Department of Physical Chemistry[1], Faculty of Pharmaceutical Sciences, Hoshi University; Department of Health and Nutrition Sciences[2], Faculty of Human Health, Komazawa Women's College, CycloChem Co., Ltd.[3]; Department of Pharmaceutical Sciences[4], Nihon Pharmaceutical University, Japan

# Effect of γ-cyclodextrin as a lyoprotectant for freeze-dried actinidin

T. Furuishi[1], T. Ishigami[1], T. Endo[1], H. Nagase[1], M. Okada[2], I. Nishiyama[2], A. Jo[3], K. Terao[3], H. Ueda[4]

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*Takayuki Furuishi, Ph.D., Department of Physical Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan*
*t-furuishi@hoshi.ac.jp*

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Actinidin (ATD) is a cysteine protease found in kiwifruit. It is used to tenderize meat and to enhance the digestion of proteins in the small intestine. However, ATD is unstable during freeze-drying, which alters its bioactivity. It is well known that sugars have the ability to protect proteins from the stress of freeze-drying. In this study, we investigated the protective effect of various saccharides on the stability of ATD during freeze-drying. The ATD activities of the samples containing γ-cyclodextrin (CyD) showed only a small decrease, and compared with trehalose and sucrose, γ-CyD was a more effective stabilizer for ATD. Secondary structural changes in freeze-dried ATD were observed by circular dichroism spectroscopy and compared with the changes in stabilized samples. There was a close relationship between the α-helix content and the stabilization. The sugars stabilized the protein by suppressing the changes in the α-helix. Fourier transform infrared spectroscopy measurement showed that the amide I band of ATD with γ-CyD was shifted to a lower wavenumber compared with other sugars. Therefore, stronger hydrogen bonds may be formed between ATD and γ-CyD than between ATD and other sugars. The suppression of changes in the protein secondary structure accompanying the formation of hydrogen bonding between the protein and the sugar also contributed to the protective effect of the sugars.

## 1. Introduction

Actinidin (EC 3.4.22.14, ATD) is a cysteine protease and contains a free sulfhydryl group essential for its activity. Arcus (1959) identified ATD as the protease in raw kiwifruit that prevents gelatin from setting. This enzyme is the major protein in most *Actinidia* (kiwi) fruits. Protein and DNA sequence data show that ATD is a member of a group of closely related cysteine proteases found in plants (e.g., papain from papayas, ficin from figs, bromelain from pineapples, and aleurain, EP-A, and EP-B from barley), slime molds, insects, and mammals (e.g., cathepsin B and cathepsin L) (Praekelt et al. 1988). The crystal structure of ATD was determined by Baker (1980). Moreover, Lewis and Luh (1988) assessed the meat tenderizing properties of ATD and characterized the molecular changes in beef muscle proteins caused by its proteolytic activity. The digestion of a variety of food proteins has been reported under small intestinal digestion conditions (Kaur et al. 2010). Furthermore, Nohno et al. (2012) reported that tablets containing ATD had an accumulative effect in reducing volatile sulfur compounds in mouth air with long-term use; accordingly, ATD has attracted attention in the food and pharmaceutical industries.

Freeze-drying has been used to enhance the long-term stability of protein pharmaceuticals when the stability of aqueous formulations is insufficient. However, some proteins become unstable during freeze-drying, which alters their bioactivity levels. Generally, freeze-drying is a three-step process, starting with cooling and freezing, followed by primary drying and secondary drying. Freeze-drying conditions exert various stresses on proteins (Crowe et al. 1990; Wang 2000; Abdul-Fattah et al. 2007). Saccharides can protect proteins from the stress of the freeze-drying process, including disaccharides such as trehalose and sucrose and polyols such as sorbitol and mannitol (Wang 2000; Chang et al. 2005; Chang and Pikal 2009). Cyclodextrins (CyDs), which are cyclic oligosaccharides of α-1,4-linked glucopyranose units, function as host compounds for inclusion complexes. The formation of inclusion complexes with CyDs could eliminate the undesirable physicochemical properties of drugs, such as poor water solubility and low stability (Brewster and Loftsson 2007). In addition, many studies have indicated that CyDs form complexes with accessible hydrophobic amino acid side chains, such as aromatic amino acids in peptides and proteins (Horský and Pitha 1994; Koushik et al. 2001; Khajehpour et al. 2004; Aachmann et al. 2012). CyDs increases the stability of proteins to thermal and chemical denaturation in aqueous solution (Tavornvipas et al. 2006; Serno et al. 2010). However, there are few studies of the effectiveness of various CyDs on stabilizing proteins during freeze-drying and preserving their activity (Brewster et al. 1991; Ressing et al. 1992). We have reported that hydroxypropyl (HP)-CyDs stabilized lactate dehydrogenase (LDH) during freeze-drying (Iwai et al. 2007). Although ATD can be used as a food additive or active pharmaceutical ingredient, it would be convenient if ATD isolated from kiwifruit could be freeze-dried and transported. However, our preliminary experiments showed that ATD becomes unstable and inactive during freeze-drying. Appropriate food or pharmaceutical additives could be used to maintain the bioactivity of ATD during freeze-drying. On this basis, we hypothesized that CyD would protect the relative activity of ATD during freeze-drying.

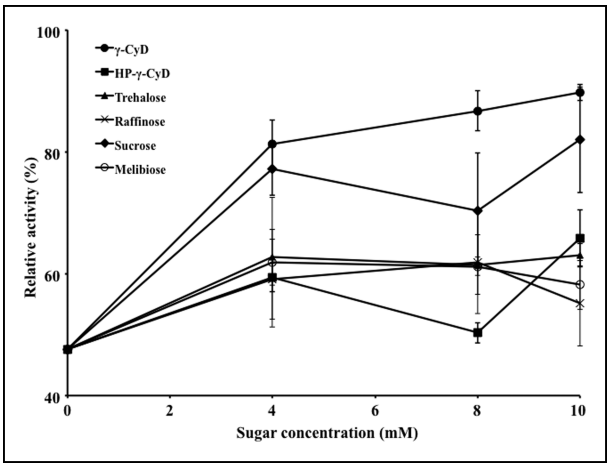


Fig. 1: Effect of concentration of various sugars on the relative activity of ATD. Each point represents the mean ± S.D. of three experiments.

In this study, the effect of γ-CyD and HP-γ-CyD on the stability of ATD during freeze-drying was investigated. Additionally, γ-CyD and HP-γ-CyD were compared with the disaccharides sucrose, trehalose, and melibiose, and the trisaccharide raffinose. Moreover, the mechanism by which the CyD system stabilized ATD during freeze-drying was elucidated in detail by Fourier transform infrared spectroscopy (FT-IR) and explained with the water replacement hypothesis.

## 2. Investigations, results and discussion

### *2.1. Effect of sugar concentration on the relative activity of ATD*

Figure 1 shows the effect of the sugar concentration on the relative activity of ATD after freeze-drying. The relative activity of the ATD sample without the saccharide decreased to approximately 48% after freeze-drying. In contrast, the addition of 4 mM of a disaccharide, trisaccharide, or HP-γ-CyD increased the relative activity of the ATD by up to 55–65%. However, the relative activity of the ATD plateaued at a saccharide concentration of 4 mM. γ-CyD was the most effective additive for stabilizing ATD during freeze-drying. The relative activity of ATD samples with γ-CyD was higher than that of the samples containing the other saccharides at concentrations of 4, 8, 10 mM; the relative activity of ATD with 10 mM γ-CyD was more than 90% of the original relative activity.

### *2.2. Relationship between ATD activity and protein secondary structure*

Freeze-dried ATD was measured with circular dichroism (CD) spectroscopy from 190 to 240 nm, and the rate of change of the secondary structure content was calculated (Fig. 2). The α-helix content of ATD after freeze-drying without a saccharide decreased to about 19%, whereas that of intact ATD was about 26%. All the saccharides in this study reduced the decrease of the α-helix content of ATD after freeze-drying, and the α-helix content of ATD with γ-CyD was approximately 94%.
Figure 3 shows the relationship between the relative activity of ATD and the α-helix content. There was a good linear correlation (r = 0.878) between the α-helix content and the relative activity of ATD after freeze-drying and rehydration. Therefore, the saccharides prevented changes in the α-helix structure during freeze-drying.

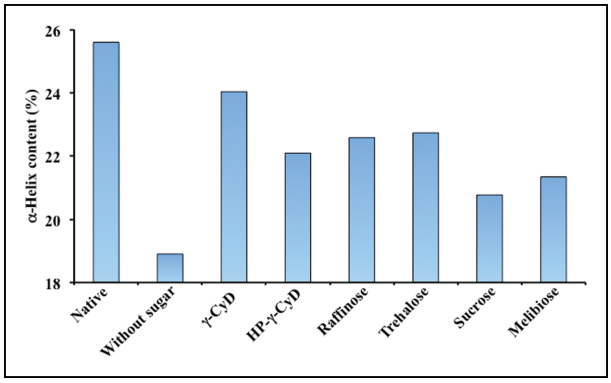


Fig. 2: Effect of α-helix content on the relative activity of ATD. The concentration of all the sugars was 4 mM.

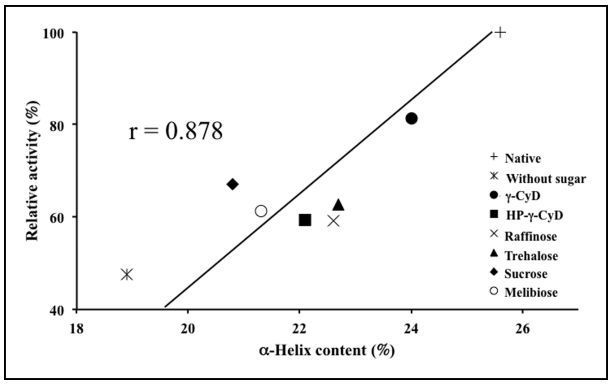


Fig. 3: Relationship between relative activity and α-helix content.

### *2.3. FT-IR spectroscopy*

To elucidate the effect of the hydroxyl groups in the saccharides on the amides in ATD, FT-IR peaks near 1670 $cm^{-1}$ (amide I) were measured with or without saccharides. We chose trehalose, γ-CyD and HP-γ-CyD as the saccharides. Trehalose is one of the most effective additives for stabilizing proteins during freeze-drying because it interacts with amide I band stretching in ATD. γ-CyD and HP-γ-CyD are more effective lyoprotectants for ATD than the other saccharides in this study. Figure 4 shows the amide I absorption peak of freeze-dried ATD with or without saccharides. The amide I peak of ATD containing saccharides was shifted to lower frequencies by approximately 13 (γ-CyD), 6 (HP-γ-CyD) and 12 (trehalose) $cm^{-1}$, respectively.
In general, the magnitude of the shift in the peak frequency of the amide I vibration absorbance arises from the hydrogen

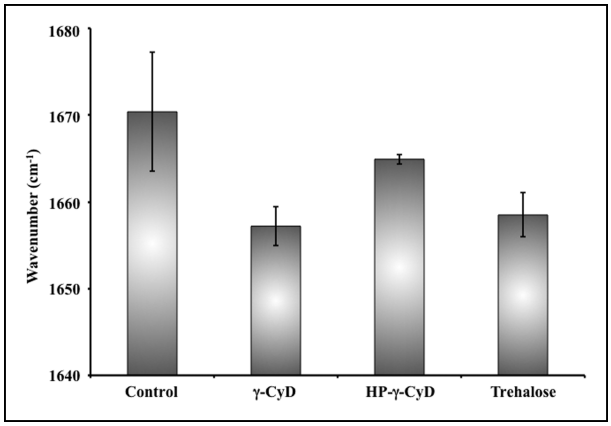


Fig. 4: Effect of various sugars on the amide I peak of ATD. Each bar represents the mean ± S.D. of three experiments.

bonding between the protein and the saccharides in the freeze-dried solids. γ-CyD induced the largest shift in the absorption peak, followed by trehalose and HP-γ-CyD. Previous reports have showed that HP-γ-CyD is a more effective lyoprotectant for LDH than γ-CyD and trehalose (Iwai et al. 2007). We did not elucidate the mechanism of interaction between HP-γ-CyD and LDH directly, but the stabilizing effect of HP-γ-CyD on the freeze-dried LDH increased as the degree of substitution (DS) of the hydroxypropyl group of HP-γ-CyD increased. In contrast, the relative activity of ATD with γ-CyD was greater than with HP-γ-CyD. In general, an increase in the DS of CyD decreases the formation of inclusion complexes because of the steric hindrance of its substituted groups (Trinadha et al. 1992). This is probably because the interaction of the amino acids of ATD with the hydroxyl group of HP-γ-CyD inhibits the formation of the inclusion complex between ATD and HP-γ-CyD.

Trehalose has been found to be the most effective of the di- and trisacchrides in terms of the functional recovery of biomaterials (Crowe et al. 1983). Two mechanisms for the interaction between trehalose and biostructures in dry systems have been proposed to account for the stabilizing effect of saccharides. Carpenter and Crowe (1989) suggested that, under dry conditions, trehalose is directly hydrogen bonded to proteins, replacing the hydration water (water replacement hypothesis). This hypothesis is supported (i) by the high flexibility of trehalose around the glycosidic bond (Ballone et al. 2000) and (ii) by the lack of internal hydrogen bonds between the two glucose rings, even at high sugar concentrations (Batta and Kover 1999). Moreover, Green and Angell (1989) suggested that the protective efficacy of trehalose is a result of its glass transition temperature ($T_g$), which is higher than that of other disaccharides. According to the glass forming hypothesis, the glassy state maintains biological structures in their native state and arrests dynamic processes that lead to a loss of activity. Evidence in the literature indicates that trehalose acts through a combination of the glass forming and water replacement mechanisms.

We investigated the stabilizing mechanism of ATD during freeze-drying using both hypotheses. We could not determine a clear $T_g$ for γ-CyD and HP-γ-CyD. Prestrelski et al. (1995) reported that conformational changes in recombinant human interleukin-2 during lyophilization were prevented by β-CyD, which has a high $T_g$ (108 °C). Therefore, the glass formation hypothesis may partially explain the stabilization of ATD by γ-CyD and HP-γ-CyD during freeze-drying.

FT-IR showed that the amide I band of ATD was shifted by the addition of γ-CyD. Therefore, the stabilization of ATD may arise from the interaction between the hydroxyl groups of γ-CyD and the amino acids of ATD, based on the water replacement hypothesis. However, the HP-γ-CyD, which has more hydroxyl groups than γ-CyD, did not preserve the relative activity of ATD after freeze-drying compared to γ-CyD. The protective effect of the γ-CyD on the ATD depended on the number of hydroxyl groups in HP-γ-CyD and on the inclusion complex of ATD with CyD. Therefore, the stabilizing effect of γ-CyD on the relative activity of ATD during freeze-drying may be explained by both the water replacement hypothesis and inclusion complexation between ATD with γ-CyD.

In conclusion, without saccharides the activity of freeze-dried ATD decreased markedly to approximately 48% of its initial value. However, γ-CyD preserved the relative activity of ATD at over 90% by protecting the α-helix structure of ATD. FT-IR measurements revealed that stronger hydrogen bonds were formed between ATD and γ-CyD than between ATD and HP-γ-CyD. Therefore, γ-CyD prevented the relative activity of ATD from decreasing during freeze-drying. This could be explained by the water replacement hypothesis and the formation of an inclusion complex of ATD with γ-CyD. These results suggest that γ-CyD can be used as an effective lyoprotectant excipient for proteins in freeze-drying formulations.

## 3. Experimental

### 3.1. Materials

Samples of ripe kiwifruit (*Actinidia deliciosa* cv. 'Hayward') were purchased at a local market in Tokyo. L-Pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilide (Pyr-Phe-Leu-*p*NA) was purchased from Peptide Institute, Inc. (Osaka, Japan). γ-CyD was purchased from Wacker Chemie AG (Munich, Germany) and HP-γ-CyD was purchased from Nihon Shokuhin Kako Co. (Tokyo, Japan). The average DS is defined here as the number of substituents per single glucopyranose unit. The DS of HP-γ-CyD was 5.5. α,α-Trehalose dehydrate, melibiose, and raffinose were purchased from Sigma Chemical Co. (St., Louis, MO.) Sucrose was purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). All other materials and solvents were of analytical grade. Milli-Q purified water was used in all experiments.

### 3.2. Purification of ATD

ATD was purified from ripe kiwifruit by covalent chromatography according to the methods published by Brocklehurst et al. (1981). Briefly, the proteins precipitated from kiwifruit juice with 60% saturated $(NH_4)_2SO_4$ were passed through a thiopropyl sepharose 6B column (GE Healthcare Bio-Sciences AB), and fully active ATD was eluted with Tris-HCl buffer (pH 8.0) containing L-cysteine and dithiothreitol. The protein concentration of purified ATD solution was determined by the method reported by Lowry et al. (1951) using bovine serum albumin (Fraction V, Sigma Aldrich Co.) as the standard.

### 3.3. Freeze-drying of samples

Freeze-dried samples were prepared as follows. ATD was diluted to a 0.1 mg/mL solution with 50 mM phosphate buffer (pH 6.0). Sugars were added to the ATD/buffer solution to adjust the sugar concentration to 4, 8, or 10 mM. Each solution was transferred into a vial, and the vials were then placed in a methanol bath at −60 °C for 20 min. Freeze-drying was performed at 4 Pa for 24 h.

### 3.4. Assays of ATD activity

ATD in the samples was assayed at 25 °C by the following method. Freeze-dried samples were rehydrated with purified water to give an ATD concentration of 0.1 mg/mL. ATD solution was added to a reaction mixture of 5 mM Pyr-Phe-Leu-*p*NA, and the absorbance increase of pNA at 405 nm was monitored immediately with a UV spectrometer (V-560, JASCO Co., Tokyo, Japan). ATD activity was estimated by the initial reaction rate.

### 3.5. CD analysis

CD spectra of ATD were measured using a spectropolarimeter (J-820, JASCO). Samples were placed in quartz cells with a path length of 1 mm and the CD spectra were measured across a wavelength range of 190–240 nm at 0.2 nm intervals. The ATD secondary structure was estimated by JASCO's protein secondary structure analysis program.

### 3.6. FT-IR spectroscopy

FT-IR measurements were performed at a temperature of 25 °C with an FT-IR spectrometer (FT-IR 4100, JASCO) by the KBr disk method. Freeze-dried ATD samples were prepared for FT-IR measurements in a dry nitrogen purged glovebox by pressing a mixture of the freeze-dried sample and KBr into a pellet as described previously. This procedure for preparing KBr pellets does not alter the structure of proteins in dry solid. The absorbance of each KBr pellet sample was collected in 32 scans at resolution of 4 $cm^{-1}$ in range of 4000–400 $cm^{-1}$.

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