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ORIGINAL PAPER

Immobilization of lysozyme on poly(*N*-isopropyl acrylamide)/2-hydroxyethyl methacrylate copolymer core–shell gel beads

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Abstract The immobilization of chicken egg white lysozyme (Lyz) molecules on poly(*N*-isopropyl acrylamide) gel beads containing 2-hydroxyethyl methacrylate (HEMA) (PGBH) was studied as a function of temperature and HEMA content. Using dynamic and static light scattering measurements, nanometer-sized PGBH particles were shown to exhibit thermo-responsive behavior, and aggregate formation occurred during temperature changes from 25 to 40 °C. The radii of PGBH and Lyz-immobilizing PGBH, the amount of immobilized Lyz and the activity of immobilized Lyz depended on both HEMA content and temperature. Moreover, the activity of immobilized Lyz also depended on the molecular size of the substrates, and the Lyz immobilized on PGBH particles with higher HEMA content showed activity toward low molecular weight substrates at 40 °C nearly equal to that of native Lyz, which indicates that no conformational change in the Lyz molecule occurred after immobilization. These results demonstrate that changes in the activity of the immobilized Lyz were due to a balance of an increase in the affinity between the substrate and Lyz resulting from concentration effects and the steric hindrance between the substrate and Lyz incorporated into the PGBH aggregates with increasing HEMA content and temperature. Furthermore, these results demonstrate that PGBH is a useful material as an enzyme immobilization carrier.

Keywords Enzyme immobilization · Lysozyme · Poly(*N*-isopropyl acrylamide) gel beads · 2-Hydroxyethyl methacrylate · Enzymatic activity

Introduction

An understanding of the formation mechanism of protein–polyelectrolyte complexes (PPCs) and their enzymatic activities could provide a better explanation for

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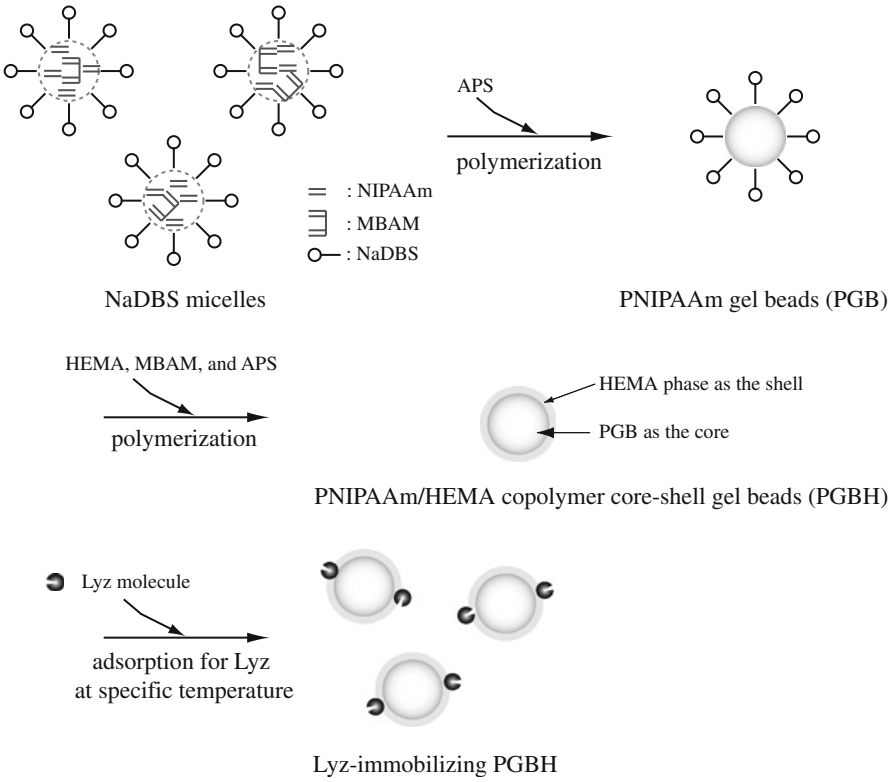
the interaction mechanism of polyelectrolytes with ionic colloidal particles and improve the molecular design of composite protein–polymer systems such as immobilized enzymes, as well as enhance the design of protein separation processes using water-soluble polymers. In previous studies, we examined the complexation of various proteins with water-soluble polymers and the enzymatic activities of the resulting PPCs [1–3]. The formed PPCs showed 20–50% of the activity of the native enzymes as has been reported by many researchers. For instance, Hamdya et al. reported that immobilized urease on copolymeric hydrogels based on 2-hydroxyethyl methacrylate (HEMA) and itaconic acid (IA) with HEMA/IA (92.5/7.5) showed 41.3% activity [4]. In another example, Shimomura et al. [5] reported that glucose oxidase immobilized on magnetic particles via graft polymerization of acrylic acid (AAc) had an activity level 50% of that observed for the native enzyme.

In our recent study, PPCs of lysozyme (Lyz) with polyelectrolytes containing hydroxyl groups such as potassium polyvinyl alcohol sulfate with a low degree of esterification and chondroitin sulfate C showed high activities that were nearly equal to that of native Lyz [6]. Similar high activity was reported by Kokufuta et al. in the PPCs of pepsin with polyethylene glycol [7]. From these results, it can be anticipated that enzyme immobilization carriers with high enzymatic activity can be prepared via introduction of hydrophilic monomers using the proper immobilization matrices and immobilization methods.

Ito et al. [8] reported that the poly(*N*-isopropyl acrylamide) gel beads (PGB) of nanometer size showed thermo-responsive behavior as well as the PNIPAAm molecule which possesses a lower critical solution temperature (LCST) of around 32 °C [9–11]. Moreover, Khan [12] reported that smart copolymer microgels consisting of a well-defined temperature sensitive core and pH-sensitive shells can be obtained from NIPAAm and AAc with different molar ratios of AAc. Taking advantage of the remarkable change in physical properties with temperature, studies on applications such as drug delivery systems for controlled-release of drugs and enzyme immobilization carriers have been widely performed using polymer particles and gels [13–15]. Kawaguchi et al. reported materials for protein separation using PNIPAAm particles and preparation of immobilized particles such as DNA and lecithin [16–19]. Furthermore, Lin et al. [20] reported the preparation of a core–shell copolymer latex with thermo-responsive properties consisting of NIPAAm and chitosan as the core side and methacrylic acid and methyl methacrylate as the shell side and its potential application as a vehicle for drug targeting. Consequently, these nanoscale materials will be excellent matrices for biotechnology and biomedical applications.

In the present study, we prepared PGB particles containing hydrophilic monomers such as HEMA (PGBH) in which the PGB served as the core of the PGBH and the HEMA as the shell and examined the molar mass and radii of various PGBH and Lyz-immobilizing PGBH as a function of HEMA content and temperature by means of dynamic and static light scattering (DLS and SLS) measurements (Scheme 1). In addition, the ratios of immobilized Lyz molecules to PGBH particles and temperature dependencies on the associated activity of the immobilized Lyz towards bacteria and low molecular weight substrates were studied as a function of the amount of HEMA.



Scheme 1 Schematic illustration for preparation procedure of PNIPAAm/HEMA copolymer core–shell gel beads (PGBH) and concept in this study. In the present study, we prepared four PGBH particles with varying amount of HEMA consisting of thermo-responsive PGB as a core and HEMA phase as a shell. Thermo-responsive property of PGBHs and their adsorption capacity for lysozyme (Lyz), and activity of the immobilized Lyz were studied as a function of temperature and amount of HEMA using DLS, SLS, absorption spectrometry, and activity measurements for Lyz-immobilizing PGBHs to evaluate a usefulness of PGBH as an enzyme immobilized carrier

Finally, the influence of the introduction of HEMA to PGBH and the usefulness of PGBH as an enzyme immobilization carrier are discussed.

Experimental section

Materials

Lysozyme from chicken egg white (Lyz, purity 95%) was obtained from Sigma-Aldrich Japan Co., Japan. *Micrococcus lysodeikticus* (*M. lysodeikticus*, Sigma-Aldrich Japan Co., Japan) and hexa-*N*-acetyl-D-glucosamine [$(GluNAc)_6$, Seikagaku Co., Japan, purity $\geq$95%] were used as bacterial and low molecular weight substrates, respectively. For preparation of the PGBH particles, *N*-isopropylacrylamide (NIPAAm) monomer was purchased from Kohjin Co., Ltd, Japan and HEMA monomer

was purchased from Wako Pure Chemical Industries Ltd, Japan. *N*,*N*'-Methylene-bis acrylamide (MBAM) crosslinking agent, sodium *n*-dodecyl benzene sulfonate (NaDBS) surfactant, and ammonium peroxodisulfate (APS) polymerization initiator were also obtained from Wako. All samples were of analytical reagent grade and used without further purification, except for NIPAAm. NIPAAm was purified by recrystallization from *n*-hexane for the removal of the polymerization inhibitor *p*-methoxyphenol. All water used in this study was deionized, twice distilled, and filtered through a Gelman (Gelman Sciences, Inc., USA) 0.22 μm filter.

Preparation of various PGBH particles

PGBH particles with varying HEMA content were prepared as follows, with detailed preparation conditions listed in Table 1. Prior to preparation of the various PGBHs 1–3, PGBH 0 (i.e., PGB) was synthesized as the core of the PGBH particles using the radical polymerization procedure reported by Ito et al. [8]. NIPAAm (0.100 mol), NaDBS (2.00×10^{-3} mol), and MBAM (1.00×10^{-3} mol) were dissolved in 200 g of water. The NIPAAm solution was then purged with nitrogen for 1 h to remove oxygen. After 2.00×10^{-4} mol of APS was added to the NIPAAm solution, polymerization was carried out under nitrogen atmosphere while stirring at 60 °C for 2 h.

For the preparation of modified PGBH particles, the PGBH 0 solutions prepared using the above procedure were incubated at 45 °C and stirred under nitrogen atmosphere for 1 h. Next, specific amounts of HEMA and MBAM (see experimental conditions for PGBHs 1–3 in Table 1) were dissolved in the PGBH 0 solution, which was then purged with nitrogen for 1 h. Then specific amounts of APS were added to the PGBH 0 solution and polymerization was carried out under nitrogen atmosphere while stirring at 45 °C for 2 h.

After the polymerization step was complete, the polymer products were purified by dialysis to remove any unreacted NIPAAm, HEMA, MBAM, APS, and NaDBS from the PGBH dispersed solutions. The purified products were then freeze-dried.

DLS and SLS measurements

DLS and SLS measurements were carried out with a Brookhaven system BI-200SM (Brookhaven Instruments Co., USA) equipped with a 256-channel digital autocorrelator

Table 1 Amount of HEMA, MBAM, and APS for the preparation of various PGBH particles

Sample	HEMA $\times 10^2$ (mol)	MBAM $\times 10^3$ (mol)	APS $\times 10^4$ (mol)
PGBH 0	0	1.00	2.00
PGBH 1[a]	1.00	0.10	0.20
PGBH 2[a]	2.00	0.20	0.40
PGBH 3[a]	3.00	0.30	0.60

[a] For the preparation of PGBH containing various amounts of HEMA, PGBH 0 (i.e., PGB) was used as a core of PGBHs 1–3

(BI-2030AT) and a 2-W Ar laser [Stabilite 2017, Spectra-Physics Lasers (Spectra-Physics, Inc., USA)]. A 400-μm pinhole aperture was employed for the EMI photomultiplier tube, and decahydronaphthalene was used as the refractive index matching fluid to reduce stray light. We analyzed the autocorrelation functions with the CONTIN program [21] and estimated the hydrodynamic radii (R_h) of various PGBH and Lyz-immobilizing PGBH samples. For SLS measurements, the optical alignment was ensured to less than 3% deviation from linearity in the $I\sin\theta$ versus θ plot over the range of $40° \leq \theta \leq 140°$. Each measurement was carried out for 1 s. We determined the Rayleigh ratio on the basis of the average of five such measurements. Changes in the refractive index with varying concentrations of different PGBHs and Lyz were measured at 25 °C with an Otsuka Model DRM-1021 electrophotometric differential refractometer (Otsuka Electronics Co., Ltd., Japan).

Measurements of Lyz adsorption by PGBH

The immobilization of Lyz on the PGBH particles was carried out using a mixture of 0.015 g of the various PGBHs with 50 cm^3 of Lyz solution (0.1 g/dm^3) at pH 6.7 stored at temperatures from 25 to 65 °C for 24 h.

The amount of immobilized Lyz was estimated as follows. After incubation for 24 h at a prescribed temperature, the solution was centrifuged at 15,000 rpm for 30 min using a Beckman Coulter Model Allegra 64R centrifuge (Beckman Coulter Co., Japan). The amount of immobilized Lyz was estimated from the absorption spectral measurements of the supernatant solution using a previously prepared calibration curve at 280 nm. The absorbance of the solution was measured using a Hitachi Model U-1000 spectrophotometer (Hitachi High-Technologies Co., Japan).

Activity measurements of immobilized Lyz toward *M. lysodeikticus* and $(GluNAc)_6$

The hydrolytic reaction of *M. lysodeikticus* at pH 6.7 was initiated by quick mixing of the *M. lysodeikticus* solution (0.08 g/dm^3, 250 cm^3) with 25 cm^3 of Lyz-immobilizing PGBH solution that had been stored at temperatures from 25 to 65 °C. The Lyz concentration in the Lyz-immobilizing PGBH solution was adjusted to 0.100 g/dm^3 by dilution with a phosphate buffer solution at pH 6.7. Changes in the absorbance of the solution at 450 nm during the reaction were measured using a Hitachi Model U-1000 spectrophotometer.

The hydrolytic reaction of $(GluNAc)_6$ was analyzed using the method of Imoto et al. [22]. A $(GluNAc)_6$ solution (4.0×10^{-5} mol/dm^3, 5.5 cm^3) was added to 2.75 cm^3 of Lyz-immobilizing PGBH solution and the resulting mixture incubated at 25 and 40 °C. A color reagent solution (2.0 cm^3, see below) was then mixed with 1.5 cm^3 of the sample solution, and the new mixture incubated in boiling water for 15 min. After cooling, the absorbance of the sample solution at 420 nm was read versus water. The color reagent solution was prepared by dissolving 0.5 g potassium ferricyanide in

1.0 dm^3 of a 0.5 M sodium carbonate solution. *N*-Acetyl-D-glucosamine (Seikagaku Co., Japan) was used to estimate the hydrolysis rate of $(GluNAc)_6$.

Results and discussion

Temperature dependence on the radii and molar mass of PGBH

Many researchers have reported that copolymers consisting of NIPAAm and AAc show pH and AAc content dependencies of the LCST [12, 23–25]. As reported by Ito et al. [8] and others [12, 20], the PGBH 0 particle used as the core of the PGBH particles has been shown to exhibit thermo-responsive properties. Therefore, to clarify the influence of these factors on the introduction of HEMA to PGBH 0, we first examined the temperature dependence of the PGBHs with various HEMA contents on the R_h and molar mass (M_w) of the particles.

The temperature dependence of various PGBH samples on R_h is shown in Fig. 1. M_w and R_h values for various PGBHs at 25 and 40 °C are listed in Table 2. The R_h values decreased with increasing temperature from 25 to 35 °C and were then nearly constant above 35 °C. In particular, an abrupt decrease in the R_h of the PGBHs was observed around 32 °C independent of HEMA content, a temperature that corresponds to the LCST of PNIPAAm. This result indicates that the PGBH 1–3 particles possess a thermo-responsive property similar to that of the PGBH 0 particle. Moreover, the M_w values of the PGBHs increased with an increase in temperature from 25 to 40 °C, indicating the formation of PGBH aggregates (see Table 2). Furthermore, the R_h values of the PGBHs increased with increasing HEMA content. The aggregation number (α), however, decreased with increasing HEMA content, a change which is thought to be caused by an increase in the

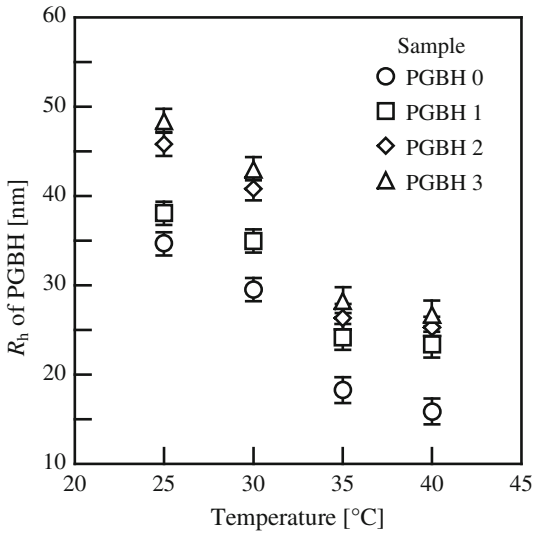


Fig. 1 Temperature dependence on R_h of PGBHs with varying HEMA content. The symbols *circle*, *square*, *diamond*, and *triangle* represent the R_h of PGBH 0, PGBH 1, PGBH 2, and PGBH 3, respectively. DLS measurements were carried out at a PGBH concentration of 0.3 g/dm^3

Table 2 Molar mass and radii of various PGBH particles at 25 and 40 °C

Sample	$M_w \times 10^{-7}$ (g/mol)[a]		Aggregation number, α (–)[a]	R_h of PGBH (nm)[b]	
	25 °C	40 °C		25 °C	40 °C
PGBH 0	0.385	1.63	4.23	34.7	16.0
PGBH 1	0.677	2.45	3.62	38.1	23.5
PGBH 2	0.850	2.61	3.07	45.8	25.3
PGBH 3	0.886	2.54	2.87	48.4	26.7

[a] At SLS measurements, molar mass of various PGBHs (M_w) at 25 and 40 °C were evaluated at PGBH concentration ranges from 0.01 to 0.3 g/dm^3. Moreover, aggregation number, α, is calculated from the following equation: $\alpha = M_w$ at 40 °C/M_w at 25 °C

[b] DLS measurements were carried out at a PGBH concentration of 0.3 g/dm^3

hydrophilic interactions as a greater number of HEMA molecules is introduced into the PGBH. Therefore, changes in the R_h of the PGBHs are attributed to the contraction of the PGBH 0 in the core of PGBHs 1–3 because the HEMA shell does not possess any thermosensitivity, and aggregation through an increase in hydrophobic and/or hydrophilic interactions between the isopropyl acrylamide groups of NIPAAm in the core and the 2-hydroxyethyl group of the HEMA molecules in the shell.

PGBH adsorption of Lyz

Considering that the PGBH particles showed R_h changes with temperature and HEMA content (see Fig. 1; Table 2), it can be anticipated that the adsorption behavior of Lyz molecules by PGBH particles should also depend on temperature and HEMA content. We next, therefore, determined the amount of Lyz immobilized by various PGBHs and the radii of those Lyz-immobilizing PGBHs as a function of HEMA content at 25 and 40 °C. The changes in the amount of Lyz immobilized by PGBH and the radii of the Lyz-immobilizing PGBHs at 25 and 40 °C with varying HEMA content are shown in Fig. 2 and Table 3. The amount of immobilized Lyz increased with increasing HEMA content, and the amount of Lyz immobilized at 40 °C (■) was higher than the amount immobilized at 25 °C (□). As can be seen in Fig. 2 and Tables 2, 3, the R_h of the Lyz-immobilizing PGBHs at 25 °C (○) also increased with increasing HEMA content and were larger than the R_h values for both the PGBHs at 25 °C and native Lyz, whereas the R_h of Lyz-immobilizing PGBH 0 nearly equaled that of PGBH 0. Moreover, the R_h of the Lyz-immobilizing PGBHs at 40 °C (●) increased with an increase in the HEMA content and were larger than the R_h values for PGBH at 40 °C, native Lyz and the R_h values for Lyz-immobilizing PGBH at 25 °C for all of the different amounts of immobilized Lyz.

These results indicate that the immobilization of Lyz at 25 °C is mainly caused by an increase in the hydrophilic interactions between PGBH, Lyz, and Lyz-immobilizing PGBH with increasing HEMA content. For the PGBH immobilized at 40 °C, however, the changes in the amount of immobilized Lyz are considered to result from the presence of lattices in highly ordered PGBH aggregates that serve as

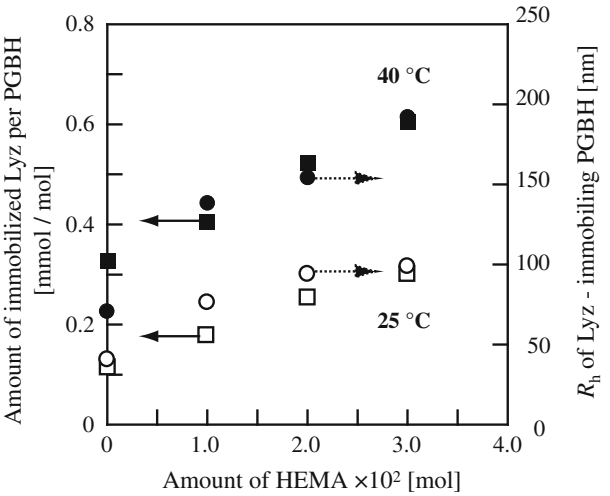


Fig. 2 Changes in the amount of immobilized Lyz and the radii of Lyz-immobilizing PGBH at 25 and 40 °C with varying HEMA content. The *solid* and *dotted arrows* refer to the amount of Lyz immobilized to PGBH at 25 °C (*open square*) and 40 °C (*filled square*) and the radii of Lyz-immobilizing PGBH at 25 °C (*open circle*) and 40 °C (*filled circle*), respectively. Measurements of the radii of Lyz-immobilizing PGBH at 25 and 40 °C were carried out at a PGBH concentration of 0.03 g/dm^3

Table 3 Radii of Lyz-immobilizing PGBH and kinetic parameters of Lyz immobilized to PGBH particles toward *M. lysodeikticus* at 25 and 40 °C

Sample	25 °C			40 °C		
	R_h (nm)[a]	$K_m \times 10^2$ (g/dm^3)[b]	V_{max} (g/dm^3 s)[b]	R_h (nm)[a]	$K_m \times 10^2$ (g/dm^3)[b]	V_{max} (g/dm^3 s)[b]
PGBH 0	40.5	4.2	5.7	70.3	3.5	9.1
PGBH 1	76.0	4.7	5.5	138.0	3.6	8.6
PGBH 2	93.7	4.9	5.1	153.8	3.7	8.4
PGBH 3	98.7	5.4	4.5	191.8	3.8	8.1

[a] DLS measurements were carried out at a PGBH concentration of 0.03 g/dm^3

[b] Measurements for *M. lysodeikticus* concentration dependencies on the initial velocity of immobilized Lyz were carried out at Lyz and *M. lysodeikticus* concentrations of 0.100 g/dm^3 and 0.02–0.20 g/dm^3

adsorption sites for Lyz in addition to the increasing hydrophobic and/or hydrophilic interactions between PGBH, Lyz, and Lyz-immobilizing PGBH that occur as the temperature and HEMA content increase.

Activity of immobilized Lyz towards different molecular-sized substrates

It is well known that the activity, storage stability, and reusability of immobilized enzymes exhibit temperature, pH, and component content dependencies that are generally influenced by the immobilization method (e.g., covalent bond, physical

adsorption, or entrapping). On the basis of the results presented in Fig. 2 and Tables 2, 3, the Lyz molecules were thought to be located in different environments at 25 and 40 °C because the immobilization of Lyz appeared to occur via different driving forces. These differences suggested that the activity of the immobilized Lyz was affected by temperature and HEMA content. Furthermore, Kawaguchi et al. reported that the activity of trypsin immobilized on particles with a PNIPAAm spacer were influenced by the molecular size of the substrates, the surface density of the trypsin and PNIPAAm on the particles and the molecular weight of the PNIPAAm spacer [16–19]. Similarly, we reported that the formation mechanism and activity of Lyz complexed with polyelectrolytes were influenced by the molecular size of the substrates (such as *M. lysodeikticus* and $(GluNAc)_6$), the surface charge density of the Lyz and polyelectrolyte, and the molecular weight of the polyelectrolyte [1–3, 6]. We therefore next evaluated the relative activity of PGBH-immobilized Lyz with varying HEMA content toward substrates of different molecular sizes for the purpose of determining the efficiency of the PGBH particles as enzyme immobilization carriers.

The changes in the relative activity of the immobilized Lyz towards *M. lysodeikticus* and $(GluNAc)_6$ at 25 and 40 °C as a function of HEMA content are shown in Fig. 3. Relative activity was calculated from the relative values of the initial hydrolytic velocity of the immobilized Lyz per initial velocity of native Lyz at optimum pH. The relative activity of the immobilized Lyz toward *M. lysodeikticus* at 25 °C (○) was nearly constant at 33.7%, whereas that of immobilized Lyz at 40 °C (●) gradually decreased from 45.2 to 33.6% with increasing HEMA content. The relative activity of the immobilized Lyz toward $(GluNAc)_6$ at 25 and 40 °C (▲ and ▲), however, increased gradually with increasing HEMA content at both temperatures. Interestingly, PGBH 3-immobilized Lyz at 40 °C showed a high activity that was nearly equal to that of native Lyz. Moreover, the relative activity of the immobilized Lyz towards the high molecular weight substrate glycol chitin

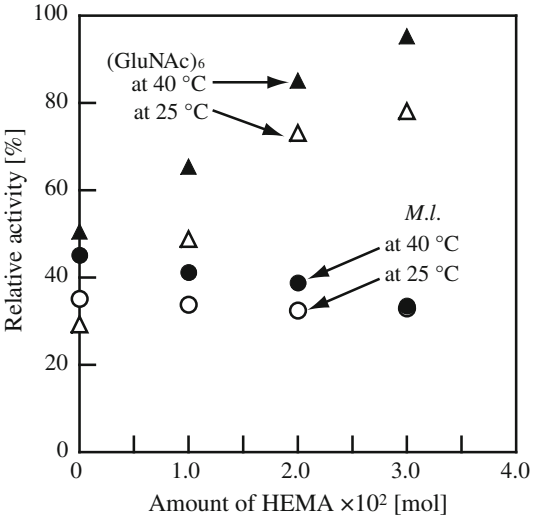


Fig. 3 Changes in the relative activity of PGBH-immobilized Lyz toward *M. lysodeikticus* and $(GluNAc)_6$ at 25 and 40 °C as a function of HEMA content. The symbols *open circle*, *filled circle*, *open triangle*, and *filled triangle* represent the relative activities of immobilized Lyz toward *M. lysodeikticus* at 25 °C, *M. lysodeikticus* at 40 °C, $(GluNAc)_6$ at 25 °C, and $(GluNAc)_6$ at 40 °C, respectively. Activity measurements were carried out at *M. lysodeikticus*, $(GluNAc)_6$, and Lyz concentrations of 0.08 g/dm^3, 4.0×10^{-5} mol/dm^3, and 0.100 g/dm^3, respectively

(M_w: 1.24×10^5 g/mol) at 25 and 40 °C, however, decreased gradually with increasing content from 55.6 to 33.0% and 57.0 to 38.8%, respectively (data not shown). Considering the HEMA content dependencies of the activities of the immobilized Lyz towards various substrates reported in previous papers [5, 6, 16–19], these results indicate that the decrease in the activity of the immobilized Lyz was mainly caused by steric hindrance between the substrates and the Lyz through PGBH aggregation and not by conformational changes in the Lyz molecules. In addition, the affinity between *M. lysodeikticus* and Lyz was not affected, because the Lyz molecules were located in the hydrophilic environment when the HEMA was introduced to the PGBHs.

To clarify the reason for the decrease in activity of the immobilized Lyz, we examined the dependence of immobilized Lyz activity on the *M. lysodeikticus* concentration and estimated the relevant kinetic parameters [apparent Michaelis constant (K_m) and maximum reaction velocity (V_{max})] to discuss the affinities between the immobilized Lyz and *M. lysodeikticus* and the steric hindrance resulting from the immobilization. The kinetic parameters for the immobilized Lyz towards *M. lysodeikticus* at 25 and 40 °C are listed in Table 3. The K_m and V_{max} values were estimated from the intercepts of each straight line on the *Y*- and *X*-axes, respectively, in the Lineweaver–Burk plots.

As shown in Table 3, the K_m and V_{max} values at 25 °C increased and decreased with an increase in the HEMA content, respectively. Moreover, the V_{max} values at 40 °C decreased with increasing HEMA content, while the K_m values at 40 °C remained constant. The K_m and V_{max} values at 40 °C, however, showed smaller and larger values than the K_m and V_{max} values at 25 °C, respectively. Furthermore, the K_m values of the immobilized Lyz at 25 and 40 °C were approximately 1.33 and 1.21 times larger than that of native Lyz, whereas the V_{max} values at 25 and 40 °C were about 1/3 (0.283 and 0.317) smaller than that of the native enzyme. Hamdya et al. reported that the K_m value of immobilized urease was approximately 2 times larger than that of the native enzyme [4]. Furthermore, Kamala et al. reported that the K_m values of glucoamylase immobilized on polypropylene-grafted polyacrylic acid and polypropylene-grafted polyacrylamide fibers were approximately 2.13 and 3.68 higher than those of the respective native enzymes [26]. In addition, in this study, the optimum temperature for immobilized Lyz was shifted 10 °C higher than that of the native enzyme at 45 °C, and the thermal stability of the activity was improved with immobilization on PGBHs in spite of the disadvantage of the steric hindrance created as a result of aggregation (data not shown), as was the case with the immobilized urease [4].

Given both the results above and the results of the activity measurements for immobilized Lyz towards $(GluNAc)_6$, it can be concluded that this current method can be useful for the preparation of an enzyme immobilized carrier in which the three-dimensional structure of Lyz is not affected by immobilization. Changes in the relative activity of the immobilized Lyz with temperature and HEMA content are thought to be caused by an increase in the affinities between *M. lysodeikticus* and Lyz ($1/K_m$ values) through reaction of several adsorbed Lyz molecules with the substrate molecules at various positions due to concentration effects and a decrease in the interactions between *M. lysodeikticus* and Lyz in the highly ordered PGBH aggregates (V_{max} values) due to steric hindrance.

Conclusions

PGBH particles with varying HEMA content were prepared and the molar mass, radii, levels of Lyz immobilization, and activity of immobilized Lyz were investigated as a function of temperature and HEMA content by means of DLS, SLS, UV–vis spectroscopy, and activity measurements. Our main conclusions are as follows. (1) All PGBH particles showed thermo-responsive behavior. (2) The radii of the PGBH and Lyz-immobilizing PGBH particles, the amount of immobilized Lyz, and the activity of the immobilized Lyz depended on the HEMA content and the temperature. (3) The relative activity of immobilized Lyz toward $(GluNAc)_6$ increased with increasing HEMA content and temperature, whereas the activity toward *M. lysodeikticus* remained constant or decreased with an increase in HEMA content at 25 and 40 °C. This result indicates that the relative activity of the immobilized Lyz also depended on the molecular size of the substrate. Moreover, Lyz immobilized on PGBH particles with a higher HEMA content (PGBH 3) at 40 °C showed a high activity that was nearly equal to that of native Lyz, which indicates that PGBH with a higher HEMA content can be used to immobilize Lyz molecules without causing any conformational changes in the Lyz molecules or decreasing their activity.

These results are due to an increase in the hydrophobic and/or hydrophilic interactions between Lyz, PGBH, and Lyz-immobilizing PGBH with increasing temperature and HEMA content, as well as the immobilization of Lyz by PGBH aggregates in which lattices play an important role by serving as adsorption sites. The changes in activity were also due in part to the increasing concentration of Lyz molecules already reacted with the substrate in the hydrophilic environment as the HEMA content increased, and the increasing steric hindrance between the substrate and the immobilized Lyz in the highly ordered PGBH aggregates as the temperature increased.

Therefore, nanometer-sized PGBH particles with a hydrophilic monomer such as HEMA can function as a useful enzyme immobilization carrier. These studies suggest that one may establish more efficient and conventional methods for the preparation of immobilized enzymes with high activities and suitable in vivo uses.

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