Thermal stability of proteins in the presence of aprotic ionic liquids

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ABSTRACT

Thermal stability of lysozyme dissolved in aqueous solutions was examined in the presence of water-miscible aprotic ionic liquids consisting of 1-ethyl-3-methylimidazolium cation and several kinds of anions. Addition of ionic liquids to an aqueous solution containing lysozyme prevented unfolded proteins from aggregating irreversibly at high temperatures. The thermal denaturation curve of lysozyme with ionic liquids was entirely shifted to higher temperature, compared with that without ionic liquids. The remaining activity of lysozyme after the heat treatment was markedly dependent upon the kind and concentration of ionic liquids. The remaining activities of lysozyme with 1.5 M 1-ethyl-3-methylimidazolium tetrafluoroborate ([emim][BF$_4$]) and 0.1 M 1-ethyl-3-methylimidazolium trifluoromethanesulfonate ([emim][Tf]) exhibited 88 and 68% after the heat treatment at 90°C for 30 min, respectively, although that without ionic liquids was perfectly lost.

Keywords: Thermal Stability; Lysozyme; Ionic Liquid; Remaining Activity

1. INTRODUCTION

In resent years, the production and applications of proteins have rapidly increased, not only in biochemical research, but also in the chemical, food, and pharmaceutical industries, since proteins can exhibit exquisite biological activities. Three-dimensional structure of proteins is kept by several weak interactions such as ionic effects, hydrogen bonds, and hydrophobic interactions. When these weak interactions are disrupted by changes of several different kinds in the environment of proteins, proteins are denatured, and inactivated via their unfolding [1-3]. In particular, modest heating can easily disrupt several of these stabilizing interactions. Thermal denaturation is a serious problem not only in the separation and

storage of proteins but also in the processes of biotransformation, drug production, and food manufacturing. Several strategies have so far been proposed in order to prevent thermal denaturation [4-11,21]. They include chemical modification, immobilization, genetic modification, and addition of stabilizing agents. The addition of stabilizing agents to an aqueous solution containing proteins is one of the most convenient methods for minimizing thermal denaturation. It has been reported that polyols, sugars, amino acids, amino acid derivatives, methylamines, and inorganic salts are available for improving protein stability. However, these additives do not sufficiently prevent irreversible protein aggregation or some of them are no longer stable at high temperatures.

Ionic solvent that is liquid at room temperature has attracted increasing attention as a green solvent for the chemical processes because of the lack of vapor pressure, the thermal stability, and the high polarity [12,13]. Chemical and physical properties of ionic liquids can be changed by the appropriate modification of organic cations and anions, which are constituents of ionic liquids. Biotransformation in ionic liquids has increasingly been studied [14,15,34]. We have found that the activity of protease is highly maintained not only in water-immiscible aprotic ionic liquids but also in water-miscible aprotic ionic liquids as well [22,23]. On the other hand, it has been reported that protic ionic liquids keep the stability of proteins in an aqueous solution at high temperatures [24,25], and amyloid fibrils of proteins are dissolved in protic ionic liquids and are refolded by dilution with an aqueous solution [32]. Moreover, aprotic ionic liquids can refold the denatured protein [33].

In our present work, we have focused on the remaining activity of proteins after heat treatment in order to address a question of whether or not water-miscible aprotic ionic liquids consisting of 1-ethyl-3-methylimidazolium cations and several kinds of anions affect the thermostability of proteins in aqueous solutions. As a model protein, chicken egg-white lysozyme has been employed, since it is well investigated regarding its structure, properties,

functions, and thermostability [16-18,24,25].

2. EXPERIMENTAL

2.1. Materials

Lysozyme from chicken egg while (EC 3.2.1.17, 46400 units/mg solid, MW=14300, pI=11.1) and *Micrococcus lysodeikticus* (ATCC No. 4698) were purchased from Sigma-Aldrich Co. (St. Louis, USA). 1-Ethyl-3-methylimidazolium trifluoromethanesulfonate ([emim][Tf]) (98% purity) was supplied from Shikoku Kasei Co. (Kagawa, Japan). 1-Ethyl-3-methylimidazolium tetrafluoroborate ([emim][BF₄]) (99% purity) and 1-ethyl-3-methylimidazolium chloride ([emim][Cl]) (99% purity) were obtained from Kanto Chemical Co. (Tokyo, Japan). The structures of ionic liquids used in the present work are shown in **Figure 1**. The other reagents were purchased from Sigma-Aldrich Co. (St. Louis, USA). All solvents used were of guaranteed grade and commercially available, and were used without further purification.

2.2. Heat Treatment of Lysozyme

In a typical experiment, the aqueous solution containing 100 μM lysozyme was prepared by dissolving lysozyme to 0.01 M phosphate buffer solution at pH 7.0. One mL of lysozyme aqueous solution with or without a requisite quantity of ionic liquids in a 4-mL screw-cap vial was placed in thermostated silicone oil bath at 90°C for 30 min.

2.3. Measurement of Remaining Activity of Lysozyme

Lysozyme catalyzes hydrolysis of the *β*-1,4 glycosidic linkage between the *N*-acetylmuramic acid and *N*-acetylglucosamine components of peptidoglycan. This causes breakdown and removal of peptidoglycan from the bacterium which results in cell bursting or lysis in natural hypotonic solutions [16]. After the heat treatment, an aqueous solution of lysozyme was cooled in thermostated water bath at 25°C for 10 min. After 10 μL of the cooled aqueous solution of lysozyme was added to 3mL of 0.01 M phosphate buffer solution at pH 7 containing 200 mg/L *Micrococcus lysodeikticus* at 25°C, the absorbance was continuously measured at 450 nm by
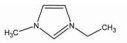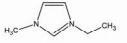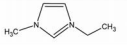
UV/vis spectrophotometer (Ubest-55, Japan Spectroscopic Co. Ltd.). Bacterial lysis obeys a first order reaction. The lysis rate constant (k) is calculated by

$$\ln\left(A^0_{450}/A_{450}\right) = kt \qquad (1)$$

where t, A^o_{450}, and A_{450} are the reaction time, the absorbance of the substrate solution at 450 nm at $T = 0$, and the absorbance of the substrate solution at 450 nm at $T = t$, respectively. The remaining activity (R. A.) is defined as

$$R.A. = 100 x k/k_0 \qquad (2)$$

where k_o and k are the lysis rate constants of native and heat-treated enzymes at 25°C, respectively. Data for remaining activity is the average of triple measurements.

3. RESULTS AND DISCUSSION

3.1. Thermal Inactivation of Lysozyme

When proteins dissolved in an aqueous solution are placed at high temperatures, most of proteins are immediately unfolded due to the disruption of weak interactions, including ionic effects, hydrogen bonds, and hydrophobic interactions, which are prime determinants of protein tertiary structures. In addition, the intermolecular aggregation among unfolded proteins, the incorrect structure formation, and the chemical deterioration reactions in unfolded proteins proceed as shown in **Figure 2** [1-3,19,20]. In particular, protein aggregation easily occurs upon the exposure of the hydrophobic surfaces of a protein, and this phenomenon becomes the major problem because of the irreversible inactivation. On the other hand, when a heated solution of denatured proteins without protein aggregation is slowly cooled back to its normal biological temperature, the reverse process, which is renaturation with restoration of protein function, often occurs. Accordingly, if stabilizing agents can sufficiently prevent irreversible aggregation of unfolded proteins, it is expected that unfolded proteins are refolded by cooling
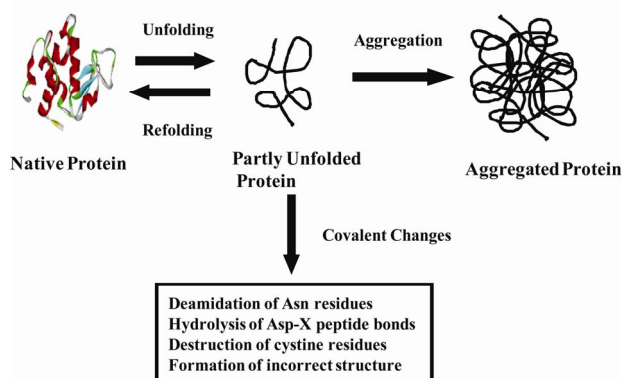
Figure 2. Schematic illustration of thermal denaturation of proteins.

Ionic liquid	Structure	m.p. (°C)	Water miscibility
[emim][Tf]	H₃C–N⁺═N–CH₃ [CF₃SO₃]⁻	-9	Miscible
[emim][BF₄]	H₃C–N⁺═N–CH₃ [BF₄]⁻	14.6	Miscible
[emim][Cl]	H₃C–N⁺═N–CH₃ [Cl]⁻	78	Miscible

Figure 1. Structures of ionic liquids used in the present work.

treatment, and the high remaining activity is obtained.

Table 1 represents the remaining activities of lysozyme in the presence of various kinds of additives after heat treatment at 90°C for 30 min. Lysozyme without additives lost its activity perfectly after heat treatment. Native lysozyme solution immedieately became turbid due to the formation of protein aggregation, as soon as heat treatment was carried out, as shown in **Figure 3(b)**. It has been reported that the precipitation due to protein aggregation is observed above 10 µM lysozyme [18]. As lysozyme concentration in the present work was 100 µM (1.4 mg/mL) which was ten times higher than that, the formation of protein aggregation was dramatically accelerated. Inorganic salts and glycerol used as a conventional stabilizing agent inhibited the formation of protein aggregation, and exhibited thermal stabilization to some extent. On the other hand, [emim][BF$_4$] and [emim][Tf] showed high remaining activities. The lysozyme solution in the presence of ionic liquids was transparent after heat treatment, as seen in

Table 1. Remaining activities of lysozyme in the presence of various kinds of additives after heat treatment at 90°C for 30 min.

Additive	Remaining activity (%)
None	0
1.0 M Sodium chloride	29
0.3 M Ammonium sulfate	41
2.8 M Glucose	8
5.4 M Glycerol	15
7 mM β-Cyclodextrin	0
0.01 M Triton-X	0
2 % Pectin	0
0.7 M Urea	6
0.1 M [emim][Tf]	68
1.5 M [emim][BF$_4$]	88
1.5 M [emim][Cl]	3.4

(a)   (b)

Figure 3. Photographs of lysozyme solutions after heat treatment at 90°C for 30 min: (a) lysozyme solution with 1.5 M [emim][BF$_4$], (b) lysozyme solution without [emim][BF$_4$].

Figure 3(a). When lysozyme solution in the presence of protic ionic liquids (alkylammonium formates) is heated at 90°C, protein aggregation is prevented, and any cloudy appearance is absent [25]. The hydrophobic core of lysozyme unfolded by heat interacts with the cation of ionic liquids, and cation adsorption results in acquisition of a net positive charge preventing aggregation via electrostatic repulsion [24].

Figure 4 shows the relationship between temperature and the remaining activity of lysozyme in aqueous solutions containing water-miscible ionic liquids after the heat treatment for 30 min. As seen in the figure, the dependence of the remaining activity on the temperature exhibited the sigmoid curve. The remaining activity of lysozyme without ionic liquids gradually decreased with an increase in temperature below 70°C, accompanied with the formation of precipitation due to protein aggregation, drastically dropped in the range from 70 to 80°C, and was then lost at temperatures of 80°C or higher. The transition temperature was exhibited around 75°C, similar to the case measured by differential scanning calorimetry [24]. On the other hand, the remaining activity of lysozyme with 1.5 M [emim][Cl] gradually decreased with an increase in temperature below 75°C, and drastically dropped in the range from 80 to 90°C. The remaining activity of lysozyme with 1.5 M [emim][BF$_4$] was highly maintained below 80°C, gradually decreased with temperature, and the remaining activity depicted 60% at 98°C. Similarly, the remaining activity of lysozyme with 0.1 M [emim][Tf] was highly retained below 80°C, gradually decreased with temperature below 92°C, drastically dropped in the range from 92 to 98°C, and was then lost at 98°C. These results indicated that the addition of aprotic ionic liquids to an aqueous solution of lysozyme effectively improved the thermal stability of lysozyme at high temperatures.
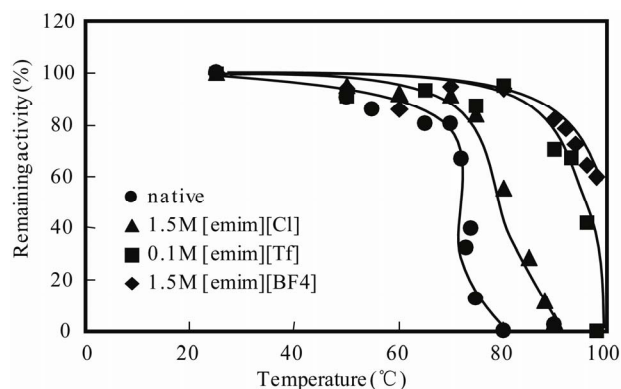

Figure 4. Thermal denaturation curves of lysozyme with or without ionic liquid. The aqueous solution of 100 µM lysozyme with or without ionic liquids was incubated in a silicone oil bath thermostated at requisite temperature for 30 min.

3.2. Refolding of Lysozyme with Ionic Liquids

Figure 5 shows the time course of remaining activity in the presence of ionic liquids at 25°C after the heat treatment at 90°C for 30 min. The remaining activity of lysozyme with 1.5 M [emim][BF$_4$] or 0.1 M [emim][Tf] increased with incubation time, and reached the equilibrium at 2 and 7 min, respectively. In thermal denaturation of lysozyme without protein aggregation, when the hydrophobic core of proteins is exposed, but the disulfide bonds keep intact, denatured proteins spontaneously refold to their native structures on cooling after thermal denaturation [26-30]. The refolding of thermally-denatured proteins is enhanced in the presence of protic ionic liquids such as alkylammonium nitrate and alkylammonium formates [24,25]. Moreover, N'-alkyl and N'-(ω-hydroxyalkyl) N-methylimidazolium chlorides refold denatured proteins such as hen egg white lysozyme and the single-chain antibody fragment ScFvOx [33].

3.3. Dependence of the Remaining Activity of Lysozyme on the Concentration of Ionic Liquids via Heat Treatment

Figure 6 shows the plot of the remaining activity of lysozyme against the concentration of ionic liquids after the heat treatment at 90°C for 30 min. The remaining activity was strongly dependent on the concentration of [emim] [BF$_4$] or [emim][Tf], while the effect of concentration of [emim][Cl] was not observed. The remaining activity in the presence of [emim][BF$_4$] increased with an increase in the concentration of [emim][BF$_4$] and reached a plateau around 0.8 M. The remaining activity in the presence of [emim][Tf] dramatically increased with increasing the concentration of [emim][Tf], the maximal remaining activity was obtained at 0.1 M [emim][Tf], and
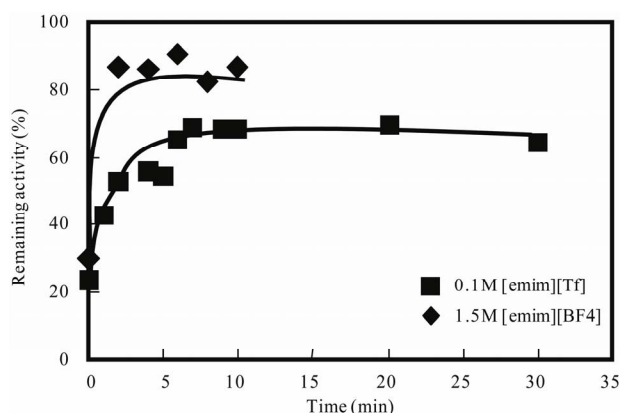
Figure 5. Time dependence of remaining activity of lysozyme with ionic liquids on cooling at 25°C after heat treatment at 90°C for 30 min. After heat treatment, the aqueous solution of 100 μM lysozyme with 0.1 M [emim][Tf] or 1.5 M [emim][BF$_4$] was incubated in a water bath thermostated at 25°C.

then decreased steeply. After heat treatment, the remaining activity of lysozyme increases with an increase in the concentration of ethylammonium formate and 2-methoxyethylammonium formate, while the remaining activity increases at low concentration of propylammonium formate, but at higher concentrations of propylammonium formate the protein spontaneously denatures [25]. Thus, the dependence of concentration of ionic liquids on the remaining activity of proteins changes by switching from one ionic liquid to another.

3.4. Dependence of the Remaining Activity of Lysozyme on the Concentration of Ionic Liquids after the Incubation at 25°C

Figure 7 shows the plot of the remaining activity of lysozyme against the concentration of ionic liquids after the incubation at 25°C for 30 min without the heat treatment. The remaining activity in the presence of [emim][Cl] or [emim][BF$_4$] was undependent on the concentration of
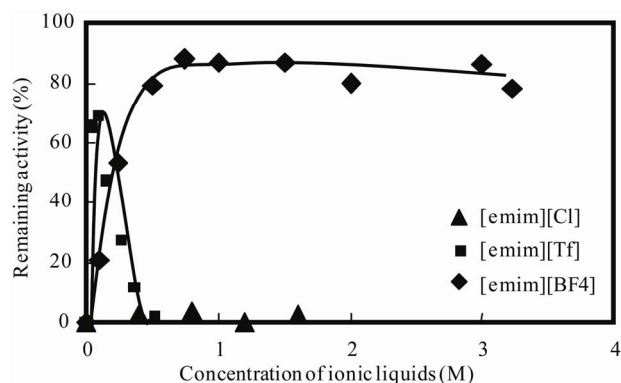
Figure 6. Effect of concentration of ionic liquids on remaining activity of lysozyme after heat treatment at 90°C for 30 min. The aqueous solution of 100 μM lysozyme with requisite concentration of ionic liquids was incubated in a silicone oil bath thermostated at 90°C for 30 min.
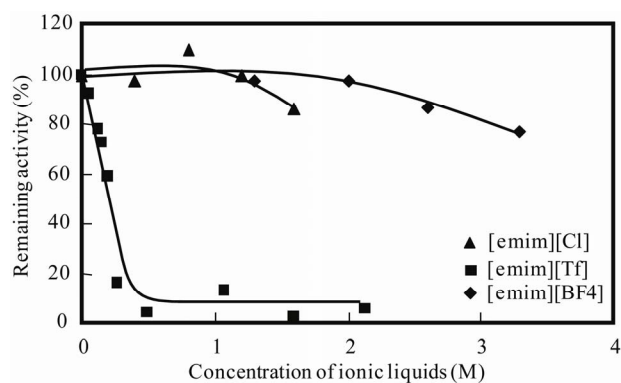
Figure 7. Effect of concentration of ionic liquids on remaining activity of lysozyme during incubation at 25°C. The aqueous solution of 100 μM lysozyme with requisite concentration of ionic liquids was incubated in a water bath thermostated at 25°C for 30 min.

ionic liquids till 1.2 M [emim][Cl] or 2.0 M [emim][BF$_4$] and gradually droped, while it in the presence of [emim][Tf] decreased with an increase in the concentration of [emim][Tf]. These results indicate that [emim][Tf] tends to strongly function as a denaturant, compared with [emim][Cl] and [emim][BF$_4$]. Electrolytes promote or inhibit the stability of proteins according to the kind of electrolytes [35]. Moreover, chemical denaturants, such as urea and guanidine hydrochloride, can promote dissolution of inclusion bodies, which are protein aggregation formed by prokaryotic expression systems [31]. Similarly, [emim][Tf] inhibits the formation of protein aggregation at low [emim][Tf] concentrations, but it mainly denatures proteins at higher [emim][Tf] concentrations.

3.5. Time Course of Remaining Activity of Lysozyme via Heat Treatment with or without Ionic Liquids

Figure 8 shows time course of remaining activity of lysozyme with or without ionic liquids through the heat treatment at 90°C. The remaining activity of lysozyme without ionic liquids dramatically decreased with an increase in time, accompanied with the formation of protein aggregation, and was almost lost at 10 min. It has been reported that the remaining activity in the thermal denaturation process accompanied with the formation of protein aggregation follows first-order kinetics [18]. As seen in the figure, the relationship of the remaining activity of proteins in the absence of ionic liquids with heat treat time could be correlated by first-order kinetics.   On the other hand, 1.5 M [emim][BF$_4$] or 0.1 M [emim][Tf] prevented the thermal inactivation of lysozyme. In the presence of ionic liquids the turbidity of solutions due to protein aggregation was not observed through heat treatment. This indicates that the thermal inactivation mainly
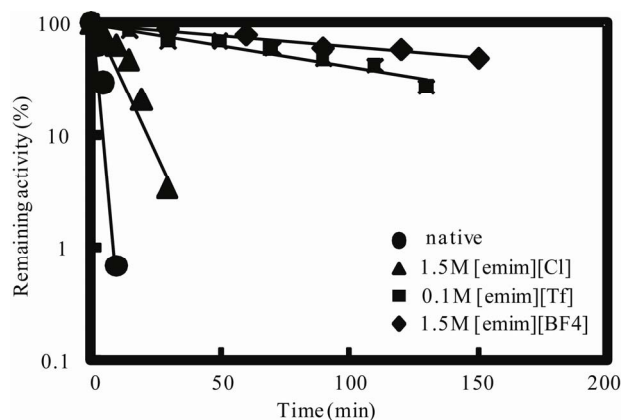
Table 2. Rate constants and half lives of inactivation of lysozyme at 90°C.

Ionic liquid	Rate constant (min^{-1})	Half life (min)
none	0.43	1.6
1.5 M [emim][Cl]	0.065	11
0.1 M [emim][Tf]	0.0081	86
1.5 M [emim][BF$_4$]	0.0049	141

results from the covalent change as shown in **Figure 2**. The plots of remaining activity versus heat treatment time on thermal inactivation of lysozyme in the presence of ionic liquids followed first-order kinetics on linearity. It has been reported that the thermal inactivation of lysozyme obeyed first-order kinetics when it irreversibly proceeded by the covalent change without the formation of protein aggregation [17]. **Table 2** represents rate constants and half lives of inactivation of lysozyme with or without ionic liquids calculated from the fitting curves. The half lives with 1.5 M [emim][BF$_4$], 0.1 M [emim][Tf], and 1.5 M [emim][Cl] were 88, 54, or 6.9 times longer than that without ionic liquids, respectively.

4. CONCLUSIONS

We have demonstrated that the remaining activity of lysozyme is sufficiently maintained after heat treatment at high temperatures, since aprotic ionic liquids prevented unfolded proteins from aggregating. The remaining activity of lysozyme markedly depended upon the kind and concentration of ionic liquids. Specifically, [emim][Tf] exhibited thermostabilization effect of proteins at low concentrations, but denatured proteins at high concentrations. When the heat treatment was carried out at 90°C, the half lives with 1.5 M [emim][BF$_4$] and 0.1 M [emim][Tf] were much superior to that with 1.5 M [emim][Cl].

Figure 8. Time dependence of remaining activity with or without ionic liquids after heat treatment at 90°C. The aqueous solution of 100 μM lysozyme with or without ionic liquids was incubated in a silicone oil bath thermostated at 90°C.

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