Conjugation of Trypsin by Temperature-Sensitive Polymers Containing a Carbohydrate Moiety: Thermal Modulation of Enzyme Activity

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Novel temperature-sensitive polymers containing glucose units in their backbone were synthesized and covalently conjugated to trypsin. A series of copolymers based on N-isopropylacrylamide (NIPAAm) and glucosyoxylethyl methacrylate (GEMA) were prepared by using 4,4′-azobis(4-cyanovaleric acid) as an initiator, which resulted in one terminal carboxylic acid group per polymer chain. The polymers were conjugated to primary amine groups of trypsin with water-soluble carbodiimide as a coupling agent, which led to a star-shaped conformation. The polymer–enzyme conjugation was confirmed and characterized by size exclusion and reversed-phase chromatography. Almost of all amine groups in trypsin available for the conjugation were consumed and, consequently, a very dense layer of copolymers was actually coated around the enzyme surface. The conjugated enzymes exhibited reversible precipitation/resolubilization behaviors over a wide range of temperatures, depending on the content of GEMA in the copolymer. They also demonstrated no detectable self-digestion (autolysis) process, but the unconjugated enzyme showed very severe autolysis that led to a rapid inactivation in aqueous solution. When bovine serum albumin was used as a substrate, the protein substrate was not attacked by the conjugated enzyme, but completely digested by the unconjugated enzyme. This result was presumably caused by a steric repulsion process of the attached polymer chains around the enzyme toward the protein substrate. However, the enzyme retained sufficient activity against a low molecular weight substrate. Interestingly, the conjugated enzymes demonstrated very peculiar enzyme activity–temperature profiles, with two apparent optimal temperatures, indicating that a temperature-controlled collapse and flocculation of the copolymers around the enzyme surface modulated the mass transfer rates of substrate to the active site of the enzyme. The conjugated enzymes also exhibited improved thermal stability with increasing the amount of carbohydrate units in the polymer chain.

Introduction

A variety of synthetic polymers have been chemically conjugated to various proteins to generate novel functional protein–polymer hybrid molecules. One of the best-known polymers is poly(ethylene glycol) (PEG), and its conjugation to various therapeutic proteins and enzymes has been extensively studied (1). PEG-conjugated proteins generally have an improved stability and exhibit a solubility increase in various organic solvents. When injected in vivo, they demonstrate protection from other proteolytic enzymes, less immunogenicity, and a longer circulation time, probably because of the steric action of hydrophilic and highly mobile PEG chains. Other synthetic water-soluble and insoluble polymers, such as polymethacrylates and polystyrene, have also been conjugated to enzymes mainly to improve their thermal stability or to increase the reaction rate in organic solvents (2, 3).

Poly(N-isopropylacrylamide) (polyNIPAAm) has been known for its unique thermally reversible solubility change in aqueous solution at its phase transition temperature, that is, lower critical solution temperature (LCST) (4). PolyNIPAAm exhibits a precipitation behavior above the LCST (31–32 °C), but redissolves below the LCST. Because of its reversible hydration–dehydration behavior over a narrow temperature range, the conjugation of polyNIPAAm to different biospecific ligands has been used for thermal affinity bioseparation of antibodies and enzymes (5–7), as well as for the thermal modulation of molecular recognition between receptors and specific ligands. Recently, it has been reported that the conjugation of polyNIPAAm in a site-specific manner to streptavidin can thermally control the binding of its biotin ligand to the recognition site (8). PolyNIPAAm has also been copolymerized with various hydrophilic/hydrophobic functional monomers to control the range of phase transition temperature (9). As more hydrophilic comonomers are incorporated in the polymer chain, the LCST shifts to higher temperature with the concomitant broadness of its phase transition.

Several polyNIPAAm-conjugated enzymes have been reported: alkaline phosphatase (6), glucoamylase (10), and trypsin (11). PolyNIPAAm can be chemically conjugated to enzymes by multiple-point attachment by...
reacting functionally activated comonomers such as N-acryloxysuccinimide in its backbone with primary amine groups of the enzyme molecules (6). An alternative way of conjugation is to first prepare polyNIPAAm with a reactive terminal end group, such as carboxylic acid, and then to conjugate it to the amine groups of the enzyme. This method generated a star-shaped conformation of the conjugated enzyme (11). The aforementioned studies, however, revealed that although the conjugated enzymes showed a reversible precipitation/redissolution behavior in response to the temperature above and below the LCST, there was insignificant effect of the temperature variation around LCST on the enzyme activity. It was postulated that above the LCST, the attached polyNIPAAm on the enzyme surface might collapse and become dehydrated and then flocculated, but the accessibility of small molecular weight substrates to the enzyme active site could not be hindered in comparison with that of the unconjugated enzyme. This inability to hinder accessibility may be caused either by incomplete coverage of the active site by the collapsed polyNIPAAm and/or by conjugation of the polymers to regions of the enzyme far away from the active site. In a previous paper, it was demonstrated that a single chain of polyNIPAAm conjugated adjacent to the active site of streptavidin can lead to an “on/off” molecular recognition of biotin in a thermally controlled fashion (8). This result suggests that the amount of polymer conjugated to the enzyme, its chain length, and its conjugation location are all important to affect thermal control of enzyme activity. In a study of polyNIPAAm-conjugated antibody, specific antigen binding affinity was dependent on the number of polymer chains conjugated to the antibody (7).

When natural and synthetic polymers are conjugated to enzyme molecules, their thermal stabilities generally tend to increase, presumably either because of the restricted tendency of protein conformational change toward unfolding at higher temperature or because of microenvironmental variations in properties such as viscosity and surface tension (12). It was suggested that a multiple point attachment of protein molecule to polymer matrices plays a major role in the stabilization of immobilized or conjugated enzymes (13). Additionally, it is known that highly water-absorbing additives, such as polyols and carbohydrates, can stabilize various proteins by changing a thermodynamic equilibrium of protein unfolding kinetics. In particular, sugars stabilize different proteins by a “preferential hydration” mechanism (14). Thus, it can be expected that the enzyme conjugated with a hydrophilic polymer containing a sugar moiety in its chain would exhibit a thermal stabilization effect because the enzyme could be in a sugar-rich microenvironment.

In the present study, a series of copolymers based on NIPAAm and a glucose containing monomer, glycosoxyethyl methacrylate (GEMA), were synthesized and conjugated to the enzyme trypsin. These copolymers, although having different amounts of sugar moiety in their backbone, showed thermally reversible behaviors at their respective LCST range, depending on NIPAAm/GEMA molar ratio in the composition of copolymer backbone. A carboxylic acid terminal end group in poly-NIPAAm-co-GEMA) was directly conjugated to primary amine groups of trypsin via a water-soluble carbodiimide coupling process, leading to a star-shaped structure of the conjugated enzymes. The conjugated enzymes thus had novel temperature-sensitive properties in addition to providing an artificial glycosylation effect on the enzyme surface. These conjugated trypsins were purified and characterized in detail: molecular weight was determined by size exclusion chromatography; surface hydrophilicity/hydrophobicity was estimated by reversed-phase chromatography; and the phase transition temperatures; the amount of polymer attached onto the enzyme surface; and the kinetic properties of the conjugated enzyme were determined as a function of temperature. In particular, two hypotheses were tested in this study. First, the temperature change below and above the LCST of the copolymers might affect the enzyme activity because the mass transfer of substrate to active site might be hindered by the collapse of polymer chains on the enzyme surface, consequently limiting the substrate accessibility. Second, the presence of conjugated polymers with glucose units in the vicinity of the enzyme surface might demonstrate improved thermal stabilization of the enzyme.

Materials and Methods

Materials. N-Isopropylacrylamide was purchased from Eastman Kodak and recrystallized with hexane. Glysosetyl methacrylate (GEMA; 52.4% in aqueous solution) was obtained from Nippon Fine Chemical Co. through professor K. Nakamae of Kobe University, Japan, and used without any further purification. 4,4’-Azobis(4-cyanovaleric acid), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), trypsin from bovine pancreas, N-α-benzoyl-ω-arginine p-nitroaniline (BAPNA), 2- (N-morpholinol) ethanesulfonic acid (MES), and bovine serum albumin (BSA) were from Sigma Chemical Co. Other chemicals were of analytical grade.

Methods. Synthesis of Poly(NIPAAm-co-GEMA). Twenty grams of NIPAAm and GEMA in varying molar feed compositions of 95:5, 90:10, and 85:15 were dissolved in 100 mL of methanol and charged into a round-bottomed flask. After purging with nitrogen gas, 100 mg of 4,4’-azobis(4-cyanovaleric acid) was added as an initiator. The polymerization was carried out at 60 °C for 4 h under nitrogen atmosphere. The copolymer was precipitated in diethyl ether and washed three times. The obtained polymer was dried under vacuum.

Conjugation of Poly(NIPAAm-co-GEMA) to Trypsin. A carboxylic acid in the terminal end group of poly(NIPAAm-co-GEMA) was conjugated to primary amine groups of trypsin by using EDAC as a coupling agent. One gram of the copolymer was dissolved first in 20 mL of MES pH 5.5 buffer, and 100 mg of EDAC was added. After 3 h of reaction, the N-acylurea-activated polymer solution was slowly added to 50 mL of the MES buffer solution containing 100 mg of trypsin. The conjugation reaction proceeded for 12 h at 4 °C. To separate the conjugated enzymes from the EDAC, as well as to change the buffer species, the aforementioned solution was dialyzed against 1 L of Tris buffer (pH 7.4) using a Spectrapore dialysis membrane (MW cutoff pore size: 10,000) overnight at 4 °C. To separate out the conjugated enzymes from unconjugated enzymes, ammonium sulfate was slowly added to the solution up to 20% (w/w) concentration, and the precipitated fraction was recovered and redissolved in buffer solution. In a separate control experiment, it was confirmed that the poly(NIPAAm-co-GEMA) was selectively precipitated in the 20% ammonium sulfate solution, whereas the native trypsin was not. The conjugated enzyme solutions were further purified by an ultrafiltration process using a membrane with a MW cutoff of 30,000 (Amicon) to remove any residual unconjugated trypsin, self-digested trypsin fragments, and low molecular weight polymers. Finally, the conjugated enzymes were freeze-dried.
**LCST of Conjugated Enzymes.** The LCST of the conjugated enzymes was determined by reading the change of transmittance value at 600 nm as the temperature was increased with a Beckman UV–vis spectrophotometer. The conjugated enzyme with 0.1% (w/v) concentration in Tris pH 7.4 buffer was used, and at each temperature reading, at least 5 min of incubation period was allowed.

**Activity Assay of Conjugated Enzymes.** The protein content in the conjugated enzymes was determined by measuring absorbance at 280 nm using a series of native trypsin as calibration standards. There was no difference in protein contents between the direct absorbance reading at 280 nm and biocinchonic acid (BCA) colorimetric protein assay. Enzyme activity of conjugated enzymes was determined with BAPNA as a substrate analogue. A substrate solution with the concentration of 3 mM in 0.1 M Tris buffer (pH 7.4) containing 0.01 M calcium chloride was used. The enzyme reaction was carried out by adding 2.5 mg of the conjugated enzymes to 3 mL of substrate solution, and the reaction was terminated by adding 1 mL of 10% (v/v) acetic acid. The absorbance was recorded at 410 nm. In the case of enzyme activity assay in a cloudy solution state, which was observed at higher reaction temperatures above the starting point of LCST change, the turbid yellowish solution was incubated in an ice–water bath until the transparent yellow solution was obtained, and then the absorbance value was determined. For the activity determination of native trypsin, 25 μg of enzyme was used and the procedure just described was followed.

**Determination of Unreacted Amine Groups in Trypsin.** The unreacted primary amino groups in the conjugated enzymes were quantitatively determined by a fluorometric method (15). As an amine-reactive fluorescent dye, fluorescamine, was used. Fluorescamine dissolved in a dimethylformamide (DMF) solution (1.5 mL with the concentration of 0.1 mg/mL) was added to 0.5 mL of conjugated enzyme solutions in 0.1 M sodium phosphate buffer (pH 8.0). After 7 min of reaction, the fluorescence was determined at an excitation wavelength of 390 nm and an emission wavelength of 483 nm with a Shimadzu spectrofluorometer. Native trypsin was used as a control in this experiment to calculate the remaining amine groups in the conjugated enzymes.

**Size-Exclusion (SEC) and Reversed-Phase Chromatography.** The conjugation between polymer and trypsin was confirmed by using SEC and reversed-phase chromatography with a Waters 600E HPLC system and UV detector at 280 nm. For the SEC, the following conditions were used: Bio-silic SEC 250-5 (Bio-Rad) column (300 × 7.8 mm dimension); 0.1 M phosphate/0.1 M sodium chloride/0.01 M sodium azide solution as a mobile phase; flow rate, 1 mL/min; injection volume size, 200 μL. To determine the molecular weight of the conjugated enzymes, the following calibration proteins were used: thyroglobulin (670 000), bovine γ-globulin (158 000), chicken myoglobin (44 000), and equine myoglobin (17 000). From the elution time of each protein, a calibration curve was constructed and approximate molecular weights of the conjugated enzymes were estimated. By considering the obtained molecular weight of the conjugated enzymes as well as the number of consumed primary amine groups in enzyme for the conjugation, molecular weights of copolymers were then calculated. For the reversed-phase chromatography, an Alltech Brownlee RP-300 (Alltech) column (250 × 4.6 mm dimension) was used with gradient pumping of the two solvents composed of water containing 0.12% trifluoroacetic acid and 0–80% (v/v) acetonitrile.

**Results and Discussion**

The synthesis of poly(NIPAAm-co-GEMA) conjugated enzymes is schematically illustrated in Figure 1. The conjugated enzymes have a star-shaped molecular structure by attaching a carboxylic terminal end group of the polymer chain to primary amine groups in trypsin. In PEG-conjugated therapeutic proteins, the same star-shaped polymer–protein conjugates were obtained. Previously, this kind of polyNIPAAm conjugation strategy has been reported for various proteins (7, 11, 16); these studies used azobisisobutyronitile (AIBN) as a free radical initiator and 3-mercaptopropionic acid as a chain transfer agent to terminate the propagation of growing chain ends in the early stage and simultaneously produce one carboxylic acid end group in the polymer chain, which was then conjugated to enzymes via a carbodiimide coupling process. In these cases, molecular weight of the
obtained polyNIPAAm was generally low (5000–6000) because of the early termination of radical polymerization. In the present study, thermally decomposing radical initiator, 4,4′-azobis(4-cyanovaleric acid), was used to generate a carboxylic acid in one starting end group of a growing polymer chain. This conjugation method is expected to yield relatively high molecular weight polymer compared with those of using 3-mercaptopropionic acid as a chain transfer agent. NMR and IR spectra of poly(NIPAAm-co-GEMA), in which the GEMA fraction incorporated in the polymer chain can be identified: broad proton peaks in glucose units appearing in the 3–4 ppm region in the NMR spectrum, and double bands of C=O stretching at 1600–1750 cm⁻¹ in the IR spectrum due to the presence of amide (NIPAAm) and ester (GEMA) groups in the polymer chain.

Figure 2 shows size-exclusion chromatograms of native trypsin, poly(NIPAAm-co-GEMA 85:15)-conjugated trypsin after ultrafiltration, and the filtrate fraction obtained from the ultrafiltration. The polymer-conjugated enzyme shows one single peak that eluted earlier than that of trypsin, and very minor trypsin and other fragment peaks, indicating that the conjugated enzyme was minimally contaminated with unconjugated trypsin. When the SEC was monitored at 214 nm instead of 280 nm to further detect the polymer fractions, the elution profiles monitored at the two wavelengths exactly overlapped, suggesting that there was insignificant amount of unconjugated polymers remaining in the conjugated enzyme. From the elution time, apparent average molecular weights of the conjugated enzymes were estimated; they range from 500 000 to 580 000 depending on the molar feed composition of NIPAAm/GEMA in the polymer backbone. In the SEC of the filtrate fraction collected from the ultrafiltration process, a trypsin peak as well as lower molecular weight fraction peak can be observed; the fraction eluted later than that of trypsin might be self-digested trypsin fragments generated during the conjugation process.

Figure 3 demonstrates reversed-phase HPLC chromatograms of a mixture of trypsin and conjugated enzymes with different GEMA amounts in the polymer...
chain. The first major peak, appearing at ~24.4 min, is trypsin, and the adjacent second peak appearing next to that of trypsin is the conjugated enzyme. In these chromatograms, it can be deduced that the polymer-conjugated enzymes are more hydrophobic than trypsin because they are eluted later. These elution profiles make sense because primary amine groups in the conjugated enzymes are modified with more or less hydrophobic poly(NIPAAm-co-GEMA). All the conjugated enzymes could be dissolved in polar organic solvents such as methanol, dioxane, and DMF, whereas the native trypsin is not soluble in these solvents. In fact, the solubility of the conjugated enzymes in polar organic solvents depends on the amount of GEMA in the polymer backbone. For example, as the GEMA content in a series of conjugated enzymes increased, the conjugated enzymes exhibited a limited solubility in more hydrophobic polar solvents (i.e., ethyl acetate, tetrahydrofuran, and acetone) than in the aforementioned solvents. This behavior can be directly evidenced in the reversed-phase HPLC chromatograms. As more GEMA is incorporated in the polymer (C chromatogram in Figure 3), the elution time of conjugated enzymes gradually shifts to that of native trypsin. This result suggests that the hydrophilic/hydrophobic surface character of the conjugated enzymes can be modulated by the copolymerization of NIPAAm and GEMA in varying molar feed ratios.

Table 1 shows a summary of the conjugation between different poly(NIPAAm-co-GEMA) and trypsin. The activities remaining after the conjugation range from 14.9 to 27.2%, which are a little bit lower than the expected values. However, considering the extent of conjugation between primary amine groups and polymers, these results are understandable. Of 15 amine groups present in trypsin, almost all amino groups are reacted, which was analyzed by a fluorescamine assay: 13–14 polymer chains per trypsin molecule were expected to densely attach onto the surface of trypsin. From the apparent molecular weights of conjugated enzymes (500 000 and 580 000), average molecular weights of three copolymers attached to the enzyme were calculated. These values were similar to those obtained from an end group titration method which yielded a molecular weight of ca. 40 000.

Because the polymer-conjugated trypsins have densely packed water-soluble polymers around the enzyme surface, it is of great interest to examine the self-digestion behavior of trypsin. It was postulated that although native trypsin, a proteolytic enzyme, digests itself with the production of smaller fragments (autolysis), the conjugated trypsin can be protected from such self-digestion by the sterically modified surface of the attached polymer chains, which might suppress the accessibility of trypsin to other trypsin molecules, and/or by polymer blocking of the active site. Figure 4 shows the SEC analysis of self-digestion behaviors for both native trypsin and polymer-conjugated trypsin, which were incubated at room temperature for 24 h. It can be seen that the native trypsin was severely digested by itself, resulting in lower molecular weight fractions that appear later than that of native trypsin. On the other hand, the conjugated enzyme shows an intact peak shape and the same elution time with generating tiny amount of smaller fragments, revealing that the polymer–enzyme conjugates have a self-protection property. The amount of conjugated enzyme used in this experiment was 10 times greater than that of native trypsin to compensate for its reduced specific activity after conjugation. To further investigate the limited action of high molecular weight substrates toward the conjugated enzymes, BSA (MW 66 000) was chosen and used as a model substrate for trypsin. Figure 5 demonstrates reversed-phase HPLC results after the incubation of the native trypsin and the conjugated trypsin with BSA for 24 h at room temperature. The results confirm that the conjugated enzymes did not attack BSA because of attached polymer chains, whereas the native trypsin completely digested BSA, with the resulting generation of many small fragments. Thus, it can be expected that the conjugation of polymer to trypsin enhances its stability in aqueous solution while concomitantly maintaining its activity for low molecular weight substrates, which can readily diffuse into and away from the enzyme active site.

Table 1. Summary of Poly(NIPAAm-co-GEMA)-Conjugated Trypsins

<table>
<thead>
<tr>
<th>poly(NIPAAm-co-GEMA)-conjugated enzymes</th>
<th>activity remaining after conjugation (%)</th>
<th>percent of amine groups reacted (no. of amine groups reacted in trypsin)</th>
<th>av MW of conjugated enzymes</th>
<th>av MW of polymer conjugated to enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIPAAm/GEMA feed molar ratio (mol %)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95:5</td>
<td>23.3</td>
<td>86.6 (12.9)</td>
<td>503 000</td>
<td>37 100</td>
</tr>
<tr>
<td>90:10</td>
<td>27.2</td>
<td>92.0 (13.8)</td>
<td>583 000</td>
<td>40 500</td>
</tr>
<tr>
<td>85:15</td>
<td>14.9</td>
<td>94.7 (14.2)</td>
<td>572 000</td>
<td>37 800</td>
</tr>
</tbody>
</table>

a These values were calculated on the basis of specific activity values compared with that of native trypsin (100%). b Native trypsin has 15 primary amine groups; the number of amine groups reacted in trypsin also indicates the number of conjugated polymer chains per trypsin molecule. c Molecular weight of native trypsin is 23 800.

Figure 4. SEC analysis of self-digestion behaviors for (A) trypsin and (B) poly(NIPAAm-co-GEMA, 85:15)-conjugated trypsin after incubation for 24 h.
Because poly(NIPAAm-co-GEMA) exhibits thermally reversible solubility change in response to temperature, it is important to determine its phase transition temperature. PolyNIPAAm exhibits its LCST at 31–32 °C, and its copolymers with other more hydrophilic monomers, such as acrylamide and acrylic acid, have LCSTs shifted to higher temperatures (9). Figure 6 shows the transmittance changes of a series of poly(NIPAAm-co-GEMA) as a function of temperature. It can be seen that with increasing the GEMA content in the copolymer backbone, the transmittance change shifts to higher temperatures, and the extent of changes gets broader over a wide range.

Figure 5. Reversed-phase chromatograms after 24 h of incubation of BSA with trypsin and poly(NIPAAm-co-GEMA, 85:15)-conjugated trypsin: (A) BSA alone, (B) BSA with trypsin, and (C) BSA with the conjugated trypsin.
of temperatures. This is likely to be caused by the fact that the hydrophobic interaction of isopropyl groups in NIPAAm units, which is a main driving force for the LCST phenomenon, is reduced by the incorporation of more hydrophilic GEMA units in the polymer chain. It is also of interest to see a two-step transmittance change in poly(NIPAAm-co-GEMA) that becomes more apparent with increasing GEMA content. The plausible explanation for this observation may be either polymer flocculation kinetic factor or intrinsic phase transition behavior originated from a nonrandom distribution of NIPAAm and GEMA along the polymer chain. The segmented block formation of NIPAAm and GEMA in the polymer backbone, which resulted from different monomer reactivity ratios, possibly exhibited such two distinct transmittance changes. The increase in transmittance values at higher temperature regions occurred by the precipitation of flocculated polymer-conjugated enzymes out of solution.

Several previous studies on polyNIPAAm-conjugated enzymes revealed that there was no apparent effect of polymer collapse above the LCST on the enzyme activities (10). This lack of effect could be attributed to the possibility that the collapsed and dehydrated polymer layer around the enzyme did not hinder the diffusion of a substrate molecule to the active site. The density of polyNIPAAm oligomers conjugated around the enzyme active site may not be high enough to block the active site; thus, the mass transfer rates of a small substrate to and product from the active site might not be different with and without conjugation. Another possibility is that collapse of homo-polyNIPAAm on the enzyme surface at the LCST could result in fully exposing the active site to aqueous environment, allowing the substrate access to the active site without any diffusion limitation. In the present study, poly(NIPAAm-co-GEMA), with a broad temperature range of polymer precipitation, was densely conjugated on the enzyme surface, as evidenced in the molecular weight and the number of reacted primary amine groups in the conjugated enzymes. Figure 7 shows temperature–activity profiles of a series of poly(NIPAAm-co-GEMA)-conjugated enzymes. The native enzyme shows a typical bell-shaped curve, whereas poly(NIPAAm-co-GEMA)-conjugated enzymes exhibit a tendency of two split-activity optimum temperatures with increasing the GEMA content in the copolymer composition. These peculiar activity–temperature profiles were reproducible three times. The conjugated enzymes with the GEMA contents of 10 and 15% show the most distinctive double peaks at 40 and 55 °C, whereas the native enzyme has its maximum activity at 55 °C.

This interesting temperature-modulated control of enzyme activity can be best explained by the process of collapse, dehydration, and precipitation of the densely conjugated poly(NIPAAm-co-GEMA) onto the enzyme surface, which occurs with increasing the temperature. This process is schematically illustrated in Figure 8. The conjugated polymers with higher GEMA contents would undergo a progressive conformational transition from random coil to a collapsed, globular shape as the temperature increased, which might cause a temperature-dependent gradual masking of the active site. In Figure 6, the transmittance change becomes broader with increasing the GEMA content, indirectly suggesting that a gradual conformational transformation of conjugated polymers occurred as the temperature was raised. This gradual change is in sharp contrast to a sudden collapse of homo-polyNIPAAm. At the lower GEMA content in the polymer (95:5 NIPAAm/GEMA ratio), the activity–temperature profiles tend to show a single bell-shaped curve followed by a small shoulder peak, supporting the postulation that the gradual polymer collapse gradually modulated the apparent enzyme activity. Thus, the accessibility of low molecular weight substrate to the catalytic site might be reduced by the steric hindrance of densely collapsed polymers, which would result in the decrease in enzyme activity between 40 and 50 °C. When the polymers completely collapse at higher temperature regions, the active site could be reexposed and the accessibility of the substrate might be regained, leading to the maximum activity seen at 55 °C; thereafter the enzyme is denatured. To test the reversibility of enzyme activity between 40 and 45 °C, the enzyme conjugated with the copolymer with an 85:15 NIPAAm/GEMA molar feed ratio was thermally cycled between the two tem-
It was found that the observed activity was reversibly cycled in response to the thermal cycling operation within the range of its time-dependent stability decay, indicating that the copolymer indeed played a role in thermally reversible "on/off" control of the enzyme activity. It should be mentioned that the conjugated enzymes did not stay as individual molecules at higher temperatures, but aggregated to each other in the state of a cloudy solution as a result of decrease in solubility. However, the flocculation of the conjugated enzymes into a microparticulate mass did not affect the enzyme activity. In the case of homo-polyNIPAAm-conjugated enzymes reported previously (6, 10), the same activity retention after flocculation occurred, but there was no detectable difference in activity—temperature profiles between native and conjugated enzymes. This result suggests that the macroscopic aggregation of the conjugated enzymes did not hinder the diffusion of substrate to the active site. A more important factor in exhibiting such two optimum activity profiles would be microscopic collapsing behaviors of conjugated polymers near the active site of enzyme. Thus, the observed activity profiles were not simply attributed to the solubility change of conjugated enzymes in response to temperature.

Because poly(NIPAAm-co-GEMA) contains a pendant glucose moiety along its backbone, it would be interesting to see if it enhances the thermal stability of the conjugated enzymes. There have been several previous studies regarding thermal stability improvement of various enzymes by chemical conjugations of natural carbohydrates and synthetic polymers (17). Another approach for stabilization would be to simply add protein stabilizers, such as polyols and low molecular weight sugars, into the enzyme solution (14, 18). Figure 9 shows the thermal stability of conjugated enzymes incubated at 50 °C. The heat stability is enhanced gradually with increasing GEMA content in the conjugated polymer chain. This result indeed supports the enhanced thermal stability mechanism by the addition of carbohydrate when the carbohydrate residues are located in the vicinity of the enzyme molecule.

In conclusion, we have synthesized a series of novel temperature-sensitive copolymers based on NIPAAm and GEMA, with pendant carbohydrate moieties along their backbone, and conjugated them to trypsin in a star-shaped conformation. These polymer-conjugated trypsin exhibited remarkable protection against self-digestion. The conjugated enzymes also exhibited two temperatures for maximum activity, presumably resulting from the gradual collapse of polymers on the enzyme surface with increasing temperature. Additionally, improved thermal stability was also noticed as a result of carbohydrate-rich microenvironment created by the GEMA component along the polymer in the vicinity of enzyme.

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References and Notes


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