The Effects of Cyclodextrins on the Thermal Denaturation and Renaturation Processes of Bovine Pancreatic Ribonuclease A in an Aqueous Solution Studied by Circular Dichroism and Fluorescence Spectroscopy.

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(Received)

key words: bovine pancreatic ribonuclease A, cyclodextrin, circular dichroism spectroscopy, fluorescence spectroscopy, inclusion, thermal stability

Abstract

Circular dichroism and fluorescence spectroscopic measurements showed that the thermal denaturation and renaturation processes of bovine pancreatic ribonuclease A (RNase A) in an aqueous solution at pH 7.0 are greatly affected by the addition of glucosyl-β-cyclodextrin (G1-β-CD). The result of circular dichroism measurements revealed that G1-β-CD lowered the thermal stability of RNase A to result in an irreversible denaturation of RNase A in the aqueous solution. The α- and γ-cyclodextrin gave less effect on the thermal stability. The ellipticity at 220 nm of the thermally denatured RNase A scarcely recovered with the re-cooling process in the presence of G1-β-CD. The temperature dependency of the fluorescence intensity at 309 nm due to six tyrosine residues of RNase A was significantly affected by the addition of G1-β-CD. The effect of the addition of CDs on the thermal stability was larger in the order of G1-β-CD > γ-CD > α-CD.

Introduction

The interaction between proteins and saccharides in aqueous media has been studied in many aspects. For example, the thermal stability of a protein in an aqueous solution is ordinarily enhanced by the addition of saccharide [1,2]. Another example is that cyclodextrins (CDs), cyclic oligosaccharides composed of more than six glucopyranosyl units, promote the renaturation process of denatured proteins in aqueous solution if they are added at an appropriate concentration [3-5]. This chaperon-like effect of CDs is most distinctive when large sized CDs consisted of more than fifty glucopyranosyl units are applied for the renaturation of denatured proteins [3-5]. These large sized CDs work on stripping denaturants or detergents which prevent renaturation of proteins.

The normal sized CDs, on the other hand, lower the thermal stability of proteins in aqueous solution when they are added excessively [6-9]. The cavity size of normal sized CDs increases in the order of α-, β- and γ-CD whose number of glucopyranosyl units are six, seven and eight, respectively. The inclusion ability of CDs varies dependent on their cavity size. The addition of 0.1 mold m^{-3} of glucosyl-β-CD (G1-β-CD), whose internal cavity size fits to the side chain of tryptophan, to the aqueous solution of chicken egg white lysozyme considerably

lowered the thermal stability of the protein [9]. The addition of α- or γ-CD to an aqueous solution of the protein gave less effect on the lowering of the thermal stability. The addition of methyl α-D-glucoside (MG), which has no hydrophobic internal cavity, raised the thermal stability of the protein. These results suggested that inclusion of tryptophan residues of lysozyme brought about the lowering of the thermal stability of the protein [9]. Such interactions between β-CD (or its derivative) and tryptophan residues have been repeatedly reported [10-13].

The effect of CD's on other aromatic amino acid residues, phenylalanine and tyrosine is not clear at present because proteins usually has some tryptophan residues, which are most likely to be included in the cavity of CDs than other aromatic amino acids. Then, we have selected bovine pancreatic ribonuclease A (RNase A), which has tyrosine residues and no tryptophan residues. A X-crystallographic study on the three-dimensional structure of the RNase A revealed that it is constructed from three paralleling β-sheets and some connecting helix structures, in which six tyrosine (Tyr-25, Tyr-73, Tyr-76, Tyr-82, Ty-97, Tyr-115) and three phenylalanine residues are involved [14]. Thus, the authors have studied the temperature dependency of circular dichroism or fluorescence spectroscopy to observe the conformational change of the main chain of RNase A or environmental change of tyrosine residues in the protein,

respectively.

Experimental

Materials

The α- and γ-CDs were supplied by Nihon Shokuhin Kako Co., Ltd. The G1-β-CD was supplied by Bioresearch Corporation of Yokohama, Ltd. Bovine pancreatic ribonulcease A (RNase A), methyl α-D-glucoside (MG) were purchased from Sigma Chemical Co. and were used without further purification. The CDs and MG were dried overnight in vacuo at 110℃. Sodium monohydrogenphosphate and potassium dihydrogenphosphate of reagent grade were used to prepare a phosphate buffer solution of pH 7.0 for spectroscopic measurements.

Circular dichroism measurements

Bovine pancreatic ribonuclease A was dissolved in a phosphate buffer solution (pH7.0) to become 5 μmol dm^{-3} in concentration. A quartz cuvette of 1cm light path length was filled with the sample solution of 3 cm^3. Circular dichroism spectra were recorded in the range from 200 to 300 nm at an interval of 0.2 nm with a JASCO J-750 spectropolarimeter equipped with a temperature controlling unit. The temperature of

the sample was raised step-by-step from 25℃ to 75℃. The accuracy of temperature was within ±0.1℃. At each temperature for measurement, the solution was secured for 10 minutes before scan to equilibrate the sample solution fully. The values of scan rate, response, band width, and sensitivity were 100 nm/min., 2 sec., 1.0 nm, and 100mdeg, respectively. Four scans were averaged to obtain one spectrum.

Fluorescence spectral measurements

The same samples used for circular dichroism measurements were used for fluorescence measurements. A quartz cuvette of 1cm light path length was filled with the sample solution of 3 cm^3. The fluorescence spectrum was measured with a Shimadzu RF-5300 fluorescence spectrophotometer equipped with a temperature adjustable cell holder. The fluorescence spectrum was measured from 300 to 400 nm with the excitation wavelength at 305nm. The temperature of sample solution was stepwisely elevated from 25 ℃ to 75 ℃ monitored by a cupper-constantan thermocouple. The accuracy of temperature was within ± 0.1 ℃. After the temperature was elevated to 75℃, the temperature of the same sample was stepwise lowered from 75℃ to 25℃. The fluorescence spectra were recorded in each step of temperature. The solution was secured for 10 minutes before scan to equilibrate the

sample solution fully.

Results and Discussion

Circular dichroism measurements

RNase A in an aqueous solution gives a negative circular dichroism band in a wavelength region shorter than 240 nm, due to a negative Cotton effect characteristic of α-helical structure of the protein [15,16]. The absolute value of the ellipticity at 220nm (Θ_{220}) is frequently utilized as a measure of the helix content of a protein (26% of a RNase A molecule is reported to form α-helix structure [17]). This value gradually decreased with elevating temperature, and abruptly decreased in the vicinity of 60℃ and become virtually constant at higher temperatures than 70℃ (Figure 1). The denaturation temperature (*Tm*) was determined to be 64℃ as a point of inflection. The *Tm* value agreed well to that measured by the temperature dependency of the absorbance change of the amide I infrared band assignable to β-sheet structure [18,19]. When the temperature was lowered from 75℃, the Θ_{220} value gradually decreased with lowering temperature. The *Tm* value was 62.0℃. The Θ_{220} value at room temperature did not fully recover its original intensity (Figure 1), suggesting that the denaturation process of the RNase A molecule is partially irreversible. Similar irreversible tendency was also reported on the absorbance change of the amide I

infrared band observed at a corresponding temperature range [18,19].

The plot for the Θ_{220} value of RNase A v.s. temperature in the presence of 0.1 mol dm^{-3} G1-β-CD in the heating process is very similar to that for RNase A without the CD, but the *Tm* value for the former was 2.0℃ lower than the latter (Figure 1). The plot for Θ_{220} value in the cooling process was strongly influenced by the addition of G1-β-CD as shown in Figure 1. The point of inflection was not clearly observed for the cooling process. This suggests that RNase A is irreversibly denatured by the addition of G1-β-CD when the temperature was raised to 75℃.

The temperature dependency of the Θ_{220} value in the presence of MG, which has no inclusion ability, is similarly shown in Figure 2. The plot for the Θ_{220} value of RNase A with MG is very similar to that for RNase A in the absence of the saccharide. This result strongly suggests that the lowering of *Tm* in the presence of G1-β-CD is due to the inclusion ability of the CD. Considering that RNase A has no tryptophan, six tyrosine and three phenylalanine residues, the lowering of *Tm* should be attributed to the inclusion of the side chains of tyrosine and phenylalanine residues by G1-β-CD. The binding constants (*Ka*) for β-CD complexes with three aromatic amino acids decrease in the order of L-tryptophan > L-tyrosine > L-phenylalanine [20,21], suggesting that G1-β-CD gives more effect on six tyrosine residues than three

phenylalanine residues.

The addition of α-CD brought about slight *Tm* lowering of less than 1.0℃. The effect of γ-CD addition on *Tm* was almost the same as that of α-CD. The effect of CD's on the *Tm* of RNase A were generally smaller than those of chicken egg white lysozyme, probably due to the lack of tryptophan residues in RNase A. The effect of α- or γ-CD on the renaturation process of RNase A has less than G1-β-CD as shown in Figure 2.

Fluorescence spectral measurements

The fluorescence spectra gave a maximum emission at 309 nm due to six tyrosine residues of RNase A [22]. The intensity of this fluorescence band changed with varying temperature of the sample solution. Figure 3 shows the temperature dependency of the fluorescence intensity at 309 nm in the absence of saccharide. The intensity linearly decreased with heating to give a minimum intensity (T_{min}) at 60℃. The intensity increased as the temperature was further raised to give a maximum intensity at 66℃ (T_{max}), and then decreased at higher temperatures than the T_{max}. The temperature was raised up to 75℃ and then lowered. The intensity increased in the cooling process to give a weaker maximum intensity at 66℃ than that observed in

the heating process. When the temperature was lowered than 60℃, the fluorescence intensity virtually traced back that of the heating process to regain the original intensity at 25℃.

The above mentioned intensity decrease in fluorescence intensity at 309 nm with heating is due to thermal quenching [23] similarly to tryptophan residues of lysozyme [9]. If no environmental change occurs on tyrosine residues, the fluorescence intensity simply decreases with a temperature increase. However, the unfolding of a RNase A molecule gradually proceeds with heating to expose tyrosine residues from the interior to the surface. The probability of the radiationless transition to the surrounding amino acid residues is large when the tyrosine residues are buried in the interior of the protein , but becomes smaller when the residues are exposed to the surface of the protein [23], resulting in the enhancement of the fluorescence due to the tyrosine residues. The two factors of thermal quenching and denaturation of the protein balance at T_{min} (60℃) to bring forth the intensity minimum of the fluorescence at 309 nm. The enhancement effect of fluorescence intensity due to exposure of tyrosine residues lasts to the point at which the tyrosine residues expose fully to the aqueous solution. This temperature corresponds to T_{max} at 66℃. If the temperature is raised more than T_{max}, the fluorescence intensity decreases again due to thermal

quenching because the protein is already fully denatured. In the cooling process, the fluorescence intensity traced back that of the heating process to recover its original intensity before heating. However, the fluorescence intensity at T_{max} was less prominent than that in the heating process. The difference in intensity is probably due to irreversible denaturation of the protein that raises the probability of radiationless transition to result in the decrease of fluorescence intensity.

The temperature dependency of the fluorescence intensity at 309 nm in the presence of G1-β-CD is also shown in Figure 3. The fluorescence intensity at 309 nm in the presence of G1-β-CD at 25℃ was virtually equal to that in the absence of the CD. However, the temperature dependency of the fluorescence intensity was significantly different: the fluorescence intensity only slightly decreased as the temperature was elevated up to 50℃, and abruptly increased at temperatures higher than 50℃ to give a distinctive intensity maximum at T_{max} (64℃). The relative fluorescence intensity is about twice of that in the absence of G1-β-CD. The fluorescence intensity lowered when the sample is heated higher than T_{max}. The drastic enhancement in intensity caused by G1-β-CD will be triggered by the inclusion of tyrosine residues within G1-β-CD cavities. The inclusion may be caused by extreme exposure of tyrosine residues to the surface of the protein. In the cooling process, the enhancement of

fluorescence intensity was less than the heating process. However, the intensity maximum was more clearly observed than that in the absence of G1-β-CD. The fluorescence intensity of the re-cooled sample, at temperatures lower than 50℃ in the presence of G1-β-CD, is far larger than before heating. This suggests that tyrosine residues are not folded into the interior of the protein to give a smaller probability of radiationless transition. As a result, the stronger fluorescence intensity may has been observed for the re-cooled sample. The similar phenomenon was observed in the fluorescence intensity of tryptophan residues of chicken egg white lysozyme in the presence of G1-β-CD [9].

The temperature dependency of the fluorescence intensity of RNase A at 309 nm in the presence of MG is depicted in Figure 4. The overall feature is very similar to that observed for RNase A in the absence of saccharide, suggesting that the addition of a saccharide with no inclusion ability affects little on the environments of tyrosine residues both in the heating and cooling processes. Thus, the environmental change of tyrosine residues brought about by the addition of G1-β-CD is attributable to its inclusion ability.

The effects of the addition of α- and γ-CD are depicted in Figure 4. The effect of α-CD was not large: the T_{max} value is unchanged by the addition of α-CD. However,

the fluorescence intensity at T_{max} was a little more enhanced compared with the condition in the absence of the CD both in the heating and cooling processes. Thus observed enhancement in fluorescence intensity by the addition of γ-CD suggests that the environment of tyrosine residues has been changed both in the heating and cooling processes. The fluorescence intensity in the presence of γ-CD at T_{max} was about 170 (arbitrary unit), that is far stronger than that in the presence of α-CD, and a little weaker than that in the presence of G1-β-CD. The fluorescence intensity at 25℃ is increased when the sample was re-cooled after the heating process only in the presence of G1-β-CD. The fluorescence intensity at 25℃ was little increased by the heating when α- or γ-CD was added to the sample solution. These results show that the effect of the addition of CD is larger in the order of G1-β-CD > γ-CD > α-CD. Considering that the fluorescence intensity at 25℃ after the heating process in the presence of α- or γ –CD is virtually equal to that without saccharide, the environments surrounding tyrosine residues in the re-cooled RNase A are very similar to that of the protein in the absence of α- or γ -CD.

A remaining problem is that the effect of the lowering of *Tm* in the presence of G1-β-CD is not considered in our present work. The highest temperature experimented in this study was commonly set at 75℃ although the *Tm* is dependent

on the saccharide added to the sample solution. The sample with a lower *Tm* may have been overheated at 75℃ to occur an irreversible denaturation of the protein. It is probable that the conformational change in the re-cooled protein in the presence of G1-β-CD may primarily be attributable to the irreversible denaturation of the protein due to overheating. The temperature dependence of the conformation of the re-cooled sample, which is heated to the appropriate temperature dependent on its *Tm*, may clarify this point. The authors are now working on this problem focused on the temperature dependence of circular dichroism spectra of proteins that are heated to various temperatures.

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Figure Captions

Figure 1. Temperature dependency of the ellipticity of Ribonuclease A at 220 nm in an aqueous buffer at pH 7.0 in the absence of saccharide (heating process : ○, cooling process : □) and in the presence of 0.1 mol dm^{-3} G1-β-CD (heating process : △, cooling process : ▽).

Figure 2. Temperature dependency of the ellipticity of Ribonuclease A at 220 nm in an aqueous buffer at pH 7.0 in the presence of 0.8 moldm^{-3} MG (heating process : ○, cooling process : □), 0.1 mol dm^{-3} α-CD (heating process : △, cooling process : ▽) or 0.1 mol dm^{-3} γ-CD(heating process : ◇, cooling process : ◆).

Figure 3. Temperature dependency of the fluorescence intensity of (0.1 mmol dm^{-3} Ribonuclease A at 309 nm in an aqueous buffer at pH 7.0 in the absence of saccharide (heating process : ○, cooling process : □) and in the presence of 0.1 mol dm^{-3} G1-β-CD (heating process : △, cooling process : ▽).

Figure 4. Temperature dependency of the fluorescence intensity of (0.1 mmol dm^{-3} Ribonuclease A at 309 nm in an aqueous buffer at pH 7.0 in the presence of 0.8 mol dm^{-3} MG (heating process : ○, cooling process : □), 0.1 mol dm^{-3} α-CD (heating process : △, cooling process : ▽) or 0.1 mol dm^{-3} γ-CD(heating process : ◇, cooling process : ◆).

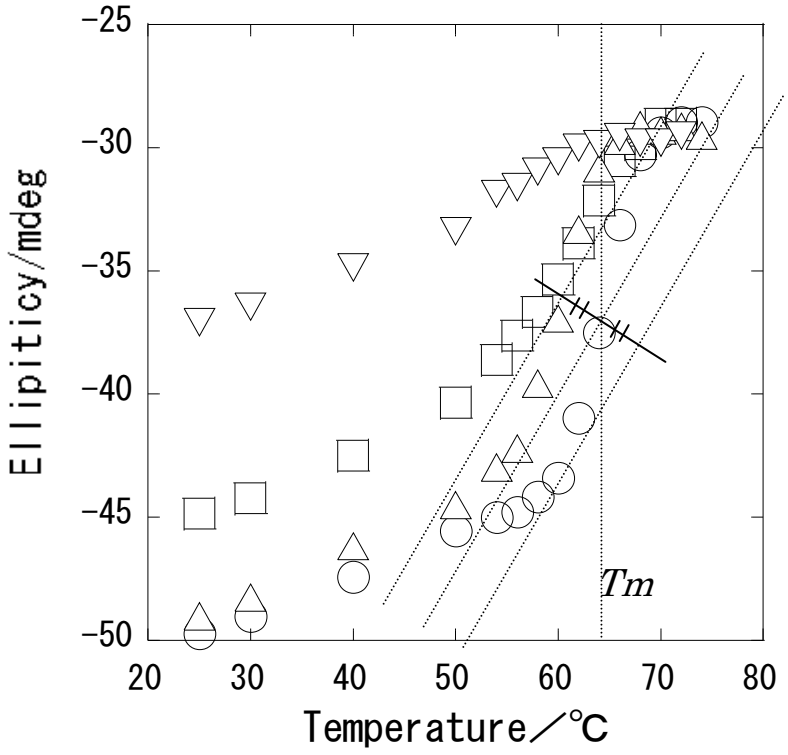
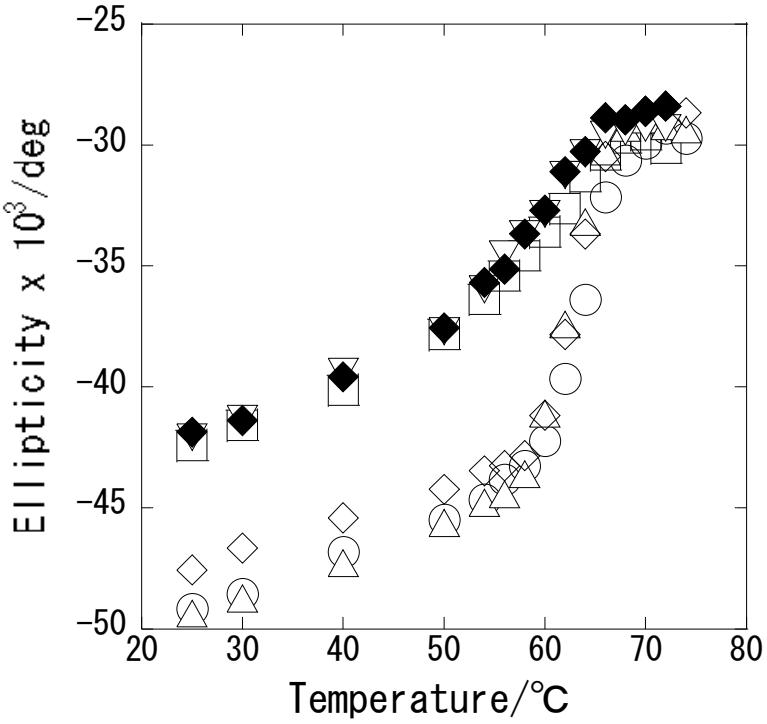


Figure 1.　Yoshikiyo et al.,



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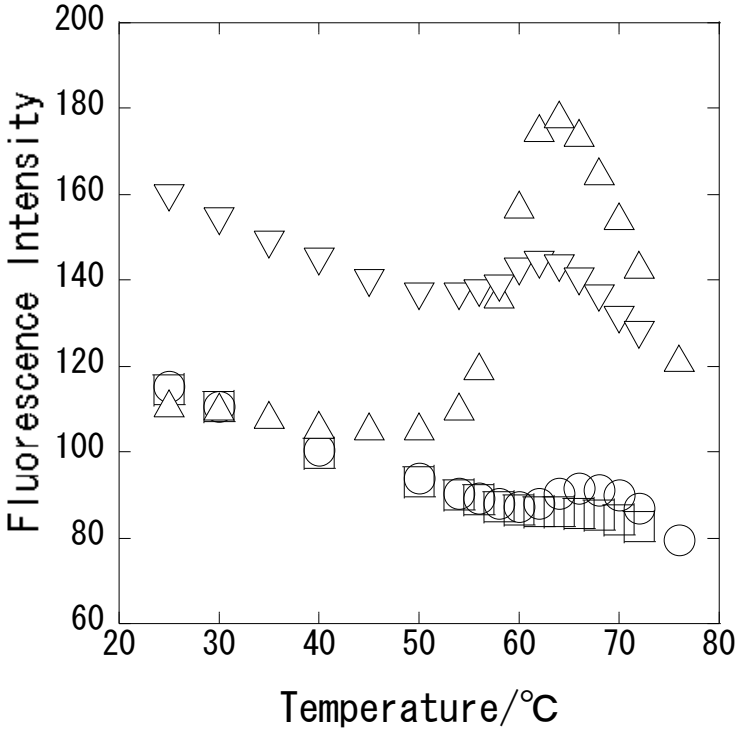

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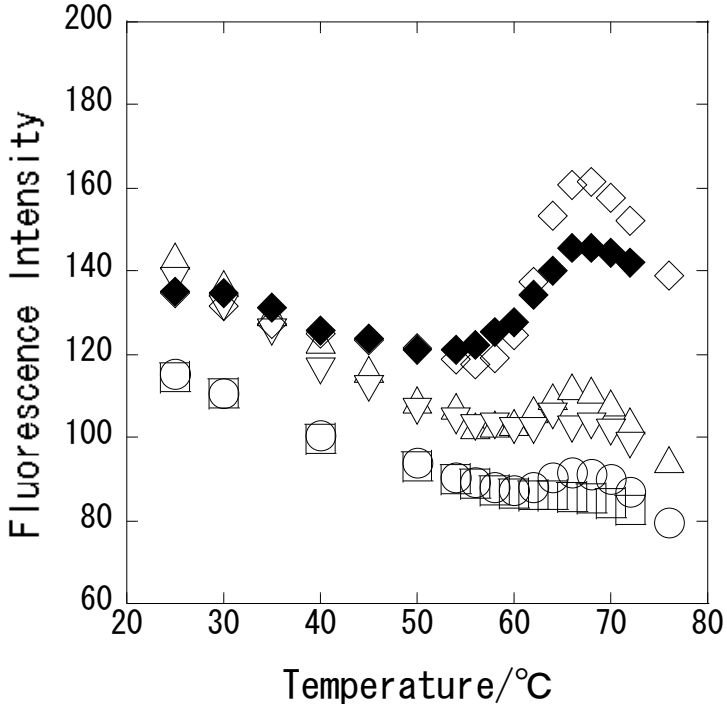

Figure 4. Yoshikiyo et al.,