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Fluorophotometric Determination of Cycloheptaamylose and Its Application to the Studies on Solubilization of the Cycloamylose with Urea[1]

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In the preceding paper[1] was reported the preparation of a complex between cycloheptaamylose (C7A) and 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride, DNS-Cl), and its application to the fluorescent labeling of proteins and a biomembrane. This C7A-DNS-Cl complex (CDC) was found to be highly soluble in aqueous urea solution and moderately soluble in aqueous media containing excess C7A. These findings were useful for dansylating proteins and a plasma membrane in a homogeneous system while DNS-Cl is usually scarcely soluble in water. Since urea alone does not increase the solubility of DNS-Cl, these phenomena are assumed to be due to solubilization of C7A by some interaction with urea.

On the other hand, the first paper of this series[3] described the enhancement of the fluorescence intensity of dansyl amino acids in aqueous media by addition of C7A and its application to assay of amino acids. Dansyl leucine (DNS-Leu) showed the greatest increment of fluorescence among the dansyl amino acids tested.[3,4] C7A was also found to largely enhance the fluorescence intensity of 1-anilinonaphthalene-8-sulfonate (ANS) by Cramer and co-workers.[5]

In the present study, fluorophotometric assay methods for C7A were devised utilizing the enhancement of fluorescence intensity of DNS-Leu and ANS. Solubility of C7A in urea solution was studied by means of ANS reagent. Some thermodynamic studies on the interaction of C7A with urea are also described below.

Materials and Methods

C7A was purchased from Hayashibara Biochemical Laboratories, Inc., and once recrystallized from water before use. Magnesium 1-anilinonaphthalene-8-sulfonate (ANS) was the gift of Tokyo Kasei Kogyo Co. DNS-Leu was purchased from Seikagaku Kogyo Co. Solubility experiments were carried out in a reaction vessel thermostated within an accuracy of 0.1° by a Sharp Thermo Electric Model TE-12K thermostat. Fluorescence intensity was measured with a Hitachi Model 103 fluorescence spectrophotometer thermostated with the same thermostat.

Fluorophotometric Determination of C7A——a) ANS Method: To 2 ml of an aqueous sample solution containing 0.4 to 3.0 mg of C7A per ml is added 2 ml of 60 mM ANS in 0.2M phosphate buffer, pH 7.7 (ANS reagent). The resultant fluorescence is measured at 22.5° at the excitation and emission wavelengths of 380 nm and 500 nm, respectively. An equivolume mixture of the ANS reagent and water is used as the reference.

b) DNS-Leu Method: The procedure is essentially the same with that of ANS method except that 400 mM DNS-Leu in 0.2M phosphate buffer, pH 7.7, is employed in place of the ANS reagent, and the excitation and emission wavelengths are 365 nm and 510 nm, respectively.

Solubility Determination——A hundred milliliter of an aqueous solution, containing 0.27 g of KH_2PO_4, 6.45 g of $NaHPO_4$, 0 to 6M urea and appropriate amount of C7A, was incubated at a proper temperature

1) This paper constitutes part III of the series entitled "Microanalysis of Proteins and Peptides"; Part II: T. Kinoshita, F. Iinuma, and A. Tsuji, *Chem. Pharm. Bull.* (Tokyo), 22, 2421 (1974).
2) Location: *Hatanodai-1, Shinagawa-ku, Tokyo.*
3) T. Kinoshita, F. Iinuma, and A. Tsuji, *Chem. Pharm. Bull.* (Tokyo) 22, 2413 (1974).
4) T. Kinoshita, F. Iinuma, and A. Tsuji, *Biochem. Biophys. Res. Commun.*, 51, 666 (1973).
5) F. Cramer, W. Saenger, and H.-Ch. Spatz, *J. Am. Chem. Soc.*, 89, 14 (1967).

for 2 hr under shaking. After the incubation, the mixture was centrifuged to remove excess C7A, adequately diluted, and the concentration of C7A was determined by ANS method. The amount of C7A employed in this experiment was a little excess than that required for saturation. The approximate saturation point was previously determined by portionwise addition of C7A to 0 to 6M urea solution buffered with the same concentration of the phosphates mentioned above.

Results and Discussion

Fluorophotometric Determination of C7A

Cramer and coworkers[5] reported the increased fluorescence of ANS induced by C7A at neutral pH. Optimum pH range for the fluorescence enhancement of dansyl amino acids was found to be 6 to 10.[3] In the present study, therefore, assay of C7A was carried out at pH 7.7.

The preceding paper[3,4] demonstrated that urea, sodium chloride, glucose and lysine little affect the fluorescence of dansyl amino acids enhanced by C7A. Since fluorescence intensity ought to be measured in the presence of urea for determining the solubility of C7A, the influence of urea on the assay of the oligosaccharide was further examined. Fig. 1 exhibits the effect of urea on the fluorescence intensity of ANS and DNS-Leu in the presence of C7A. DNS-Leu method appears to be less affected by urea of high concentration. However, the assay methods are sensitive enough so that the fluorescence is measured at urea concentration below 0.1M in the solubility study. Either assay method is not practically affected by such low concentration of urea.

Fig. 2 displays the standard curves for C7A by ANS method and DNS-Leu method. The fluorescence intensities of reagent blanks in both methods corresponded to about 0.2 mg/ml of C7A. Since the standard curve for ANS method showed more excellent linearity than that for DNS-Leu method, the former method was employed in the following solubility study.

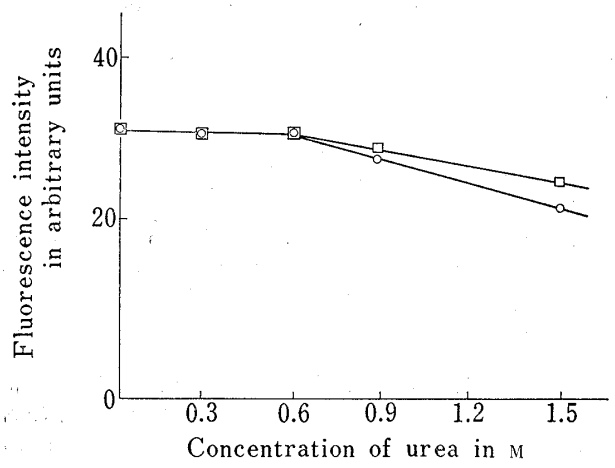

Fig. 1. Effect of Urea on the Fluorescence Intensity of ANS (○) and DNS-Leu (□) in the Presence of C7A (1.5 mg/ml)

For concentration of the fluorochromes, refer to the text.

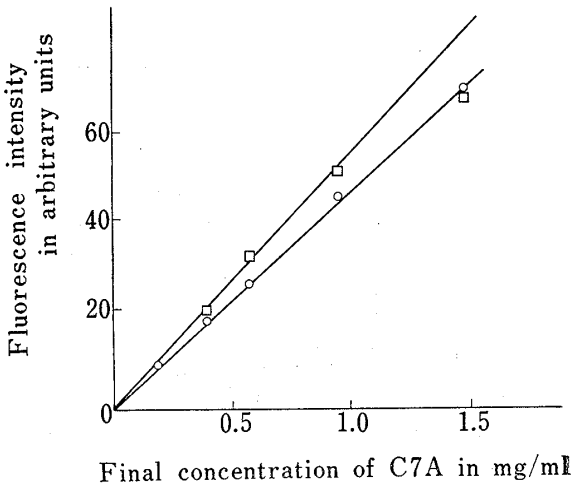

Fig. 2. Standard Curves for C7A in ANS Method (○) and in DNS-Leu Method (□)

Solubility Study

Solubility of C7A in aqueous media is comparatively low as an oligosaccharide. Fig. 3 indicates that its solubility is markedly enhanced by addition of urea and that the increment of the solubility increases with the concentration of urea. In 6M urea is dissolved 142 mg and 200 mg of C7A per ml at 24.3° and 39.5°, respectively.

The solubility data in Fig. 3 was further analysed thermodynamically as follows. When considering the process where C7A is transferred from water to urea solution, the following

equation for obtaining free energy (ΔG) of partitioning of C7A between water and urea phases may be written.[6]

$$\Delta G = -2.3RT \log (N/N_0)$$

where N_0 and N indicate mole fractions of C7A in water and that in urea solution, respectively. Enthalpy (ΔH) for partitioning can be determined according to van't Hoff equation and entropy (ΔS) can be calculated from ΔG and ΔH.[6,7] The results thus obtained are summarized in Table I.

The mechanism of solubilization by urea seems to be affected by complicated factors.[8] Complex formation between urea and the guest molecule solubilized is not always substantiated. Feldman and Gibaldi[6] investigated thermodynamically the solubilization of benzoic and salicylic acid by urea and observed small negative free energy, small positive entropy, and enthalpy of approximately zero. These data do not support hydrogen bond formation and, in addition, rule out the possibility of complex formation between urea and these aromatic acids. Solubilization of these substances was therefore assumed to be due to the break up of water clusters.

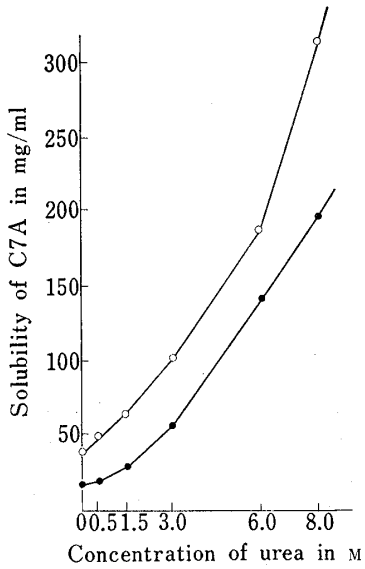

Fig. 3. Solubility of C7A as a Function of Urea Concentration at 24.3° (●) and 39.5° (○)

Table I. Thermodynamic Data for C7A in Urea Solution

Concentration of urea (M)	ΔG (cal·mol^{-1}) 24.3°	ΔG (cal·mol^{-1}) 39.5°	ΔH (cal·mol^{-1})	ΔS (cal·deg^{-1}·mol^{-1})
0.5	− 124	− 126	− 92	+ 0.1
1.5	− 395	− 345	−1364	− 3.3
3.0	− 781	− 731	−1756	− 3.3
6.0	−1451	−1208	−6205	−16.0
8.0	−1715	−1693	−2147	− 1.5

On the contrary, sizable thermodynamic values were obtained in the present study. The free energy change at urea concentration of 6M is in the range of −1.2 to −1.5 kcal·mol^{-1}, indicating a spontaneous process for the solubilization. This ΔG is larger than that for hydrogen bond formation (−0.5 to +1 kcal·mol^{-1}).[9] The enthalpy and entropy at urea concentration of 6M are −6.2 kcal·mol^{-1} and −16.0 cal·deg^{-1}·mol^{-1}, respectively. These values are far greater than the enthalpy ranging −1.5 to 0 kcal·mol^{-1} and the entropy ranging −3 to −5 cal·deg^{-1}·mol^{-1} reported for hydrogen bond formation in aqueous media.[9] These results substantiate the involvement of hydrogen bond in the solubilization of C7A. Moreover, markedly large values for the enthalpy and entropy strongly suggest the formation of a complex between C7A and urea. The enthalpy and entropy in 8M urea are unexpectedly small. This may partly be due to the high viscosity of the solution.

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7) I. Klotz, "The Proteins," Vol. 1. ed. by H. Neurath and K. Bailey, Academic press Inc., New York, 1953, pp. 727—806.
8) F. Cramer, "Einschlussverbindungen," Springer-Verlag, Heidelberg, 1945; K. Takemoto, "Inclusion Compound," Tokyo Kagaku Dojin, Tokyo, 1969, p. 5.
9) K. Shibata, "Chemical Methods for the Investigation of Protein Conformation," University of Tokyo Press, Tokyo, 1969, p. 10.

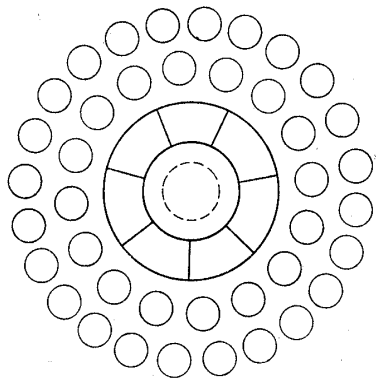

Fig. 4. Posturated Structure for C7A-Urea Adduct and the Mode of Inclusion of DNS-Cl in the Hydrophobic Cavity of C7A

: C7A ○: urea, ◌: DNS-Cl

There are two possible mechanism for the interaction of urea with C7A. The first is that urea interacts with the inside of the ring of C7A to give an inclusion compound, and the second is that urea forms hydrogen bond with hydroxyl groups outside the ring to construct a cluster around the C7A molecule. If the first assumed mechanism is the case, urea may compete with other substrates which form inclusion compound with C7A. Perchlorate and benzoate have been proved to be included in the cavity of cycloamyloses by Cramer and co-workers.[3] We have demonstrated that the fluorescence of dansyl valine enhanced by addition of C7A is quenched by 55 and 75% by each 0.25 M of perchlorate and benzoate, respectively.[4] This may be due to competition of these anions with dansyl valine for the binding site of C7A molecule. On the other hand, the fluorescence of the same dansyl amino acid was not affected by addition of 0.25 M urea.[4] Similarly, the fluorescence of ANS and DNS-Leu in the presence of C7A was not quenched by 0.3 M urea in the present study (Fig. 1). In addition, inclusion of a molecule bearing polar group(s) in C7A cavity has been proved to show positive entropy change.[4,5,10] Apparently, this is not the case with urea which shows large negative entropy. These facts rule out the first mechanism. The second assumed mechanism therefore seems more reasonable and the following interpretation of the thermodynamic data may be conceivable.

According to Cramer and Hettler,[11] all the glucose residues in C7A are present in C-1 chair form, the CH-groups of carbons 3 and 5 of each unit forms the inside of the ring, and primary and secondary hydroxyl groups are therefore projecting outside the ring. If this conformation is correct, then hyrogen bond formation to give a considerably bulky urea cluster around the C7A ring would readily occur, and, moreover, the hydrogen bonding would little affect the small molecule included inside the cavity of C7A. The structure of the complex may be posturated as depicted in Fig. 4.

Although DNS-Cl is hardly soluble either in water or urea solution, it is highly soluble in aqueous solution containing both C7A and urea.[1] C7A-DNS-Cl complex (CDC) is also excellently soluble in urea solution.[1] These facts suggest that C7A exerts, in a sence, "Einschleppeffekt."[8] C7A appears to provide a hydrophobic region, like proteins, in aqueous urea solution and solubilize hydrophobic substrate such as DNS-Cl. C7A-urea system is expected to be effective for solubilization of various reagents or pharmaceuticals.

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