Papain Immobilization onto Porous Poly(γ-methyl L-glutamate) Beads

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SYNOPSIS

Water-insoluble papain was prepared by immobilizing papain onto the surface of porous poly(γ-methyl L-glutamate) (PMLG) beads with and without spacer. The mode of the immobilization between papain and porous PMLG beads was covalent fixation. The relative activity and the stability of the immobilized papain was investigated. The retained activity of the papain covalently immobilized by the azide method was found to be excellent toward a small ester substrate, *N*-benzyl L-arginine ethyl ester (BAEE), compared with that of the peptide binding method. The values of the Michaelis constant K_m and the maximum reaction velocity V_m for free and immobilized papain on the PMLG beads were estimated. The apparent K_m was larger for immobilized papain than for the free enzyme, while V_m was smaller for the immobilized papain. The thermal stability of the covalently immobilized papain was higher than that of the free papain. The initial enzymatic activity of the covalently immobilized papain remained approximately unchanged with storage time, when the batch enzyme reaction was performed repeatedly, indicating the excellent durability.

INTRODUCTION

Since the recovery yield and the reusability of free enzymes as industrial catalysts are quite limited, attention has been paid to enzyme immobilization,[1] which offers advantages over free enzymes in choice of batch or continuous processes, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reaction mixture, and adaptability to various engineering designs.[2-4] A concerted or sequential reaction of several enzymes is also obtainable by the use of mixed or stratified beds. Furthermore, the interest in the immobilized enzymes and their application to bioprocessing,[5,6] analytical systems,[7] and enzyme therapy[8] has steadily grown in the past decade. Thus, many approaches to the preparation of water-insoluble enzymes have been explored in recent years[9-12] to study the enzyme reaction in biphasic systems similar to those existing *in vivo*.

In this study, papain is selected as a hydrolytic enzyme and the polymer support employed is a porous poly(γ-methyl L-gluta-mate) (PMLG) bead that has very narrow pore size distribution around 0.1 μm[13] to get larger surface area per weight of beads compared to that of nonporous beads. Oligoglycine with different chain lengths is used to study the effect of spacer length on the hydrolytic activity of the immobilized papain. *N*-benzyl L-arginine ethyl ester (BAEE) is selected as a low molecular weight substrate, as well as casein as a high molecular weight substrate, for the enzyme reaction in this study. The stabilities and durabilities of the immobilized enzyme are also investigated.

EXPERIMENTAL

Materials

The monomer, *N*-carboxyanhydrides (NCA) of γ-methyl-L-glutamate was prepared according to the method reported in the previous paper[14] and purified by recrystallization from an ethyl acetate solution with the addition of petroleum ether. Recrystalli-

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zation was repeated more than three times. PMLG was synthesized by the NCA method. The polymerization was initiated with triethylamine (TEA) at an NCA-to-TEA molar ratio of 25. PMLG was purified and fractionated as described in the previous paper.[14] The molecular weight of PMLG sample was determined in *m*-cresol at 25.0°C by the sedimentation equilibrium method using a MOM 3170-b type ultracentrifuge equipped with a Reyleigh interference optical system and a 12-mm double sector cell. The molecular weight was estimated to be: M_w = 95,000.

The porous PMLG bead was prepared by the method described earlier.[13] Figure 1 shows the scanning electron micrographs (SEM) of the porous PMLG bead. Papain (3.5 m Anson μg/mg, Merck), BAEE, and other chemicals were purchased from Nacalai Tesque (Kyoto, Japan). Casein was purchased from Wako Chem. Co. Ltd (from Bovine Milk, Sigma C7891), being first heated in the 2% aqueous suspension adjusted to pH 8.0 for 30 min in a boiling water bath.

Immobilization of Papain

Papain molecules were covalently immobilized on the porous PMLG beads by the azide method, as well as the peptide binding method as shown in Figure 2, depending on the direct fixation (Method-1, (1)) or the indirect fixation with oligoglycine spacers (Method-2, (2)-(3)). In the azide method, an example of the preparation of PMLG–papain conjugate is as follows: PMLG beads [I] were immersed in a mixture of 80% hydrazine hydrate and ethanol (1 : 1) at 37°C for 1 h. The obtained PMLG hydrazides [II] were separated by filtration and washed with methanol. The washed polymers [II] were rinsed twice with 0.05*N* HCl and then with water until the pH of the washings was neutral. Then, the PMLG hydrazides [II] were immersed in an ice-cold mixed solution of 0.1*N* HCl and 3% sodium nitrate (5 : 1). The mixture was stirred gently at 0°C for 20 min. The polymers were separated by filtration and washed several times with ice-cold 50% ethanol and then rinsed once with 0.05*M* PBS used in the coupling reaction for papain. The resulting PMLG acyl azides [III] were immediately immersed in an ice-cold 0.05*M* PBS containing 3–5 mg/mL papain, and the mixture was shaken gently at 4°C for 24 h. The washing of the PMLG–papain conjugate [IV] was repeated until no residual papain could be detected in the last PBS phase by UV spectrum measurement. The final product was stored at 4°C after lyophilization.

The immobilization of papain on PMLG beads inserting oligoglysine chains as spacer was per-

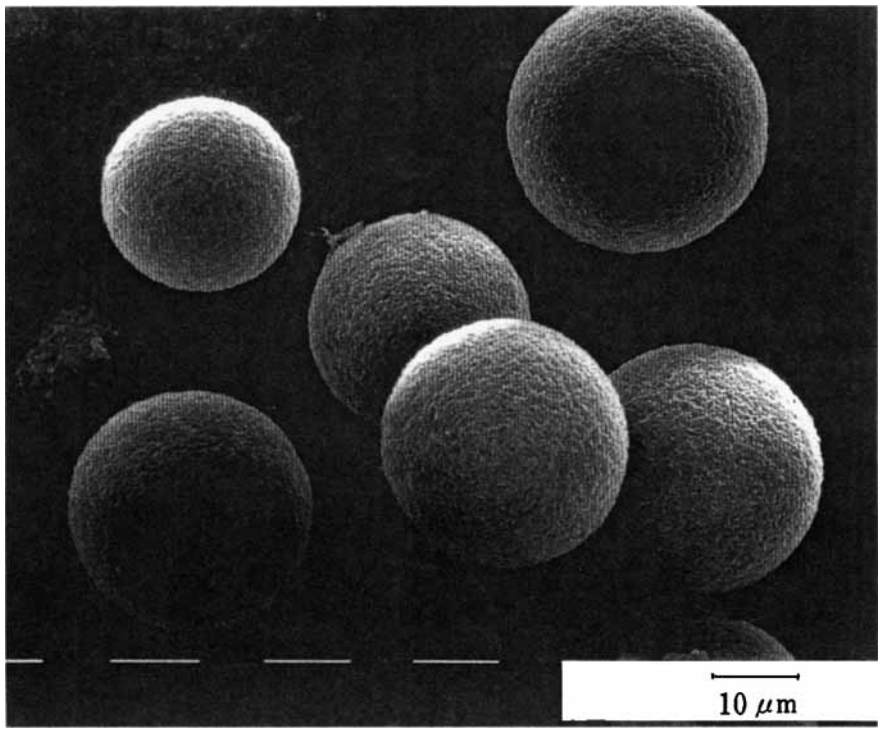

Figure 1 SEM plots of PMLG microspheres.

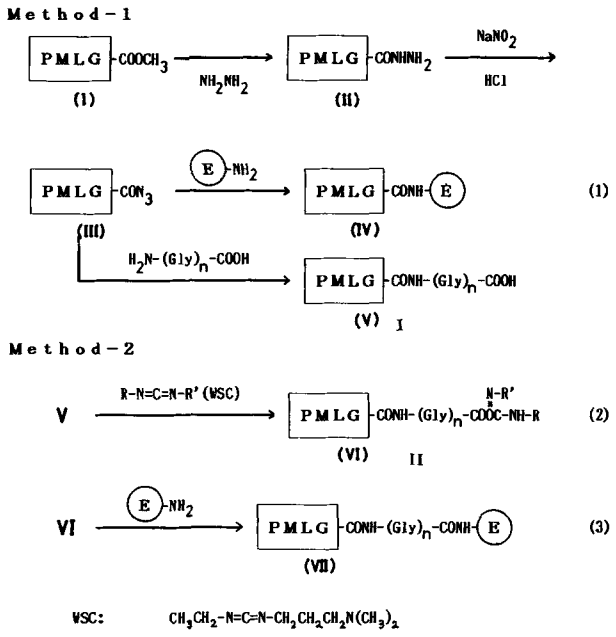

Figure 2 Scheme of the immobilization reactions.

formed via two steps as described in Figure 2. After PMLG–oligoglycine conjugate [V] was prepared by the azide method as described above, the coupling of papain molecules to the PMLG–oligoglycine [PMLG-G(n)] [V] was performed by the peptide binding method. In this case, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (WSC) was used in 0.05*M* PBS at pH 5.75. The coupling reaction mediated by the WSC is, in most cases, rapid due to the high degree of activation afforded by *O*-acylisourea azlactone derivatives.[15]

The amount of papain immobilized was determined by UV measurement (280 nm) after hydrolysing the conjugate with 6*N* HCl at 110°C for 24 h. In all experiments, PMLG beads without immobilized papain or the PMLG–G(n) were used as "control" in determination of the amount of the immobilized papain.

Activity Measurements

The hydrolytic activity of free and immobilized papain were determined using BAEE as a low molecular weight substrate. After predetermined periods of time, the enzymatic activity was calculated from the initial rate of BAEE hydrolysis by determining KOH consumed within the given period of time.

The caseinolytic determinations were performed according to Bergmeyer.[16] The activities of free and immobilized papain were determined in the following way: The reaction mixture consisted of 2 mL 0.01*M* PBS at pH 8.0 and 1.0 mL free enzyme solution or the immobilized enzyme suspension in 0.05*M* PBS, which contained 2 mM EDTA, 5 mM cystein, and 1.0 mL 2.0 wt % casein solution. The reaction mixtures were stirred vigorously at 37°C for 20 min, followed by termination with the addition of trichloroacetic acid to a concentration of 3.0 wt %. The absorbance of the solution or the supernatant at 280 nm was plotted against the enzyme weight to evaluate the enzymatic activity.

The Michaelis constants, K_m and V_m, of the papain immobilized on PMLG beads were estimated employing BAEE ranging from 1.0–10.0 mM.

Stability Measurements

The thermal stability of the immobilized papain was evaluated by measuring the residual activity (ZA) of papain exposed to various temperatures in 0.05*M* PBS of pH 7.4 for various periods of time. After heating, the samples were cooled quickly and assayed for enzymatic activity at 37.0°C immediately or after storage at 4°C. Storage before the assay (30 min–48 h) did not alter the measured activities. The residual activities were related to the original activities (assayed at 37.0°C without heating). The kinetics and thermal inactivation were investigated by determining the residual activity of the free and immobilized papain after incubation at various temperature. The first-order inactivation rate constants, k_i, were estimated by the equation

$$\ln A = \ln A_o - k_i t, \qquad (1)$$

where A_o is the initial activity and A is the residual activity after t min of the temperature effect.[17]

To determine the enzyme stability with pH, the free and immobilized papain were incubated in PBS at 37.0°C including a definite amount of BAEE substrate and various pH regions for 20 min.

To evaluate durabilities of the immobilized papain when repeatedly used, the dried immobilized papain was washed in 0.05*M* PBS twice and then resuspended in a fresh reaction mixture to measure the enzymatic activity. This cycle was repeated on the same sample. To check the possibility of any leakage of enzyme molecules under washing, the amount of the immobilized papain was determined after the last batch test. The storage stability of the free and immobilized papain was evaluated by placing the enzymes in 0.05*M* PBS pH 7.4 at 25°C for various periods of time and the activity was assayed.

RESULTS AND DISCUSSION

Immobilization of Papain

In the case of the coupling reaction mediated by WSC, isourea intermediate rearranges rapidly to the unreactive *N*-acyl urea in the aqueous environment, leading to loss of the activated end group to be used for the successive coupling reactions with the amino group of papain molecules. Thus, there must be an optimum reaction time to activate the carboxyl group of the PMLG–G(n). Figure 3 shows the effect of the reaction time of WSC activation on the relative activity of PMLG–G(3)–papain for BAEE hydrolysis. The result indicates that the relative activity, which is a ratio of the specific activity of the immobilized papain to that of the free enzyme, is strongly affected by the activation time and becomes highest at the activation time around 45 min.

Effect of Surface Concentration on Activity

Figure 4 shows the surface concentration of papain immobilized onto PMLG beads by the azide method at different enzyme concentrations. In the low-concentration region (less than 4.0 mg/mL), the amount of immobilized papain is almost proportional to the initial papain concentration. In all the sequent experiments, the initial papain concentration was kept to 5.0 mg/mL unless otherwise stated. The maximum amount of immobilized papain onto PMLG beads was 0.68 wt %. Figure 5 illustrates the effect of the surface concentration of papain immobilized onto PMLG beads on the relative activity of BAEE hydrolysis. The relative activity of the immobilized papain without spacer decreases gradually with decreasing surface concentration of the immobilized proteases, especially at the surface concentration below 0.1 wt %, whereas the immobilized

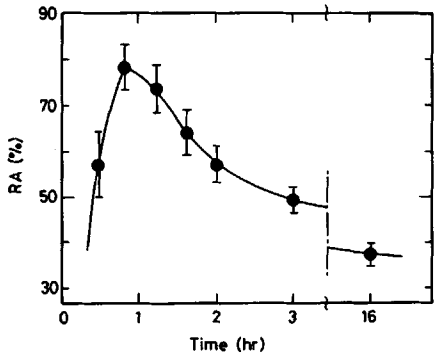

Figure 3 Effect of the WSC activation time on the relative activity (RA) for PMLG–G(3)–papain-1. Hydrolysis: BAEE, pH 8.0 and 37.0°C.

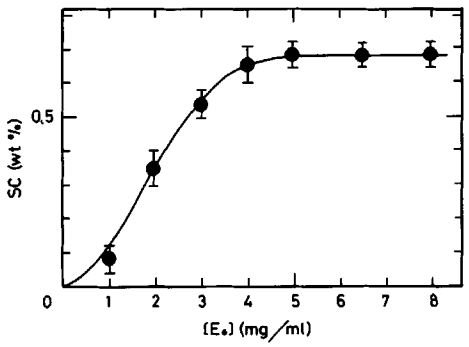

Figure 4 Effect of papain concentration (E_0) on the saturated amount of the immobilized papain on the PMLG–papain (24 h, pH 7.4, 25°C).

papain with oligoglycine spacers give an approximately constant relative activity in the whole concentration region studied. These results may be explained in terms of structural deformation of the immobilized papain molecule. The immobilized enzyme is likely to undergo significant conformational deformation, especially in the lower surface concentration region without spacer, whereas the immobilized enzyme molecule with spacer may be protected from the structural deformation even in the lower surface concentration region owing to the spacer effect. This is consistent with similar behavior observed in the case of the immobilized proteases onto the surface of the acrolein microspheres.[18]

Determination of Michaelis Constant and Maximum Reaction Velocity

All enzymatic hydrolysis reactions yield data that can be analyzed in the framework of the Michaelis–

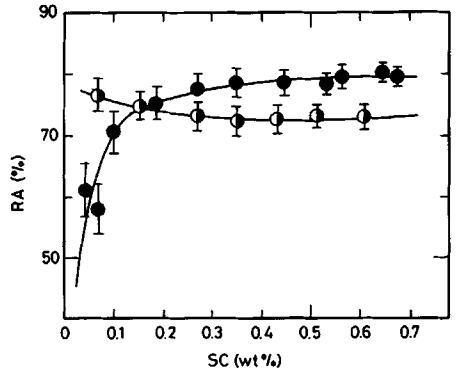

Figure 5 Effect of the surface concentration of the immobilized papain on the relative activity (RA). Hydrolysis: BAEE, pH 8.0 and 37.0°C. (●), PMLG–papain; (◑), PMLG–G(3)–papain.

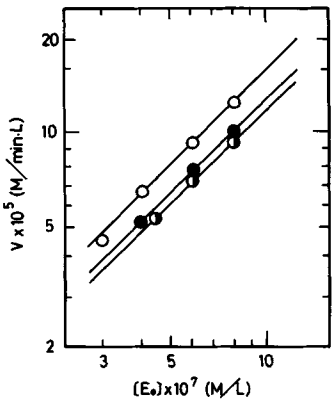

Figure 6 Effect of the papain concentration on the reaction velocity. Hydrolysis: BAEE, pH 8.0 and 37.0°C. (○), free papain; (◑), PMLG–G(3)–papain-1; (●), PMLG–papain-1.

Menten mechanism. The rate of hydrolysis was expected to be the first order in enzyme concentration.

Figure 6 shows the expected experimental results of BAEE hydrolysis by the free and the immobilized papain, indicating first-order behavior with respect to papain concentration.

Initial reaction rates were determined at different initial BAEE concentrations ranging from 1.00–10.0 mM. Figure 7 shows Lineweaver–Burk plots for the free and the immobilized papain. The values of the Michaelis constant K_m and the maximum reaction velocity V_m for the free and the immobilized papain on PMLG bead (PMLG–papain-1) are estimated from Figure 7 and tabulated in Table I with the experimental results for PMLG–G(3)–papain-1.

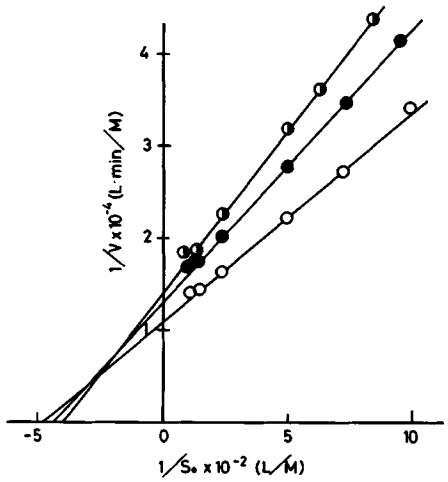

Figure 7 Lineweaver–Burk plots of $1/V$ vs. $1/S$. (○), free papain; (◑), PMLG–G(3)–papain-1; (●), PMLG–papain-1.

Table I Michaelis Parameters K_m and V_m at pH 8.0, 37°C

Sample Code	[E] (m/L)	K_m (m/L)	V_m (m/min, L)
Papain	6.0×10^{-7}	2.0×10^{-3}	9.4×10^{-5}
PMLG-Pap-1	6.0×10^{-7}	2.4×10^{-3}	7.9×10^{-5}
PMLG-G(3)-Pap-1	6.0×10^{-7}	2.8×10^{-3}	7.4×10^{-5}

The apparent K_m values of the immobilized enzyme were higher than that of the free enzyme. This may be due to the limitation of diffusion resistance. On the other hand, the V_m values of the immobilized enzymes were lower than that of the free one, suggesting the relative activity of the immobilized enzyme decreased in the course of the covalent fixation.

Furthermore, it is pointed out that the V_m values of the covalently immobilized papain by the azide method was found to be higher than that of the peptide binding method. This advantage may be due to the use of the mild azide method for coupling.[19]

Effect of Spacer on Activity

The effect of the spacer length on the relative activity of the hydrolysis of substrate was investigated at the almost same surface concentration of the immobilized enzymes. Table II summarizes the experimental results, which show that the immobilized enzymes retain hydrolytic activity toward the low molecular weight BAEE substrate, but are less active toward casein, a high molecular weight substrate. The low activity toward casein probably reflects the difficult approach of casein to the active site of the papain because of steric hindrance caused by the enzyme immobilization and the large size of the macromolecular substrate.

In addition, it is apparent in Table II that an

Table II Effect of the Length of the Spacer on the Relative Activity (RA) (%) of the Immobilized Enzyme

		Papain	
G(n)	wt %	BAEE	Casein
2	0.65	70.3 ± 1.5	24.5 ± 2.0
3	0.60	78.0 ± 1.8	28.0 ± 2.4
4	0.56	76.5 ± 1.2	31.5 ± 2.2
6	0.48	72.8 ± 1.5	33.5 ± 2.1

optimum spacer length exists for the immobilized enzyme toward the hydrolysis of the low molecular weight substrate. The highest activity was obtained with PMLG-G(3)-papain. On the other hand, the enzymatic activity toward the high molecular weight substrate increased with increasing spacer length at least in the length range examined. This result indicates that the inclusion of a spacer to the carrier surface may reduce steric interference with the substrate binding process, especially toward high molecular weight substrates.

Thermal Stability of the Immobilized Enzymes

The thermal stability of immobilized enzymes is one of the most important criteria of their application. The activity of immobilized enzymes, especially in covalently bound system, is generally more resistant against heat and denaturing agents than that of the soluble form.[20,21] The effect of temperature on the stability of the immobilized papain in PBS is shown in Figure 8.

Figure 8 illustrates the residual activity ($ZA = A/A_0$) of papain with BAEE hydrolysis at pH 8.0 and 37.0°C after the preheat treatment for 1 h (pH 8.0 in PBS) at the given temperature. The immobilized papain is more stable than the free papain in the range of higher temperatures. The immobilized papain at 70°C after 60 min effect exhibited activity two to three times that of the free one. The kinetic curve of thermal inactivation of the cases of PMLG-papain and PMLG-G(3)-papain at 75°C reveals a two-stage process characterized by the following constants, i.e., $k_1 = 4.0 \times 10^{-2}$ min^{-1} and $k_2 = 1.0 \times 10^{-2}$ min^{-1} for PMLG-papain and $k_1 = 4.5 \times 10^{-2}$ min^{-1} and $k_2 = 1.2 \times 10^{-2}$ min^{-1} for PMLG-G(3)-papain (Fig. 9). The free papain loses 90% of its initial activity at 75°C within 45 min.

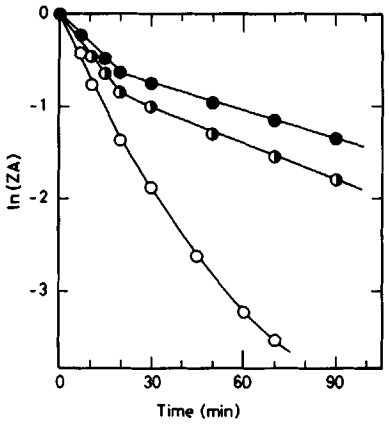

Figure 9 Kinetics of temperature inactivation at 75°C of BAEE hydrolysis at pH 8.0 and 37.0°C. (○), free papain; (◑), PMLG-G(3)-papain-1; (●), PMLG-papain-1.

The immobilized papain onto PMLG beads with spacer is slightly less stable than that onto PMLG beads directly (Fig. 8), suggesting that the direct immobilization of papain onto PMLG beads stabilizes the papain molecule. This may be due to the multipoint attachment of the papain molecule to the surface of PMLG beads with reduced molecular mobility of the papain compared with the case with spacer.

Durability for Repeated Use

Durability of immobilized enzymes is also very important in applications because they are subjected to repeated hydrolysis reactions. Figure 10 illustrates

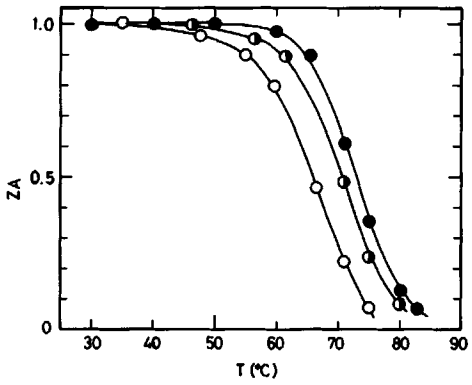

Figure 8 Effect of the heat treatment at the given temperature and pH 8.0 for 1 h on the residual activity (ZA) of BAEE hydrolysis at pH 8.0 and 37.0°C. (○), free papain; (◑), PMLG-G(3)-papain-1; (●), PMLG-papain-1.

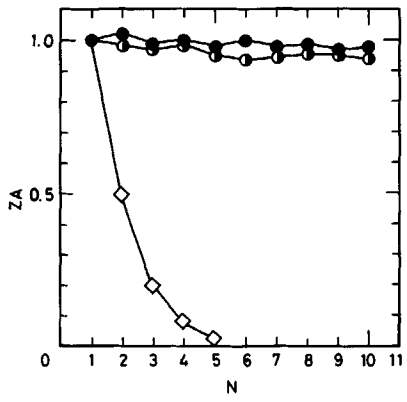

Figure 10 Thermal inactivation of PMLG-papain-1 at 70°C in the presence of urea. Hydrolysis: BAEE, pH 8.0 and 37.0°C. (○), without urea; (⊙), 1.0M urea; (⊕), 3.0M urea; (⊕), 4.5N urea; (◑), 6.0M urea; (●), 8.0M urea.

the effect of repeated use on the residual activity of BAEE hydrolysis by the immobilized papain. The activity is seen to be retained, irrespective of the spacer interposition, even if the batch reaction is repeated at least 10 times.

It was found that the amount of the immobilized papain after the last batch reaction was equivalent to the original amount within experimental error. This suggests that no leakage of the immobilized papain occurred as a result of the repeated washing. This high stability was in marked contrast with the rather poor durability of the papain adsorbed on the surface of PMLG beads.[22]

Storage Stability

As shown in Table III, aqueous suspensions of the immobilized papain could be stored at 4°C for six months without a significant loss of activity, whereas the corresponding free papain lost more than 30% of their initial activity under the same condition. The higher stability of the immobilized papain can be attributed to the prevention of autodigestion and thermal denaturation as a result of the fixation of papain molecules on the surface of PMLG beads. It is of interest to point out that there is a similarity between the thermal and storage stabilities. These findings can be accomodated in a general framework by considering the state of the covalent fixation between the carrier material and the enzyme molecules. It is reported that hydrophilic carriers such as Sephadex, Sepharose, and polyacrylamide yield enzyme derivatives of high lyophilization and thermal stabilities.[23,24]

To examine the enzymatic stability in the continuous reaction system under a higher-temperature condition, the effect of the storage in PBS of pH 7.4 at 37.0°C was studied for the immobilized papain. Under this condition, the residual activity of papain may decrease due to autodigestion. Figure 11 illustrates the experimental results obtained with the residual activity for BAEE hydrolysis. It is apparent that the immobilized papain is much more stable than the free one.

Table III Residual Activity (ZA) after Storage at 4°C for Six Months in Aqueous Suspension

Sample Code	SC (wt %)	ZA (BAEE)
Papain	—	0.70
PMLG–Papain	0.56	0.99
PMLG–G(3)–Papain	0.60	0.97

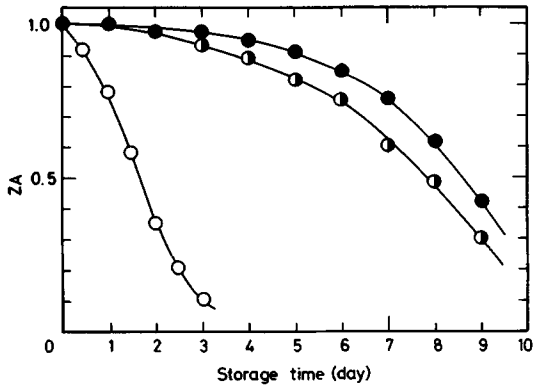

Figure 11 Effect of the storage in PBS at pH 7.4 and 37.0°C on the residual activity (ZA) of BAEE hydrolysis at pH 8.0 and 37.0°C. (○), free papain; (◑), PMLG–G(3)–papain-1; (●), PMLG–papain-1.

CONCLUSION

The immobilized papain onto the surface of porous PMLG beads with and without any length of spacer by covalent fixation gave rather high activity toward small ester substrates, but still low toward casein, a high molecular weight substrate. The relative activity of the immobilized papain without spacer decreased gradually with the decreasing surface concentration of the immobilized papain. On the other hand, the immobilized papain with oligoglycine spacers gave an almost constant activity for the substrate hydrolysis with the surface concentration region studied. The apparent K_m values were larger for immobilized enzymes than for the free enzyme, while V_m values were smaller for the immobilized enzymes.

The thermal and storage stability of the immobilized papain were higher than those of the free ones. The initial enzymatic activity of the immobilized papain was maintained, almost unchanged, without any elimination and inactivation of papain, indicating the excellent durability of the bound enzyme.

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