Supramolecular Assembly between Nanoparticles of Hydrophobized Polysaccharide and Soluble Protein Complexation between the Self-Aggregate of Cholesterol-Bearing Pullulan and α-Chymotrypsin

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ABSTRACT: Complexation between α-chymotrypsin (Chy) and the self-aggregate of cholesterol-bearing pullulan (CHP) was studied by size exclusion column chromatography (SEC), fluorescence spectroscopy, circular dichroism (CD), and differential scanning calorimetry (DSC). The CHP self-aggregate strongly complexed with the Chy dimer and formed colloidally stable nanoparticles (R_G = 12 nm) at pH 4.2 and 25 °C. Enzymatic degradation of the CHP–Chy complex by pullulanase suggested that Chy may locate deeply inside the matrix of the CHP self-aggregate hydrogel. Upon complexation, the bulk structure of the CHP aggregate changed, the helix content of Chy increased (from 9 to 29%), and the β-form content decreased (from 34 to 21%). V_{max} of the complexed Chy at pH 8.0 decreased up to 1/88 compared with that of free Chy at pH 8.0, while K_m did not change much. Chy was released from the complex by the addition of bovine serum albumin (BSA). Released Chy had almost the same enzymatic activity as that of free Chy before the complexation. The secondary structure of Chy in the complex did not change much even after the complex was treated for several hours at 92 °C. Even after the heating, Chy was released from the complex by adding BSA and still had 74% of the original enzyme activity. No thermal unfolding of Chy in the complex was suggested by DSC and fluorescence spectroscopy over the temperature range 20–80 °C. These results indicated that the thermal stability of Chy dramatically increases upon complexation with the CHP self-aggregate.

Introduction

Solution properties of hydrophobized polymers have been extensively studied from the viewpoint of industrial applications such as oil recovery and latex paint technology.[1] However, most previous studies have focused on their association behavior in water[2] and the interaction with relatively small molecules such as fluorescence probes[3] or surfactants[4] but scarcely on the interaction with other macromolecules. We have recently reported that hydrophobized polysaccharide derivatives, such as cholesterol-bearing pullulan (CHP, Figure 1), form stable nanoparticles by self-aggregation (diameter 20–30 nm) and complexes with various small hydrophobic molecules.[5] The hydrophobized polysaccharides also interact with various molecular assembly systems such as liposomes,[6] lipid monolayers,[7] black lipid membrane,[8] and O/W emulsion.[9]

In a previous communication, we reported that hydrophobized polysaccharides complex even with various globular proteins.[10] Interaction between proteins and water-soluble polymers has attracted much attention with respect to the understanding of many biological systems as well as the immobilization of proteins in biotechnology.[11] On the concept of supramolecular assembly, cluster formation through hydrogen bonding, host–guest complexation, and self-organization of small amphiphilic molecules have been extensively investigated.[12–14] However, supramolecular assembly in macromolecules such as synthesized polymers and proteins or enzymes has scarcely been investigated.[15] The complex between proteins and the self-aggregate of hydrophobized polysaccharide is one example of a supramolecular assembly in macromolecules. In this paper, we describe a basic study of the macromolecular

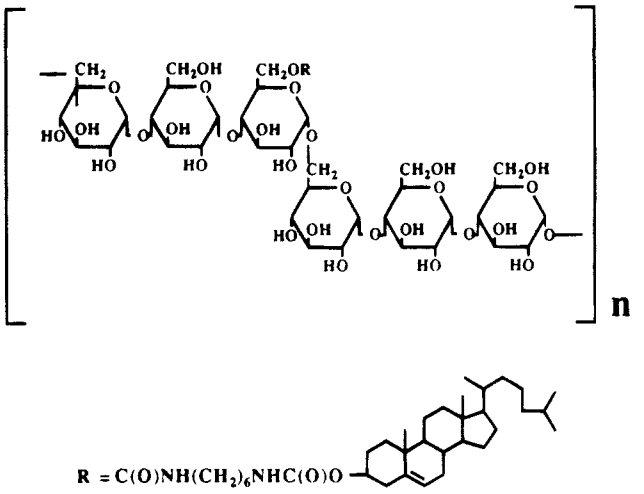

Figure 1. Structure of cholesterol-bearing pullulan.

complexation between the CHP self-aggregate and a soluble enzyme, α-chymotrypsin (Chy), using size exclusion column chromatography (SEC), fluorescence spectroscopy, circular dichroism (CD), and differential scanning calorimetry (DSC).

Materials and Methods

Materials. A pullulan (M_w = 5.5 × 10^4, M_w/M_n = 1.6) derivative carrying two cholesterol groups per 100 glucose units (coded as CHP-55-2) was synthesized according to the method previously reported.[5c] The cholesterol groups are incorporated at random in the parent pullulan.

α-Chymotrypsin (Chy, Type I-S originating from bovine pancreas, Sigma, St. Louis, MO) and pullulanase (*Klebsiella pneumoniae*, Hayashibara Biochemical Lab Inc., Okayama) were commercially available and used without further purification. Neopullulanase was a kind gift of Dr. Hidetaka Okada, Ezaki Glico Co. Ltd., Osaka. Fatty acid bearing bovine serum albumin (BSA) (Seikagakukogyo Co. Ltd., Tokyo) was

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employed, and its thiol groups were treated with iodoacetamide to prevent dimerization of BSA.[16] Magnesium 1-anilinonaphthalene-8-sulfonate (ANS) (Nacalai Tesque, Inc., Kyoto), 2-(*N*-morpholino)ethanesulfonic acid (MES) (Dojindo Lab, Kumamoto), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (Dojindo Lab, Kumamoto), *N*-[tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid (TAPS) (Dojindo Lab, Kumamoto), *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (CTN) (United States Biochemical Corp.), and 4-phenylspiro-[furan-2(3*H*')-1'-futalan]-3,3'-dione (fluorescamine, Fluka BioChemica) all were commercially available and used without further purification.

Preparation of CHP Solution.[5c] CHP (1.0–5.0 mg/mL) was suspended and swelled in 5 mM MES containing 200 mM NaCl (pH 4.2) under stirring for 12–24 h at 50–60 °C to give a milky suspension. The resulting suspension was sonicated using a probe type sonifier (TOMY, UR-200P, Tokyo) at 40 W and room temperature for 15 min, and an optically clear solution was obtained. Reproducibility of the sample preparation was carefully checked by high-performance size exclusion column chromatography (HPSEC).[5c]

High-Performance Size Exclusion Column Chromatography (HPSEC) Measurements. The HPSEC system (Tosoh Ltd., Tokyo) was composed of a CCPD dual pump, an RI-8010 refractive index detector, a UV-8010 UV detector, and a Chromatocorder 12 data processing system with a GPC extension module. SEC was performed with Ultra Spherogel SEC4000 (Beckman, 0.75 × 30 cm column) or Superdex 200 HR 10/30 (Pharmacia, 1.0 × 30 cm column). A sample was eluted with 5 mM MES buffer containing 200 mM NaCl (pH 4.2) at a flow rate of 0.5 mL/min and 25 °C. The HPSEC system was calibrated with a standard sample set of various size pullulans (P-82, Showa Denko, Tokyo). To estimate the radius of gyration (R_G) as a function of molecular weight (M) determined by HPSEC, an empirical equation for standard pullulan proposed by Kato et al.[17] was adopted: $R_G = 1.47 \times 10^{-2} M^{0.58}$.

A given amount of Chy powder (final concentration 0.2–4.0 mg/mL) was added to a CHP suspension (1.0 mg/mL). The resulting mixture was stirred and incubated at 25 °C. At a given interval, an aliquot (100 μL) was submitted to HPSEC. The time course of the complexation was monitored by a UV detector at 280 nm.

Isolation of the CHP–Chy Complex. Chy (17.5 mg) was dissolved in 7.0 mL of 5 mM MES buffer (pH 4.2) containing CHP (5.0 mg/mL). After incubation for 12 h at 25 °C, the mixture so obtained was subjected to preparative gel chromatography (Sephacryl S300-HR, 1.8 × 30 cm column), and the CHP–Chy complex was isolated. For all the samples fractionated, the concentration of CHP was determined by the phenol–sulfuric acid method,[18] while the concentration of Chy was determined by the fluorescamine method.[19]

Fluorometric Study. The natural fluorescence of Chy and the CHP–Chy complex was recorded with a Hitachi F-3010 fluorescence spectrometer equipped with a thermoregulated cell compartment. Binding of the CHP self-aggregate or the CHP–Chy complex with a fluorescent probe such as ANS was also fluorometrically investigated. The emission maximum and the relative fluorescence intensity of ANS were determined as a function of the concentration of CHP or CHP complexed with Chy at 25 °C. A stock solution (0.01 mL) of ANS (1.0×10^{-4} M) was mixed with 0.99 mL of an aqueous suspension of the CHP self-aggregate (0.1–1 mg/mL) or the same concentration of CHP complexed with Chy.

Enzymatic Degradation of the CHP Self-Aggregate and the CHP–Chy Complex by Pullulanase and Neopullulanase. A given amount of an aqueous pullulanase or neopullulanase solution (final concentration 50 μg/mL) was added in 5 mM MES buffer (pH 4.2) to CHP (3.65 mg/mL) or CHP (4.32 mg/mL) complexed with Chy (0.36 mg/mL) and incubated for 2 h at 25 °C. At a given interval, 100 μL of the mixture suspension was pipetted out and submitted to HPSEC. The elution was monitored by refractive index and UV (at 280 nm) detectors.

Enzymatic Activity Assay. For the activity assay of Chy, CTN was used as the substrate. A 100 μL dioxane solution of CTN ($(1.0–6.0) \times 10^{-5}$ M) was added to 2.8 mL of 50 mM HEPES buffer (pH 8.0). Reaction was initiated by adding 100 μL of a solution of free Chy (1.5×10^{-7} M) or Chy (2.0×10^{-6} M) complexed with CHP to 2.9 mL of the CTN solution. The initial hydrolysis rate was spectrophotometrically monitored at 30.0 °C by following the release of *p*-nitrophenolate at 400 nm ($\epsilon_{400} = 18750\ M^{-1}\ cm^{-1}$).[20]

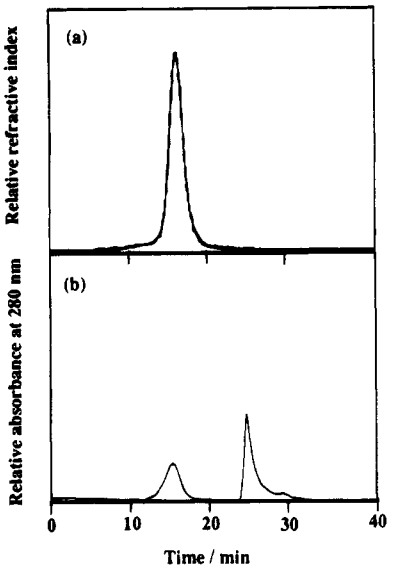

Figure 2. HPSEC chromatograms of the CHP self-aggregate alone (a) and after incubation with Chy for 12 h (b) in 5 mM MES buffer containing 200 mM NaCl at pH 4.2 and 25 °C.

Interaction between the CHP–Chy Complex and BSA. Release of Chy from the complex upon addition of BSA was investigated by HPSEC, and the enzymatic activity of Chy released was simultaneously followed. A given amount of BSA (1.2 or 4.8 mg) was added to a 1200 μL solution of the CHP–Chy complex in 5 mM MES buffer containing 200 mM NaCl (pH 4.2) (CHP, 335 μg/mL; Chy, 36.4 μg/mL). The mixture was incubated for 72 h at 25 °C. An aliquot was submitted to HPSEC (Pharmacia, Superdex 200 HR 10/30, 1.0 × 30 cm column) at a given time, and the enzymatic activity of Chy released was determined.

Circular Dichroism (CD). The change of the secondary structure of Chy complexed with the CHP self-aggregate was investigated by using CD. CD spectra were obtained on a JASCO J-600 at 25 °C. Relative contents of the α-helix and β-form of Chy were computationally calculated over a range between 210 and 240 nm at 1.0 nm intervals using the program of Takeda et al.[21]

An aqueous solution of the isolated CHP–Chy complex (CHP, 174 μg/mL; Chy, 20 μg/mL) or free Chy (20 μg/mL) was kept at 92 °C for a given period of time. The sample solution was cooled to 25 °C and the CD was measured. The structural change of Chy was studied from the ratio of the mean residual ellipticity at 222 nm before (θ_0) and after the thermal treatment (θ_t).

High-Sensitivity Differential Scanning Calorimetry. High-sensitivity differential scanning calorimetry of free Chy and the CHP–Chy complex was performed on a Microcal MC-2.

Results and Discussion

Complexation between the CHP Self-Aggregate and Chy. Complexation between Chy and the CHP-55-2 self-aggregates was investigated by HPSEC. The pH of the medium was adjusted to 4.2 to suppress the self-digestion of Chy[22] and also to keep Chy as the dimer.[24] Figure 2a indicates that CHP forms relatively monodisperse aggregates; the radius of gyration (R_G) is approximately 13 nm, and the apparent polydispersity of the nanoparticles is 1.2. The aggregation number of CHP was approximately 10 as determined by static light

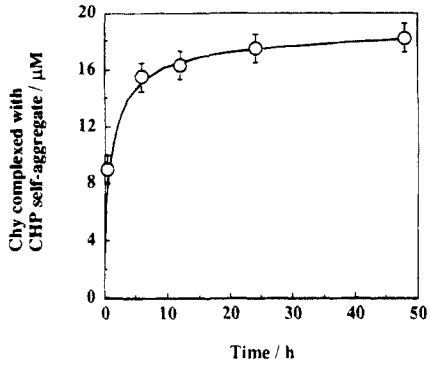

Figure 3. Complexation of Chy (119 μM) with the CHP self-aggregate (8.6 μM) as a function of time in 5 mM MES buffer containing 200 mM NaCl at pH 4.2 and 25 °C.

scattering measurements.[5c] Figure 2b shows a chromatogram of the CHP self-aggregate (8.6 μM) coincubated with excess Chy (119 μM) for 12 h at 25 °C. Free Chy that eluted at R_t = 24.6 min separated from the complex (R_t = 15.0 min). The peak of the complex overlapped that of the parent CHP self-aggregates. The complexation nearly reached an equilibrium after approximately 48 h (Figure 3). Beyond 50 h, it was difficult to obtain reliable data concerning further interaction, because of the self-digestion of free Chy accompanied by precipitation during longer incubation. The CHP–Chy complex was also isolated by preparative gel chromatography. HPSEC measurements showed that the isolated CHP–Chy complex was nearly monodisperse, and R_G of the complex particle (approximately 12 nm) was not much different from that of the parent CHP self-aggregate (R_G = 13 nm). Judging from the molecular size of the Chy dimer (hydrodynamic diameter approximately 4 nm[23]), the Chy dimer seems to be incorporated inside the CHP self-aggregate. The complexation was very strong, and Chy did not dissociate from the complex even after a week at 25 °C. The molar ratio of Chy to CHP self-aggregate in the complex was 2.1 after incubation for 48 h at 25 °C (Figure 3). This means that one Chy dimer (M_w = 49000) must be complexed by one CHP self-aggregate, because Chy usually exists as a dimer under the conditions employed (in 0.2 M NaCl at pH 4.2).[24] As previously reported, one CHP self-aggregate particle complexes with one BSA molecule (M_w = 67000)[10b] or three myoglobin molecules (M_w = 17000).[10a] The CHP self-aggregate may provide a limited capacity for binding these soluble and globular proteins.

The interaction of the CHP–Chy complex with ANS[5c,25] was fluorospectroscopically investigated to understand the structural change of the host CHP self-aggregate after complexation of the protein. The emission maximum and the intensity of ANS upon interaction were plotted as a function of the concentration of free CHP and CHP complexed with Chy (Figure 4). The emission maximum reveals the microscopic polarity around the probe.[26] A blue shift of the emission maximum indicates that ANS locates in the less hydrophilic domain. ANS did not complex with free Chy at all under the same conditions. The binding isotherm of ANS significantly changed for the CHP–Chy complex. ANS bound more strongly to the CHP–Chy complex than to the original CHP self-aggregate. For the parent CHP self-aggregate, ANS locates in the polysaccharide corona of the aggregate.[5c] It is, therefore, suggested that the complexation with Chy could have an influence on the microenvironment of the polysaccharide corona.

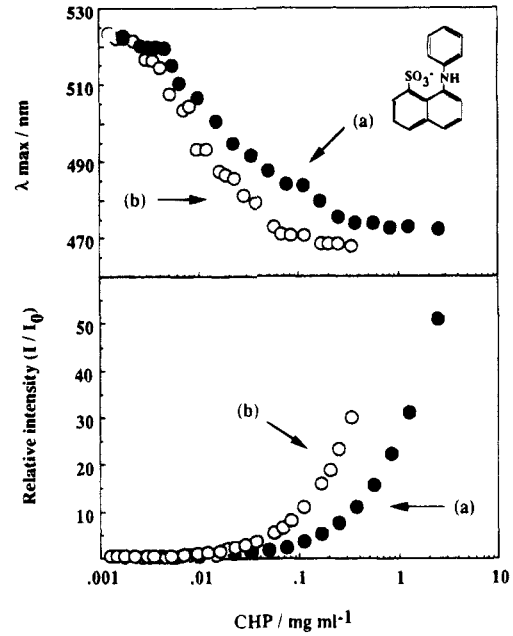

Figure 4. Emission maximum (top) and relative fluorescence intensity, I/I_0, (bottom) of ANS (1.0×10^{-6} M) as a function of the concentration of free CHP (a) and the complexed CHP (b) in 5 mM MES buffer containing 200 mM NaCl at pH 4.2 and 25.0 °C. The relative fluorescence intensity is the ratio between the intensity in the presence (I) and absence (I_0) of a given amount of CHP or CHP–Chy complex.

The free energy change of the complexation between BSA and the CHP self-aggregate was exothermic in the direct measurement by isothermal titration microcalorimetry.[10b] The thermodynamic parameters indicated that hydrogen bondings were multiply formed between the polysaccharide skeleton and BSA upon complexation.[10b] Similarly to the case of BSA, the hydrogen bondings may concern the complexation between Chy and CHP. It would affect the structure and stability of the complex (*vide infra*).

Enzymatic Lysis of the CHP in the Complex. Enzymatic degradation of the polysaccharide skeleton of the complex was carried out by using pullulanase and neopullulanase to understand the location of Chy in the complex. Figure 5a shows the chromatograms of the CHP self-aggregate after pullulanase treatment (50 μg/mL) for 2 h at 25 °C; peaks appeared at R_t = 18.0 and 25.4 min. The former and smaller peak corresponded to approximately 20% of the total CHP, and the particle size (R_G) was approximately 7 nm. On the other hand, the latter and larger peak corresponded to approximately 80% of the total CHP and was attributed to the smaller saccharides enzymatically degraded. The enzymatic degradation of the polysaccharide skeleton of the CHP self-aggregate did not proceed more than 80%. The rest always remained undigested at this concentration of pullulanase. Of course, the parent pullulan without cholesterol groups was completely hydrolyzed under the same conditions. This may suggest that only the corona, and not the inner core, of the polysaccharide aggregate is digested by the enzyme. The same result was also obtained when neopullulanase was employed for pullulanase (though data are not shown). Even in the case of the CHP–Chy complex, enzymatic degradation of the polysaccharide skeleton did not exceed more than 85% of the total CHP (Figure 5b). Figure 5c shows the chromatogram of the enzymatically treated sample, which is detected by UV to determine the distribution of Chy. More than 98% of the Chy still remained in

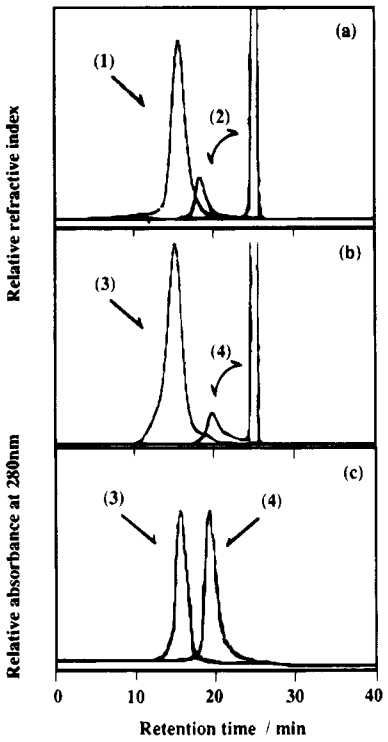

Figure 5. Results of HPSEC carried out after the pullulanase treatment of the CHP self-aggregate (a) and the CHP–Chy complex (b and c) for 2 h at 25 °C: (1) the CHP self-aggregate; (2) products after the enzymatic digestion of (1); (3) the CHP–Chy complex; (4) products after the enzymatic digestion of the CHP–Chy complex. In (a) and (b), eluents were detected by RI, while in (c), eluents were detected by UV (see details in text, Materials and Methods).

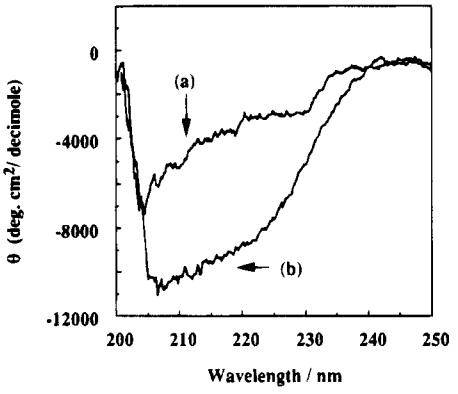

Figure 6. CD spectra of free Chy (a) and complexed Chy (b) at pH 4.2 and 25 °C in 5 mM MES buffer containing 200 mM NaCl.

the complex even after 85% degradation of the polysaccharide skeleton of the complex. These results suggest again that the Chy dimer may be entrapped deeply inside the matrix of the aggregate, neither in the region close to the surface nor in the corona of the CHP self-aggregate.

Structure of Chy Complexed with the CHP Self-Aggregate. The CD spectrum of the CHP–Chy complex is shown in Figure 6. The secondary structure of Chy changed upon complexation. A negative CD band of free Chy around 230 nm disappeared upon complexation. This band was attributable to the interaction between the His-40 and Trp-141 of Chy[26] and is sensitive to small conformational changes in the active site of the enzyme.[26] We must consider at least two possibilities for the increase in the ellipticity at 222 nm. One is due to a conformational change of the secondary structure of Chy, and the other is due to the induced CD of the aromatic residues of Chy in the chiral environment of the sugar moiety. The first possibility may be more plausible because the secondary structural change of Chy is well understood in the achiral environment such as a micelle. The helix content of Chy increases upon addition of SDS;[21,26] for example, the helical content is 29% and the β-form content is 12% in 6 mM SDS.[21] The helix and β-form contents of Chy calculated by X-ray analysis are 9 and 34%, respectively.[27] Chy consists of three polypeptide chains, namely, A-chain (Cys1-Leu13), B-chain (Ile16-Tyr146), and C-chain (Ala149-Asn245).[27] Takeda et al.[21] reported that the helical content in the B- and C-chains increases by hydrophobic binding of SDS to the exposed aromatic side chain, though the detailed mechanism is not clear yet.[26] Grandi et al.[28] also found that the helical content of lysozyme was 34% in water, and it changes to 48% in reverse micelles. This conformational change is due to the anomalous character of water in the reverse micelle. Such change might strengthen hydrogen bondings within the lysozyme and increase the helical content of the protein.[28] If we assume that the secondary structure of Chy changes by the complexation with the CHP self-aggregate, 29% helix and 21% β-form will be estimated from the computational analysis of the CD spectra of the CHP–Chy complex.[21] This means that the helix content drastically increases from 9 to 29%, while the β-form content decreases from 34 to 21% upon complexation. Both the hydrogen bonding with the polysaccharide skeleton and the hydrophobic interaction with the cholesterol groups of CHP might be responsible for the increase of the helical content.

The natural fluorescence of the tryptophan moiety of the protein is sensitive to the conformational change of the protein.[29] Chy fluoresces at 337 nm (at pH 4.2 and 25.0 °C), while the CHP–Chy complex fluoresces at 338 nm with almost the same intensity as that of free Chy. Chy has eight tryptophan moieties, and most of those are in the hydrophobic environment that is not exposed to the bulk aqueous phase.[29] The complexation with the CHP self-aggregate may not necessarily affect the hydrophobic region of the Chy molecule.

To investigate the structural change at the active site of Chy, the enzymatic activity of the complexed Chy was determined at pH 8.0 using CTN as the substrate. K_m and V_{max} for free Chy were 1.7×10^{-6} M and 3.5×10^{-2} mol min^{-1} mg^{-1} just after the preparation of the sample solution, while they were 1.1×10^{-6} M and 4.0×10^{-4} mol min^{-1} mg^{-1} after complexation. The V_{max} of complexed Chy decreased by 1/88 compared with that of free Chy; nevertheless, K_m changed little. No significant change of the K_m value suggests that the small hydrophobic substrate, CTN, which is different from a large macromolecule such as protease,[30] still can penetrate into the hydrogel matrix of the CHP–Chy complex.

Interaction between the CHP–Chy Complex and BSA. Although no spontaneous dissociation of Chy from the complex was observed even after a week at 25 °C, Chy was released from the complex by the addition of BSA at 25 °C. For example, 5% of the complexed Chy was released by the interaction with 4.0 mg/mL of BSA for 72 h at 25 °C. The released Chy was monitored by HPSEC after the coincubation with BSA. This may be an exchange between the two proteins due to the difference in the binding strength between BSA and Chy. The enzymatic activity of Chy was determined after the release from the complex by adding BSA. The released Chy showed the same activity (1.23×10^{-5} M

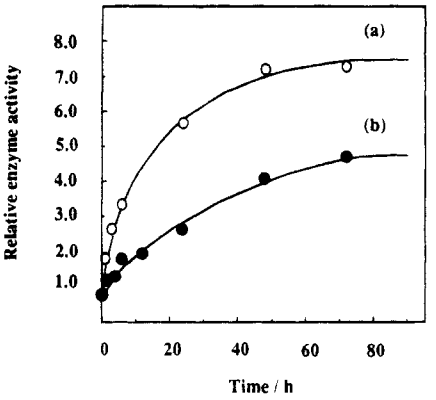

Figure 7. Apparent enzymatic activity of Chy released from the complex in the presence of BSA at pH 4.2 and 25 °C as a function of incubation time in 5 mM MES buffer containing 200 mM NaCl: (a) with 4.0 mg/mL of BSA; (b) with 1.0 mg/mL of BSA. The enzymatic activity was represented by the relative value to that in the absence of BSA.

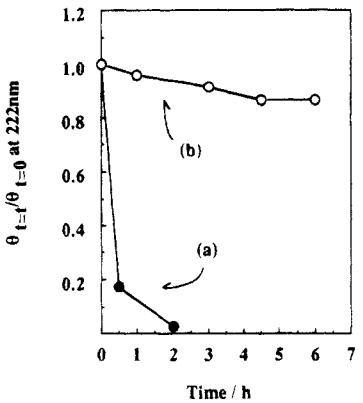

Figure 8. Relative change of the mean residual ellipticity of free Chy (a) and complexed Chy (b) at 222 nm after keeping at pH 4.2 and 92 °C in 5 mM MES buffer containing 200 mM NaCl.

mg^{-1} s^{-1}) as that observed before the complexation (1.22×10^{-5} M mg^{-1} s^{-1}). The complexed Chy was released keeping the native conformation. The dissociation of Chy from the complex was followed by monitoring the enzymatic activity of Chy that was released to the bulk phase (Figure 7). The protein exchange was not so fast, and it took almost 80 h until equilibrium was attained, though binding of BSA to the vacant CHP self-aggregate was accomplished within 3 h. The tight complexation between Chy and the aggregate with multiple hydrogen bondings might be responsible for the retardation of the protein exchange.

Thermal Stability of the CHP–Chy Complex. The thermal stability of the secondary structure of Chy was investigated by CD. Figure 8 shows the mean residual ellipticity change at 222 nm of free Chy and the CHP–Chy complex kept at 92 °C for 6 h. The ellipticity of free Chy was completely lost within 2 h at the elevated temperature, while that of the complex still remained more than 85% of the original ellipticity even after heating for 6 h. The residual enzymatic activity of Chy was again examined after heating. Free Chy completely lost the activity at 92 °C for 1 h, while the complexed Chy still kept the activity even though it decreased up to 7% of the original activity (Table 1). Chy was released also by the addition of BSA from the once heated complex. It is noteworthy that the released Chy still kept 74% of the original activity even after the heating (Table 1). These results indicate that the thermal denaturation of the protein was drastically

Table 1. Relative Enzyme Activity of Free Chy and Complexed Chy Evaluated by the Initial Reaction Velocity[a]

	under usual conditions	after keeping at 92 °C
Chy	100.0	0
complexed Chy[b]	1.1	0.07
Chy released from the complex[c]	99.0	74.0

[a] Initial velocity was measured at pH 8.0 and 30.0 °C by using CTN as the substrate in 50 mM HEPES buffer. [b] After being incubated at 92 °C for 60 min, the sample solution was cooled to room temperature prior to the measurement of the enzyme activity. [c] Initial velocity was measured after treatment with 4.0 mg/mL of BSA at 25.0 °C for 24 h. The amount of Chy released was determined by HPSEC.

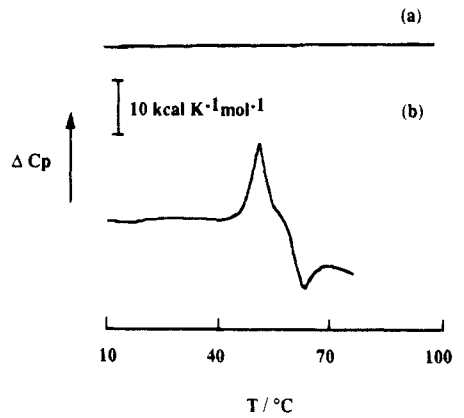

Figure 9. Excess heat capacity (ΔC_p) change *vs* temperature (T): (a) Chy (0.36 mg/mL) complexed with the CHP self-aggregate (4.32 mg/mL); (b) free Chy (0.99 mg/mL) in 5 mM MES buffer containing 200 mM NaCl at pH 4.2.

prevented upon the complexation with the CHP self-aggregate.

Thermal denaturation of protein is usually considered a two-step mechanism:[31]

$$N \rightleftharpoons D \rightarrow I \quad (1)$$

where N, D, and I are the native, reversibly denatured, and irreversibly denatured forms of the protein. Thermal inactivation starts with a reversible unfolding of the protein followed by an irreversible process such as oligomerization of the unfolded protein. DSC measurements can provide direct information about the process of the thermal unfolding of a protein.[31] To understand the thermal stability of complexed Chy, high-sensitivity differential scanning calorimetry was carried out. Free Chy gave both an endothermic peak at 52.8 °C (the midpoint temperature) (Figure 9b) due to the thermal unfolding of Chy[32] and a subsequent exothermic peak due to aggregation of the unfolded Chy. Surprisingly, the CHP–Chy complex did not give any peak (Figure 9a) in the DSC over the range 10–100 °C. In addition, the sample showed neither CD spectral change nor dissociation of Chy from the complex even after the DSC measurement. The fluorescence of the tryptophan moiety of the protein is also sensitive to the conformational change with the thermal unfolding of the protein.[29] The emission maxima of free Chy shifted by approximately 10 nm above 50 °C, which corresponds to the transition temperature of unfolding of Chy (Figure 10). In the case of the CHP–Chy complex, however, no spectral change was observed at least up to 80 °C. These results indicate that the thermal unfolding of Chy (the preequilibrium in eq 1) is largely retarded upon complexation. Several water-soluble

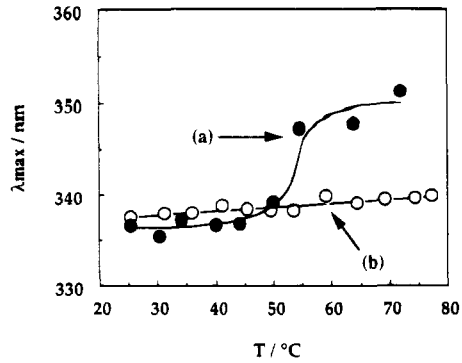

Figure 10. Change in natural fluorescence of free Chy (50.2 μg/mL) (a) and complexed Chy (50.2 μg/mL) (b) as a function of temperature in 5 mM MES buffer containing 200 mM NaCl (pH 4.2).

polymers and various hydrogels promote thermal stabilization of soluble proteins.[33,34] The unfolding temperature of a protein increases by 5–15 °C in the presence of water-soluble polymers or in a hydrogel matrix.[33–36] To our knowledge, however, there is no report that such a drastic retardation of unfolding of a protein occurs in water as seen in this work. We have previously reported a similar thermal stabilization of BSA upon the complexation with the CHP self-aggregate.[10b] The multipoint interactions between the protein and the CHP self-aggregate must be the principal driving force of the stabilization.[33]

The self-aggregate of a hydrophobized polysaccharide constructs a unique supramolecular assembly with a soluble protein in water, accompanied by the enhancement of thermal stability in both the structure and the enzymatic activity of the protein. In this sense, the hydrophobized polysaccharide nanoparticle is promising as a unique host even for a macromolecular guest. Such a property of hydrophobized polysaccharides seems to be attractive in enzyme engineering.

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References and Notes

(1) (a) *Water-Soluble Polymers*; ACS Symposium Series 467; Shalaby, S. W., McCormick, C. L., Butler, G. B., Eds.; American Chemical Society: Washington, DC, 1991.
(2) (a) Landoll, L. M. *J. Polym. Sci., Polym. Chem. Ed.* **1982**, *20*, 443. (b) Strauss, U. P.; Chiao, Y.-C. *Macromolecules* **1986**, *19*, 355. (c) Kobayashi, K.; Sumitomo, H.; Ichikawa, H. *Macromolecules* **1986**, *19*, 529. (d) Schultz, D. N.; Kaladas, J. J.; Maurer, J. J.; Bock, J.; Pace, S. J.; Schultz, W. W. *Polymer* **1987**, *28*, 2110. (e) Chu, D.-Y.; Thomas, J. K. *Macromolecules* **1987**, *20*, 2133. (f) Winnik, F. M.; Winnik, M. A.; Ringsdorf, H.; Venzmer, J. *Langmuir* **1991**, *95*, 2583. (g) Ezzell, S. A.; McCormick, C. L. *Macromolecules* **1992**, *25*, 1881.
(3) (a) Binana-Limbele, W.; Zana, R. *Macromolecules* **1990**, *23*, 2731. (b) Seo, T.; Take, S.; Miwa, K.; Hamada, K.; Iijima, T. *Macromolecules* **1991**, *24*, 4255. (c) Schild, H. G.; Tirrell, D. A. *Langmuir* **1991**, *7*, 1319.
(4) (a) Dualeh, A. J.; Steiner, C. A. *Macromolecules* **1990**, *23*, 251. (b) Briggs, S.; Selb, J.; Candau, F. *Langmuir* **1992**, *8*, 838. (c) Iliopoulos, I.; Wang, T. K.; Audebert, R. *Langmuir* **1991**, *95*, 617. (d) Magny, B.; Iliopoulos, I.; Audebert, R.; Piculell, L.; Lindman, B. *Prog. Colloid Polym. Sci.* **1992**, *89*, 118. (e) Tanaka, J.; Meadows, J.; Williams, P. A.; Phillips, G. O. *Macromolecules* **1992**, *25*, 1304.
(5) (a) Akiyoshi, K.; Yamaguchi, S.; Sunamoto, J. *Chem. Lett.* **1991**, 1263. (b) Akiyoshi, K.; Sunamoto, J. In *Organized Solutions*; Friberg, S. E., Lindman, B., Eds.; Marcel Dekker: New York, 1991; p 289. (c) Akiyoshi, K.; Deguchi, S.; Moriguchi, N.; Yamaguchi, S.; Sunamoto, J. *Macromolecules* **1993**, *26*, 3062.
(6) (a) Takada, M.; Yuzuriha, T.; Katayama, K.; Iwamoto, K.; Sunamoto, J. *Biochim. Biophys. Acta* **1984**, *802*, 237. (b) Sunamoto, J.; Iwamoto, K. *CRC Crit. Rev. Ther. Drug Carrier Syst.* **1986**, *2*, 117. (c) Sunamoto, J.; Sato, T.; Taguchi, T.; Hamazaki, H. *Macromolecules* **1992**, *25*, 5665.
(7) (a) Baszkin, A.; Rosilio, V.; Albrecht, G.; Sunamoto, J. *Chem. Lett.* **1990**, 299. (b) Baszkin, A.; Rosilio, V.; Albrecht, G.; Sunamoto, J. *J. Colloid Interface Sci.* **1991**, *145*, 502.
(8) Moellerfeld, J.; Prass, W.; Ringsdorf, H.; Hamazaki, H.; Sunamoto, J. *Biochim. Biophys. Acta* **1986**, *857*, 265.
(9) (a) Carlsson, A.; Sato, T.; Sunamoto, J. *Bull. Chem. Soc. Jpn.* **1989**, *62*, 791. (b) Fukui, H.; Akiyoshi, K.; Sato, T.; Sunamoto, J.; Yamaguchi, S.; Numata, M. *J. Bioact. Compat. Polym.* **1993**, *8*, 305.
(10) (a) Akiyoshi, K.; Nagai, K.; Nishikawa, T.; Sunamoto, J. *Chem. Lett.* **1991**, 1727. (b) Akiyoshi, K.; Nishikawa, T.; Shichibe, S.; Mitui, Y.; Miyata, T.; Kodama, M.; Sunamoto, J., submitted to *Macromolecules*.
(11) For example: (a) Ahlers, M.; Muller, W.; Reichert, A.; Ringsdorf, H.; Venzmer, J. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 1269. (b) Xia, J.; Dubin, P. L.; Kokufuta, E. *Macromolecules* **1993**, *26*, 6688.
(12) (a) Lehn, J.-M. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 89. (b) Ringsdorf, H.; Schlarb, B.; Venzmer, J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 113.
(13) (a) Cram, D. J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 1009. (b) Rebek, J., Jr. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 245.
(14) (a) Kunitake, T. *J. Macromol. Sci., Chem.* **1979**, *A-13*, 587. (b) Fendler, J. H. *Membrane Mimetic Chemistry*, John Wiley & Sons: New York, 1982.
(15) (a) Wangner, N.; Dose, K.; Koch, H.; Ringsdorf, H. *FEBS Lett.* **1981**, *132*, 313. (b) Hamachi, I.; Noda, S.; Kunitake, T. *J. Am. Chem. Soc.* **1991**, *113*, 9625. (c) Naka, K.; Ohki, A.; Maeda, S. *Chem. Lett.* **1991**, 1303.
(16) Sogami, M.; Foster, J. F. *Biochemistry* **1968**, *7*, 2172.
(17) Kato, T.; Katsuki, T.; Takahashi, A. *Macromolecules* **1984**, *17*, 1726.
(18) Dubios, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350.
(19) Bohlen, P.; Stein, S.; Dairman, W.; Undenfrien, S. *Arch. Biochem. Biophys.* **1973**, *155*, 213.
(20) Martin, C. J.; Golubow, J. G.; Axelrod, A. E. *J. Biol. Chem.* **1959**, *234*, 294.
(21) Takeda, K.; Wada, A.; Moriyama, Y. *Colloid Polym. Sci.* **1990**, *268*, 612.
(22) Hofstee, B. H. *Arch. Biochem. Biophys.* **1965**, *112*, 224.
(23) Birktoft, J. J.; Blow, D. M. *J. Mol. Biol.* **1972**, *68*, 187.
(24) (a) Morimoto, K.; Kegeles, G. *Biochemistry* **1967**, *6*, 3007. (b) Aune, K. C.; Timasheff, S. N. *Biochemistry* **1971**, *10*, 1609.
(25) Stryer, L. *J. Mol. Biol.* **1965**, *13*, 482.
(26) Hunt, A. H.; Jirgensons, B. *Biochemistry* **1973**, *12*, 1609.
(27) Blow, D. M. *Biochem. J.* **1969**, *112*, 261.
(28) Grandi, C.; Smith, R. E.; Luisi, P. L. *J. Biol. Chem.* **1981**, *256*, 837.
(29) Wu, A. C. M.; Eitenmiller, R. R.; Powers, J. *Lebensm-Wiss. Technol.* **1976**, *9*, 102.
(30) Cebra, J. J.; Givol, D.; Silman, H. I.; Katchalski, E. *J. Biol. Chem.* **1961**, *236*, 1720.
(31) *Thermostability of Enzymes*; Gupta, M. N., Ed.; Springer-Verlag, Narosa Publishing House: New Delhi, 1993.
(32) Takahashi, K.; Fukuda, H. *Biochemistry* **1985**, *24*, 3084.
(33) Klibanov, A. M. *Anal. Biochem.* **1979**, *93*, 1.
(34) Monsan, P.; Combes, D. *Methods Enzymol.* **1988**, *137*, 584.
(35) Fujita, Y.; Iwasa, Y.; Noda, Y. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 1896.
(36) Koch-Schmidt, A.-C.; Mosbach, K. *Biochemistry* **1977**, *16*, 2105.