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Investigation of xenon and natural gas hydrate as a storage medium to maintain the enzymatic activity of the model proteins

Juyoung Shin*,*, Songlee Han**,***, and Seong-Pil Kang***,†**

*Department of Chemical Engineering, Hanyang University, 222, Wangsimni-ro, Seongdong-gu, Seoul 04763, Korea
**Department of Chemical Engineering, Chungnam National University, 99, Daehak-ro, Yuseong-gu, Daejeon 34134, Korea
***Climate Change Research Division, Korea Institute of Energy Research, 152, Gajeong-ro, Yuseong-gu, Daejeon 34129, Korea
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Abstract−To medically use and store proteins like enzymes, long-term maintenance of their activity must be considered. We examined the effectiveness of several methods for preserving the activity of three model-protein solutions. Solutions of catalase, *L*-lactate dehydrogenase, and carbonic anhydrase were used to form gas hydrates with xenon and natural gas. These enzyme aqueous solutions showed inhibitory effects on hydrate formation, and exhibited significant differences in induction time as well. The hydrates formed of enzyme solutions with xenon or natural gas are expected to have a better preservation effect than storage at room temperature and in liquid nitrogen. Changes in the activity of enzymes stored under different conditions were measured in relation to storage time. Storage in hydrate was good for maintaining the activity of catalase and *L*-lactate dehydrogenase. For carbonic anhydrase, the activity at room temperature was generally similar to that after storage in gas hydrate, but storing it in liquid nitrogen produced better results. For certain enzymes, storage in gas hydrates is expected to be a more effective method of maintaining activity than protein storage methods like freeze-drying, which causes mechanical damage to the protein.

Keywords: Gas Hydrate, Protein Storage, Liquid Nitrogen, Activity Preservation

INTRODUCTION

An enzyme is a biochemical catalyst and the efficiency of enzymatic reaction is about 8-14 times greater than that of non-enzymatic chemical reactions. Moreover, enzymes have characteristics like specificity and selectivity, in addition to their high efficiency, which makes them valuable and widely utilized in a number of fields. The industrial use of enzymes started with the production of rennet (the milk-clotting enzyme from the fourth stomach of a calf) to make cheese [1]. Now, it is used in increasingly wider areas, including the manufacturing of food and chemicals and the production of medicines. Many of the biochemical and medical products including enzymes are used as aqueous solutions. However, they are very unstable, so if they are to be stored for a long time, a method like freeze-drying may be used to freeze the solutions (−20 to −70 °C) [2]. Alternatively, more stable solids may be used. Aqueous solutions like medical proteins and vaccine products are often stored frozen, but doing this for a long time does not guarantee stability, and protein aggregation occurs [3,4]. Attention must be paid to prevent loss of activity during the thawing process [5]. At present, storing proteins and enzymes solidified by freeze-drying (at 0 °C or higher, most commonly 2-8 °C) is the most common storage method. The samples are frozen and dried at the same time, under vacuum. In this process, due to various stress factors (e.g., low temperature, and physical pressure at the ice/water interface during freezing) the stability and activity of protein deteriorate. Although freeze-drying is most frequently used for manufacturing medicines, its use is only practical when the products are high-value-added materials, which offsets the low economic efficiency. Freeze-drying requires considerable investments in manufacturing facilities and high operation, so it is not practical for large-scale protein production. There is a clear demand for alternative technologies. When attempts were made to use gas hydrates for activity preservation of a protein, it was confirmed that gas hydrates could maintain the activity of enzymes for a long time [6].

Gas hydrates are crystalline compounds with a three-dimensional lattice structure formed by the hydrogen bonds of water molecules, which contain gas molecules (e.g., methane, ethane, CO_2) small enough to fit into niches formed within the crystal structure [7]. Depending on what substances are involved, this might occur at relatively high pressure and low temperature condition. Gas hydrates form one of three structures (I, II, or H), depending on the type and size of the gas molecules. Gas hydrates have been studied for diverse applications in the fields of environment and energy. Of particular interest here are various studies connected with the self-preservation effect of gas hydrates. The self-preservation effect refers to the fact that gas hydrates may not be dissociated into their original constituents (i.e., water and gas), but could remain as solid hydrate for a long time under temperature and pressure conditions in which gas hydrates cannot be maintained [8,9]. Gudmundsson and Borrehaug conceived using the self-preservation effect for transporting natural gas as hydrates [10]. They experimentally confirmed

†To whom correspondence should be addressed.
E-mail: spkang@kier.re.kr
‡This article is dedicated to Prof. Huen Lee on the occasion of his retirement from KAIST.

that the hydrates lasted for ten days. Stern et al. experimented with the self-preservation effect of methane hydrates, and as a result, confirmed that methane hydrate was not completely dissociated in the range of −20~0 °C, and that more than 50% of the initial volume of the hydrates was maintained for about 15 days (the pressure and temperature necessary to maintain the gas hydrates was excluded from their report) [11]. However, if it is possible to realize the self-preservation effect to maintain hydrates for a long time; it should be possible to store the hydrates at a relatively high temperature (−20 to −10 °C). Moreover, at 1 bar, gas hydrates could be used as a more economic method for storing and transporting gases such as methane, natural gas, and CO_2.

Booker et al. measured the thermodynamic properties of the process (i.e., the formation and dissociation of Xe hydrates), and confirmed that they do not greatly affect the activity of the *L*-lactate dehydrogenase (LDH) enzyme solution [6]. There was a slight activity loss, but it was found that the LDH could be stored in Xe hydrates as its activity was maintained. In the field of food research, workers attempted to store ascorbic acid in gas hydrates while suppressing its oxidation [12,13]. However, the interaction between bio-products and gas hydrates has not yet been properly explained, and only the results were interpreted.

In this study, the self-preservation effect of gas hydrates was applied to evaluate experimentally the performance of protein storage methods. This gas hydrate method was checked by forming gas hydrates with three enzyme solutions (to serve as model proteins). The gases used were Xe and simulated natural gas that forms structure I and II hydrates, respectively. Xe is one of the noble gases, and is colorless, dense, and odorless. In particular, xenon is generally unreactive and non-toxic for humans. It does not have any mutagenic effect. These reasons make it possible to use Xe in medicine or medical application, so we chose Xe as a possible guest for biotechnology application. Natural gas consists of methane, ethane, propane, butane, CO_2, H_2S, and so on. Among these components, some are toxic for humans and can even make mutagenic proteins under long time exposure. Thus, we synthesized a simulated natural gas that only contained methane, ethane, and propane. The model enzymes were catalase, L-lactate dehydrogenase, and carbonic anhydrase. Natural gas forms structure II hydrates and they are known to exhibit the self-preservation effect when they form gas hydrates [15]. In fact, Xe hydrate does not show self-preservation effect, and it has been suggested that Xe from dissociated hydrate penetrates easily through ice [16]. However, Xe hydrate prevents the mechanical stress of ice on protein surface even after the dissociation, because the remaining ice is different from the usual freezing ice in its surface and it could play a role of thermal insulant.

EXPERIMENTAL METHODS

We used Xe gas (99.999%) and simulated natural gas (C_3H_8 2.92 mol%, C_2H_6 4.60 mol%, CH_4 balanced) as the guest gases for forming gas hydrates. Enzymes, such as *L*-lactic dehydrogenase (600-1,200 units/mg) extracted from the muscle of a rabbit, catalase (2,000-5,000 units/mg) extracted from the liver of a cow, and carbonic anhydrase (≥2,500 units/mg) originating from the red corpuscles of a cow, were purchased from Aldrich. Sucrose (≥99.5%), *L*-histidine (≥99%), *L*-histidine monohydrochloride monohydrate (≥98%), Trizma® base (≥99.9%, $NH_2(CH_2OH)_3$), hydrochloric acid (37%, HCl), 4-nitrophenyl acetate (*p*-NPA, $CH_3CO_2C_6H_4NO_2$), acetonitrile (≥99.8%, CH_3CN), potassium dihydrogen phosphate (≥99.0%), potassium hydroxide (≥85%) and hydrogen peroxide

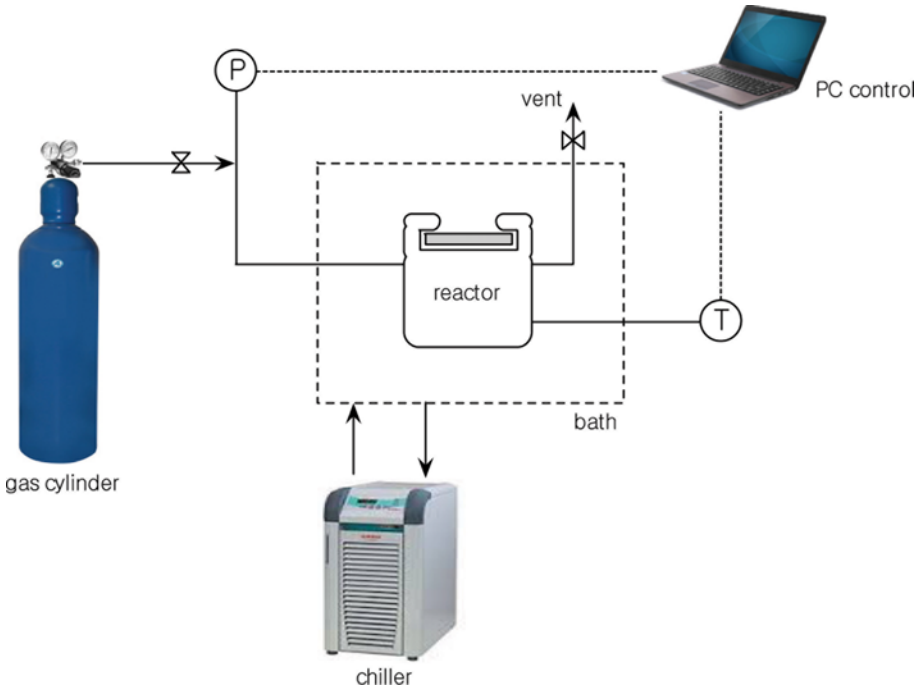

Fig. 1. Schematic diagram of the apparatus.

(30% w/w), which were necessary for preparing the enzyme aqueous solution, were also purchased from Aldrich. The LDH assay kit was purchased from Biovision.

Catalase 11 mg was melted in 55 ml of 50 mM potassium phosphate buffer (pH 7.4) to make a 0.2 mg/ml (400-1,000 units/ml) solution. *L*-lactate dehydrogenase 8.2 mg (661 units/mg solid) was melted in 41 ml of the *L*-histidine buffer to make 0.2 mg/ml (132.2 units/ml). Then, 10% sucrose, 0.68 mg/mol *L*-histidine and 3.27 mg/ml of *L*-histidine monohydrochloride monohydrate were melted to make sure that the L-histidine buffer was at pH 5.5. Next, 5 mg (3,090 units/mg protein) of carbonic anhydrase was melted in 20 ml of the Tris buffer to make the 0.25 mg/ml (772.5 units/ml) enzyme solution. The Tris buffer was 50 mM, and pH 8.1 hydrogen chloride was used to adjust the pH of the Tris buffer.

To use aqueous solutions containing enzymes to form gas hydrates, 5-7 ml of enzyme solutions was injected into a high-pressure cell. Tempered glass 45 mm in diameter was installed at the top of a stainless steel cell with an internal volume of about 20 ml so that it was possible to observe the inside. To measure the temperature inside the cell, a 1/16″ K-type thermocouple (OMEGA) was installed. To control the temperature, it was placed in a bath connected to a circulating chiller. Fig. 1 depicts the schematic diagram of the experimental apparatus.

First, the temperature of the water bath was adjusted by circulating the coolant in the chiller to keep the temperature of the high-pressure cell at 30 °C. When the temperature became stable, a vacuum pump was used to remove the residual air from the cell. The Xe gas was purged two or three times to 20 bar, and then the Xe gas was injected until the desired experimental pressure was obtained. Then it was left for sufficient time so that the gas would dissolve into the solution. When the pressure stabilized, the temperature decreased at the rate of 2 °C/h to 5 °C. The temperature and pressure were recorded, and a magnetic stirrer was started to stir the solution. It was left alone until the temperature decreased to the desired point and the pressure inside the cell was stable, and then the experiment was commenced. Changes in the pressure were monitored to detect if gas hydrates were formed. The experiment to form gas hydrate using simulated natural gas was conducted in the same way, at 55 bar. Finally, when the experiment was over, the gas hydrate samples obtained after opening the high-pressure cell were put in several vials and stored in a freezer at −20 °C.

As for the activity of catalase (CAT), the changes in absorbance were measured at 240 nm using the UV/VIS spectrophotometer at intervals of 10 secs for 5 min after the reactive solution was added to the enzyme solution. The time when the absorbance decreased from 0.45 to 0.40 was obtained, and the activity was measured using the following formula. Activity was measured after storage for one day, for each condition, and converted into a percentage. As for the reactive solution, H_2O_2 was diluted in the potassium phosphate buffer to make up 0.035-0.05%, and then the concentration of H_2O_2 was measured along with the absorbance, so that the initial absorbance would start over 0.45.

$$\text{units/ml enzyme} = 3.45 \times (\text{dilution factor}) / (\text{time, min}) \times 0.1$$

Biovision's Lactate Dehydrogenase Activity Assay Kit was used to measure the activity of *L*-lactic dehydrogenase (LDH). The 50 µl buffer solution, including 2 µl substrate solution, was mixed with 50 µl diluted sample solution, which was used to make two measurement solutions. Before culture, the absorbance (T_1) was measured and the samples were cultured at 37 °C for 30 min. The absorbance (T_2) was measured at 450 nm and the change in nicotinamide adenine dinucleotide (NADH) concentration was obtained by using the standard curve. The LDH activity was converted with the following formula.

$$\text{LDH Activity} = \frac{B}{(T_2 - T_1) \times V} \times \text{Sample dilution}$$

B: change in NADH between T_1 and T_2 (nmol)
T_1: absorbance measured before culture
T_2: absorbance measured after 30 min of culture
V: volume of added sample (ml)

As for measurement of the activity of carbonic anhydrase (CA), changes in *p*-NP (4-nitrophenol), generated due to CA in the decomposition of *p*-NPA, were measured in real time for 10 min at 25 °C. Because *p*-NPA decomposes, it was completely melted in 10% acetonitrile just before the experiment. Then 3 ml of UV cell was used to set the solution, in which 2 ml of Tris buffer solution was mixed with 1 ml of *p*-NPA, as the blank. Then, 1.9 ml of Tris buffer, 1 ml of *p*-NPA, and 0.1 ml of diluted solution were mixed, and change in absorbance was measured at 400 nm for 10 min. Changes in the concentration of *p*-NP, generated due to hydrate-preservation *p*-NPA were excluded as background. The measured absorbance was converted to the concentration of p-NP using the *p*-NP standard curve and compared with the increased rate of *p*-NP. All enzyme solutions were prepared under conditions (at room temperature, in liquid nitrogen, and in the gas hydrate form with either Xe or natural gas), before being stored under their respective conditions. The activity of the enzymes in each sample was measured three times, and then compared with the others in relation to their period of gas storage. Because the measurement methods and calculation methods differed, the enzyme activity of the samples in each of the four conditions at the start of the experiment was set as 100%, and the relative activity of each sample was obtained by normalization.

RESULTS AND DISCUSSION

To determine whether the enzymatic activity was better maintained by certain of the storage methods, first, the Xe gas and simulated natural gas were used to form gas hydrates. Fig. 2 illustrates the pressure profile along time during Xe hydrate formation. As the temperature decreased, gas dissolution occurred and crystal nuclei appeared. The pressure decreased gradually as the enzyme solution containing CAT was mixed with Xe gas by stirring. Because the solubility of Xe gas in water is very low [17], the pressure decrease due to dissolution was also very small, and the pressure change due to the temperature decrease seemed very small. Then, when the formation of nuclei and crystal growth started, gas inclusion in the hydrate crystal sharply increased, and the pressure inside the cell decreased abruptly. As illustrated in the figure, the pressure decreased from 22 bar to 8.5 bar for about 60 min during Xe

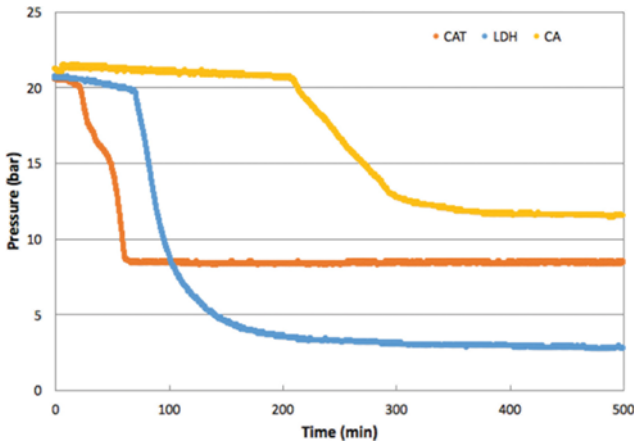

Fig. 2. Pressure profile in relation to time during Xe hydrate formation.

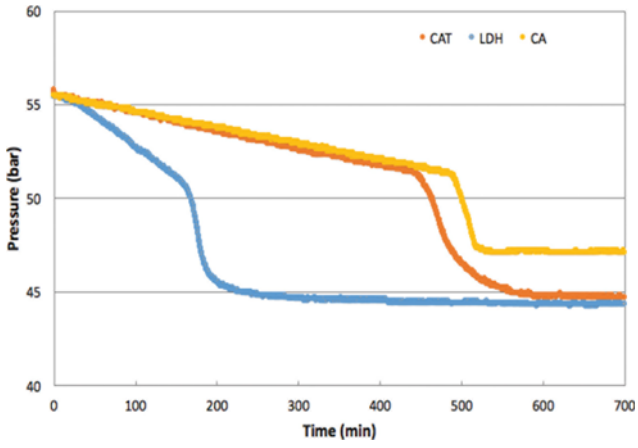

Fig. 3. Pressure profile in relation to time during natural gas hydrate formation.

hydrate formation and growth. This physical reaction was mostly over after about 60 min, and the pressure stabilized at equilibration. The Xe hydrate containing LDH required about 70 min for nucleus formation, and then as the hydrate crystals began to grow, the pressure sharply dropped and stayed low for about 200 min. Afterwards, the pressure went down to 2.8 bar and equilibrium was reached. As for the CA-Xe hydrate, except for a slight decrease in temperature due to the temperature change, for about 200 min after stirring began, there was no decrease in temperature due to the formation of hydrates. The time from the start of the stirring to the beginning of the formation of hydrates is called the induction time [18]. It was confirmed that after enough hydrates had formed (at about 11.5 bar), the reaction was completed. The results from repeating the same experiment under the same conditions were generally consistent. By comparing the time it took for the Xe hydrate to be formed and grow, a kinetic inhibition effect was shown. The formation Xe hydrates was inhibited in the order of CA>LDH>CAT.

Fig. 3 illustrates changes in the pressure of each enzyme solution when simulated natural gases were used to form hydrates. As for CAT-simulated natural gas hydrates, as the pressure of the cell decreased by about 5 bar due to the stirring, the induction time was about 450 min, and then as the hydrates grew the pressure dropped. The pressure declined abruptly and remained low for about 150 min. At about 43 bar, the crystals stopped growing. As for the formation of LDH-simulated natural gas hydrates, after an induction time of about 160 min, gas hydrate was formed. The pressure decreased about the same as in the case of CAT. As for CA-simulated natural gas hydrates, the induction time was about 480 min, after which the pressure changed due to the formation of hydrates. In the case of simulated natural gases, the overall consumption of gas corresponded to about 10 bar, not a big difference, and the pressure decrease was relatively small. Examining the time it took the simulated natural gas hydrate to be formed and grow, the induc-

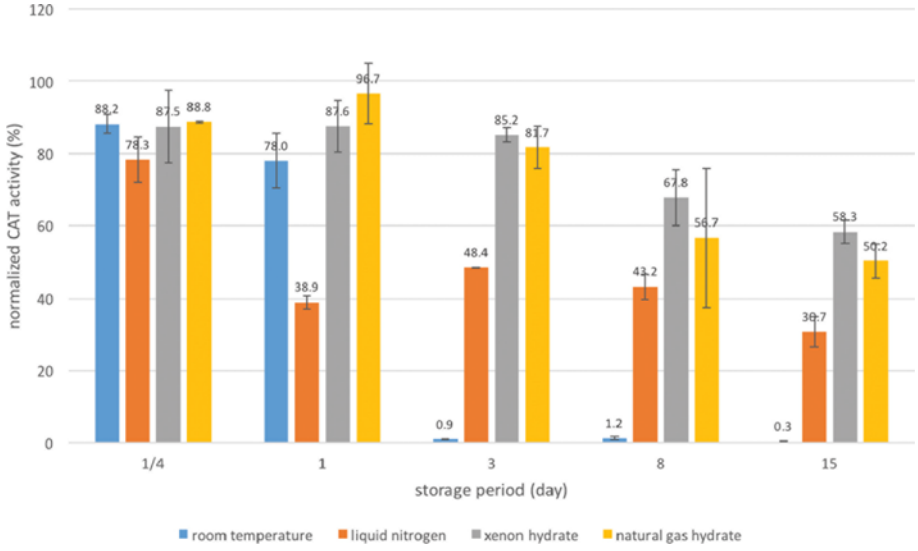

Fig. 4. Normalized optical absorbance via UV/VIS spectrophotometry at 240 nm, of catalase (CAT) in solutions under different storage conditions.

tion time was in order of CA>CAT>LDH, and there was a kinetic inhibition effect with regard to the formation of hydrates with simulated natural gas. The degree of decrease in pressure was similar for all enzymes, and it can be said that a similar amount of water was converted into hydrates. Compared to the case of the Xe hydrates in Fig. 3, however, the Xe hydrates showed a similar degree of pressure drop with regard to CAT and CA, but showed a high degree of pressure drop with regard to the LDH enzyme solution. This means that the conversion rate into hydrates was relatively high. In Xe or simulated natural gas hydrates, LDH was formed relatively more quickly and showed a high conversion rate.

Figs. 4 to 6 illustrate changes in enzyme activity in relation to the storage method and period. Fig. 4 shows the result of activity measurement for CAT. After the enzyme solution was stored at room temperature for one day, activity decreased by about 78%. By day 3 and afterwards, the activity had almost disappeared. For CAT stored in liquid nitrogen, the activity dropped by more than half on day 1, and then the activity slowly decreased, but generally remained constant. For samples with Xe and natural gas hydrates that were stored in freezer, the activity decreased by about 20% by day 3, but generally the activity was much higher than that of enzymes stored in liquid nitrogen. The activity of enzymes stored in hydrates also tended to decrease over time, but remained above 50% after 15 days. Thus, hydrate preservation is expected to be a good candidate method for protein storage.

Fig. 5 illustrates the result of the experiment conducted for the LDH enzyme solution under the same conditions as in Fig. 4. Because the activity of the LDH enzyme solution samples was generally unstable, the deviations among the same sample were rather large. This made it difficult to compare their activity over a long period. When LDH was melted in the *L*-histidine buffer solution (pH 5.5) and stored at room temperature, the activity dropped abruptly after day 1 to <50% of the initial activity, and there was almost no activity on day 3. The samples that were quickly frozen and stored in liquid nitrogen also had lost 50% of their activity on day 1, similar to the samples stored at room temperature, and then their activity decreased abruptly. LDH had a great loss of activity when it was stored at room temperature and at low temperature. However, these enzymes stored Xe and natural gas hydrates slowly lost their activity. LDH in Xe hydrates retained 50% of its activity after 15 days. Samples in natural gas hydrate may also be stored for a long time, but the level of residual activity was lower than that for storage in Xe hydrate.

Fig. 6 illustrates changes in activity when CA enzymes were stored under several conditions. Unlike the previous two enzyme samples, the activity of the CA melted in the 50 mM Tris buffer solution (pH 8) and stored at room temperature, slowly decreased but maintained a certain level of activity. After 15 days, it retained only 30% of its activity. In further contrast to the two previous samples, CA did not lose as much activity if it was stored at room temperature or in liquid nitrogen. The CA quickly frozen and stored in liquid nitrogen retained 70% or more of its activity after 15. As for the samples stored in Xe and natural gas hydrates, activity slowly decreased, and after 15 days, it dropped below 50%. In Xe hydrate, sample activity declined to about 57% in the first three days. Then, over the next twelve days (to Day 15) the activity decreased by 26%. The loss of activity was slow after the initial drop in activity. The CA samples stored in natural gas hydrate slowly lost activity at first, but the activity abruptly dropped after Day 8. Storing CA enzymes in liquid nitrogen was the most effective way to maintain their activity. Unlike for the results of experiments on the previous samples, the level of activity was relatively high for all storage methods.

One of the things necessary for interpreting the experimental results in Figs. 4 to 6 is to examine the properties of each enzyme. First, CAT is an enzyme found in nearly all living things exposed to oxygen, and serves as a catalyst in decomposing hydrogen peroxide into water and oxygen [19]. CAT is an enzyme protein that has strong resistance to thermal denaturation. When it is stored frozen at −20 °C, its activity is maintained for about a month in

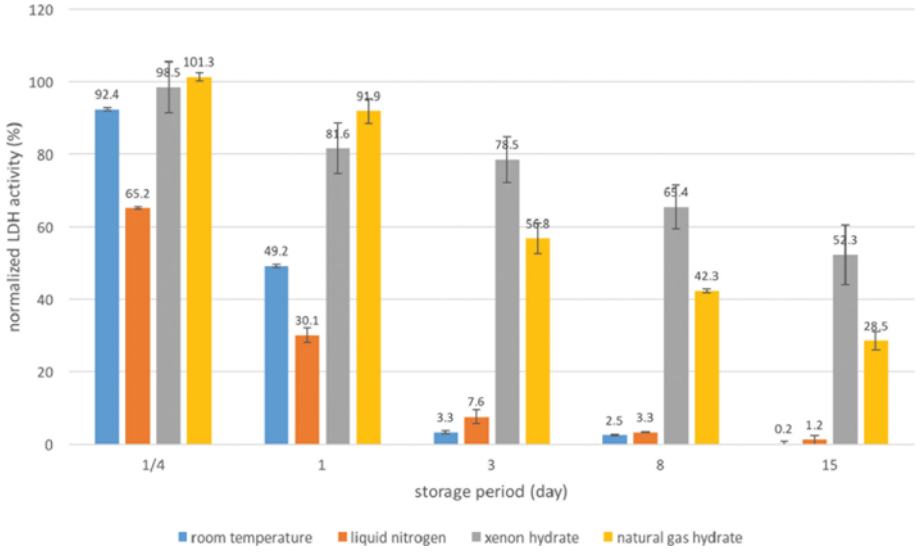

Fig. 5. Normalized optical absorbance via UV/Vis spectrophotometry at 450 nm, of lactate dehydrogenase (LDH) in solutions under different storage conditions.

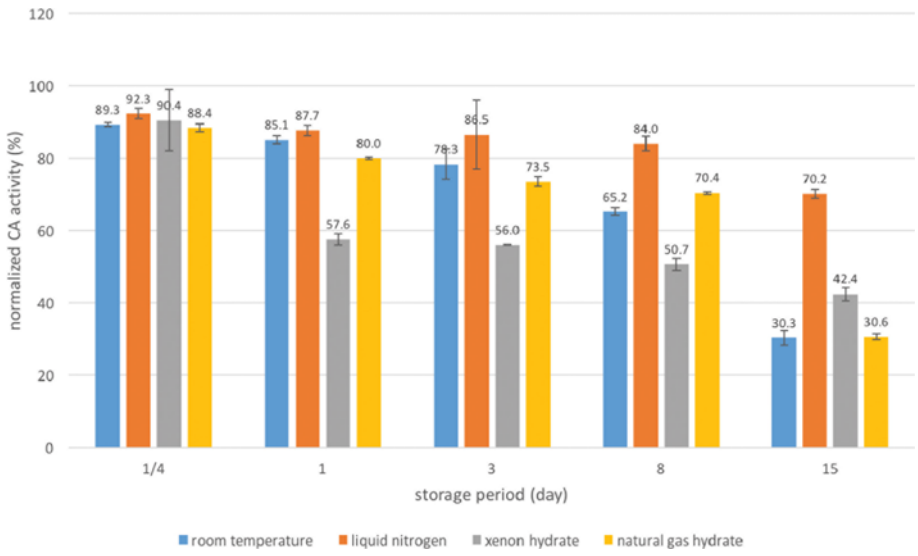

Fig. 6. Normalized optical absorbance via UV/Vis spectrophotometry at 400 nm, of carbonic anhydrase (CA) in solutions under different storage conditions.

general, so it is stable at a low temperature. As found in Fig. 4, except when it is stored at room temperature, the other three methods of storing the enzyme (low temperature) maintained a certain level of activity, which is attributed to this property. Storage in liquid nitrogen showed better results than storage in hydrates.

Because *L*-LDH in Fig. 5 can denature tissue easily even at the low temperature used in the freeze-drying process, it is very unstable. Accordingly, it is often used for studies on thermal denaturation. Because it is very unstable, and thus very responsive to thermal stimulus, it loses activity easily when stored at room temperature and even in liquid nitrogen. However, when it is stored using hydrate, it maintains its activity. In particular, Xe hydrates were more effective in maintaining the activity than natural gas hydrates.

Illustrated in Fig. 6 is CA, an inexpensive and widely used enzyme, which is also used in studies on CO_2 fixation. Many studies are being conducted on its use for industrial purposes. Because it has a high level of thermal stability, it is stable if stored at 4 °C. In general, it is stored at −18 °C or lower, with addition of a carrier protein. As it is very stable against heat; regardless of how it is stored, it maintains sufficient activity. However, it was less capable of maintaining activity when stored in hydrates.

It is difficult to draw general conclusions from these experiments because the number of samples tested was quite small and the experiments were performed under restricted conditions. Even so, storage of thermally enzymes like LDH in gas hydrates should be effective. This conclusion is based on the levels of activity maintained after storage under the different conditions studied in this work. Moreover, storing thermally stable enzymes like CAT at a relatively higher temperature in hydrate seems more effective in maintaining activity than storing them frozen at a very low temperature (as in liquid nitrogen). This should save the energy needed to store them at very low temperature. As for thermally stable enzymes like CA, all storage methods were effective in maintaining activity, except storage at room temperature. Compared to the results of Booker et al. [6], who experimented on LDH samples with Xe hydrate, the samples in hydrates showed a higher level of activity than samples stored in liquid nitrogen, and showed a similar level of activity to samples stored at room temperature. This trend was similar to the results of this experiment for the early 6 h from the beginning of storage. LDH is known to exhibit cold denaturation at −28 °C due to unfolding of the protein [20]. For this reason, storing it below the denaturation temperature as in liquid nitrogen may be the direct cause of its decreased activity. Accordingly, this may be why storing it above the denaturation temperature in hydrate, produced better results.

Putting these results together, it is clear that for certain enzymes storage within hydrate can be beneficial. One of the reasons why protein is denatured during freezing is that ice crystals exert mechanical stress at the water/protein interface and damage the protein surface. More specific studies on effect of the crystal structure of hydrates on the enzyme protein in solution must be conducted. Furthermore, to draw more general conclusions, it appears that experiments involving a greater variety of hydrate formers will be necessary.

CONCLUSIONS

Experiments were conducted to evaluate proposed enzyme storage methods using gas hydrates. This work was intended to provide alternatives that might solve such problems as thermal stress of biomaterials, and aggregation generated by various factors. To this end, three model proteins (CAT, LDH, and CA) with different thermal denaturation properties were selected, and solutions including these enzymes were stored under four conditions to examine how effective each method was in maintaining enzyme activity: at room temperature, in liquid nitrogen, and in gas hydrate (with Xe or natural gas). The experimental enzyme solutions were induced to form gas hydrate by decreasing the system temperature from 30 °C to 5 °C (at 20 bar for Xe and 55 bar for simulated

natural gas). The hydrates formed were either stored at atmospheric pressure or at −20 °C in a freezer. During the storage period, changes in enzyme activity were measured in relation to the period of storage. It was confirmed that the hydrate induction time of all enzyme solutions was shorter when the guest molecule was Xe, rather than natural gas, and the conversion of enzyme solution into hydrate was more uniform when natural gases were used than when Xe gas was used. Looking at how well the activity of each enzyme was maintained, we found that the activity of CAT decreased less when it was stored in gas hydrate than when it was stored at room temperature or in liquid nitrogen. The activity of LDH was also higher when the samples were stored in gas hydrate than at room temperature or in liquid nitrogen. The activity of CA decreased more when it was stored at room temperature and in gas hydrate, than when it was stored in liquid nitrogen. Thus, depending on the types of enzymes and their thermal denaturation properties, appropriate methods of maintaining activity vary. Moreover, for some enzymes, storage in gas hydrate may provide an alternative to the enzyme damage caused by freeze-drying. In the future, to apply the method of storing enzymes using gas hydrates, extensive studies must be conducted on appropriate methods of hydrate formation, maintaining stable pH, selection of appropriate temperature and pressure conditions, and the physical influence of the crystalline structure of hydrates on enzyme proteins.

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