Stabilization of Insulin upon Supramolecular Complexation with Hydrophobized Polysaccharide Nanoparticle

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The hydrogel nanoparticle that was formed by self-assembly of cholesterol-bearing pullulan complexed with 5 - 10 insulin monomers in water. Biocolloidal and thermal stability of insulin drastically increased upon the supramolecular assembly with the hydrophobized polysaccharide.

Efficient delivery of bioactive peptides or proteins to a target cell or organ has received considerable attention in biotechnology and medicine. Insulin (Ins) self-associates to oligomers and polymeric aggregates depending on the concentration, ionic strength, pH, temperature, solvent composition and / or shear stress.[1] The biocolloidal and thermal stabilization of insulin in solution is one of important factors to successful development of insulin delivery system.[1,2] Various attempts have been made to overcome this problem by addition of an organic solvent, an amino acid, a water soluble polymer or a surfactant.[2] We have reported that the hydrophobized polysaccharide such as cholesterol-bearing pullulan (CHP) forms a hydrogel nanoparticle upon self-assembly in water.[3] The hydrogel nanoparticle spontaneously complexes with various soluble proteins inside the hydrogel matrix.[4] This is a unique hybrid assembly between an amphiphilic polysaccharide and a soluble protein. The supramolecular assembly of proteins with amphiphilic polymers has attracted interest as a new methodology for functionalization of proteins.[4,5] We describe herein biocolloidal and thermal stabilization of insulin upon the complexation with CHP nanoparticle.

CHP was synthesized according to the same method as that reported previously.[3] Pullulan (M_w = 5.5 × 10^4, M_w/M_n = 1.54) was substituted by 1.0, 1.7, and 3.4 cholesterol moieties per 100 glucose units. They were coded as CHP-55-1.0, -1.7, and -3.4. An aqueous suspension of CHP was prepared by a sonication method.[3] The CHP self-aggregates are relatively monodispersive; their radii of gyration (R_G) determined by high performance size exclusion column chromatography (HPSEC)[3] were 16 nm for CHP-55-1.0, 14 nm for CHP-55-1.7, and 12 nm for CHP-55-3.4. The aggregation number, which was determined by the static light scattering measurement, was approximately 10 for all CHP self-aggregates studied in this work.[3] The water content of one hydrogel nanoparticle was estimated[3] to be 84 wt % for CHP-55-1.0, 79 wt % for CHP-55-1.7, and 50 wt % for CHP-55-3.4.

The complexation between Ins (Bovine pancreas, Sigma, M_w = 5700) and the CHP nanoparticle was investigated by the HPSEC method. The complexation proceeded very fast and reached an equilibrium within 10 min. This is much different from the case of other relatively larger proteins such as α-chymotrypsin (M_w= 24500), bovine serum albumin (M_w = 66000), and myoglobin (M_w = 17000), that take several hours to reach an equilibrium.[4] Figure 1 shows the complexation isotherms for various CHP nanoparticles. The concentration profile of the complexed Ins showed a saturation phenomenon with an increase in the initial concentration of Ins. Considering the aggregation number of the CHP self-aggregate, the maximum number of the complexed Ins was estimated to be 5 for CHP-55-1.0, 8 for CHP-55-1.7, and 10 for CHP-55-3.4. The number of complexed Ins increased with an increase in the substitution degree of cholesterol group of CHP. HPSEC indicated that the CHP-Ins complex was also monodispersive, and the R_G of the complexes was not affected by the complexation even though the number of complexed Ins increased. Therefore, Ins would be complexed deeply inside the amphiphilic hydrogel matrix of the nanoparticle, in which the hydrophobic microdomain of the associating cholesterol forms non-covalent cross-links of gel structure.[3] The number of the cross-links of one nanoparticle increases with an increase in the number of substitution degree of cholesterol, leading to an increase in the binding site for Ins.

The CD spectra of free and complexed Ins are shown in Figure 2. Ins is in an equilibrium between monomer and dimer

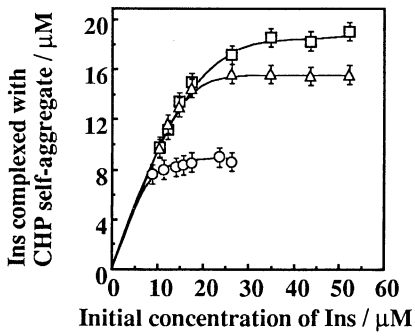

Figure 1. Complexation isotherm of Ins to various CHP self-aggregates (1.8 μM) in 100 mM phosphate buffer (pH 8.0) at 25 ° C; CHP-55-1.0 (○), CHP-55-1.7(△), and CHP-55-3.4(□).

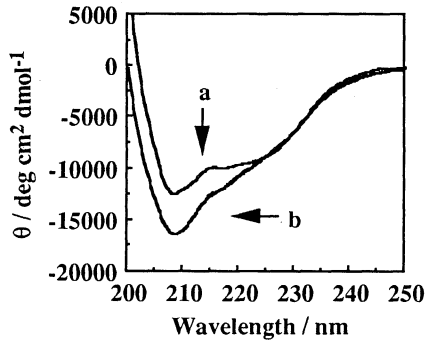

Figure 2. CD spectra of free Ins (10.5 μM) (a), and Ins (10.5 μM) complexed with CHP-55-1.7 self-aggregate (2.7 μM) (b) in 100 mM phosphate buffer (pH 8.0) at 25 ° C.

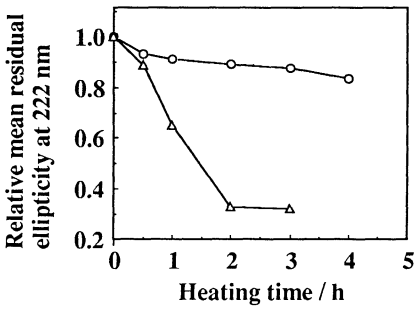

Figure 3. Change of relative mean residual ellipticity at 222nm of free Ins (10.0 μM) (Δ) and Ins (10.0 μM) complexed with CHP-55-1.7 self-aggregate (2.0 μM) (O) as a function of heating time at 90 °C.

depending on its concentration in water. The ratio of the molar ellipticity at 223 nm to that at 208 nm is comparable to the monomer-dimer ratio of Ins.[6] Pocker, *et al.* reported that 93 % of Ins exists as the monomer in 0.1 μM (M = mol dm^{-3}) solution, where $[\theta]_{208}/[\theta]_{223}$ = 1.52, and 90 % of Ins as the dimer in 10 μM solution, where $[\theta]_{208}/[\theta]_{223}$ = 1.35 (Figure 2).[6] The $[\theta]_{208}/[\theta]_{223}$-values of every CHP-Ins complexes, which were prepared by simple mixing of Ins (10.5 μM) and the CHP self-aggregate (2.7 μM), were very similar to that of Ins monomer. The $[\theta]_{208}/[\theta]_{223}$-value was 1.52 for all the CHP-Ins complexes. Most of Ins would be complexed with the CHP nanoparticle as the monomer form under the conditions employed.

The complex showed an excellent colloidal stability without further aggregation and any precipitation. Ins did not dissociate from the complex at all even after keeping for a month at 25 °C. Aggregation or precipitation of Ins with the self-association was largely prevented upon the complexation. Figure 3 shows the change of the mean residual ellipticity at 222 nm for free and complexed Ins as a function of time after heating at 90 °C. The CD spectra were measured at 25 °C for the samples once heated after filtration through a 0.2 μm pore size filter. The ellipticity of free Ins was lost within 2 h by heating. This is mainly due to the precipitation by the aggregation of unfolded Ins. For complexed Ins, on the other hand, more than 85 % of the original ellipticity still remained even after 4 h heating. The self-association of Ins also would not occur in the complex because the $[\theta]_{208}/[\theta]_{223}$ value (1.50) did not change at all even after heating. Unfolding of Ins occurs at 63 °C in water (pH 3).[7] The process is not fully reversible upon the aggregation of unfolded Ins and the thermal destruction.[7] Such the irreversible denaturation of Ins would be effectively prevented by the complexation with the CHP nanoparticle.

The supramolecular assembly of a soluble protein with a hydrophobized polysaccharide is effective for the protein to gain biocolloidal and thermal stability. The biodegradable polysaccharide nanoparticle is promising in biotechnology and medicine because it is possible to endow functional moiety such as cell specificity[8] or thermoresponsibility[9] to the polysaccharide.

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