Circular Dichroism and Fluorescence Spectroscopy Studies of the Effect of Cyclodextrins on the Thermal Stability of Chicken Egg White Lysozyme in Aqueous Solution.

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Abstract

Circular dichroism spectroscopy revealed that the thermal stability of chicken egg white lysozyme in an aqueous buffer solution is significantly lowered by the addition of 6-*O*-α-D-glucosyl-β-cyclodextrin (G1-β-CD), whereas it is raised by the addition of methyl α-D-glucopyranoside. The α- and γ-cyclodextrin also lowered the thermal stability, although the effects were less prominent than that of G1-β-CD. Fluorescence spectroscopy suggested that cyclodextrins include the side chains of tryptophan residues within their internal cavities to lower the thermal stability of lysozyme. The fluorescence intensity of a sample, re-cooled to 25℃ after thermal denaturation at 75℃ in the presence of G1-β-CD, was stronger than that observed for native lysozyme. The fact that the fluorescence intensity of the re-cooling sample was stronger than that of the native one indicates that G1-β-CD persists in binding to the side chains of tryptophan residues of the re-cooled lysozyme.

1. Introduction

The inclusion effects of cyclodextrins (CDs) on the conformations of proteins are recent strong concerns in the field of cyclodextrin science. One of such effects is an artificial chaperone-like function of CDs [1,2]. Proteins prepared *in vitro* are occasionally in aggregated and denatured state. Attempts to renaturate the denatured proteins have been reported with various combinations of denaturants and detergents [3]: The denaturants cause unfolding of denatured proteins and the detergents prevent re-aggregation of unfolded proteins. These reagents are removed by succeeding dialysis to obtain natural proteins. However, the detergent molecules frequently persist in sticking on proteins and prevent prompt renaturation. It was reported that high molecular weight CDs with more than fifty glucosyl residues effectively accelerate the renaturation of proteins [1,2]. Such large size CDs promote the renaturation by stripping off the sticking detergents from the unfolded proteins by inclusion [2].

The normal size CDs, such as α-, β- and γ-CDs, on the other hand, show weaker effect on the promotion of protein renaturation. They rather lower the thermal stability of protein, when they are excessively added to its aqueous solution [4-6]. Suppressing or accelerating effects of some β-CD derivatives on the aggregation

of protein are also reported [7-9]. The interaction of Alzheimer β-amyloid peptide with β-CD is reported by NMR and circular dichroism measurements [10]. These curious phenomena are interpreted that the side chains of aromatic amino acid residues such as tryptophan, phenylalanine or tyrosine are included in the internal cavity of CDs. When the side chains of aromatic amino acid residues are included in CDs, the hydrophobicity of the protein core is diminished and its thermal stability is lowered [4,5]. The effect of cyclodextrin on the dissociation of bovine insulin oligomers [6] and of β-CD dimers on disrupting aggregation of L-lactate dehydrogenase [11], are reported in aqueous solution. The binding constants (K_a's) of CDs with free aromatic amino acids inform us that β-CD includes them stronger than α- or γ-CD [12-14]. A ^{13}C NMR study on the formation and molecular dynamics of CD complexes with phenylalanine also gave the same result with regard to the size of CD cavity [15]. Such strong interaction between aromatic side chain and CD is utilized for stabilizing the helix structure of peptides[16,17].

The aim of the present work is to clarify how CDs affect the thermal stability of protein and also which parts of the protein they include. We have selected chicken egg white lysozyme for this study, because the three dimensional structure is clear and it involves many aromatic amino acid residues [18]. The conformation of the peptide

main chain was analyzed by circular dichroism measurements [19,20]. It is also known that the local condition of six tryptophan residues of chicken egg white lysozyme is reflected on fluorescence spectra [21-23]. Thus, here, we have utilized circular dichroism and fluorescence spectroscopies in order to clarify the mechanism of decrease in the thermal stability of lysozyme with the addition of CDs.

2. Experimental

Materials

The α- and γ-CD were supplied by Nihon Shokuhin Kako Co., Ltd. The 6-*O*-α-D-glucosyl-β-CD (G1-β-CD) was supplied by Bioresearch Corporation of Yokohama, Ltd. Chicken egg white lysozyme, methyl α-D-glucopyranoside (MG) and L-tryptophan 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 buffer solution of pH 7.0 for circular dichroism measurements. Acetic acid and sodium acetate of reagent grade were used to prepare a buffer solution of pH 5.5 for fluorescence measurements.

Circular dichroism measurements

Chicken egg white lysozyme was dissolved in a phosphate buffer (pH 7.0) to become 5 μmol dm^{-3} in concentration. A quartz cuvette of 1 cm light path length was filled with 3 cm^3 of the sample solution. 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 80 ℃. The error 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^{-1}, 2 s, 1.0 nm, and 0.1 deg, respectively. Four scans were averaged to obtain one spectrum.

Fluorescence spectral measurement

Chicken egg white lysozyme was dissolved in an acetate buffer (pH 5.5) to become 0.1 mmol dm^{-3} in concentration. A quartz cuvette of 1 cm light path length was filled with 3 cm^3 of the sample solution. 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 305 nm. The temperature of sample solution was elevated step by step from 25 ℃ to 75 ℃. The error of temperature was within 0.1 ℃. After the temperature was elevated to 75 ℃, the temperature of the same sample was lowered step by step from 75 ℃ to 25 ℃. The fluorescence spectra were recorded in each step of temperature elevating and lowering processes. The solution was secured for 10 minutes before scan to equilibrate the sample solution fully.

3. Results and Discussion

Circular dichroism measurements

Figure 1 shows the circular dichroism spectra of lysozyme at pH 7.0 without CD at 25 ℃ (solid line) and 80 ℃ (dashed line). A negative circular dichroism band was observed in a wavelength region shorter than 240 nm. The band is due to a negative Cotton effect characteristic of helical structure [19,20]. The ellipticity at 220 nm (Θ_{220}) is a standard measure of helical content of a protein and was used to estimate the secondary structural change of the protein. The negative band at 220 nm decreased in intensity with elevating temperature, and the absolute value of the intensity was minimum at 80 ℃. The temperature dependency of the Θ_{220} value was

plotted in Figure 2. In a temperature region below 60 ℃, the Θ_{220} value was almost constant to make a flat baseline. Then, the absolute value of Θ_{220} gradually decreased followed by an abrupt decrease, and finally leveled off at 80 ℃. The denaturation temperature (*Tm*) of lysozyme was determined to be 72 ℃ as the point of inflection of this curve as depicted in Figure 2.

The effects on *Tm* value with the addition of CDs and MG were examined. The addition of the carbohydrates had little effect on the feature of the Θ_{220} value vs. temperature curve except for *Tm* value : The minimum and maximum Θ_{220} values were unchanged. This suggests that the secondary structures of lysozyme in the native and denatured state are not affected by the addition of the carbohydrates. Figure 3 illustrates the plots of the *Tm* values of lysozyme vs. the ratios (***R***) of molar concentrations of the carbohydrates to that of lysozyme. Within the observed *R* range of carbohydrates, the *Tm* values of lysozyme continuously decreased proportional to *R* value without saturation. This suggests that the net K_a value of lysozyme for the complexation with carbohydrates are not large. The strict discussion of K_a seems difficult because a lysozyme molecule has multiple binding sites for compexation with CDs and the number of binding sites is possible to change in the course of denaturation and/or renaturation processes. Thus, we just focus on the fact that the value of *Tm*

decreased with increasing $\boldsymbol{R}$ value. This fact agrees with that of a DSC study on the systems composed of four proteins (lysozyme, ribonuclease A, ubiquitin, and yeast PGK) and α-CD [6]. The decrease in *Tm* with the addition of CD was most prominent for G1-β-CD. The lowering effects of α- and γ-CD were less than that of G1-β-CD. This order in the magnitude of decrease in *Tm* value (G1-β-CD >> α-CD ≒ γ-CD) agrees with the order of K_a values for the complexation of aromatic amino acids or oligopeptides involving aromatic amino acids with α-, β-, γ-CD and some β-CD derivatives [12-15]. The addition of MG, on the other hand, slightly raised the *Tm* value. MG has no ability to include the aromatic amino acid residues. These results strongly suggest that the decrease in *Tm* value of lysozyme is due to the inclusion of aromatic amino acid residues by CDs.

It has been discussed with regard to the effects of carbohydrates with no inclusion ability on thermal stability of proteins in aqueous solution from the structural viewpoint of water molecules surrounding proteins [24,25]. Such carbohydrates were pointed out to enhance the hydrogen bond network of aqueous solution, because their equatorial hydroxyl groups well interact with water molecules [25]. This effect is responsible for enhancement in the thermal stability of lysozyme to raise *Tm* value with increasing MG.

We have also measured circular dichroism spectra for sample solutions adjusted at pH 5.5, where lysozyme is reported to be most stable [22]. Thus, the change in pH value from 7.0 to 5.5 resulted in the increase of *Tm* value about 2 ℃. However, the minimum and maximum Θ_{220} values were little affected by the pH change.

Fluorescence measurement

The fluorescence spectrum of lysozyme in an aqueous solution at pH 5.5 with the excitation wavelength at 305 nm, had a peak at 342 nm without CD at 25 ℃. This fluorescence peak exclusively arises from six tryptophan residues of lysozyme [21,22]. The intensity of this peak decreased with elevating temperature, whereas the value of λ_{max} changed little. The fluorescence intensity increased again with re-cooling. However, the fluorescence intensity of the re-cooled sample at 25 ℃ was weaker than that of the original one at 25 ℃. Changes in fluorescence intensity at 342 nm with temperature in elevating and lowering processes are plotted to give Figure 4. The intensity decreased with temperature elevation till 68 ℃ (T_{min}), at which the fluorescence intensity became local minimum. The T_{min} value at 68 ℃ is lower than the T_{min} value, where the helix content of lysozyme is 50% of the native state, and

corresponds to a temperature where the Θ_{220} value begins to change. The intensity then increased till 75 ℃. When the temperature was succeedingly lowered in a step by step manner, the intensity decreased till T_{min} and then increased with lowering temperature. However, the fluorescence intensities in the re-cooling process below 60 ℃ were weaker than those of temperature elevation process, suggesting that the renaturation of the re-cooled protein is imperfect.

It seems that the decrease in the fluorescence intensity with temperature elevation observed at lower temperatures is not relevant to conformational change of lysozyme, because our circular dichroism measurements suggested that the secondary structure of lysozyme is kept unchanged till about 60 ℃ as shown in Figure 2. The most probable cause is the thermal quenching with elevating temperature. In order to estimate the effect of thermal quenching with elevating temperature, we measured the fluorescence spectra of 0.6 mmol dm^{-3} L-tryptophan in an aqueous solution at pH 5.5. The concentration of L-tryptophan corresponds to the total concentration of tryptophan residues involved in 0.1 mmol dm^{-3} lysozyme. The fluorescence intensity of L-tryptophan at 25 ℃ was stronger than that of lysozyme. In the native state, tryptophan residues of lysozyme are folded in the interior of the protein and surrounded by many amino acid residues [18]. In such a state, the electronically

excited side chain of the tryptophan residues easily relaxes to the ground state via various radiationless transition processes to give weaker fluorescence than the free tryptophan. The fluorescence intensity of L-tryptophan continuously decreased till 75 ℃ in a similar manner as lysozyme between 25 ℃ and 64 ℃. Such decrease in intensity due to temperature elevation is known as thermal quenching of fluorescence. This phenomenon is caused by increase of the probability of intermolecular collisions and non-radiative transitions [26, 27]. The extent of thermal quenching can be empirically estimated by the following analysis. In order to confirm the thermal quenching, we have plotted the logarithm of fluorescence intensity (log F) against the reciprocal value of absolute temperature for L-tryptophan (Figure 5). The data for L-tryptophan were well fitted to a straight line as follows:

$$\log F = 792.1/T - 0.5425, \quad n = 17, r = 0.998,$$

where T, n, and r are absolute temperature, number of data, and correlation coefficient, respectively. The linear relationship between log F and $1/T$ for L-tryptophan supports the interpretation that the intensity decrease is due to thermal quenching, because factors relevant to fluorescence quenching (collision of molecules, internal conversion and intersystem crossing) are proportional to $\exp(-\Delta E/kT)$ (ΔE : activation energy, k: Boltzmann constant) [28]. The similar linear relationship were obtained for

lysozyme at the temperature region between 25 ℃ and 64 ℃ as follows (Figure 5):

$\log F = 634.7/T - 0.1746$, $n = 11$, $r = 0.994$ (temperature elevating process),

$\log F = 474.6/T - 0.2981$, $n = 11$, $r = 0.999$ (re-cooling process).

These results show that the decrease in fluorescence intensity of lysozyme between 25 ℃ and 64 ℃ is also due to thermal quenching. We do not discuss more about the implication of the activation energy. We consider that the slope corresponds to the relative probability of fluorescence against intermolecular collisions and other non-radiative transitions. The fact that the slopes of the tangent line for G1-ß-CD added lysozyme changes indicates that the optical environments of tryptophan residues are strongly changed by the existence of the cyclodextrin, thus increase the probability of fluorescence. In the temperature region above 64 ℃, the log F values gradually increased with temperature elevation. The lysozyme molecule starts to unfold above 64 ℃, and the buried tryptophan residues are gradually exposed to the solvent. This causes a decrease in the probability of the radiationless transition of energy to surrounding residues and enhances the fluorescence quantum yield. The two factors of thermal quenching and unfolding finally balance at T_{min} (68 ℃) to make a local minimum in fluorescence intensity. At temperatures higher than T_{min}, the factor of unfolding overwhelms that of thermal quenching, and fluorescence intensity increases

at higher temperatures than T_{min}.

The fluorescence spectra of lysozyme in the presence of 0.8 mol dm^{-3} MG at pH 5.5 was similarly measured (Figure 6). The addition of MG caused a raise in T_{min} value about 1 ℃. This agrees with the result of circular dichroism measurements. The temperature dependency of the fluorescence intensity was drastically changed by the addition of G1-β-CD as shown in Figure 7. The intensity decreased till T_{min} (60℃), then increased till 70 ℃ (T_{max}) and finally decreased at higher temperatures than T_{max}. The intensity decrease at higher temperatures than T_{max} is attributed to thermal quench of fluorescence, since the quantum yield of fluorescence has already been enhanced to the full at T_{max}, at which all tryptophan residues are exposed to the solvent. In the re-cooling process, the fluorescence intensity went back to follow the trace of temperature elevating process with weaker maximum and minimum intensities at T_{max} and T_{min}, respectively. It is noticeable that the fluorescence intensity of the re-cooled sample at 25 ℃ was stronger than that of the pre-heated sample. This tendency was reproducibly observed. Temperature dependency of the fluorescence intensity of lysozyme in the presence of α-CD is shown in Figure 8. Although less prominent than the case of G1-β-CD, the overall feature of temperature dependency of fluorescence intensity is similar to Figure 7: The intensity decreased

till T_{min} (64 ℃), then increased till T_{max} (72 ℃), and decreased at higher temperatures than T_{max}. In the course of re-cooling process, the intensity roughly traced back that of the temperature elevating process. The intensity of the re-cooled sample at 25 ℃ was just the same as that of the pre-heated sample. This fact might suggest that α-CD promotes the renaturation of the protein in the re-cooling process. However, as will be mentioned below, the comparable intensity in the re-cooling process with that in the temperature elevating process will be brought about by α-CD which persists in including the side chains of tryptophan residues. The addition of γ- CD gave less effect than α-CD on temperature dependency of fluorescence intensity (Figure 9). The temperature dependency was more similar to that observed in the absence of CD than that in the presence of α-CD. However, T_{min} (66 ℃) was about 2 ℃ lower than that observed in the absence of CD. This shows that the addition of γ- CD gives an effect to some extent on thermal stability of lysozyme. The fluorescence intensity of the re-cooled sample at 25 ℃ was slightly weaker than that of the pre-heated sample

Table 1 shows effects of CDs and MG on the fluorescence intensity of L-tryptophan at 25 ℃. The fluorescence intensity was apparently enhanced by the addition of CDs. The magnitude of the effect increased in the order of MG < α-CD ≒ γ-CD << G1-β-CD, indicating that the inclusion of tryptophan by CDs, especially by

G1-β-CD, enhances the fluorescence intensity. The enhancement may be due to a change in the environment of tryptophan from hydrophilic to hydrophobic [26]. Hence, the excess recovery of fluorescence intensity in re-cooling process in the presence of CDs will be recognized as the effect of inclusion of tryptophan residues by CDs. The conformational changes of lysozyme with temperature elevating and re-cooling processes in the presence of CDs are schematically summarized in Figure 10. Strong interaction of G1-β-CD with indole ring may hinder the renaturation of protein in the re-cooling process.

As a conclusion, the present study revealed that the thermal stability of lysozyme is lowered by the addition of CDs, which include the tryptophan residues of the protein to diminish the hydrophobicity of the hydrophobic core of lysozyme. Some CDs persisting in including the side chain of tryptophan residues may retard the full renaturation of lysozyme, which causes the excess recovery of the fluorescence intensity. However, it remains to be solved whether CDs interact with other amino acid residues of lysozyme or not. In order to solve this problem, it is necessary to use spectroscopies other than fluorescence spectroscopy. We are now working with NMR spectroscopy.

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Table 1. The relative fluorescence intensity of L-tryptophan (0.6 mmol dm^{-3}, pH 5.5, 25 ℃) at 342 nm with an excitation wavelength at 305 nm in the absence and presence of carbohydrates

Carbohydrate (conc./ mmol dm^{-3})	Relative Fluorescence Intensity / %
None(−)	100
MG (0.8)	102
G1-β-CD (0.1)	189
α-CD (0.1)	131
γ-CD (0.1)	133

Figure Captions

Figure 1. Circular dichroism spectra of 5 μmol dm^{-3} lysozyme in an aqueous buffer (pH 7.0) at 25 ℃ (solid line) and 80 ℃ (dashed line).

Figure 2. Temperature dependency of the ellipticity at 220 nm (Θ_{220}) of lysozyme in aqueous buffer at pH 7.0. The *Tm* value was determined as the point of inflection in this curve.

Figure 3. The *Tm* values of lysozyme in the presence of CDs (▲: α-CD, ○: G1-β-CD, □: γ-CD) and MG (×) in various molar concentration ratios (***R***). The actual molar concentration ratio of MG is multiplied by eight. The straight lines are fitted lines for data.

Figure 4. Temperature dependency of the fluorescence intensity at 342 nm of (0.1 mmol dm^{-3} lysozyme in an aqueous buffer (pH 5.5) in temperature elevating process (○) and re-cooling process (▲).

Figure 5. The log *F* vs. 1000/*T* plots for L-tryptophan (●), lysozyme (temperature elevation process : ○), and lysozyme (re-cooling process : ▲), respectively. Three straight lines represent fitted lines as described in the text.

Figure 6. Temperature dependency of the fluorescence intensity at 342 nm of 0.8 mmol dm^{-3} lysozyme in an aqueous buffer (pH 5.5) in the presence of MG in

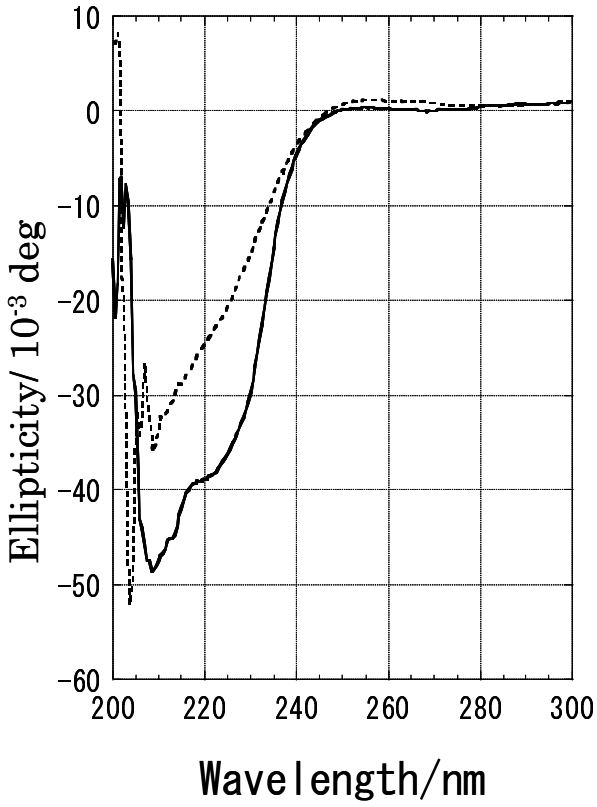
temperature elevating process (○) and re-cooling process (▲).

Figure 7. Temperature dependency of the fluorescence intensity at 342 nm of 0.1 mmol dm^{-3} lysozyme in an aqueous buffer (pH 5.5) in the presence of G1-β-CD in temperature elevating process (○) and re-cooling process (▲) .

Figure 8. Temperature dependency of the fluorescence intensity at 342 nm of 0.1 mmol dm^{-3} lysozyme in an aqueous buffer (pH 5.5) in the presence of α-CD in temperature elevating process (○) and re-cooling process (▲).

Figure 9. Temperature dependency of the fluorescence intensity at 342 nm of 0.1 mmol dm^{-3} lysozyme in an aqueous buffer (pH 5.5) in the presence of γ-CD in temperature elevating process (○) and re-cooling process (▲).

Figure 10. A schematic picture representing the conformational change of lysozyme with temperature elevating (solid arrow) and re-cooling (dashed arrow) processes in the presence of CDs. The ellipses and bucket-like figures represent the side chains of tryptophan residues and cyclodextrins, respectively (the real number of ellipses should be six, but is reduced by half to avoid becoming mixed-up). Some cyclodextrin molecules persist in binding to the side chains of tryptophan residues to retard the renaturation of lysozyme, which causes the excess recovery of the fluorescence intensity after the re-cooling process.



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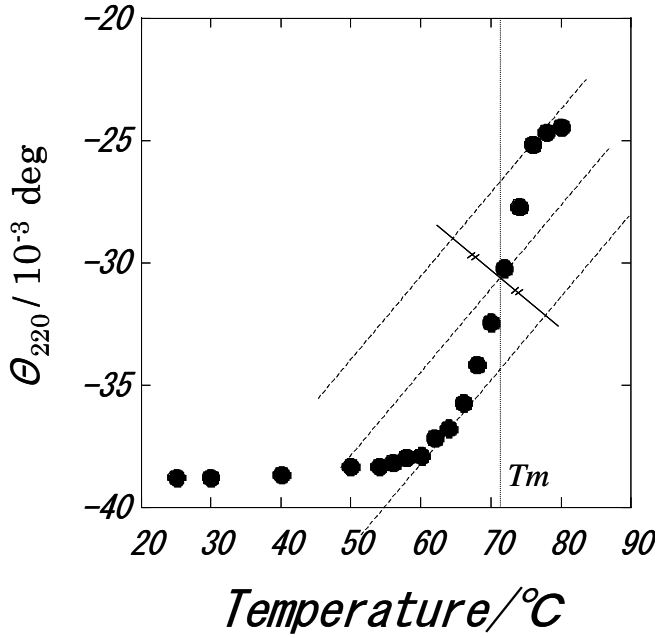

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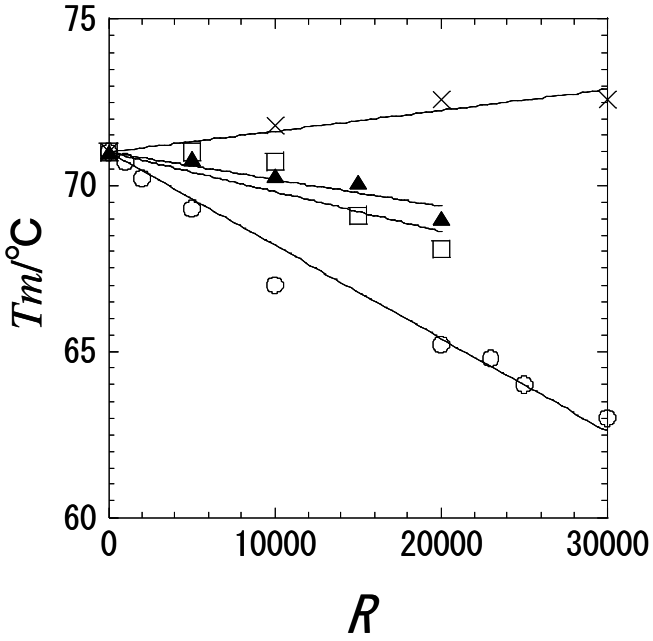

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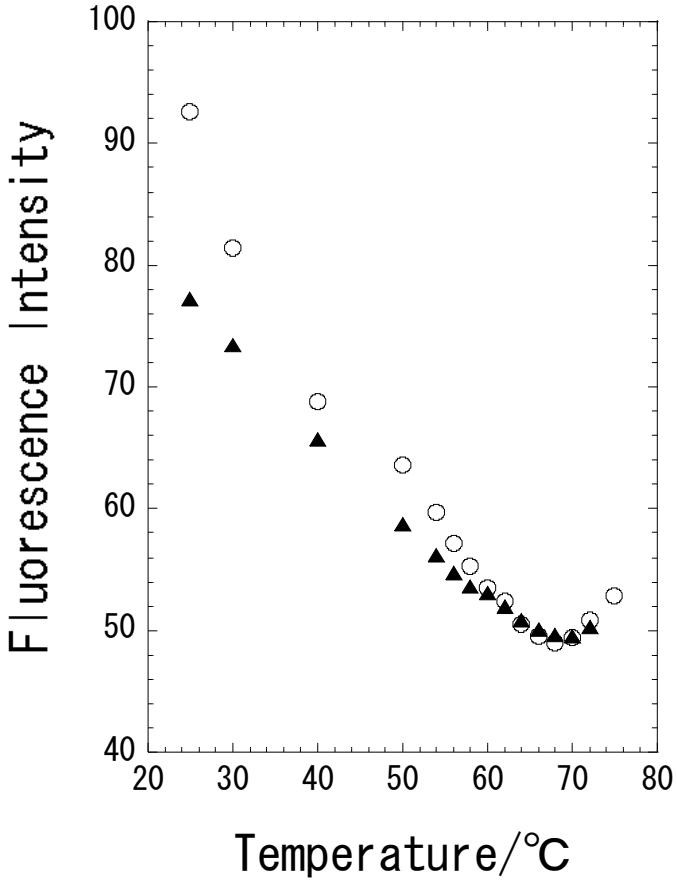

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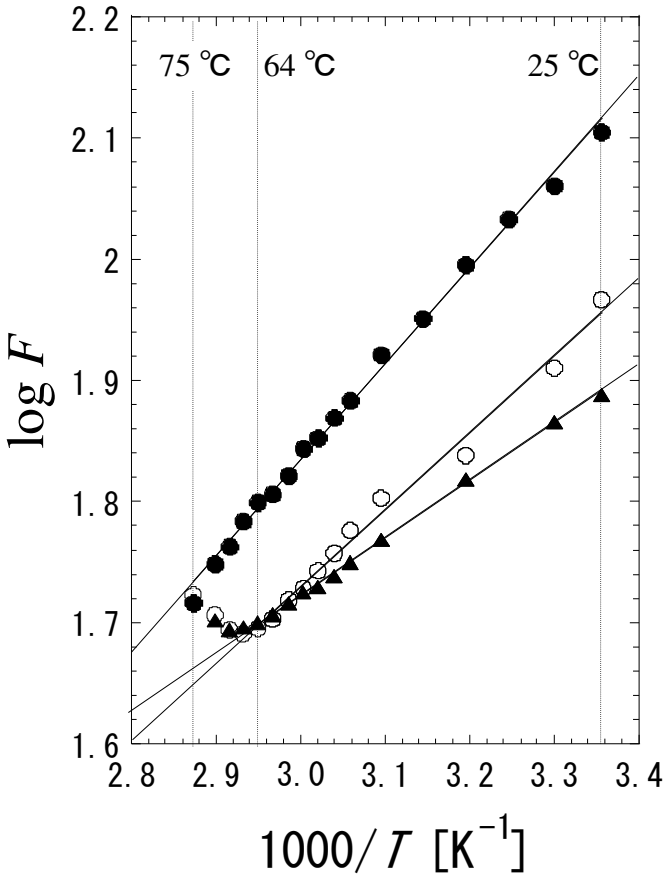

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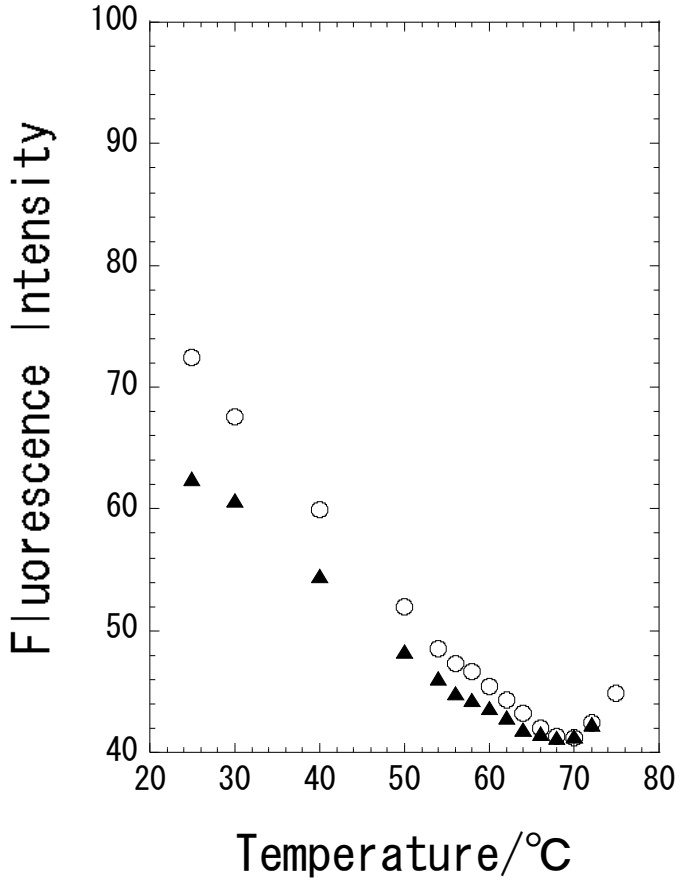

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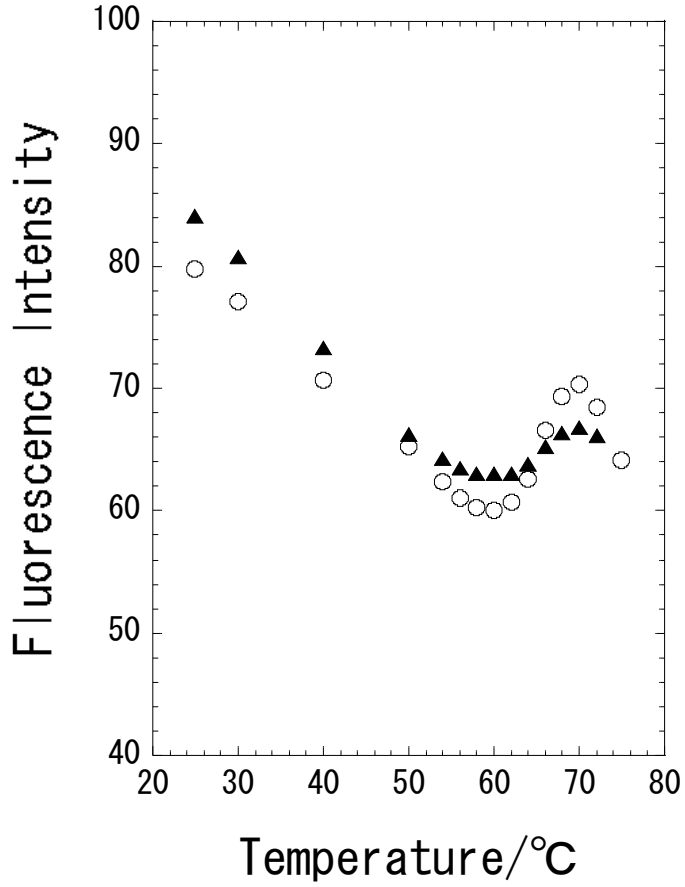

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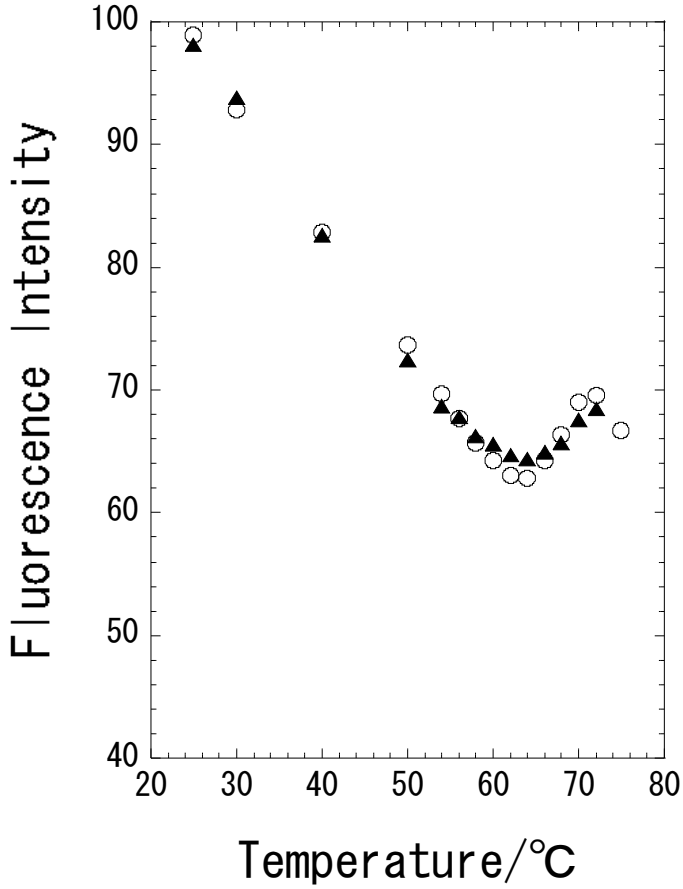

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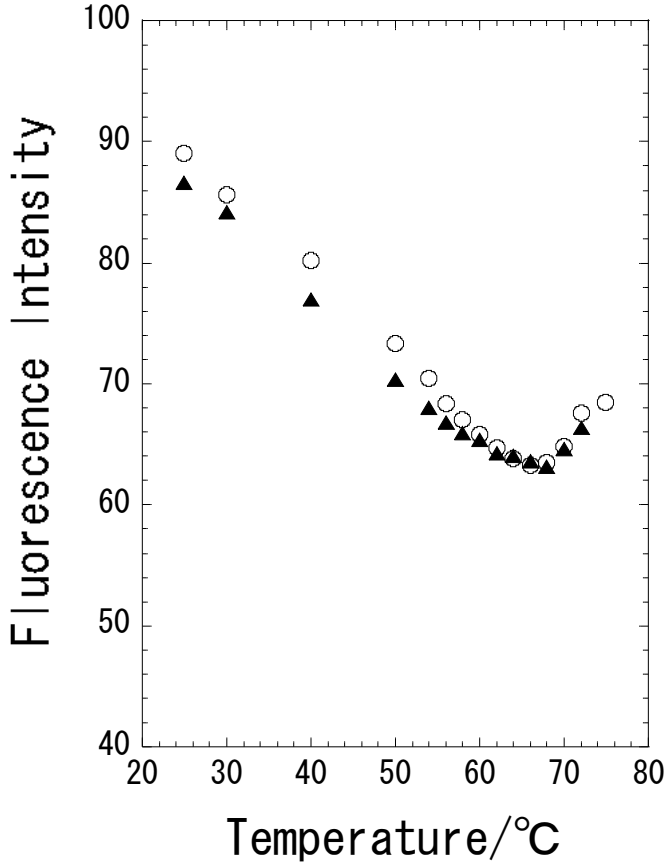

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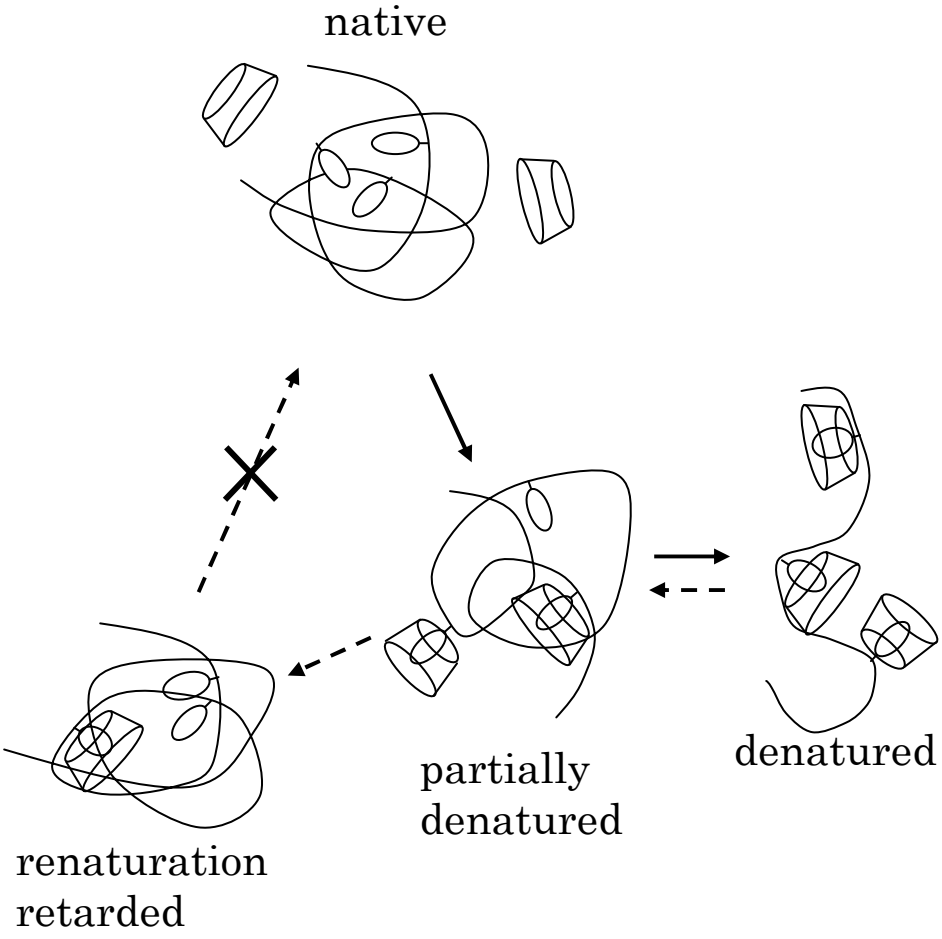

Figure 10. T. Yamamoto, et al.