Enzyme Encapsulation with Crystal Transformation of Anhydrous Maltose or Anhydrous Trehalose

(Received June 27, 2005; Accepted January 13, 2006)

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Abstract: The encapsulation of protein drugs in powdery forms is quite important in order to improve the stability, as well as to expand the application. When a protein solution is added to anhydrous sugars such as anhydrous maltose or anhydrous trehalose, water molecules are incorporated into sugars as water of crystallization, resulting in a protein encapsulated into sugar powders. This study investigated the enzyme encapsulation with crystal transformation of anhydrous maltose or anhydrous trehalose. The activity of the encapsulated enzyme depended on the crystal transformation rate of the anhydrous sugars. The remaining activity of the encapsulated alcohol dehydrogenase increased with the use of amorphous anhydrous trehalose and the addition of hydroxypropyl-β-cyclodextrin into the enzyme solution.

Key words: trehalose, maltose, encapsulation, crystal transformation

Crystallization of amorphous sugar in food has been studied as a moisture- and temperature-dependent phenomenon during water absorption. Maltose is arguably the most important sugar for the food industry.3 Maltose has three crystal forms: anhydrous α-maltose, hydroxyl β-maltose and anhydrous β-maltose. Anhydrous β-maltose is an unstable form of the anhydrous crystal. Anhydrous maltose has unique properties such as high solubility and emulsifying ability. Yoshii et al.2 investigated crystal transformation from anhydrous α-maltose to hydroxyl β-maltose and from anhydrous trehalose to hydrous trehalose. Trehalose is a non-reducing disaccharide of glucose found in several organisms that are able to survive drying. Hosoi et al.10 found that anhydrous maltose and hydrous trehalose.3 The encapsulation of protein drugs into powdery form is quite important in order to improve the storage stability, as well as to expand the applications. When a protein solution is added to anhydrous sugar such as anhydrous trehalose, water molecules are incorporated into sugars as water of crystallization, resulting in a protein encapsulated easily into the sugar powders. Lactose exhibits mechanical properties which are directly related to the crystallization process. Relatively slow crystallization produces single crystals of α-lactose monohydrate, whilst rapid crystallization results in aggregates of anhydrous α- and β-lactose micro crystals.8 The crystallization data are important in the modeling of crystallization phenomena and prediction of stability of sugar-containing food and pharmaceutical materials. Fält and Bergenstahl10 investigated the surface coverage of sodium caseinate in the surface composition of spray-dried food powders due to lactose crystallization with ESCA and SEM. Trehalose has two crystal forms and a high glass transition temperature in the dried state and is an efficient stabilizer of proteins on storage. Terebiznik et al.11 investigated the thermal stability of dehydrated α-amylase in trehalose matrices in relation to its phase transitions. They found that removal of water greatly enhanced thermal stability of α-amylase and partial crystallization of the matrix could remove water from the amorphous phase of trehalose, increasing its glass transition temperature and leading to enhanced enzyme stability. Solla-Penna and Meyer-Fernandes8 indicated that the evidence that the higher efficiency of stabilization of enzymes by trehalose was a consequence of its larger hydrated volume. Rossi et al.9 investigated the effect of various saccharides such as trehalose, sucrose or lactose, poly(vinylpyrrolidone), and maltodextrin on the stability of a dried restriction enzyme, EcoRI. Lam et al.12 discussed that trehalose and lactose decreased relaxation rates in the lysozyme-sugar systems while hydration increased relaxation rates that were correlated with changes in aggregation and activity of the protein by a solid-state NMR study.

In this study, the encapsulation of enzyme with crystal transformation from anhydrous maltose or anhydrous trehalose to a hydrous one was investigated as the encapsulation method without extra-drying or freezing. We report the characterization of this encapsulation method to get higher enzyme-encapsulation efficiency in trehalose or maltose.

MATERIALS AND METHODS

Materials. Anhydrous α-maltose and hydrous β-maltose, analytical reagent grade (maltose content, 92%) and special purified grade (maltose content, 99%), and anhydrous trehalose and hydrous trehalose (trehalose content, 99%) were donated by Hayashibara Co., Ltd. (Okayama, Japan). Yeast alcohol dehydrogenase (ADH),
Diaphorase from *Clostridium kluyveri*, Malate dehydrogenase (MDH) from pig heart, NADH and NAD⁺ (99%) were obtained from Oriental Yeast Co., Ltd. (Osaka, Japan). Hen egg-white lysozyme, six times crystallized, was obtained from Seikagaku Corporation (Tokyo, Japan). *Micrococcus lysodeikticus* cells were from Sigma (Sigma-Aldrich Japan K.K., Tokyo). Hydroxypropyl-β-cyclodextrin (HP-β-CD) was purchased from Wacker Biochem (Tokyo, Japan). Other reagents were of reagent grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Preparation of the encapsulated enzyme powder.**

Two hundred fifty milligrams of anhydrous crystal trehalose or anhydrous maltose were weighed in glass vials (10 φ × 80 mm) and mixed with 27 μL of an enzyme solution (4 mg/mL), such as alcohol dehydrogenase (ADH), lysozyme, malate dehydrogenase (MDH) or diaphorase, by a vortex mixer. HP-β-CD (0.19 mg/mL) was used as a stabilizing agent in the enzyme solution. The thermal stability of the enzyme-encapsulated powder was measured in a water bath at 25°C. At prescribed intervals, the glass vials were taken out, and the enzyme-encapsulated powder was re-dissolved in the buffer. The remaining enzyme activity was measured by a UV spectrophotometer. The X-ray diffractogram pattern of trehalose powder was measured with a Rigaku X-ray diffractometer (RAD-II, Tokyo).

**Measurement of β-anomer content of maltose and hydrous trehalose.** Twenty milligrams of maltose were solubilized in 1 mL of pyridine. Fifty micro-litters of 1-(Trimethylsilyl) imidazole (TMSI), N,O-bis (trimethylsilyl) acetamide (BSA) and chlorotrimethylsilane (TMCS) were mixed sequentially in the test tube (8φ × 40 mm). This solution was used as a solvent of trimethylsilylization (TMS) of maltose. One hundred micro-litters of maltose in pyridine was added to the TMS solution and this tube was incubated at 60°C for 30 min.

The β-anomer content of maltose was measured by FID gas chromatography on the trimethylsilyl (TMS) ester, prepared by reaction with trimethylsilylimidazole in DMSO. TMS derivatives were injected in an FID gas chromatograph (GC-14A) equipped with 5% OV-17 on Chromosorb W. The carrier gas was nitrogen. Column temperature was 210°C, and injection and detector temperature was 250°C. X-ray diffractometry also was used to measure the mutarotation. The weight change of trehalose was measured by the weight balance as the degree of crystal transformation. The X-ray diffractogram pattern of the maltose or trehalose powder was measured with a Rigaku X-ray diffractometer (RAD-II, Tokyo). The dried powder was densely packed in an aluminum sample holder. The operation conditions of the diffractometer were as follows: X-ray target, Cu; filter, Ni CuKα radiation; voltage 30 kV; time constant, 1 s; scanning speed, 2°/min; chart 20 mm/min; divergence slit, 1 mm; receiving slit, 0.5 mm; count full scale, 4000–8000 c/s.

**Enzyme assays.** Enzyme activities were measured by the spectrophotometer (Shimazu UV-2200, Kyoto, Japan) at 25°C. Lysozyme activity was assayed with the turbidity decrement at 450 nm of 0.25 mg/mL *Micrococcus lysodeikticus* in 0.1 M Tris/HCl buffer, pH 8.0. Diaphorase was assayed by the oxidation of 2.7 mM 2,6-dichlorophenolindophenol at 600 nm in 0.1 M potassium phosphate buffer containing 9 mM NADH. Malate dehydrogenase was assayed by NADH decrement at 340 nm in 0.1 M potassium phosphate buffer, pH 7.5 containing 10 mM oxaloacetate. Alcohol dehydrogenase was assayed with NADH formation at 340 nm in 11 mM sodium pyrophosphate buffer, pH 8.8 containing 1.13 mM ethanol and 10 mM NAD⁺.

**RESULTS AND DISCUSSION**

**Effect of the enzyme molar-mass on enzyme encapsulation.**

Lysozyme, diaphorase, ADH and MDH encapsulated with anhydrous crystal maltose or trehalose powders were incubated at room temperature. Figure 1 shows the decrease of the encapsulated enzyme activities in the maltose powder (99% of maltose) during crystal transformation of maltose. The time courses of relative enzyme activities in Fig. 1 were the results in the addition of 0.43 mg of enzyme to 1 g of anhydrous maltose powder and water at 2 : 1 molar ratio to maltose. Enzyme activity of lysozyme, diaphorase, or ADH except MDH corresponded to the conversion of crystal transformation until 16 h and become constant after a complete crystal transformation. The constant values of relative enzyme activities in maltose were about 80% for lysozyme, 30% for Diaphorase, and 0% for ADH and MDH.

Figure 2 shows the decrease in the encapsulated enzyme activities in the trehalose powder (99% of trehalose) during crystal transformation of trehalose. The enzyme activities in trehalose continued to deactivate until about 3 h and became constant. The constant values of relative enzyme activities in trehalose were about 80% for lysozyme, 60% for MDH, 40% for Diaphorase, and 0% for ADH. The loss of the encapsulated enzyme activity depended on the molar mass of the enzyme by means of the crystal transformation of the anhydrous sugars except for MDH activity. Kaushik and Bhat investigated the effect of trehalose on the thermal stability in aqueous solutions of five well-characterized proteins differing in their various physico-chemical properties. They showed that an in-
crease in the stability of proteins in the presence of trehalose depends upon the length of the polypeptide chain. Chang et al. investigated the mechanism of protein stabilization by sugar during freeze-drying and storage with FTIR and thermal activity monitor measurements and indicated a specific interaction between stabilizer such as trehalose and protein might be responsible for the preservation of native structure. Now, many researchers are investigating the mechanism of protein stabilization with trehalose. In the encapsulation of an enzyme with crystal transformation of anhydrous sugar, the enzyme might be denatured with solidification and this inactivation depends on the size of enzyme. Malate dehydrogenase (MDH) from pig heart has a dimeric quaternary structure. Jensen et al. investigated the effect of potassium salts of acetate, chloride and bromide at a concentration of 1 M on the quaternary conformation of phm-MDH. They suggest that MDH has high stability under hydrophobic conditions such as a high salt solution. The interaction of MDH and trehalose might affect the stability of enzyme protein during the crystallization of anhydrous trehalose to hydrous trehalose.

**Effect of crystal-transformation rate on the encapsulated enzyme activities in maltose.**

Figures 3 and 4 show time-courses of the encapsulated lysozyme activities in crystal transformation of 99% and 92% pure anhydrous α-maltose. In 99% pure of anhydrous α-maltose, crystal transformations finished in about 9 h with 4 mol of water to maltose and in 16 h with 2 mol of water to maltose. The encapsulated lysozyme activities reached 85% in 4 mol of water to maltose and 80% in 2 mol of water to maltose. In 92% pure anhydrous α-maltose, crystal transformations finished in about 24 h with 4 mol of water to maltose and in 44 h in 2 mol of water to maltose. These encapsulated lysozyme activities were 80 and 70%, respectively. These results suggest the encapsulated enzyme activity depends on the crystal transformation rate.

**Encapsulation of lysozyme with amorphous anhydrous trehalose.**

Figure 5 shows the decrease of the encapsulated lysozyme activities in the trehalose powder during crystal transformation from anhydrous trehalose to hydrous trehalose. In Fig. 5, the conversion of crystal transformation was measured by the weight change. The conversion was about 90% right after the water addition. Crystal transformation of trehalose was very rapid. Therefore, the encapsulated lysozyme activities were about 85% with 4 mol of water to maltose after 2 h and about 82% with 2 mol of water to maltose after 6 h. An amorphous anhydrous-
Effect of addition method of HP

Fig. 7. Effect of addition method of HP-β-CD on the ADH activity in trehalose.

The black bars indicate the data without HP-β-CD. The diagonally striped bars indicate the data with 2wt% HP-β-CD. Incubation temperature was 25°C.

trehalose could give high encapsulated enzyme activity. Crystal transformation of amorphous anhydrous-trehalose to hydrous trehalose finished in about 2 h in a desicciator at 50°C and relative humidity 75%. That of crystal anhydrous-trehalose finished in about 8 h. The encapsulated lysozyme activity was obtained almost 100% with amorphous anhydrous-trehalose, as shown in Fig. 5. Rapid crystal transformation of anhydrous trehalose could give high encapsulated enzyme activity.

Effect of additive, HP-β-CD into anhydrous trehalose to encapsulate ADH.

Closed symbols, HP-β-CD added (Trehalose powder contained 20 mg HP-β-CD in 1 g of trehalose); open symbols, no addition. Incubation temperature was 25°C.

Cyclodextrins have been reported to stabilize proteins against aggregation, precipitation and heat inactivation. Brewster et al. showed that hydroxypropyl-β-cyclodextrin (HP-β-CD) was able to solubilize and prevent aggregation of lyophilized interleukin-2 upon reconstitution with water. The encapsulated ADH activity with crystal anhydrous trehalose was 0%. To have high encapsulated ADH activity, the effect of HP-β-CD on protein stability of ADH encapsulation was investigated with crystal transformation of anhydrous trehalose. To improve the encapsulation efficiency of ADH, an amorphous type of anhydrous trehalose was used and 2 wt% HP-β-CD to trehalose was added as the enzyme stabilizer. The activity of the encapsulated ADH increased about 85% with amorphous anhydrous trehalose with the addition of HP-β-CD into the enzyme solution, as shown in Fig. 6. This result suggests that the crystal disorder could arise in relation to the change of physicochemical properties of trehalose as a result of the incorporation of an impurity of HP-β-CD as a protein stabilizer.

Effect of mixing method of ADH into trehalose powder containing HP-β-CD on the encapsulation yield.

Figure 7 shows the ADH encapsulation yields for various mixing method of ADH into trehalose powder. Four mixing methods were done as follows: (1) Addition of anhydrous amorphous trehalose and 2 wt% HP-β-CD to ADH solution, (2) Addition of anhydrous amorphous trehalose containing 2 wt% HP-β-CD to ADH solution, (3) Addition of anhydrous crystal trehalose to ADH solution containing 2 wt% HP-β-CD, (4) Addition of anhydrous amorphous trehalose to ADH solution containing 2 wt% HP-β-CD. The activity of encapsulated ADH could be obtained about 85% with anhydrous amorphous trehalose with the addition of 2 wt% HP-β-CD into ADH solution. The encapsulation yield and stability of ADH with anhydrous trehalose were improved by HP-β-CD addition as a co-encapsulating agent. Encapsulation method of protein with crystal transformation has merits such as low cost because drying is unnecessary and the simple method doesn’t require special instruments. On the other hand, the demerits of this encapsulation method are the low amount of encapsulation protein in crystallized sugar, the addition of impurities such as HP-β-CD to stabilize protein, and dependence of protein stability on the crystal size and sugar type.

CONCLUSIONS

Anhydrous trehalose could be a useful material to encapsulate proteins with crystal transformation to hydrous trehalose. The activity of the encapsulated enzyme depended on the molar mass of the enzyme by means of the crystal transformation of the anhydrous sugars. The remaining activity of the encapsulated alcohol dehydrogenase increased with the use of amorphous anhydrous trehalose and the addition of hydroxypropyl-β-cyclodextrin into the enzyme solution. To use the protein encapsulation method with the crystal transformation of anhydrous sugar as a powder formation process, the additive as impurities, the crystal-size control, and the removal method for the excess water to crystal transformation should be investigated.

This study was financially supported partly by a Grant-in-Aid for
REFERENCES


無水マルトースまたは無水トレハロースの結晶変換法による酵素包括粉末化

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生理活性蛋白質の包括粉末化は、蛋白質の安定化改善のために非常に重要な操作である。蛋白質溶液を無水マルトースまたは無水トレハロースのような無水糖に添加した場合、水分子は結晶水として糖に組み込まれる。そのため、糖質粉末内に蛋白質が包括される。本研究は、無水マルトースまたは無水トレハロースの結晶変換法による酵素蛋白質の包括粉末化特性について検討した。無水糖の結晶変換によって包括粉末化された酵素活性は、無水糖から含水糖への結晶変換速度に依存した。包括粉末化されたアルコールデヒドロゲナーゼの残存活性は、ヒドロキシル-β-シクロデキストリンを加えた酵素水溶液をアモルファス無水トレハロースに添加した場合高い包括酵素活性粉体を得た。