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Kinetic analysis of freeze denaturation of soyprotein by a generalized theoretical model for freeze-acceleration reaction

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ABSTRACT

A generalized *n*-th order reaction model was proposed to explain the reaction rate acceleration in the frozen state in dilute systems. The mechanism for the rate acceleration existed in the freeze-concentration effect, which was represented by a concentration factor, α. The α was theoretically related to the freezing point depression and was estimated from the analysis on the fraction of frozen water. The theoretical model was effectively applied to describe the freeze-denaturation of soyprotein. Changes in native and denatured protein concentrations during freeze-preservation were described by the model to obtain kinetic parameters, which suggested the reaction order very likely to be 2. When the preservation temperature was changed from −5 to −40 °C, the denaturation equilibrium was the highest at −5° C but the apparent kinetic parameter was the highest at −18° C. The addition of sucrose proved theoretically and experimentally to be very effective to suppress the freeze-denaturation of protein as a cryoprotectant.

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1. Introduction

It has been known that some chemical reactions in aqueous solutions are accelerated in the frozen state in dilute systems (Fennema, 1974; Poulsen and Lindelov, 1981). The acid catalyzed hydrolysis of *N*-acetyl-*N′*-formylkynurenineamide was reported to have accelerated by 60 times in the frozen state at −8 °C as compared with the corresponding super cooled state (Yamasaki et al., 1976). The alkaline hydrolysis of alkyl-4-hydroxybenzoate esters was accelerated by 20 times when the temperature changed from +30 to −9 °C (Shija et al., 1992). The oxidation rate of ascorbic acid by hydrogen peroxide increased substantially when it was frozen (Hatley et al., 1986). The oxidation rate of nitrile by oxygen increased by freezing more than 10^5 times (Takenaka et al., 1992).

As for reactions involving macromolecules, the acid denaturation rate of ricin D was reported to show its maximum at −29 °C (Yamasaki et al., 1988). The aggregation of bovine serum albumin by formaldehyde took place only in a frozen state (Sotelo and Mackie, 1993). The aggregation rate of actin and myosin in carp myofiblils was the highest at −4 °C (Kitazawa et al., 1997). Hashizume et al. (1971) studied the formation mechanism of kori-tofu, which is a frozen-insolubilized soyprotein gel, and showed that the major mechanism for protein insolubilzation is intermolecular S—S bonds and also that this reaction proceeds faster at −5 °C than at −20 °C in frozen state. Lozinsky et al. (2000) synthesized thiol-containing polyacrylamide and showed that the polymerization reaction of this material through S—S bonds accelerated substantially in a frozen state.

One of the major mechanisms of the rate acceleration in a frozen state has been reported to exist in the “concentration effect” of aqueous solutions through the ice crystal formation. To explain this rate acceleration process theoretically, Pincock and Kiovsky (1966) analyzed the reaction of ethylene chlorohydrin with hydroxyl ion. As this reaction was known to strictly follow the second order kinetics, they proposed the relationship between the observed 2-nd order rate constant, k_{2obs}, and the intrinsic constant, k_2, as follows.

$$k_{2obs} = k_2(C_F/C_{UF}) \tag{1}$$

where C_F and C_{UF}, respectively, are the concentrations of the reactant in frozen and unfrozen states.

In the previous paper (Urai and Miyawaki 2000), we theoretically extended the above 2-nd order reaction model to the *n*-th order reaction and successfully described the decrease of native protein in the freeze-preservation of soyprotein and the effect of

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glucose as a cryoprotectant. In the present paper, we generalize this theoretical model to investigate the freeze denaturation process of soyprotein further by analyzing both native and denatured proteins under various conditions of protein concentration, temperature, and concentration of sucrose as a cryoprotectant. The concentration factor in the freeze-induced concentration is also estimated theoretically by analyzing the freezable water and the fraction of frozen water.

2. Theoretical

The freeze-denaturation of soyprotein can be described by a model in which n native protein molecules react with one another to precipitate (Urai and Miyawaki 2000).

$$nC_N \rightarrow C_D\downarrow \tag{2}$$

where C_N and C_D are the molar concentrations of native and denatured proteins, respectively. The reaction order is assumed to be n-th order so that the reaction rate is:

$$-(\mathrm{d}C_N/\mathrm{d}t) = kC_N^n \tag{3}$$

where t is time and k is the intrinsic reaction rate constant. When a protein solution is frozen, the protein as a solute is concentrated through the ice crystal formation. Because of this "concentration effect", the protein concentration will increase by a factor of α while the reaction volume will decrease by a factor of $1/\alpha$ where α is the concentration factor in the freeze-induced concentration. Then, Eq. (3) will be rewritten as follows:

$$-(\mathrm{d}C_N/\mathrm{d}t) = (1/\alpha)k(\alpha C_N)^n = k\alpha^{n-1}C_N^n \tag{4}$$

The physicochemical meaning of α is considered to be as follows.

$$\alpha = [\text{freezable water}]/[\text{unfrozen water}] \tag{5}$$

Eq. (4) shows that the reaction rate will be accelerated by the concentration effect when $n > 1$ and decelerated when $n < 1$.

Eq. (4) can be integrated in the case of $n \neq 1$ as follows.

$$C_N/C_{N0} = \frac{1}{\left\{1+(n-1)k\alpha^{n-1}\mathrm{t}C_{N0}^{n-1}\right\}^{(1/(n-1))}} = \frac{1}{\{1+Kt\}^{(1/(n-1))}} \tag{6}$$

$$K = (n-1)k\alpha^{n-1}C_{N0}^{n-1} \tag{7}$$

where C_{N0} is the initial concentration of the native protein to be freeze-denatured and K is an apparent rate constant. When $n = 1$, the reaction is the standard first order reaction so that the integrated form will be:

$$C_N/C_{N0} = \exp(-kt) \tag{8}$$

In this case, no concentration effect is expected.

In practice, the experimentally observed change in the native protein concentration during the freeze-denaturation process will be as follows.

$$\left(C_N - C_{Nf}\right)\Big/\left(C_{N0} - C_{Nf}\right) = \frac{1}{\{1+Kt\}^{(1/(n-1))}} \tag{9}$$

where C_{Nf} is the final concentration of the native protein after the freeze-denaturation process at $t \rightarrow \infty$. The C_{Nf} can be assumed to be proportional to the initial protein concentration as follows.

$$C_{Nf} = \beta C_{N0} \tag{10}$$

where β has a meaning of the final intact ratio of the native protein at $t \rightarrow \infty$. Then, Eq. (9) can be rewritten as follows.

$$C_N/C_{N0} = \frac{1-\beta}{\{1+Kt\}^{(1/(n-1))}} + \beta \tag{11}$$

As for the denatured protein C_D, which increases with time, its change can be assumed to be proportional to the change in the native protein with γ as a proportionality constant as follows.

$$\begin{aligned}\Delta C_D &= C_D - C_{D0} = \gamma(C_{N0} - C_N)\\ &= \gamma C_{N0}(1-\beta)\left[1 - \frac{1}{\{1+Kt\}^{(1/(n-1))}}\right]\end{aligned} \tag{12}$$

where, C_{D0} is the initial concentration of the denatured protein. The denatured protein concentration is described by:

$$C_D/C_{D0} = 1 + \delta\left[1 - \frac{1}{\{1+Kt\}^{(1/(n-1))}}\right] \tag{13}$$

where δ is defined by:

$$\delta = \gamma(1-\beta)(C_{N0}/C_{D0}) \tag{14}$$

The δ has a meaning of the final increase ratio of the freeze-denatured protein at $t \rightarrow \infty$.

3. Experimental method

3.1. Materials and sample preparation

Soyprotein isolate (SPI; Fujipro E) was gifted by Fuji Oil Co. Ltd. (Osaka). The SPI was dissolved in distilled water at a concentration of 8 wt%. The suspension was homogenized for 5 min at 15,000 rpm (PH91, SMT, Tokyo) and centrifuged at 10,000 rpm for 15 min at 4 °C (6930 with rotor RA-3, Kubota Corporation, Tokyo). The supernatant was diluted with water by 62.5, 125, and 250-fold, respectively, and used as samples to be frozen with or without addition of sucrose as a cryoprotectant.

3.2. Measurement of freezing point of SPI solution

The freezing point of SPI solution (8%) after centrifugation was measured according to our previous method (Miyawaki et al., 1997) with a slight modification. The sample (3 mL) in a plastic tube (15 mm in diameter), equipped inside with a thermistor (0.01 °C in accuracy: D641, Takara Thermister, Yokohama), was frozen completely at −20 °C in a cooling bath (NCB-3200, Eyela, Tokyo) and then warmed to melt in the atmosphere at room temperature by stirring it with a vortex mixer. The change in the temperature was recorded by a recorder (PRR-5021, Toa DKK, Tokyo). The freezing point depression was determined from the melting curve. The freezing point of sucrose solution was obtained from the literature (Weast, 1974).

3.3. Measurement of freeze denaturation of SPI protein

The diluted SPI sample was divided into many small plastic tubes (10 ml) and frozen in refrigerators at −10, −18, and −50 °C, respectively. The frozen sample tubes were taken out at intervals, thawed at room temperature and the concentration of denatured protein, as a precipitate, was firstly measured from the turbidity at

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