, it can be clearly discerned that the higher addition concentration favors the increase of thermal denaturation temperature of lysozyme crystals. The determined characteristic peaks and enthalpy values for denaturation varied from 66 to 72 °C and from 80 to 371 J/mol, respectively, and characteristic peak temperature and enthalpy values for denaturation increase with increasing $[C_4mim]Cl$ addition concentration. Additionally, it is also noted that the denaturation enthalpy value (80.03 J g^{1-}) of lysozyme in the absence of $[C_4mim]Cl$ is very low, and the dependence of denaturation enthalpy values on $[C_4mim]Cl$ addition concentration becomes not significant after the addition concentration reaches 3%. With regard to degradation, enthalpy values increase,

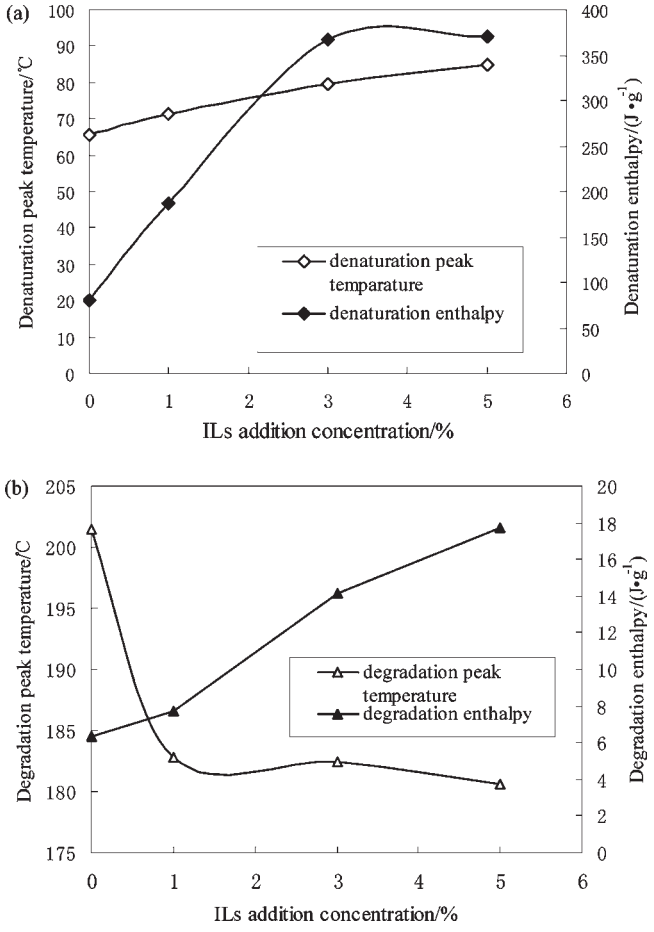


Figure 1. Change of thermal behavior of lysozyme crystals with [C_4mim]Cl addition concentration: (**a**) denaturation; (**b**) degradation.

Table 3. Summary for Dependence of Lysozyme Thermal Stability on IL [C_4mim]Cl Addition Concentration

IL	denaturation peak (°C)	denaturation ΔH (J g^{-1})	degradation peak (°C)	degradation ΔH (J g^{-1})	IL content (wt %)
[C_4mim]Cl	71.44	187.1	182.8	7.760	1
[C_4mim]Cl	79.69	366.8	182.5	14.17	3
[C_4mim]Cl	84.80	371.1	180.6	17.71	5
no additive	65.63	80.03	201.5	6.330	0

whereas degradation peak temperatures markedly decrease with the rise of IL addition concentration. The effect of ILs on the thermal behavior of lysozyme crystal can be related to change interaction among lysozyme molecules in solution.

To identify the effect of IL addition concentration on lysozyme crystallization, the growth morphology observation of crystal was carried out for comparison with the result of the experiments. Compared with no IL, the selected IL [C_4mim]Cl efficiently influences crystallization behavior of lysozyme by providing larger crystals and improving crystal morphology. The morphology of lysozyme crystals obtained under different addition levels is shown in **Figure 3**. From **Figure 3**, it can be seen that crystals were platelike in morphology under IL addition. Obviously, the crystal size was biggest under 1% addition concentration, and the crystal size became smaller and smaller with increasing [C_4mim]Cl addition concentration. Optimization of initial IL addition conditions resulted in significant improvements in crystal size and morphology. Correlation of the observed improvements in crystal morphology with DSC analysis indicated that there can be concomitant results of the lysozyme in solution

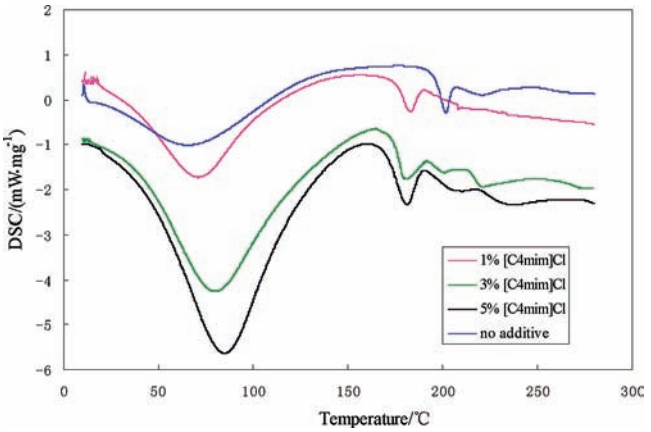


Figure 2. DSC plots of lysozyme crystals under addition of [C_4mim]Cl at different concentrations.

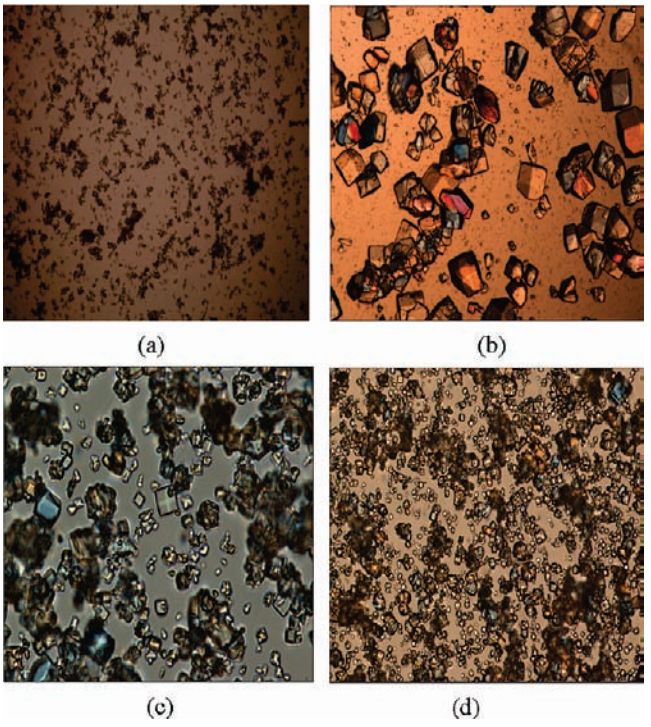


Figure 3. Polarizing micrographs of lysozyme crystals obtained under addition of [C_4mim]Cl at different concentrations: (**a**) no IL; (**b**) 1%; (**c**) 3%; (**d**) 5%.

when ILs are present by enhancing crystal contacts, changing conformational stability, or interaction among molecules.

To further confirm the effect of ILs on the thermal behavior and crystal morphology of lysozyme, [C_4mim]BF_4, [C_4mim]Br, and [bmim]I were chosen as additives to perform crystallization experiments. The DSC curves of lysozyme crystals obtained in the presence of different types of ILs at 1% addition concentration are illustrated in **Figure 4**. As expected, the thermal events are similar in each curve. The characteristic peaks and endothermic enthalpy for denaturation and degradation from DSC curves in the presence of different ILs are listed in **Table 4**. From **Figure 4** and **Table 4**, it can be seen that the thermal stability of lysozyme crystals obtained was markedly influenced by adding ILs during crystallization. The determined characteristic peak temperature for denaturation in the presence of any one of ILs is more than the case of no IL, and the endothermic enthalpy value for denaturation is far more than the case of no IL. With regard to degradation, peak value temperatures with ILs are lower than the case of

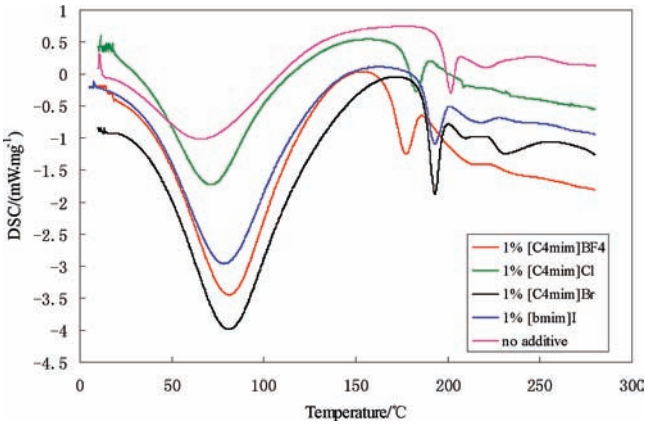


Figure 4. DSC plots of lysozyme crystals obtained in the presence of 1% IL addition concentration.

Table 4. Summary for Effect of IL Types on Lysozyme Thermal Stability

IL	denaturation peak (°C)	denaturation ΔH (J g^{-1})	degradation peak (°C)	degradation ΔH (J g^{-1})	IL content (wt %)
[C_4mim]BF_4	81.21	−229.5	176.6	−9.930	1
[C_4mim]Cl	71.44	−187.1	182.8	−7.760	1
[C_4mim]Br	81.19	−224.8	192.7	−11.42	1
[bmim]I	78.23	−275.2	192.6	−10.47	1
no additive	65.63	−80.03	201.5	−6.330	0

no ILs and peak value temperature with [C_4mim]BF_4 is significantly lower than the case of no IL, whereas endothermic enthalpy values of lysozyme crystals in the presence of ILs are slightly more than the case of no IL. These results signify that a calorimetric approach can be applied to determine useful initial IL addition conditions for prescreening ILs that induce and stabilize the macromolecule crystals.

The effect of ILs on the thermal behaviors of lysozyme crystals can be attributed to change-specific intermolecular interactions among lysozyme molecules or individual ILs and lysozyme by enhancing crystal contacts and changing molecule conformational stability in solution during crystallization, which can influence the nucleation of lysozyme and subsequent crystal growth and eventually influence the crystal morphology. The influence of different ILs on the morphology of lysozyme is illustrated in **Figure 5**. Compared with no ILs, the morphology and size of lysozyme crystals were significantly improved in the presence of ILs. Platelike crystal morphology of lysozyme in the presence of [C_4mim]BF_4, [C_4mim]Cl, and [C_4mim]Br was observed, corresponding to panels **a**, **b**, and **c** of **Figure 4**, respectively. The crystal morphology of lysozyme in the presence of [C_4mim]Cl was the prettiest. As for adding [bmim]I, it is noted that the case is completely different from adding [C_4mim]BF_4, [C_4mim]Cl, and [C_4mim]Br in crystal morphology as shown in **Figure 4d**. Needlelike lysozyme crystals in morphology are observed. The difference between cations [C_4mim] and [bmim] in aqueous solution might be responsible for the shape transition of lysozyme crystal from plate to needle.

The effect of the ILs on thermal stability and morphology of protein crystals is particularly interesting for biological macromolecule material. Macromolecules are structurally dynamic, changing conformation in the presence of ligands. More subtly, macromolecular interactions can be affected by the presence of various added compounds. According to the report by Judge et al. (*10*), ILs themselves do not appear to be salting out the crystal structure of protein. Our present work on the performance of ILs in crystallization solution indicates that ILs in aqueous

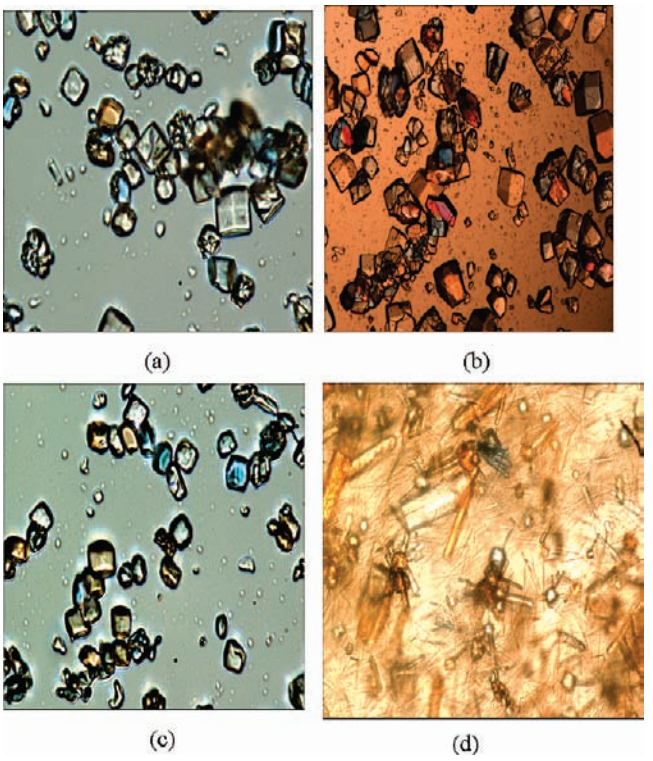


Figure 5. Polarizing micrographs of lysozyme crystals obtained with 1% IL addition concentration: (**a**) [C_4mim]BF_4; (**b**)[C_4mim]Cl; (**c**) [C_4mim]Br; (**d**) [bmim]I.

solutions would play an efficient role in the crystallization of protein. The DSC and morphology analysis may provide an indication of how the native lysozyme behaves under addition at various IL conditions. Shifts in denaturation and degradation peak temperature in the presence of ILs can be used to "quantitate" the effects of the ILs and help to determine optimal concentrations and types of ILs to use for enhancing crystallization. According to the results obtained, the thermal stability and the crystal morphology for protein could be controlled by adding ILs, confirming our initial idea of using the ILs as crystal growth template. The mechanism behind the controlled crystallization by ILs is still far from being fully understood, although some interesting work has been reported in the literature. It is difficult to monitor the solution structure and crystallization process in situ.

The crystallization control of thermal behavior and morphology of protein by ILs follows a promising strategy. In this study, although with a limited set, the range of ILs and their influence on the thermal stability and morphology of lysozyme crystals were examined; the use of instrumental approaches such as the ones discussed here, in conjunction with detailed observation of crystallization behavior, can result in more methodical and ultimately more efficient means of determining crystallization conditions and identifying useful additives so that crystallization of macromolecules can be more rationally defined. This study is especially helpful in providing instruction for conservation of protein and gaining initial insight into potential crystallization conditions for macromolecules. Whether the ILs had any effects on the biological activities of protein will be investigated in later work.

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