Glucose-Binding Property of Pegylated Concanavalin A

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Purpose. Concanavalin A (Con A) has been used in the development of sol-gel phase-reversible hydrogels for modulated insulin delivery. The usefulness of Con A has suffered from its poor aqueous solubility and stability. The goal of this study was to modify Con A with poly(ethylene glycol) (PEG) and examine the water solubility and stability of the PEGylated Con A.

Methods. Con A was PEGylated using monomethoxy poly(ethylene glycol) *p*-nitrophenol carbonates, and the extent of PEGylation was determined by the fluorescamine method. The stability of the PEGylated Con A was examined by measuring the time-dependent absorbance at 630 nm. The binding affinities of glucose and allyl glucose to native- and PEGylated-Con A were measured by the equilibrium dialysis method.

Results. The total number of PEG molecules that can be grafted to Con A was 10. As the number of grafted PEG chains per each Con A was increased up to 5, the binding affinity of glucose was gradually increased and reached the maximum. The solubility and stability of PEGylated Con A were improved significantly over those of native Con A. The binding affinity of allyl glucose to Con A was not changed much by PEGylation. When the extent of PEGylation was excessive (i.e., the number of grafted PEG chains per each Con A was larger than 5), however, the binding affinities of both glucose and allyl glucose were decreased significantly.

Conclusions. PEGylation of Con A resulted in improved aqueous solubility and stability of Con A. The binding affinity of glucose increased and reached the maximum when the extent of PEGylation was 50%. Advantages of PEGylated Con A over native Con A are improved aqueous solubility, enhanced long-term stability, and higher glucose sensitivity.

KEY WORDS: concanavalin A; PEGylation; glucose binding affinity; allyl glucose; solubility; stability.

INTRODUCTION

Hydrogels undergoing phase transition between sol and gel in response to changes in the environmental glucose concentration were developed for modulated insulin delivery (1–3). The glucose-sensitive phase-reversible hydrogel system was made of glucose-containing polymers and concanavalin A (Con A). Con A is a glucose-binding protein obtained from the jack bean plant, *Canavalia ensiformis*. At physiological pH, Con A exists as a tetramer in the presence of calcium ions (4–6). Each monomer has a glucose-binding site in a shallow pocket near the surface of the protein (7). Glucose binding sites of Con A are known to interact with glucose molecules through the segment containing C3-C6 (8). Thus, glucose maintains Con A binding ability even after modification at the C1 position. To synthesize glucose-containing polymers, glucose was modified with an allyl group at the C1 position. Our previous studies showed that the allyl glucose (AG) showed much higher binding affinity to Con A (2). The tetrameric Con A functions as a crosslinking agent for the glucose-containing polymers to form a gel in the absence of free glucose. The system becomes a sol in the presence of free glucose, which competes with polymer-bound glucose molecules for the same binding sites on Con A. The system undergoes cyclic sol-gel phase transitions by alternating the environmental glucose concentration between 1 mg/ml and 4 mg/ml.

Although the Con A-containing hydrogel system responds effectively to changes in environmental glucose concentration for modulated delivery of insulin (3), the system suffers from the poor aqueous solubility and stability of Con A. The typical Con A concentration used in the gel-sol phase-reversible hydrogels ranges from 50 mg/ml to 100 mg/ml (1,2). The solubility of Con A in the physiological condition, however, is not as high, and the Con A solution becomes turbid even at 50 mg/ml (with the absorbance of 0.15 at 630 nm). Sometimes the low solubility and stability of Con A has led to formation of insoluble materials in the system. A more stable and homogeneous phase-reversible hydrogel system is expected from the increased solubility and stability of Con A. The homogeneity in the sol-gel phase transition is important for accurate and reproducible control of the insulin release rate. For this reason, we tried to increase the solubility and stability of Con A by conjugating with poly(ethylene glycol) (PEG), a process commonly known as PEGylation. The solubility, stability, and glucose-binding properties of PEGylated Con A were determined and compared with those of native Con A.

MATERIALS AND METHODS

Materials

Con A (Type IV, essentially salt-free, lyophilized powder) and fluorescamine were purchased from Sigma Chemical Co. (St. Louis, MO). Monomethoxy poly(ethylene glycol) *p*-nitrophenol carbonates (MPEG-NPC, MW 5,000) were obtained from Shearwater Polymers, Inc. (Huntsville, AL). 2,2′-azobisisobutyronitrile and 1-vinyl-2-pyrrolidinone were purchased from Aldrich Chemical Co. (Milwaukee, WI). 1-vinyl-2-pyrrolidinone was distilled under reduced pressure before use. Spectra/Por® dialysis membranes were purchased from Spectrum Medical Industries, Inc. (Los Angeles, CA). All other chemicals were of reagent grade and used without further purification. AG was synthesized following the procedure described previously (1,9). All aqueous solutions were made using distilled-deionized water (DDW). 0.1 M Tris buffer solution (pH 7.4) with 0.1 M NaCl, 1 mM $CaCl_2$, and $MnCl_2$ was used in all experiments related to characterization of Con A and modified Con A.

Conjugation of Con A with Poly(ethylene glycol) (PEG)

Con A was conjugated with PEG using MPEG-NPC, which is active toward primary amines (10,11). Con A (100 mg) was dissolved in 10 ml of 0.1 M borate buffer, pH 8.5, and the solution was kept in an ice bath. Con A solution was stirred using a magnetic stirrer without making air bubbles or foam. MPEG-NPC was slowly added to the Con A solution. To vary the molar ratio of PEG and Con A in the reaction solution, the concentration of MPEG was changed while the

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Con A was kept constant at 12 mg/ml. The molecular weight of Con A used was 25,000 g/mole. The temperature of reaction mixture was gradually increased from 4°C to room temperature, and the reaction was allowed to continue for 20 h. At the end of reaction, the solution was dialyzed by using a dialysis tube (MWCO = 50,000) against DDW for 5–7 days with daily changes of water. During dialysis, the presence of *p*-nitrophenol was examined in the medium by measuring absorbance at 400 nm to find the endpoint of dialysis. The dialysate was lyophilized and the obtained Con A conjugates in white powder form were stored in sealed vials at −20°C.

Analysis of PEGylated Con A

The extent of modification of Con A was examined using fluorescamine, 4-phenylspiro[furan-2(3H),1′-phthalan]-3,3′-dione. Primary amine groups could be detected in as little as 50 ng of protein due to the high sensitivity of fluorescamine fluorescence (12). The intensity of fluorescence of the Con A solution with fluorescamine decreased as Con A was modified with PEG. As more PEG molecules were attached to Con A, fewer primary amine groups were available for fluorescamine. Con A solutions were prepared in 0.1 M phosphate buffer, pH 8.0. Each test tube contained between 0 and 4.5 μg of Con A in 1.5 ml solution. To each test tube, 0.5 ml of fluorescamine solution (0.3 mg/ml in acetone) was added and the whole solution was vortex mixed. After 7 min or more the fluorescence in the solution was measured using a SLM-Aminco® 8000 fluorescence spectrophotometer (Spectronic Instruments, Inc., Rochester, NY) with an excitation wavelength of 390 nm and emission at 475 nm. The extent of modification was determined by calculating the slope ratio between modified and unmodified Con A samples from the fluorescence intensity as a function of the Con A concentration. Then, the number of PEG molecules attached to Con A was calculated by prorating the extent of modification to the maximal number of primary amine groups in Con A.

To describe the number of grafted PEG chains in PEG–Con A conjugates, the notation "PEG#–Con A" was used. For example, PEG–Con A conjugates with five grafted PEG chains were described by PEG5–Con A. The molecular weight of the PEGylated Con A was calculated by adding the molecular weight of a PEG chain (which is 5,000) to the molecular weight of Con A (which is 25,000). Thus, the molecular weight of PEG5-Con A was assumed to be 50,000.

Solubility and Stability of Con A Conjugates

The solubility and stability of PEGylated Con A (i.e., PEG-Con A conjugates) were determined by measuring the absorbance at 630 nm of the conjugates in 0.1 M Tris buffer solution (pH 7.4) with 0.1 M NaCl, 1 mM $CaCl_2$ and $MnCl_2$. The experiments were performed by using Corning Disposable Assay Plates that contained 96 flat bottom wells filled with 100 μl of solutions of Con A or PEG-Con A conjugates. Serial dilutions were made to match the molar concentrations between Con A and the conjugates. The concentrations of Con A and the PEGylated Con A in the wells ranged from 0.05 mM to 4 mM. The absorbance of each well was measured at 630 nm using EL 311 Microplate Autoreader (Bio-Tek Instrument, Winooski, VT). For the stability study, the microplates filled with Con A or PEGylated Con A solutions were sealed with polyethylene film and stored at room temperature. The absorbance was measured at timed intervals for 10 days.

Binding Affinity of PEG–Con A Conjugates to Glucose and AG

The binding constants of glucose and AG to Con A and PEGylated Con A were determined by the equilibrium dialysis technique. The binding affinity of glucose-containing polymers to Con A or PEG–Con A conjugates could not be obtained directly using the technique due to the inability of the glucose-containing polymers diffusing through the dialysis membrane. For this reason, AG was used to estimate the binding affinity of glucose-containing polymers to PEGylated Con A. The equilibrium dialyzer (EMD101B Equilibrium dialyzer, Hoefer Pharmacia Biotech Inc., San Francisco, CA) consists of eight-well dialysis module and a dialysis membrane with a molecular weight cut-off of 6,000–8,000. The dialysis membrane divided each well into two chambers and the volume of each chamber was 0.5 ml. The buffer used in the dialysis experiments was 0.1 M Tris buffer (pH 7.4) with 0.1 M NaCl, 1 mM $CaCl_2$ and $MnCl_2$. Glucose and AG solutions (60~500 μg/ml) were placed in one chamber. Con A or PEGylated Con A was loaded into the other chamber and its concentration was fixed at 1% (w/v). The dialysis module was gently rotated at a speed of 20 rpm at room temperature. Preliminary measurement of the glucose and AG concentrations at timed intervals indicated that the equilibrium for diffusion of glucose and AG was reached in 20 h. At the end of experiments, aliquots were taken from both chambers of the dialysis device. The absorbance of Con A was measured at 280 nm and the change of Con A concentration was calculated by comparing the absorbance value with that of the original solutions. Aliquots from both sides were diluted sixfold, and the concentrations of glucose and AG were determined by the phenol-sulfuric acid assay (13).

RESULTS

Extent of PEG Grafting to Con A

Con A has 12 lysine residual groups and one terminal amine group. Thus, the maximal number of PEG molecules that can be grafted to one Con A molecule is 13. Because the three-dimensional structure showed that most lysinyl groups of Con A were located on the surface of Con A, a large number of PEG molecules were expected to be grafted. The number of PEG molecules grafted to each Con A molecule was determined so that a relationship between the extent of PEGylation and the properties of Con A conjugates, such as solubility, stability, and glucose-binding activity, could be found. In this study, the fluorescamine method was used to determine the extent of PEGylation of Con A. The number of PEG molecules grafted to each Con A molecule was measured as a function of the molar ratio of PEG/Con A in the reaction mixture. As shown in Fig. 1, the number of grafted PEG molecules increased almost linearly up to 10 PEG molecules for each Con A molecule, as the molar ratio of PEG to Con A increased to 10. The maximal number of PEG grafted to Con A was 10 even when the [PEG]/[Con A] ratio was increased to 15. The 77% modification of the total available

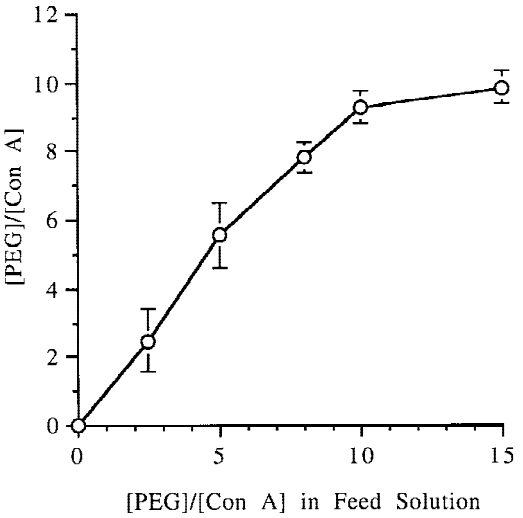

Fig. 1. Number of PEG molecules conjugated to each Con A as a function of the feed ratio (n = 3).

sites on Con A by PEG is rather high considering the possible steric hindrance between PEG chains.

Increased Solubility and Stability of Con A by PEG Grafting

The stability of PEG–Con A conjugates was examined by measuring the absorbance at 630 nm. Absorbance was measured as the concentrations of Con A, PEG2.5–Con A, and PEG5–Con A were increased up to 200 mg/ml. This concentration was equal to 8 mM, 5.3 mM, and 4 mM for Con A, PEG2.5–Con A, and PEG5–Con A, respectively (Fig. 2). The increase in absorbance was the largest with the native Con A. The absorbance increased sharply to 0.14 as the Con A concentration increased to 1.6 mM. Above this concentration, the absorbance increased slowly to 0.16. The absorbance increase for PEG2.5–Con A and PEG5–Con A, on the other hand, was relatively very small. At a concentration of 1.6 mM, the absorbance of the PEG2.5–Con A and PEG5–Con A increased only to 0.04 and 0.02, respectively. Even at a concentration of 4 mM, PEG2.5–Con A and PEG5–Con A showed much improved stability with absorbance of 0.06 and 0.03, respectively.

To examine the time-dependent changes in stability, the absorbance of Con A solutions was measured at a concentration of 4 mM at room temperature (Fig. 3). The native Con A showed continuous linear increase in the absorbance value to 1.6 during the first 6 days. The solution turned very opaque after only 4 days. On the other hand, the absorbance of the PEG–Con A solutions increased only slightly during the 10-day period, with slightly better results with the PEG5–Con A. Clearly, PEGylation resulted in higher stability of Con A as compared with the native Con A.

Binding Affinity of Modified Con A to Glucose and AG

The binding constants of glucose and AG to native Con A and PEG–Con A conjugates were determined using the equilibrium dialysis technique. The equilibrium dialysis data were analyzed using the Scatchard plot (14):

$$\frac{r}{c} = Kn - Kr$$

where r is the moles of the bound ligand (i.e., glucose or AG) per mole of the protein (i.e., native Con A or PEG-Con A conjugates), c is the molar concentration of free ligand, n is the number of binding sites on the protein, and K is the association constant for the ligand-protein complex. Fig. 4 shows examples of the binding of glucose and AG to native Con A and PEG5–Con A conjugates. In all cases, the number of binding sites (n) was 4. This indicates that PEGylation did not affect the total number of binding sites for glucose and AG. The PEGylation, however, affected the binding affinity significantly. In Fig. 4, the binding affinity of glucose to Con A was 727 M^{-1}, which is the same as the literature values. The glucose binding affinity to PEG5–Con A, however, was 2,353 M^{-1}, and this is a more than triple increase in the binding affinity over native Con A. The affinity of AG to Con A was also very high at 2,705 M^{-1}. This increase in the binding affinity of AG was known from our previous studies (1–3). The binding affinity of AG to PEG5–Con A was even higher at

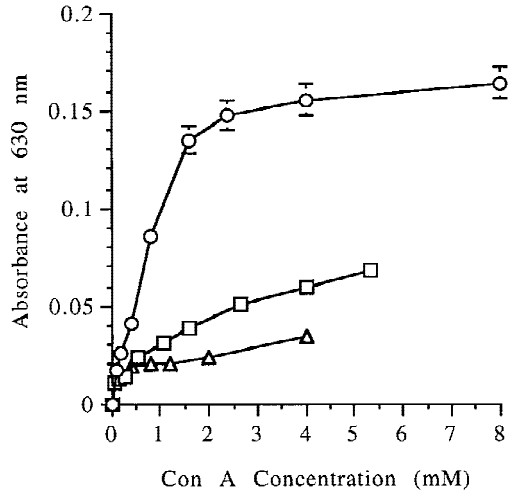

Fig. 2. Absorbance at 630 nm of Con A solutions as a function of the Con A concentration. Native Con A (△), PEG2.5–Con A (□), and PEG5–Con A (○) (n = 3).

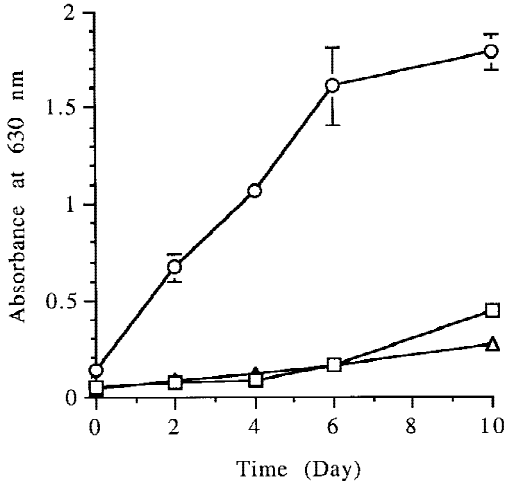

Fig. 3. Absorbance at 630 nm of Con A solutions (4 mM) as a function of time. Native Con A (△), PEG2.5–Con A (□), and PEG5–Con A (○) (n = 3).

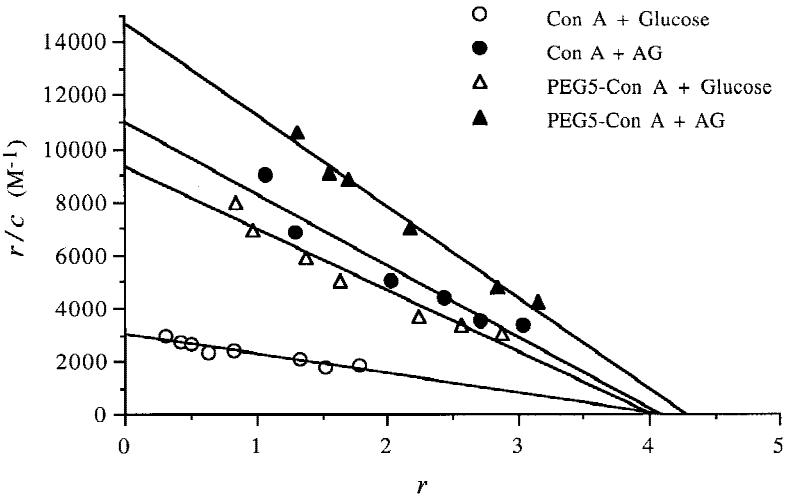

Fig. 4. Scatchard plots showing binding of glucose (open symbols) and AG (closed symbols) to native Con A (circles) and PEG5–Con A (triangles).

3,405 M^{-1}. This increase is consistent with increased binding affinity of glucose to PEG5–Con A. The main observation made from Fig. 4 is that PEGylation increased the binding affinity of glucose to Con A without altering the number of binding sites.

The binding constants of glucose and AG to all PEG–Con A conjugates are shown in Fig. 5. The effect of free MPEG molecules on the binding of glucose and AG to Con A was examined using MPEG of the same molecular weight as the one used in PEGylation. In the presence of free MPEG molecules in the Con A solution, the glucose binding constant to Con A increased slightly from 733±8 M^{-1} to 1,077±270 M^{-1}. The binding affinity of glucose to MPEG2.5–Con A and MPEG5–Con A increased significantly to 1,476±291 M^{-1} and 2,589±333 M^{-1}, respectively. The binding affinity of AG remained the same for PEGylation up to 5 PEG molecules per each Con A. The binding affinity of glucose and AG was statistically the same for PEG5–Con A. When the extent of PEGylation was higher than 5, the binding affinity decreased precipitously. The binding affinities of glucose and AG to PEG10–ConA were only 173±130 M^{-1} and 335±77 M^{-1}, respectively. Modification of Con A with more than 5 lysinyl groups of a Con A molecule virtually resulted in the loss of its binding property to glucose and AG.

DISCUSSION

PEG is one of the most widely used biocompatible polymers (15). PEG is a nontoxic, water-soluble, and highly flex-

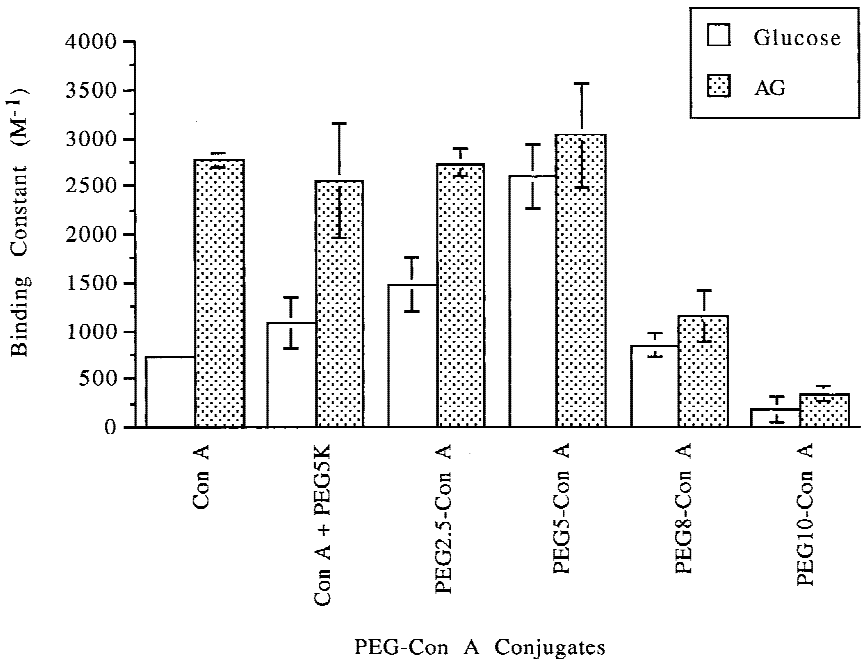

Fig. 5. Binding constants of glucose and AG to Con A and modified Con A. Simple physical mixture of Con A and MPEG (MW 5,000) (PEG5K) solution was used as a control to study the effect of free MPEG molecules on the binding of saccharides to Con A (n = 3).

ible polymer with large excluding volume. The low toxicity, immunogenicity, and antigenicity of PEG have led to its extensive biological applications including pharmaceutical, biomedical, and industrial applications (16,17). One of the major applications of PEG is modification of biologically active proteins for improved pharmacokinetics. Major benefits of PEG-protein conjugates are reduced immunogenicity and antigenicity, increased stability, increased resistance to proteolytic inactivation and circulating lives, and increased solubility (18–24). Although PEG is not biodegradable, it is readily excreted via the kidneys when its molecular weight is less than 6,000 in humans (25). MPEG coupling can only occur only at one end of the PEG molecule, and MPEG of varying molecular weights is commercially available. The polydispersity (M_w/M_n) is low, typically no greater than 1.05 for molecular weights up to 10,000. The narrow molecular weight distribution of MPEG not only simplifies analytical characterization of its protein conjugates but also makes the conjugate products reliable in quality. PEGylation of Con A increased its water solubility and stability, as expected. The improved stability is most likely due to the large excluding volume and high flexibility of the grafted PEG chains leading to reduced intermolecular interactions among Con A molecules.

The location of the saccharide binding site of Con A was determined by several research groups. The amino acid residues that may participate in the Con A–glucose binding are known to be 14–16, 97–99, 168–169, 207–208, 224–228, and 235–237 (26), or 12–18, 98–102, 205–208, and 226–229 (27,28). The information in the literature suggests that only one lysine (Lys101) is involved in the binding site of Con A. Because the glucose binding site of Con A is far from the other lysinyl groups according to the primary structure, the original glucose binding activity is expected to be retained after PEGylation of Con A. The reserved saccharide binding activity was also reported for succinyl-Con A derivatives (29). Grafting of Con A to poly(vinylpyrrolidone-co-acrylic acid) by covalent bonding also maintained the glucose binding property of Con A (30). The data in Figs. 4 and 5, however, indicate that PEGylated Con A not only preserved the glucose binding property but also increased the binding affinity substantially at moderate PEGylation. Con A has been known to bind hydrophobic monosaccharide derivatives with higher affinity (31–33). The higher binding affinity of AG to Con A than that of glucose also supports those observations. Because the total number of binding sites for glucose and AG remained the same after PEGylation (Fig. 4), the increase in the binding affinity may be due to conformational changes of Con A, but more study is necessary to prove such conformational changes. The linearity of the Scatchard plots in Fig. 4 indicates that the glucose binding sites of PEGylated Con A remain identical and independent, as in native Con A. Linear plots with n nearly equal to 4 also indicate that PEGylated Con A maintained the tetrameric form. The PEGylated Con A was able to form gels in the presence of glucose-containing polymers, and the formed gels were more homogeneous and stable than the gels formed by Con A (34). The hydrogels prepared by Con A had a tendency to show inhomogeneity due to poor solubility of Con A. The PEGylated Con A eliminated such a problem.

One interesting observation in this study is that PEGylation increased the binding affinity, i.e., bioactivity of a protein. The overall *in vivo* biological activity of a PEGylated protein is usually increased significantly as compared with that of the native protein. This is mainly due to the substantial increase in the circulation half-life of a PEGylated protein, even though the bioactivity of the PEGylated protein usually decreased (24). Covalent attachment of PEG to β-glucosidase and α-galactosidase resulted in reduction of their catalytic properties and masking of specific determinant sites on the enzymes. The enzymatic activity was almost lost after modification of only 30% of the ϵ-amino groups of α-galactosidase (35). In many instances, however, PEGylation did not cause any decrease in the bioactivity of a protein (36). In some cases, PEGylation even increased the bioactivity of the modified proteins. PEGylated chymotrypsin showed increased enzymatic activity compared with the native enzyme toward low molecular weight substrates (37). In our study, the glucose binding affinity of Con A was increased by PEGylation for 50% of the lysinyl groups available for modification. Because PEGylation is not expected to cause significant conformational changes to proteins, the increase in the activities of PEGylated proteins toward small molecules may be due to either slight changes in conformation around the binding site or a PEGylation-induced change in microenvironment (37). The observation that the glucose binding affinity of PEG5–Con A is the same as the binding affinity of AG to native Con A suggests that PEGylation contributed to the increase in hydrophobic interaction. The reduction in glucose binding affinity of PEG8-Con A and PEG10-Con A may be due to increased steric repulsion by the grafted PEG chains against the incoming glucose and AG, or further changes in local conformation around the binding site.

The increased binding affinity of glucose to PEGylated Con A had an unexpected beneficial effect. Our application of PEGylated Con A is to prepare more stable sol-gel phase-reversible hydrogels for modulated insulin delivery. The phase-transition of the Con-A containing hydrogels relies on the competitive binding of polymer-bound glucose and free glucose to Con A. The binding constants of glucose and AG to native Con A were 733±8 M^{-1} and 2759±77 M^{-1}, respectively. The binding affinity of AG was about four times larger than that of glucose, and thus, the concentration of free glucose necessary to induce gel-to-sol transition had to be four times larger than that of polymer-bound glucose. If the binding affinity of glucose is increased when PEGylated Con A is used as a physical crosslinker, then the glucose responsiveness will be changed. The increase in binding affinity of free glucose to PEGylated Con A means that the threshold concentration of free glucose for gel-to-sol transition will become much lower than that for native Con A. Thus, the sol-gel system will become more sensitive to small changes in the concentration of free glucose. The use of PEGylated Con A is expected to provide a new means to control glucose sensitivity in a number of systems, such as sol-gel reversible hydrogels, release of glycosylated insulin (30), viscometic affinity assays for glucose (38), and glucose biosensors (39).

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