

Effect of Freezing and Thawing Rates on Denaturation of Proteins in Aqueous Solutions

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Abstract: The freeze denaturation of model proteins, LDH, ADH, and catalase, was investigated in absence of cryoprotectants using a microcryostage under well-controlled freezing and thawing rates. Most of the experimental data were obtained from a study using a dilute solution with an enzyme concentration of 0.025 g/l. The dependence of activity recovery of proteins on the freezing and thawing rates showed a reciprocal and independent effect, that is, slow freezing (at a freezing rate about 1°C/min) and fast thawing (at a thawing rate >10°C/min) produced higher activity recovery, whereas fast freezing with slow thawing resulted in more severe damage to proteins. With minimizing the freezing concentration and pH change of buffer solution by using a potassium phosphate buffer, this phenomenon could be ascribed to surface-induced denaturation during freezing and thawing process. Upon the fast freezing (e.g., when the freezing rate >20°C/min), small ice crystals and a relatively large surface area of ice-liquid interface are formed, which increases the exposure of protein molecules to the ice-liquid interface and hence increases the damage to the proteins. During thawing, additional damage to proteins is caused by recrystallization process. Recrystallization exerts additional interfacial tension or shear on the entrapped proteins and hence causes additional damage to the latter. When buffer solutes participated during freezing, the activity recovery of proteins after freezing and thawing decreased due to the change of buffer solution pH during freezing. However, the patterns of the dependence on freezing and thawing rates of activity recovery did not change except for that at extreme low freezing rates (<0.5°C/min). The results exhibited that the freezing damage of protein in aqueous solutions could be reduced by changing the buffer type and composition and by optimizing the freezing-thawing protocol. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* 82: 684–690, 2003.

Keywords: protein freezing; freeze denaturation; freezing rate; thawing rate; buffer type; lactate dehydrogenase; cryomicroscope

INTRODUCTION

Freezing or freezing-drying is common unit operation in the production of therapeutic proteins and enzymes as well as cell biomass. However, freezing can induce several stresses that are capable of denaturing proteins (Franks, 1985), such as cold temperature, ice formation, solute concentration due to the crystallization of water, eutectic crystallization of buffer solutes, and resultant pH changes. Much research has been reported (Anchordoquy and Carpenter, 1996; Arkawa et al., 1993; Tamiya et al., 1985) emphasizing the employment of different cryoprotectants to increase the thermodynamic stability of the native state of the protein. The use of the protectants can sometimes be problematic in affecting the quality and purity of the products.

Quick freezing of enzymes in liquid nitrogen was often recommended in order to preserve the enzymatic activity (Nema, 1993; Shikama and Yamazaki, 1961), but several studies have reported that rapid freezing in liquid nitrogen failed to prevent some proteins from freezing denaturation (e.g., Eckhardt et al., 1991; Schwartz and Batt, 1980). More recent studies on freezing of protein solutions (Chang et al., 1996; Jiang and Nail, 1998; Strambini and Gabellieri, 1996) showed that fast freezing produced more damage to proteins and gave lower recovery of activity after freezing and thawing, the phenomenon being explained as the ice-induced partial unfolding of proteins. However, there is a lack of systematic study of the freezing rate dependence of protein denaturation and very little work (e.g., Jiang and Nail, 1998) has addressed how to reduce freezing damage during freezing and thawing through the optimization of operation conditions.

In the present study, the effect of the freezing and thawing rates on the activity of enzymes in aqueous solutions was systematically examined in the absence of cryoprotectants. A cryomicrostage system was employed to provide a precise control of freezing and thawing rates. Three enzymes, LDH, ADH, and catalase, were used as model proteins. In addition to the cooling and thawing rates, the effect

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of buffer conditions was also experimentally studied. This study aimed at obtaining a better understanding on the different mechanisms for protein denaturation in the freezing and thawing process and providing a guidance in the selection and optimization of freezing and thawing operation conditions.

MATERIALS AND METHODS

Materials and Preparation

Lactate dehydrogenase (LDH) (Type II, from rabbit muscle), alcohol dehydrogenase (ADH) from baker's yeast, catalase from bovine liver, and the bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO). The LDH product, a crystalline suspension in 3.2 *M* (NH₄)₂SO₄ solution, was used directly in the experiment without removing the ammonia sulfate. Five buffer solutions were employed in this study: potassium phosphate, sodium phosphate, citrate, HEPES, and Tris-HCl. All chemicals were reagent grade and obtained from Sigma Chemical.

The stock protein solution was prepared by dissolving a certain amount of well-mixed enzyme suspension or powder in 1 ml of buffer solution to form a protein concentration of approximately 1 mg/ml, which was incubated for 40 min at 37°C to ensure dissolution of crystals and powders. Sequential assays, carried out at 10-min intervals, showed that the enzyme solutions reached full activity after 30 min and did not change for 2 days. The protein concentration of the stock solution was determined by using the Bradford reagent, with BSA used as standard. The stock solution was further diluted in one or two steps to the final concentration desired. The diluted solutions exhibited about a 10% decrease in activity but were stable thereafter for a few hours.

Enzyme Activity Assays

LDH was assayed at 25°C using the procedure described by Worthington Biochemical Corporation (Lakewood, NJ) using a spectrophotometer (UNICAM uv4 series). The assay is based on the conversion of pyruvate to lactate. During the reaction, an equimolar amount of NADH and pyruvate is oxidized to NAD. The oxidation of NADH results in a decrease in absorbance at 340 nm. The reaction mixture consisted of 2.8 ml of 0.2 *M* Tris-HCl buffer (pH 7.3), 0.1 ml of 30 mM sodium pyruvate, and 0.1 ml of 6.6 mM NADH. The reaction was initiated by the addition of 0.1 ml of properly diluted enzyme solution. The enzyme activity was evaluated based on the measured decrease in absorbance at 340 nm.

ADH activity was measured using the protocol provided by Sigma, based on the reduction of NAD by ethanol to NADH, which results in an increase in absorbance at 340 nm. The reaction mixture consisted of 0.05 *M* sodium pyrophosphate (pH 8.8) 1.3 ml, 95% (v/v) ethanol 0.1 ml, and 0.015 *M* β-NAD 1.5 ml. The reaction was started by the

addition of 0.1 ml of properly diluted enzyme solution. The enzyme activity was calculated from the measured increase in absorbance at 340 nm.

Catalase activity assay was conducted using the procedure provided by Sigma, based on the first-order decomposition of peroxide by catalase. The reaction was initiated by the addition of 0.1 ml of diluted catalase solution to 2.9 ml of 0.036% (w/w) hydrogen peroxide in 0.05 *M* potassium phosphate buffer (pH 7.0). The catalase activity was determined from the measurement of absorbance at 240 nm.

Cryomicroscope System

The cryomicroscope system consists of an Olympus BX-50 microscope and a Linkam BCS 196 cryostage (Linkam Scientific, Tadworth, Surrey, U.K.). The cryostage consists of a silver block, 22 mm in diameter, and a temperature monitor and control system. Liquid nitrogen (LN₂) is introduced directly from a 2-liter Dewar through a small-bore tube, via a filter, by a cooling pump (Linkam LNP). The heating element is cast into the silver block to form an integral heater and sensor assembly. A platinum resistor mounted near the surface of the cryostage provides a stable and accurate temperature signal. A small quantity of vaporized gas is recycled from the pump and passed over the top surface of the cryostage window to prevent condensation. Sample carriers have been designed to hold either a Ø16-mm cover slip for freezing a thin-layer sample, or a Ø16-mm quartz crucible for freezing large samples, which can be loaded on to the silver block from the top of the stage by removing the lid or from the side of the stage via the side door.

The BCS 196 cryostage is directly mounted on the microscope substage for stability and allows standard objective lenses to be used. All the essential controls for the operation of the temperature programmer are performed by Link 2.0 Programmer Control software. A real time video and measurement system (RTVMS) was used to display and to record the real-time video through a video camera (JVC TK C1381).

Before the experiment, the temperature accuracy of the cryostage and the temperature difference between the stage and the large volume of sample (55 µl) were examined. Approximately 3 µl of aqueous solution of NaCl (23.3% wt) or KCl (19.75% wt) was pipetted to the sample crucible at room temperature, and a Ø13-mm glass cover slide was placed over the sample to form a sandwich. The sample was first cooled at a rate of 40°C/min to -45°C, held for 2 min, and was then warmed at a rate of 40°C/min to about 5°C below the eutectic temperature of the sample. The further heating in 1°C/min was carried out manually until eutectic melting was observed. The temperature indicated was recorded compared with the eutectic point of the solution. It was found that at the eutectic melting points, the indicated (cryostage) temperatures were very close to the values of eutectic melting of the samples.

For measuring the temperature difference between the stage and sample, 55 µl of buffer solution was loaded on to

the quartz crucible. A calibrated fine wire thermocouple (T-type) was embedded in the buffer solution. The sample was cooled in rates of 1–60°C/min from room temperature to –20 to –40°C, respectively. The data from the thermocouple were collected by a Datalogger (HP34970A, Ampli-con Liveline Ltd., Brighton, U.K.) and used to compare with these from the cryostage readout. The difference between the stage target temperature and the sample temperature was found to be quite close, and more importantly the sample cooling rate is identical to that of the indicated.

Freezing–Thawing

The cooling–warming protocol was made up by defining 3 ramps: (1) Pre-cooling: the samples were pre-cooled at 30°C/min to 0°C and held for 2 min to obtain same initial temperature conditions. (2) Freezing: the samples were cooled at a desired cooling (or freezing) rate to a target temperature and then held for 2 min to achieve uniform temperature. The rate of temperature change in this ramp is the freezing rate as the actual solidification, with seeding, occurred at this temperature change rate, controlled by the cryostage controller. (3) Thawing: the samples were thawed at a designed warming (or thawing) rate to 10°C to ensure the sample is completely thawed. Similarly the rate of warming in this ramp is the thawing rate.

Unless otherwise stated, freezing and thawing experiments were performed using a quartz crucible. A protein concentration of 0.025 mg/ml was selected for most experimental work in this study following a preliminary investigation (see next section); 55 µl of protein solution was transferred to the crucible which was then covered with a glass slip over the top for the better temperature accuracy. After a predefined freezing–thawing cycle, 50 µl of thawed sample was collected and kept in an ice–water mixture and assayed within a maximum of 10 min. No significant change in enzyme activity was observed during this period. Non-frozen controls containing the same amount of the protein solution was kept in the same ice–water mixture and assayed before and after each experiment. Activity recovery was expressed as a percentage of the activity prior to freezing.

In the case of seeding, samples were pre-cooled to 1°C below the freezing point of the solution and held for 2 min in the first ramp. A thin glass hollow sample tube (0.3 mm inside diameter) with a fine sealed tip was held over the surface of liquid nitrogen for ~30 s. The thin glass tube was quickly moved to the surface of the sample through the opened cryostage window at the end of the 2-min holding time and was brought in contact with the sample with the cooled tip. The second ramp was followed thereafter and the stage window was closed as quickly as possible.

RESULTS AND DISCUSSION

Preliminary Observations

The effects of protein concentration and freezing rate were firstly examined without seeding. The dependence of freeze

denaturation on enzyme concentration in 50 mM potassium phosphate buffer (pH 7) is shown in Fig. 1. The freezing and thawing protocols are given in the figure. For all three enzymes studied, significant activity loss (i.e., activity recovery < 100%) was observed at low concentrations, and activity recovery was increased with the increase of enzyme concentrations. It was also found that the activity recovery is nearly 100% for enzyme solutions with a concentrations of 1 mg/ml (results not shown in the figure). The increased activity recovery of proteins with increasing concentration during freezing and thawing has long been recognized (Fishbein and Winkert, 1977; Izutsu et al., 1994). The effect of concentration was believed to come from the poorer stability of the proteins at the lower concentrations (Tamiya et al., 1985), but the mechanism has not been systematically investigated. It has been reported that the protective effect at lower protein concentrations can be achieved by the addition of macromolecules such as BSA, PVP, polyethylene glycol, etc. (Anchordoquy and Carpenter, 1996), which are preferentially excluded from the surface of proteins and increase the overall thermodynamic stability of the native proteins.

On the basis of the results shown in Fig. 1, a protein concentration of 0.025 mg/ml was chosen for the rest of the experimental work. Proteins at this concentration are sensitive to freezing rate, and this concentration is also sufficiently high to minimize errors in solution transfer and handling, e.g., due to protein adsorption onto the surface of pipette tips.

The recovery of activity of LDH in potassium phosphate buffer after freezing at different rates was then examined under the conditions without seeding at fixed thawing rates, the results were illustrated in Fig. 2. A slight downward trend in activity recovery at higher applied freezing rates

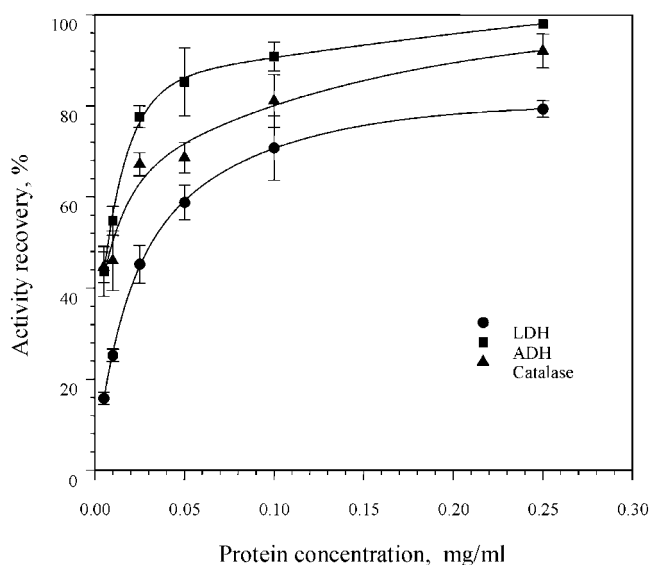


Figure 1. Effect of concentration on activity recovery of LDH, ADH, and catalase after a freezing–thawing cycle (protocol: applied freezing rate 10°C/min to –30°C without seeding, thawing rate 15°C/min).

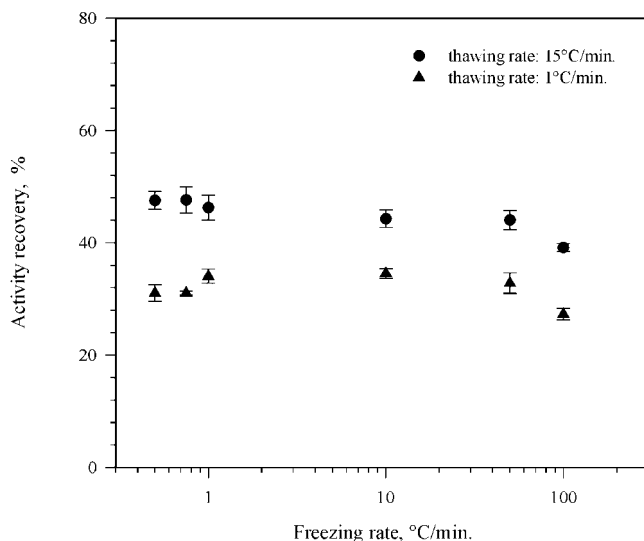


Figure 2. Effect of the applied freezing rate on the activity recovery of LDH (potassium phosphate buffer, without seeding).

(when $>10^{\circ}\text{C}/\text{min}$) was observed, but there was no statistically significant dependence of freeze denaturation on the applied freezing rate. Lower recovery of protein activity was observed when slow thawing ($1^{\circ}\text{C}/\text{min}$) was applied.

It should be pointed out that the applied freezing rate (i.e., cooling rate in the second ramp) is not the actual freezing rate when freezing without seeding due to subcooling. It was observed during the examination of the temperature measurement of the cryostage, that subcooling became pronounced as the cooling rate decreased. Spontaneous nucleation occurred at -6 to -8°C when the cooling rate exceeded $30^{\circ}\text{C}/\text{min}$, but not until -12 to -14°C when the cooling rate was $1^{\circ}\text{C}/\text{min}$. All samples exhibited a freeze exotherm at the supercooling point, and the sample temperature rose instantly to $\sim 1^{\circ}\text{C}$ then dropped to the temperature close to that of the stage with most of the liquid solidified, as observed on the monitor. If dividing this temperature difference by the time interval, the actual rates of freezing in samples in the initial solidification process were found to be -47.5 , -61.4 , -70.0 , and $-100.0^{\circ}\text{C}/\text{min}$ corresponding to the stage cooling rates of -1 , -10 , -30 , and $-60^{\circ}\text{C}/\text{min}$, respectively. The lower the applied cooling rate, the higher the subcooling, and the lower the actual nucleation temperature. This larger solidification driving force results in a more rapid phase change when it occurs. The actual “freezing rate” in random nucleation freezing, i.e., without seeding, is much higher than the applied cooling rate, and not so sensitive to the latter. Hence the activity recovery is not so sensitive to the applied cooling rate, either.

It is hence revealed the importance of seeding. Seeding must be applied in order to achieve a controllable freezing rate. Further studies were conducted all with seeding, at the temperature of around 1°C below the freezing temperature of the buffer solution. The freezing rate can then be defined and controlled precisely.

Freezing and Thawing in Potassium Phosphate Buffer

Freezing and thawing of protein solutions was investigated, with seeding, using 50 mM potassium phosphate buffer at pH 7. The effect of thawing rate on the recovery of LDH activity was examined first. LDH solutions were frozen at fixed rates of 0.8 and $60^{\circ}\text{C}/\text{min}$ and thawed at different thawing rates from 0.5 to $100^{\circ}\text{C}/\text{min}$. The results are shown in Fig. 3. The inactivation of LDH after freezing and thawing markedly decreased when the thawing rate was increased from 0.5 to $10^{\circ}\text{C}/\text{min}$, at which point a plateau was reached. This trend was similar regardless of the difference in freezing rates, although the actual activity recovery was lower when a faster freezing rate was used. This also demonstrated that fast cooling ($60^{\circ}\text{C}/\text{min}$) is more damaging than slow cooling ($0.8^{\circ}\text{C}/\text{min}$) at this protein concentration.

The effect of the freezing rate was then investigated for LDH and ADH at two thawing rates: $1^{\circ}\text{C}/\text{min}$ (in the sensitive region) and $15^{\circ}\text{C}/\text{min}$ (in the plateau region). The pattern of freezing rate dependence, as seen in Fig. 4, exhibited again a reciprocal effect of freezing and thawing rates, compared to Fig. 3. The data illustrated that the recovery decreased remarkably with the increase of the freezing rate and that the maximum recovery occurred with slow freezing (around $1^{\circ}\text{C}/\text{min}$) and fast thawing. A more severe damage to proteins happened when fast freezing ($>5^{\circ}\text{C}/\text{min}$) and slow thawing (e.g., $1^{\circ}\text{C}/\text{min}$) were applied. Microscopic observations showed that the frozen state of the protein solutions was transparent and with large ice crystal textures at slow freezing. With fast freezing, fine ice crystals were formed and the frozen samples became dim. It was also noted that, although the dependence of freezing rate was similar for LDH and ADH, ADH exhibited less sensitivity to the freezing rate than did LDH.

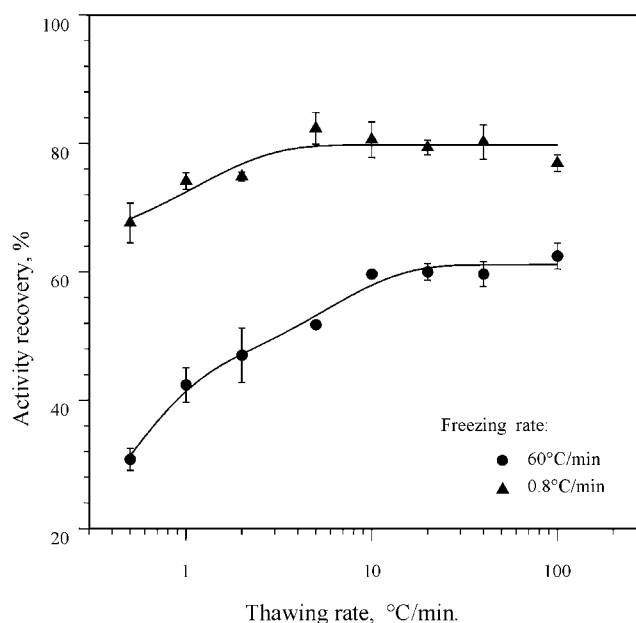


Figure 3. Effect of thawing rate on LDH activity recovery (potassium phosphate buffer, seeding temperature -1°C in freezing).

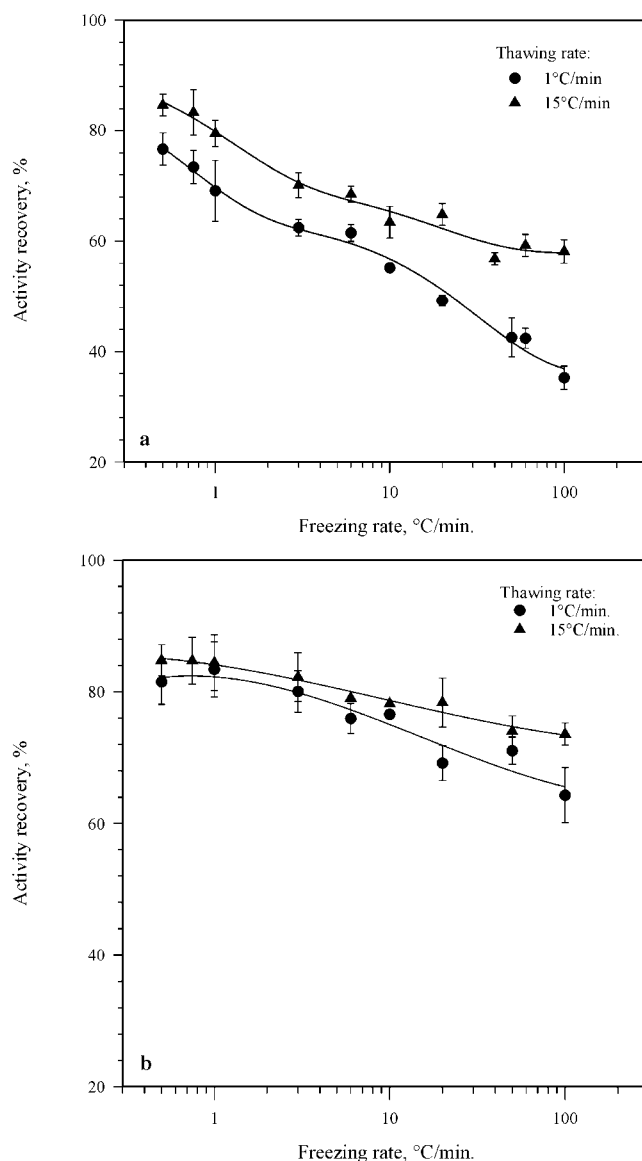


Figure 4. Effect of freezing rate on activity recovery of LDH (a) and ADH (b) (potassium phosphate buffer, seeding temperature -1°C , freezing to -30°C).

It is known that denaturation of proteins during freezing is related to the physical and chemical changes of the local environment around the protein molecules during the process (Franks, 1985). The change of local physical parameters, such as ice crystal size and morphological microstructures, and chemical parameters, such as solute concentration and pH, are thought to depend on the freezing rate. Generally, slow freezing causes large, nonuniform ice crystals to be formed. Freeze concentration occurs, in which solutes, possibly including protein molecules, are pushed into non-frozen regions, producing a large increase in solute concentration and a pH shift due to the buffer salt crystallization, causing conditions which may lead to dehydration, dissociation, or distortion of proteins and hence inactivation (Franks, 1985). However, fast freezing is believed to prevent extensive ice crystal growth and to substantially hinder

the freezing concentration and, hence, the possible denaturation of protein. However, the present study showed an opposite tendency, namely, that at a fixed thawing rate, the high recovery of activity was observed in slow freezing (about $1^{\circ}\text{C}/\text{min}$) and the fast freezing ($>10^{\circ}\text{C}/\text{min}$) caused more damage. Potassium phosphate buffer with a low concentration and neutral pH does not change its pH significantly (<1 Unit) during freezing (van den Berg, 1959), hence pH shift is not the main mechanism for the observed protein denaturation. The strong correlation of activity recovery with the well-defined freezing rate suggested that the surface denaturation is the major cause to the protein damage during freezing. This confirmed the hypothesis of Strambini and Gabellieri (1996) and Chang et al. (1996). Protein molecules are adsorbed on the surface of ice crystals, which undergo unfolding and structural change as identified by Strambini and Gabellieri (1996). Protein damage is hence directly correlated to the surface area of the ice crystals and the texture of ice. In fast freezing (e.g., when the freezing rate $> 10^{\circ}\text{C}/\text{min}$) a large area containing fine ice crystals was formed, which generates a large surface area readily adsorbing proteins and causing surface denaturation. At slow freezing rates (e.g., when freezing rate $< 1^{\circ}\text{C}/\text{min}$), ice crystals formed were much larger and produced less surface area, resulting in less significant surface denaturation.

The dependence on the thawing rate is just the opposite of the freezing rate (see the trend in Figs. 3 and 4). Slow thawing ($<10^{\circ}\text{C}/\text{min}$) caused more damage to proteins than fast thawing ($>10^{\circ}\text{C}/\text{min}$), especially significant to the fast frozen samples. In the fast frozen samples, many tiny ice crystals exist, partly as a result of rapid quenching. The system is not at thermodynamic equilibrium. External disturbances such as temperature variation would trigger an internal ice structure and texture change to reduce the system energy, typically characterized by simultaneous melting and growing of small ice crystals into larger ones (Fransen et al. 1986)—a process called recrystallization. Recrystallization imposes additional perturbation to protein molecules at the changing ice–solution interface and causes more damage. At slower thawing rate, recrystallization becomes more significant and hence results in more severe damage to proteins. If the thawing rate is fast enough, for example, when it is greater than $20^{\circ}\text{C}/\text{min}$ (see Fig. 3), the melting rate outweighs the recrystallization process and hence less protein denaturation is expected.

On the other hand, recrystallization during thawing of slow frozen samples may play a less important role. This is because a thermodynamic equilibrium is largely maintained during freezing within the system. The enzyme activity is hence less sensitive to thawing rate (see Fig. 3).

Freezing and Thawing in Different Buffer Solutions

The effect of different buffer solutions on freezing damage of LDH was examined with sodium phosphate, Tris-HCl,

HEPES, and citrate buffers (0.05 M, pH 7). Samples were seeded at -1°C and frozen at the rates of 0.75 and $30^{\circ}\text{C}/\text{min}$ with thawing rates of 1 and $15^{\circ}\text{C}/\text{min}$, respectively. The activity recovery of LDH at different buffers after freezing and thawing was showed in Fig. 5. Compared with Figs. 3 and 4, the dependence on the freezing and thawing rates for all the buffers follows the same pattern. As seen in Fig. 5, the buffer did affect activity recovery, and sodium phosphate buffer gave the lowest recovery at given freezing and thawing protocols.

The significant pH shift of sodium phosphate buffer, due to the precipitation of the buffer salt during freezing, may contribute to the freeze denaturation of LDH, as LDH is pH sensitive assembly (Anchordoquy and Carpenter, 1996). It was observed that the pH of sodium phosphate solution can drop more than 3 units during freezing. In comparison, Tris, citrate, and potassium phosphate buffers change pH by +1, -0.4, and +0.6 during freezing (Jiang and Nail, 1998; Larsen, 1973; Orii and Morita, 1977; van den Berg and Rose 1959). It is therefore not surprising to see LDH in sodium phosphate suffers the most damage buffer during freezing.

This effect was further demonstrated by the addition of NaCl into potassium phosphate buffer. Potassium phosphate and sodium chloride form Na_2HPO_4 , which can precipitate at low temperature and then change the pH of buffer dra-

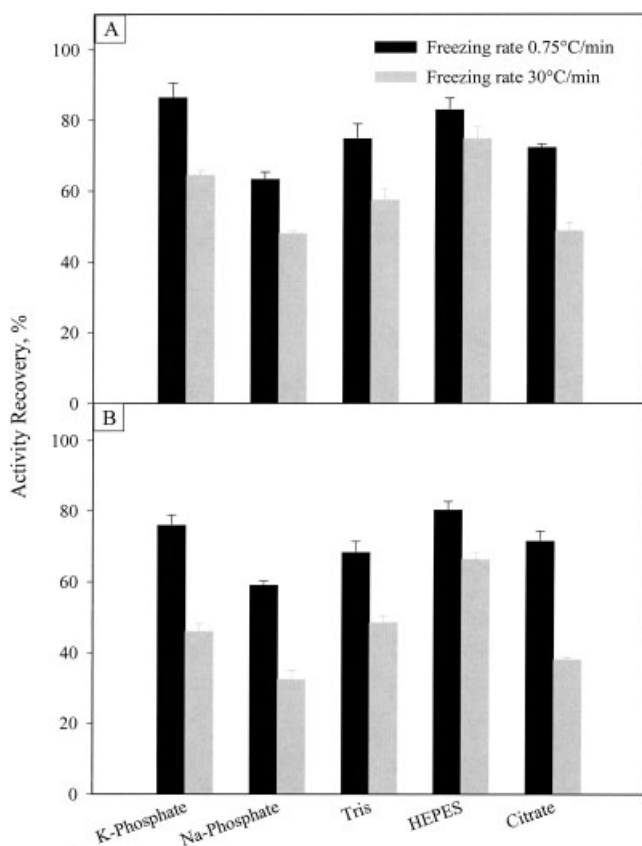


Figure 5. Effect of buffer types on LDH activity recovery at thawing rate of $15^{\circ}\text{C}/\text{min}$ (A) and $1^{\circ}\text{C}/\text{min}$ (B).

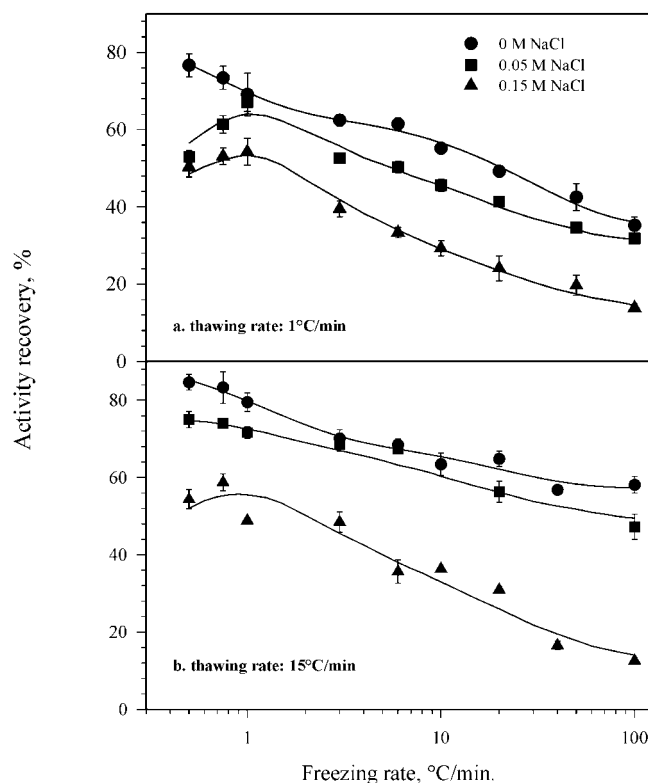


Figure 6. Effect of freezing rate on activity recovery in potassium phosphate solution with added NaCl (a, LDH thawing rate $1^{\circ}\text{C}/\text{min}$; b, ADH thawing rate $15^{\circ}\text{C}/\text{min}$).

matically (Chilson et al., 1965; Murase and Franks, 1989). The experimental results are shown in Figs. 6 and 7, which give similar patterns of dependence of activity recovery on freezing and thawing rates to those in sodium phosphate buffer. Freezing damage increased with addition of salt in the whole range of the freezing rate studied, similar to the observation of Fishbein and Winkert (1977) in their experimental work with catalase. It is quite possible that the in-

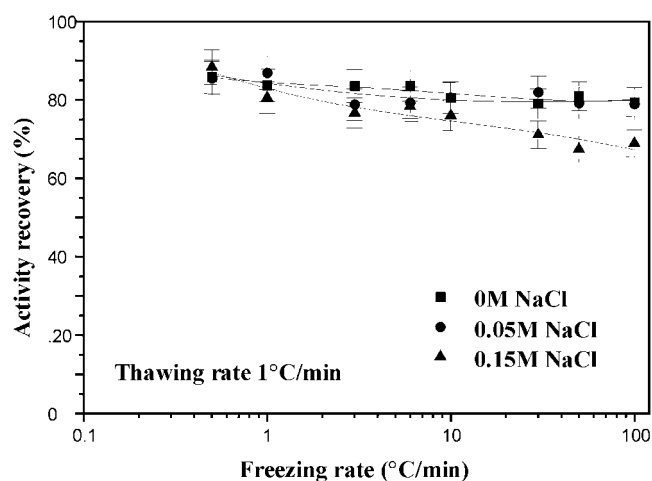


Figure 7. Effect of NaCl addition on the activity recovery of catalase (thawing rate $1^{\circ}\text{C}/\text{min}$).

crease of the concentration of sodium chloride leads to more crystallization of Na_2HPO_4 during freezing and therefore a further pH drop in the unfrozen buffer (Larsen, 1973). The decreased activity recovery at given rates of freezing and thawing may be attributed to the acidification of the buffer solutions when the concentration of sodium chloride is increased (see Figs. 6 and 7).

From Fig. 6a it can be seen that an optimal freezing rate seems to exist around $1^\circ\text{C}/\text{min}$ when NaCl is added into the potassium buffer. When the freezing rate is lower than this, protein damage is increased as the freezing rate decreases. This phenomenon is not observed with potassium phosphate without NaCl addition, therefore it should be related to the increased salt precipitation and pH shift when the freezing rate is below $1^\circ\text{C}/\text{min}$. It is likely that at such a low freezing rate, a chemical and thermodynamic equilibrium is achieved, leading to maximum salt precipitation and pH change. Such an optimal freezing rate may exist for ADH but it is not so obvious (see Fig. 6b), and it is not observed for catalase (Fig. 7).

Based on this work, it could be concluded that protein denaturation during freezing, in the absence of protecting chemicals, is mainly caused by surface denaturation and related to exposure to the ice–water interface. It is advisable to use a relatively slow freezing (about $1^\circ\text{C}/\text{min}$) and fast thawing process ($>10^\circ\text{C}/\text{min}$). Sodium phosphate buffer and other solutions with a tendency of salt precipitation during freezing should be avoided. An optimal freezing rate may exist that depends on the solution conditions.

It should be pointed out that the above conclusions are drawn from the experimental data using of a dilute protein concentration (0.025 g/l), the effect of protein concentration should be studied in future. Also the effect of salt type and cryoprotecting chemicals on freezing denaturation of proteins should be quantified in future research.

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