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Polymeric Switch on Lysozyme Activity: Role of Hydrophobic and Electrostatic Interactions

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ABSTRACT

Enzymes have attracted potential applications in both medicine and biotechnology. In our present study, we show a strategy for switching the enzymatic activity of lysozyme by the complex formation with a cationic smart copolymer. PAMA-*g*-PEG graft copolymers suppressed the enzymatic activity of lysozyme without any conformational change, indicating the formation of complex and covering the active site of lysozyme by copolymers. The addition of polyanion, poly(acrylic acid) (PAAc), recovered the suppressed enzymatic activity of the lysozyme/polymer complex efficiently. These finding suggest that that hydrophobic interaction coupled with electrostatic interactions has a great role for the complexation and decomplexation of the lysozyme/polymer complex. Circular dichroism (CD) spectral analysis indicated that the conformation of the enzymes maintained largely during the course of the complexation.
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Key word: PAMA-*g*-PEG copolymer, lysozyme, enzymatic activity, hydrophobic and electrostatic interactions

INTRODUCTION

Enzymes have attracted great attention in biomaterials education because they offer very important industrial applications. In nature, the application of enzymes would increase drastically if we can able to regulate their enzymatic activity. There are several techniques have been developed such as metal dependent switch for regulating the enzymatic activity,[1] by introducing temperature[2] and photo[3] responsive polymers to the active site of enzymes, whose activity was then switched by changes in temperature and light conditions, enzyme-polymer hybrids, which are created by nonspecific[4] or specific[5] site-directed modifications of synthetic polymers, etc. However, metal binding site or polymers into the enzymes at the active site or to the enzyme surface is required for these technologies. Tailored nanoparticle surface is another way to control the enzymatic activity[6] although tailoring of the nanoparticle or conjugation with polymers is required which makes a complex process such as combination of the several preparative steps. It is noteworthy to note that the cost effective and easy procedure is long awaited. As reported on several previous studies[6,7,8] attractive and repulsive electrostatic interactions have been considered very important for controlling or

switching the enzymatic activity. In addition, polymeric compounds carrying charged are particularly smart agents for complexation with enzymes.[9,10] Based on the recent development of complexation between enzyme and charged polymer, for the first time, we succeeded in regulating the enzymatic activity of lysozyme[11] through a simple process, the external addition of a polycationic polyamine-poly(ethylene glycol) (PEG) graft copolymer, poly(*N*,*N*-diethylaminoethyl methacrylate)-*graft*-PEG (PEAMA-*g*-PEG). They might form a complex despite the fact that both are positively charged. In the present study, effect of another polyamine-PEG copolymers, polycationic poly(*N*,*N*-dimethylaminoethyl methacrylate)-*graft*-PEG (PAMA-*g*-PEG) on the enzymatic activity of lysozyme was investigated. It should be noted that PAMA-*g*-PEG was used in this work due to the presence of low hydrophobic factor (di-ethyl to di-methyl group) (Figure 1) in PAMA-*g*-PEG as compared with PEAMA-*g*-PEG to investigate the mechanism of interaction between enzyme and polymer. These finding suggest that complexation between lysozyme and polyamine-PEG copolymers might driven not only by electrostatic interaction but also by hydrophobic interaction.

EXPERIMENT

Materials

PAMA-*g*-PEG was synthesized as described in the previous article and was a kind gift from Mr. Shinya Matsumoto[12]. PAMA-*g*-PEG was used before HPLC purification. *Micrococcus lysodeikticus* for activity assay and PAAc (M_n = 5,000 g/mol) were purchased from Wako (Osaka, Japan). Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) was obtained from Nacalai Tesque Inc. (Kyoto, Japan). All chemicals used were of high-quality analytical grade. The water used in this study was purified using the Milli-Q system (Nihon Millipore Co., Tokyo, Japan).

Enzymatic Activity Measurements

The enzymatic activity of lysozyme was measured based on bacteriolysis reaction with *M. lysodeikticus.*[8] Lysozyme concentration was determined by measuring the absorbance at 280 nm with an appropriate blank, using an extinction coefficient of 2.63 mL mg^{-1} cm^{-1}.[13] *M. lysodeikticus* suspension (substrate solution) was prepared by mixing 0.5 mg/mL of *M. lysodeiktictus* with 50 mM sodium phosphate buffer at pH 7.0. All the stock solution for additives and protein were dissolved in 50 mM phosphate buffer solution (pH 7.0). A 10 µL aliquot of the sample was added to a 1490 µL of *M. lysodeikticus* solution and decrease in turbidity of the solution was monitored at 600 nm for 60 s using a UV-vis spectrophotometer model V-550 (Japan Spectroscopic Co., Tokyo, Japan) at room temperature. The absorbance decay plots from 10 to 20 sec were fitted to a linear equation and then the enzymatic activities were determined from the slope of the fitted line.[14]

CD Spectra Measurements

Far-ultraviolet CD spectra were monitored using a Jasco spectropolarimeter (JASCO J-720W, Jasco Ltd., Tokyo, Japan). A cuvette with 0.1 cm path length was used and photomultiplier voltage did not exceed 600 V in the measurements. The results are directly obtained from CD spectrophotometer.

RESULT AND DISCUSSION

Hen egg white lysozyme (pI=9.20[15]) was used as the model enzyme in this study. In our previous study, it was found that the enzymatic activity of lysozyme was suppressed by PEAMA-*g*-PEG (pK_a 7.4[Error! Bookmark not defined.]) owing to capping of the active site of lysozyme, which involved an electrostatic interaction between the negatively charged active site of lysozyme and the positively charged amine moiety of PEAMA-*g*-PEG at neutral pH.[11] Moreover, it was anticipated that hydrophobic interaction may take part for the formation of PEAMA-*g*-PEG/lysozyme complex. It is observed that PEG-MA had almost no effect on the enzymatic activity of lysozyme.[11] PAMA-*g*-PEG was used in the present study and compared with PEAMA-*g*-PEG due to the difference in their hydrophobic factor (di-ethyl and di-methyl groups respectively). Figure 1 shows the chemical structure of PEAMA-*g*-PEG and PAMA-*g*-PEG.

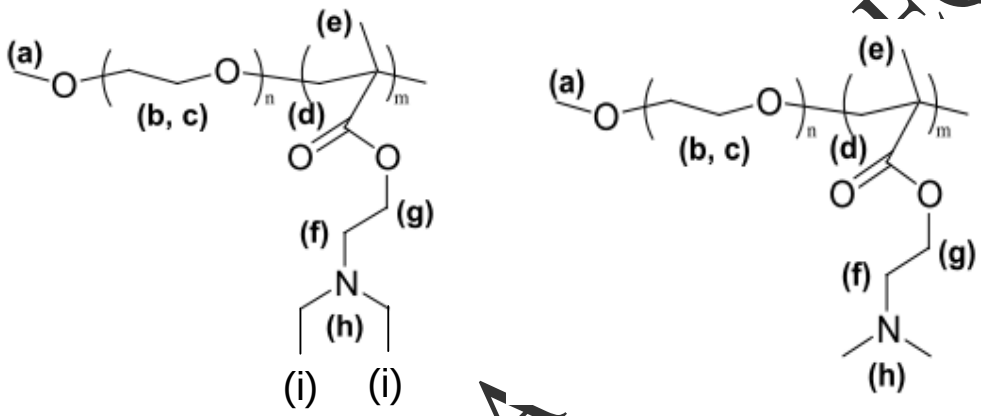


Figure 1. Chemical structure of *graft* copolymers (left) PEAMA-*g*-PEG, (right) PAMA-*g*-PEG.

Table 1 shows characterization report of both PEAMA-*g*-PEG and PAMA-*g*-PEG. Even the molecular weight of polymers has large difference but the number average molecular weight ratio of PEAMA or PAMA to PEG is of close value.

Table 1. Characteristics of PAMA-*g*-PEG in comparison with PEAMA-*g*-PEG

Name of polymer	*M*n (g/mol)	*M*n of PEG (g/mol)	Number of PEG per polymer	*M*n of PEAMA/PAMA (g/mol) [number of EAMA/AMA per polymer]	*M*n ratio of PEAMA or PAMA/PEG
[a]PEAMA-*g*-PEG	28,000	4,700	2	19,000 [104]	2.02
[b]PAMA-*g*-PEG	84,000	4,700	7	49,600 [315]	1.32

[a]Reference number 11; [b]Reference number 12

Figure 2 shows the enzymatic activity of lysozyme in the presence of a synthetic polymer, PAMA-*g*-PEG. The polycation PAMA-*g*-PEG showed a significant effect on the enzymatic activity of lysozyme when mixed with lysozyme.

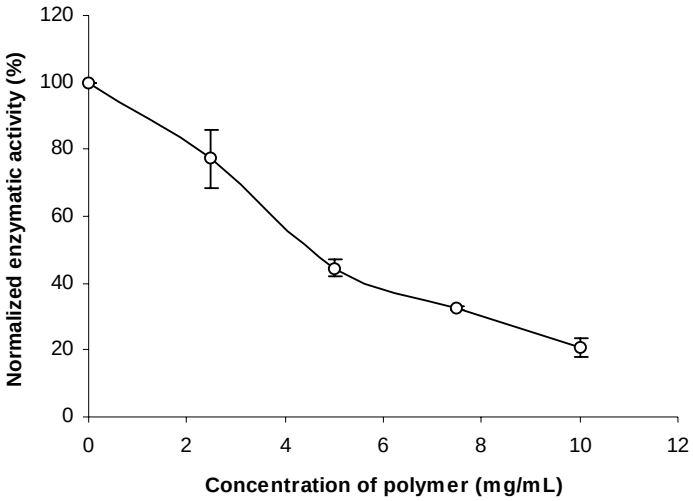


Figure 2. Changes in the normalized enzymatic activity of lysozyme in the presence of PAMA-*g*-PEG, as a function of the polymer concentration. The samples contained 0.5 mg/mL lysozyme. The enzymatic activities were measured at room temperature and pH 7.0.

The enzymatic activity was suppressed in the presence of PAMA-*g*-PEG. As comparing the present findings with the previous results, it is observed that the rate of lysozyme inactivation by PEAMA-*g*-PEG is powerful than PAMA-*g*-PEG. For example, at the 10 mg/mL concentration of PEAMA-*g*-PEG, the lysozyme activity is disappeared (0.12±2.81%)[11] but at the same concentration of PAMA-*g*-PEG, the lysozyme activity is still remaining (20.59±2.61%). The same data obtained in the every concentration of PAMA-*g*-PEG used in this experiment. These finding indicate that PAMA-*g*-PEG interact with lysozyme weakly than PEAMA-*g*-PEG due to the low hydrophobic factor which means that hydrophobic interaction also take part to form polyamine-PEG copolymers with lysozyme. It is noteworthy to mention that we showed that PEAMA-*g*-PEG prevents heat inactivation of another enzyme, ribonuclease A (RNase A), which has a positive net charge at neutral pH and a positively charged active site which is opposite to that of lysozyme[16]. Regarding the enzymatic activity of RNase A, PEAMA-*g*-PEG had negligible effects under the present experimental conditions[16]. These findings suggest that the involvement of an electrostatic repulsion between the positively charged active site of RNase A and positively charged amine moiety of PEAMA-g-PEG. As a result, the enzymatic activity was remained close to 100% as does native RNase A (See Figure 2a)[16]. Taken together, it can be noticed that electrostatic attraction is one of the dominant factor to form PAMA-*g*-PEG/lysozyme complex due to the presence of negatively charged active site lysozyme and positively charged amine moiety of graft copolymers. So that electrostatic interaction coupled with hydrophobic interaction might play the key role for the complexation. These results illustrate that the interaction between PAMA-*g*-PEG and lysozyme is weaker than between PEAMA-*g*-PEG and lysozyme due to the presence of low hydrophobic factor in the case of PAMA-*g*-PEG (di-methyl) than PEAMA-*g*-PEG (di-ethyl). It is suggested that the complexation can be accomplished by the interaction between PEAMA/PAMA segment and amino acid residues on the enzyme surface and active site, whereby the hydrophobic and electrostatic interactions

take place on the polymer-enzyme interface even though other noncovalent interactions can not be ruled out at this moment.

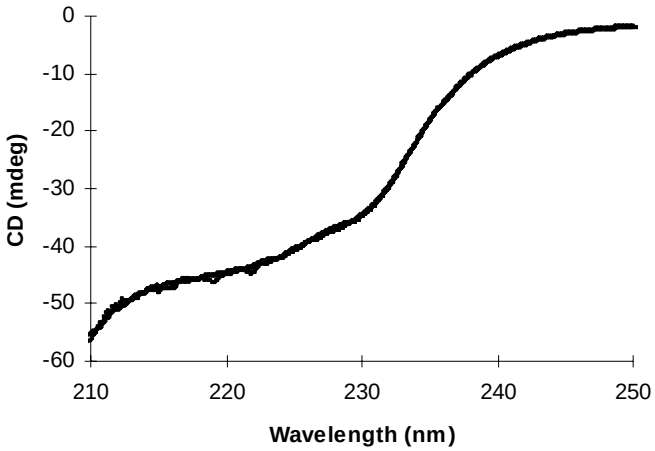


Figure 3. CD spectra of lysozyme and the lysozyme/PAMA-*g*-PEG complex at 25 °C. The samples contained 0.5 mg/mL lysozyme and 10 mg/mL PAMA-*g*-PEG.

In order to obtain information on the conformational change in the complexation step of lysozyme with PAMA-*g*-PEG, a CD spectrum of the lysozyme and its polymer complex was obtained (Figure 3). Secondary structure of protein reflects from the far-UV CD spectrum.[17] There was no difference in the CD spectra between lysozyme and the lysozyme/PEAMA-*g*-PEG complex,[11] indicating no substantial changes in the secondary structure of lysozyme in the complexation step with PEAMA-*g*-PEG. The results are in line with lysozyme and lysozyme/PAMA-*g*-PEG complex. No distortion of the spectrum was obtained indicate no conformational change.

Since PAMA-*g*-PEG has a positively charged segment at neutral pH, it is suggested that the electrostatic interaction between the negatively charged residues at the active site of lysozyme and the amine segments of PAMA-*g*-PEG leads to the inhibition of the substrate attack into the active site of lysozyme, resulting in a decrease in lysozyme activity. The hydrophobic interactions also play the key role here. These results indicate that PAMA-*g*-PEG interacted with lysozyme effectively, resulting in the inactivation of its enzymatic activity, but that the structure of lysozyme was maintained in the lysozyme/PAMA-*g*-PEG complex.

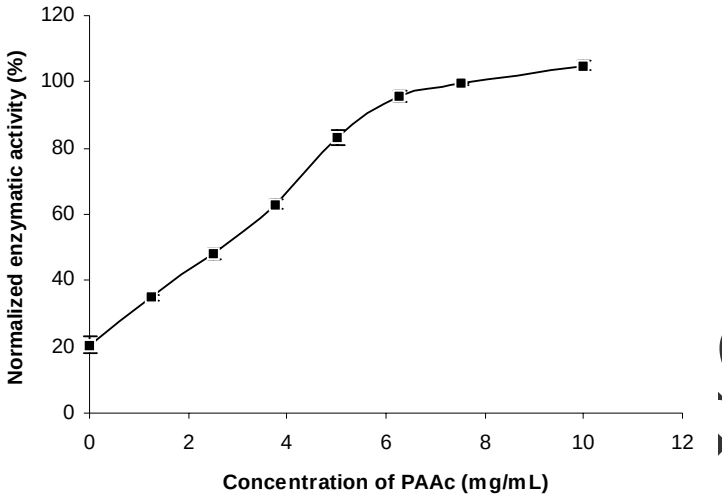
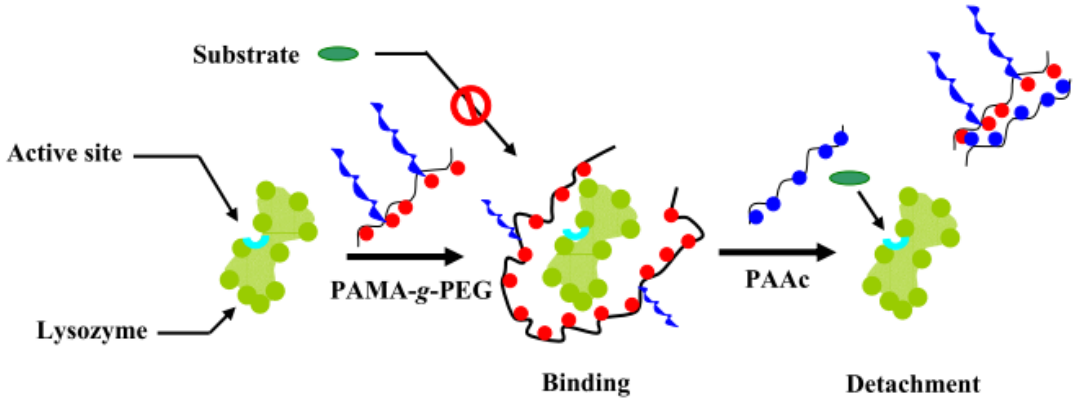


Figure 4. Recovery of the enzymatic activity of the lysozyme/PAMA-*g*-PEG complex upon the addition of PAAc. All samples contained 0.5 mg/mL lysozyme with 10 mg/mL PAMA-*g*-PEG, and measurements were carried out at room temperature and pH 7.0.

In our previous article, we showed that the enzymatic activity of lysozyme in the lysozyme/PEAMA-*g*-PEG complex completely recovered to the level of the native lysozyme upon the addition of PAAc.[11] These results indicate that the lysozyme was released from the lysozyme/PEAMA-*g*-PEG complex due to the poly-ion complex (PIC)[18,19] formation of PEAMA-*g*-PEG with PAAc. In order to evaluate again, the electrostatic interaction between lysozyme with PAMA-*g*-PEG, an exchange reaction with the anionic polymer PAAc was carried out and the variations in the enzymatic activity of the lysozyme/PAMA-*g*-PEG complex were monitored is as shown in Figure 4. It was observed again that the enzymatic activity of the lysozyme in the complex recovered upon the addition of PAAc. The recovery of enzymatic activity is fast in the present case, lysozyme/PAMA-*g*-PEG complex as compared with the previous results, lysozyme/PEAMA-*g*-PEG[11] complex. For example, at the 10 mg/mL concentration of PAAc, the lysozyme activity is recovered at up to the native level (100.7±5.17%)[11] but at the lower concentration of PAAc (5 mg/mL and 6.25 mg/mL), the lysozyme activity was recovered almost completely (83.25±2.20% and 95.51±1.57) even though attain constant value (100%) at higher concentration in both case as expected. These results indicate that due to low hydrophobic factor of PAMA-*g*-PEG than PEAMA-*g*-PEG, the interaction between lysozyme and PAMA-*g*-PEG is weaker which leads to the quick recovery of enzymatic activity upon the addition of PAAc. Taken together, it can be assumed that a hydrophobic interaction has a great role for the complexation. In this work, new additives PAMA-*g*-PEG with PAAc switch the enzymatic activity of lysozyme and two mechanisms were proposed; these data suggest that complexation and decomplexation of lysozyme/PAMA-*g*-PEG as well as the switching of enzymatic activity depends on not only the electrostatic interaction between PAMA-*g*-PEG molecules interacting with enzyme molecules but also the hydrophobic interaction between hydrophobic surfaces of enzyme

molecules with hydrophobic factor of PAMA-*g*-PEG. Complexation and decomplexation mechanism between enzyme and polymer were depicted in scheme 1.



Scheme 1. Schematic illustration of complexation and decomplexation of lysozyme and PAMA-*g*-PEG.

CONCLUSION

The smart copolymer, PAMA-*g*-PEG and polyanion PAAc, switched the enzymatic activity of lysozyme dramatically. It was interesting to note that PAMA-*graft*-PEG polymers suppressed the enzymatic activity of lysozyme, and that PAAc easily restored the lysozyme activity by means of electrostatic and hydrophobic interactions. Binding behavior between polymer and enzyme in addition to maintaining their secondary structure offers new prospects. Such switching of enzyme activity using a polymer-enzyme system may able to expand the application of enzymes in the industrial field including enzyme delivery, enzyme separations, biosensor, and bio-nanoreactors etc. This same strategy might be extended to regulate the enzymatic activity of other enzymes or the binding affinity of enzymes to other polymers, DNA or other proteins.

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