# Protease Immobilization onto Copoly(ethylene/Acrylic Acid) Fiber

SHINGO EMI and YASUHIRO MURASE, *Fiber & Textile Research Lab, Teijin Ltd., 4-1, Minohara 3-chome, Ibaraki City, Osaka 567, Japan,* TOSHIO HAYASHI, *Research Center for Medical Polymers and Biomaterials Kyoto University, Sakyo-ku, Kyoto 606, Japan,* and AKIO NAKAJIMA, *Department of Applied Chemistry, Osaka Institute of Technology, Asahi-ku, Osaka 535, Japan*

## Synopsis

Water-insoluble proteases were prepared by immobilizing papain and chymotrypsin onto the surface of the copoly(ethylene/acrylic acid) fiber. The effect of the surface area of the copolymer fiber on the amount of the immobilized protease was investigated. The mode of the immobilization between protease and copolymer such as a covalent fixation or an ionic interaction on the enzymatic activity and the stability of the immobilized protease was also investigated. The stability of the immobilized protease in this study meant thermal stability, durability for repeated use, stability in urea, and durability for repeated washing. The activity of covalently immobilized proteases was found to be still higher toward small ester substrates, but rather low toward casein, a high molecular weight substrate. The covalently immobilized proteases onto the copolymer fiber gave an almost constant specific activity, suggesting less structural deformation of the protease molecule than the conventional immobilized enzyme system in the lower surface concentration. The thermal stability of the immobilized proteases was higher than that of the respective ionic interaction or native proteases. The initial enzymatic activity of the covalently immobilized proteases was maintained almost unchanged without any elimination and inactivation of proteases when the batch enzyme reaction was performed repeatedly, indicating 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 the 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 immobilized enzymes and their application to bioprocessing,[5,6] analytical system,[7] and enzymatic 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.*

However, effects of polymer supports on the activity of enzyme have not been studied in detail until now. In this study, papain and chymotrypsin are selected as hydrolytic enzymes and the polymer support employed is copoly(ethylene/acrylic acid)(EAA)[13] fibers which have very large surface area

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TABLE I
Characterization of EAA Fibers

| Sample code | Mode | —COOH (mol %) | Denier | Surface area ($cm^2/g$) |
|---|---|---|---|---|
| EAA-1 | FA[a] | 3.7 | 17.0 | 950 |
| EAA-2 | FA[a] | 3.7 | 3.5 | 2100 |
| EAA-3 | FB[b] | 3.5 | — | 4000 |

[a] FA = modified shape of cross section of fiber.
[b] FB = burst treatment for making porous surface.

and carry about 3.7 molar percent of carboxyl group in EAA copolymer. A low molecular weight and a high molecular weight compound are used as substrates for the enzyme reaction. The stability and durability will be also described for the immobilized proteases.

## EXPERIMENTAL

### Materials

EAA fiber was spun by the modified melt-spinning method at 200°C.[14] Table I summarizes the mode and the average surface area per unit weight of the

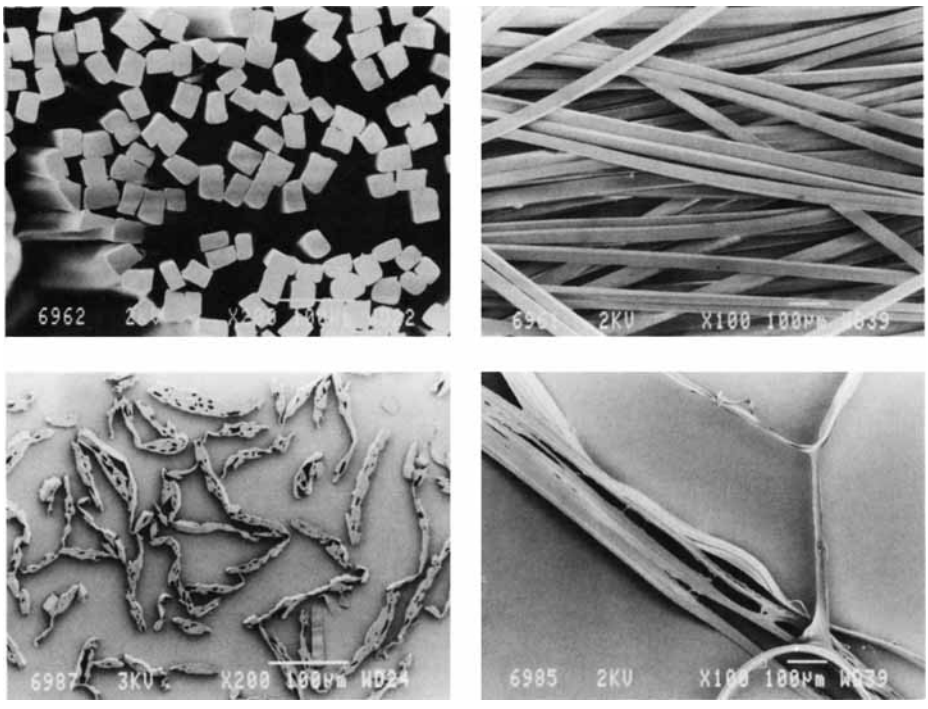


Fig. 1. SEM of EAA fibers.

EAA fibers used in this study. Papain (3.5*m* Anson $\mu$g/mg, Merck) and chymotrypsin (3× cryst. bovine pancrease, salt free, Sigma) were purchased from Nakarai Chem. Co. Figure 1 shows the scanning electron micrographs (SEM) of the EAA fibers. *N*-acetyl-L-tyrosine ethyl ester (ATEE) and *N*-benzyl-L-arginine ethyl ester (BAEE) as low molecular weight substrate were purchased from Nakarai Chem. Co.

Casein, as a high molecular weight substrate, prepared according to Hammarstein (Wako Chem. Co.), was first heated in the 2% aqueous suspension adjusted to pH 8.0 for 30 min on a boiling water bath.

## Immobilization of Proteases

Protease molecules were covalently immobilized on the EAA fibers through several steps as shown in Figure 2, depending on the direct fixation (Scheme 1) or the indirect fixation with oligoglycine spacers (Figure 2), as well as the ionic adsorption system:

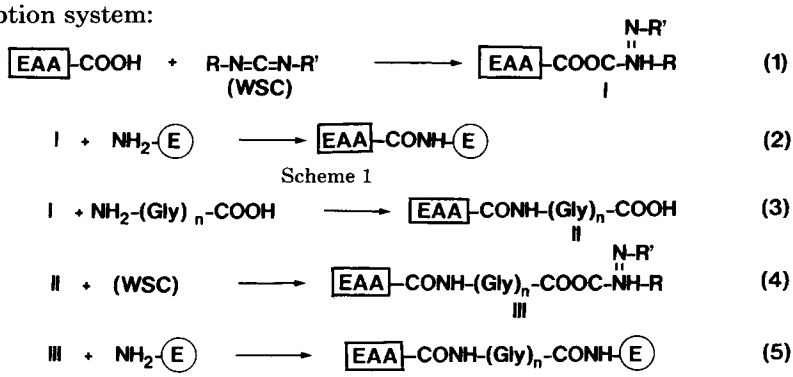


Fig. 2. Scheme of the immobilization reactions.

The coupling between the carboxyl group of the EAA fibers and the amino group of protease molecules was effected by carbodiimide, as shown in Figure 2.

As a water soluble carbodiimide (WSC), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was used in 0.05 *M* PBS at pH 5.75. The coupling reaction effected by the carbodiimide is, in most cases, rapid, due to the high degree of activation afforded by the *O*-acylisourea azlactone derivatives.[15] However, the isourea intermediate rearranges rapidly to the unreactive *N*-acyl urea in aqueous environment, leading to loss of the activated end group to be used for the successive coupling reactions with the amino group of protease molecules. Thus, there must be an optimum reaction time to activate the carboxyl group of the EAA fibers. Figure 3 shows the effect of the reaction time of WSC activation on the relative activity of EAA-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 one, is strongly affected by the activation time and becomes highest at an activation time around 45 min.

A typical immobilization procedure by covalent bond without spacer is as follows: 100 mg of EAA fibers was immersed in 10 mL of 0.05 *M* phosphate buffer (PBS) at pH 7.4. A given amount of WSC was added to the medium under vigorous stirring with a magnet in cold room, and the mixture was kept

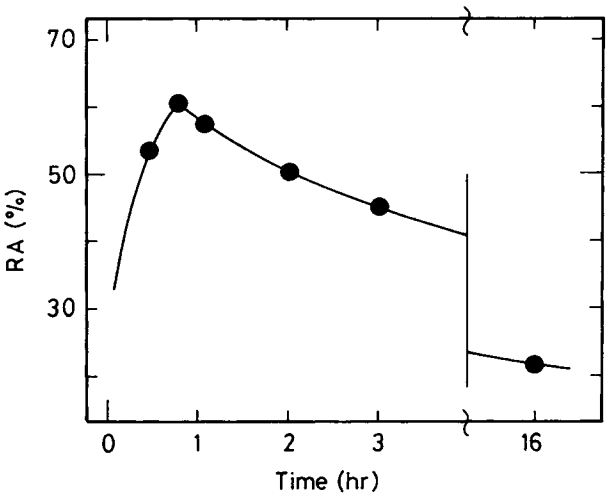


Fig. 3. Effect of the WSC activation time on the relative activity (RA) for EAA-3-papain. Hydrolysis; BAEE, pH 8.0, and 37.0°C.

at 4°C for 45 min under stirring. The fiber samples were separated from solution by using a suction technique through a glass filter, and repeated washing by 0.05 *M* PBS twice to remove the remaining WSC and other byproduct completely. After that, a given amount of protease was added to the 5 mL of 0.05 *M* PBS including the activated EAA fiber under stirring at 4°C and was kept for 16 h under stirring. The washing of the resulted sample of EAA-protease was performed the same way. No residual protease 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 proteases on EAA fibers, having inserted oligoglycine chains as spacer, was performed via two steps as described earlier.[16] The amount of proteases immobilized was determined by the classical ninhydrin method after hydrolysing the immobilized proteases with 6 *N* HCl at 110°C for 1 h. The EAA fibers were stable in 6*N* HCl at 110°C. In all the experiments, the EAA fibers without immobilized protease were used as a control in determination of the amount of the immobilized proteases.

## Titrimetric Determination of Papain Activity

The hydrolytic activity of the free papain and the immobilized papain was determined using BAEE as substrate; 2.5 mL of aqueous BAEE solution was added to 2.5 mL of papain solution or 2.5 mL of the immobilized papain medium, both in 0.1 *M* PBS at pH 8.0. 2 m*M* EDTA and 5 m*M* cystein were added in PBS to activate papain.[17] The final reaction mixture had $2.92 \times 10^{-3}$ *M* as the substrate concentration. The reaction mixture was maintained at 37.0°C under constant stirring, pH being kept at 8.0 by an addition of 0.05*N* KOH using a microburet titration. After a predetermined period of time, the enzymatic activity was calculated from the initial rate of BAEE hydrolysis by assaying the amount of KOH consumed within the given period of time. To obtain reproducible activity values, vigorous stirring was necessary, especially for the im-

mobilized papain medium. Correction was made for non-enzymatic hydrolysis. Three or four different amounts of free papain and the immobilized papain were used in each activity determination. The activity of the immobilized enzymes was expressed as the relative activity percent based of free enzymes.

### Assay of Chymotrypsin Activity

Free and immobilized chymotrypsin were assayed using 2 m*M* of 0.01M ATEE in 0.05 *M* PBS at pH 8.0. After incubating the reaction mixture under stirring for 20 min at 37.0°C, the enzyme was inactivated by raising the temperature to 100°C for 5 min. The absorbance of the solution or the supernatant at 256 nm were plotted against the enzyme weight in the reaction mixture. The slope of the initial part of the curve was used to evaluate the activity.

### Caseinolytic Activity Determination

The caseinolytic determinations were done essentially according to Bergmeyer,[18] with minor modifications to overcome some special problems encountered with the insoluble conjugates. The activities of free and immobilized chymotrypsins were determined in the following way. The reaction mixture consisted of 2 mL of 0.01 *M* PBS at pH 8.0, 1.0 mL of the free enzyme solution or the immobilized enzyme medium in 0.1 *M* PBS and 1.0 mL of 2.0 wt % casein solution. The reaction mixtures were vigorously stirred at 37.0°C for 20 min, followed by termination with trichloroacetic acid additions to have a concentration of 3.0 wt %. The absorbance of the solution or the supernatant at 280 nm were plotted against the enzyme weight to evaluate the enzymatic activity.

The hydrolytic activity of papain series was determined in a similar manner, except that the assay medium contained 2 m*M* EDTA and 5 m*M* cystein.

### Stability Measurements of Immobilized Proteases

The thermal stability of the immobilized proteases was evaluated by measuring the residual activity (ZA) of enzymes exposed to various temperatures in 0.05 *M* PBS of pH 8.0 for various periods of time. After heating, the samples were quickly cooled and assayed for enzymatic activity at 37.0°C immediately or after storage at 4°C. Storage before the assay (30 min to 48 h) did not alter the measured activities significantly. The kinetics and thermal inactivation were investigated by determining the residual activity of the free and immobilized proteases after incubating at various temperature. The first-order inactivation rate constants, $k_i$, were estimated by the equation

$$\ln A = \ln A_0 - k_i t \tag{1}$$

where $A_0$ is the initial activity and $A$ is the activity after $t$ min at various temperatures.[19]

To determine the pH stability, the free and immobilized papain were incubated in BAEE–PBS at 37.0°C and various pH regions for 20 min. To evaluate durabilities of the immobilized proteases when used repeatedly, the dried immobilized proteases were washed in 0.05 *M* PBS twice and then suspended again in a fresh reaction mixture to measure the enzymatic activity. This cycle

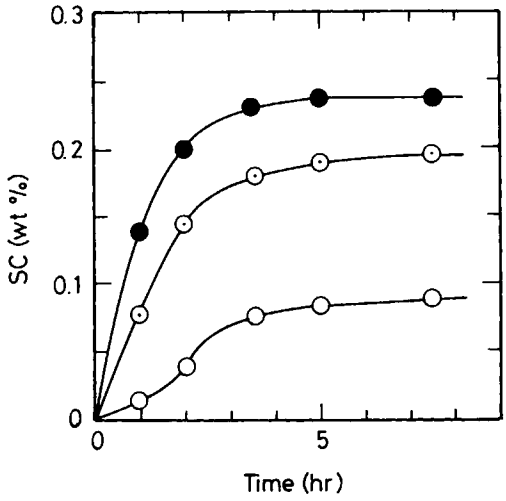


Fig. 4. Immobilization of papain on EAA-3 fibers at different papain concentrations, pH 7.4, and 25°C under slow constant stirring: (●) 5.0 mg/mL; (⊙) 3.0 mg/mL; (○) 1.5 mg/mL.

was repeated on the same sample. To check the possibility of any leakage of protease molecules under washing, the amount of the immobilized LPL was determined after the last batch test. The storage stability of the free and immobilized proteases was evaluated by placing the proteases in 0.05 *M* PBS of pH 7.4 at 25°C for various periods of time, and the activity was assayed using the above-mentioned techniques.

## RESULTS AND DISCUSSION

### Effect of Surface Concentration on the Activity

Figure 4 shows the surface concentration of papain immobilized onto EAA fibers at different enzyme concentrations. It is seen that 6 h is sufficient for

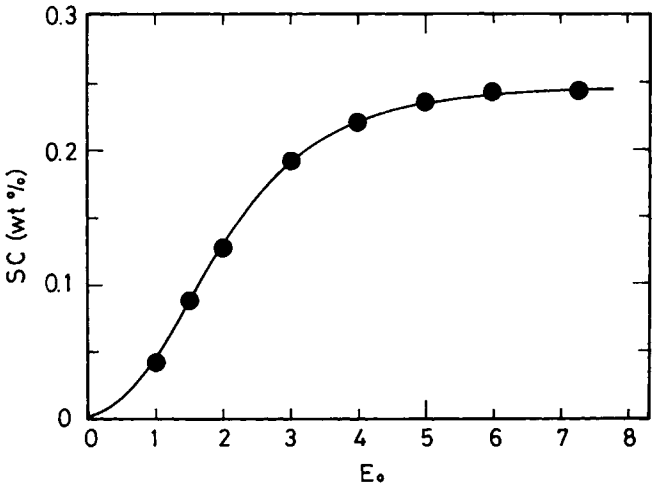


Fig. 5. Effect of papain concentration ($E_0$) on the amount of the immobilized papain on the EAA-3 fibers of for 16 h.

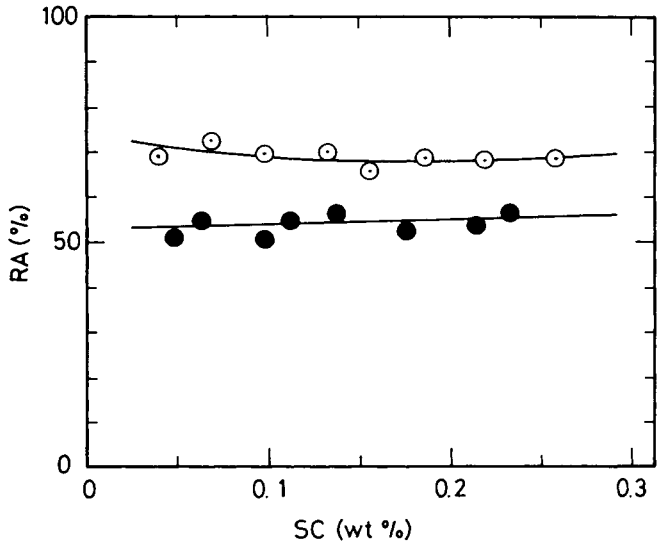


Fig. 6. Effect of the surface concentration of the immobilized papain on the relative activity (RA). Hydrolysis; BAEE, pH 8.0, and 37.0°C. (●) EAA-papain; (⊙) EAA-3-G(3)-papain.

the reaction to level off, independent of the concentration of papain, although the saturated surface concentration of the immobilized papain apparently depends on the initial papain concentration of the reaction mixture. The effect of the initial concentration of papain on the saturated surface concentration of immobilized chymotrypsin was also studied, allowing the reaction to proceed

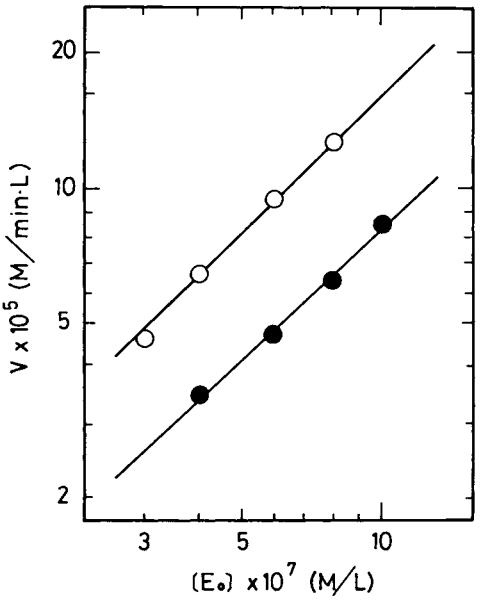


Fig. 7. Effect of the papain concentration on the reaction velocity. Hydrolysis; BAEE, pH 8.0, and 37.0°C. (○) Free papain; (●) EAA-3-papain.

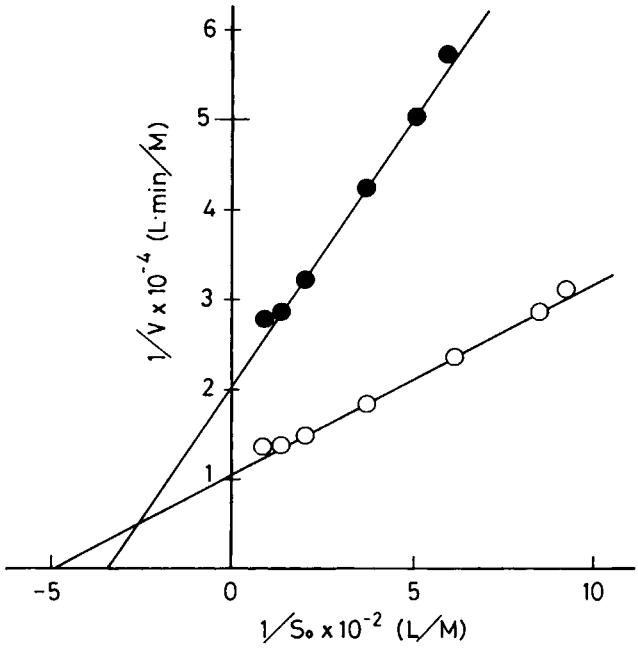


Fig. 8. Lineweaver–Burk plots of $1/V$ vs. $1/S$: (○) free papain; (●) EAA-3–papain.

for 12 h. As seen in Figure 5, the amount of immobilized papain is almost proportional to the initial papain concentration, at least in the low concentration level below about 5.0 mg/mL. In all the following experiments, the initial papain or chymotrypsin concentration was kept to 5.0 mg/mL, unless otherwise mentioned. The maximum amount of immobilized papain or chymotrypsin on EAA fibers was 0.24 or 0.22 wt %. Figure 6 illustrates the effect of the surface concentration of papain immobilized onto EAA-3 fibers on the relative activity of BAEE hydrolysis. It is clearly shown that the relative activity of the immobilized papain with and without spacer is independent of the surface concentration and gives a constant value over the whole concentration region studied. The former gives slightly higher relative activity than the latter. This result may be explained in terms of the structural deformation of the immobilized enzyme molecules as well as the diffusion of substrate. Generally, the structure of the enzyme molecule immobilized by covalent fixation should undergo strong de-

TABLE II
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 | $6.0 \times 10^{-7}$ | $2.0 \times 10^{-3}$ | $9.4 \times 10^{-5}$ |
| EAA-3–pap. | $6.0 \times 10^{-7}$ | $2.9 \times 10^{-3}$ | $5.0 \times 10^{-5}$ |
| Chymotrypsin | $8.0 \times 10^{-7}$ | $1.8 \times 10^{-3}$ | $8.0 \times 10^{-6}$ |
| EAA-3–chym. | $8.0 \times 10^{-7}$ | $3.2 \times 10^{-3}$ | $2.4 \times 10^{-6}$ |

formation, especially in the lower surface concentration region without spacer, being apt to decrease the relative activity with decreasing the surface concentration. Thus, the constancy of the relative activity of the immobilized papain without spacer observed here may be due to the rather low surface concentration of the carboxyl group of the EAA fiber. On the other hand, the immobilized enzyme molecule with spacer must be protected from the heavy structural deformation, even in the lower surface concentration region owing to the spacer effect.

## Determination of Michaelis Constant and Maximum Reaction Velocity

All enzymatic hydrolysis reactions yield data which can be analyzed in the framework of the Michaelis-Menten mechanism. The rate of hydrolysis was expected to be the first order in enzyme concentration. It was necessary to confirm this so that the rate could always be measured, since the enzyme concentration varied over a wide range. Figure 7 shows the expected experimental results of BAEE hydrolysis by the free and the immobilized papain; they indicate the first order behavior with the papain concentration.

Initial reaction rates were determined at different initial BAEE concentrations ranging from 1.00 to 10.0 m$M$. Figure 8 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 EAA fibers are estimated from Figure 8 and tabulated in Table II together with the experimental results for chymotrypsin.

The apparent $K_m$ values of the immobilized proteases without spacer were higher than those of the immobilized proteases with spacer and the free one. This may be due to the limitation of diffusion resistance. The $V_m$ values of the immobilized proteases were lower than that of the free one, suggesting the relative activity of the immobilized protease decreased in the course of the covalent fixation, especially in the case of without spacer.

## Effect of Spacer on the Activity

The effect of the spacer length on the relative activity was investigated at almost the same surface concentration of the immobilized proteases. Table III

TABLE III
Effect of the Length of $G(n)$ Spacer on the Relative Activity (RA) of the EAA-3 Immobilized Proteases

| | Papain | | | Chymotrypsin | | |
|---|---|---|---|---|---|---|
| $n$ | wt % | BAEE | Cas. | wt % | ATEE | Cas. |
| 0 | 0.23 | 55.8 | 18.7 | 0.20 | 28.0 | 4.0 |
| 2 | 0.25 | 60.3 | 25.5 | | | |
| 3 | 0.26 | 68.0 | 28.8 | 0.22 | 37.0 | 11.8 |
| 4 | 0.24 | 66.5 | 30.4 | | | |
| 6 | 0.20 | 62.8 | 34.0 | 0.19 | 34.5 | 18.0 |

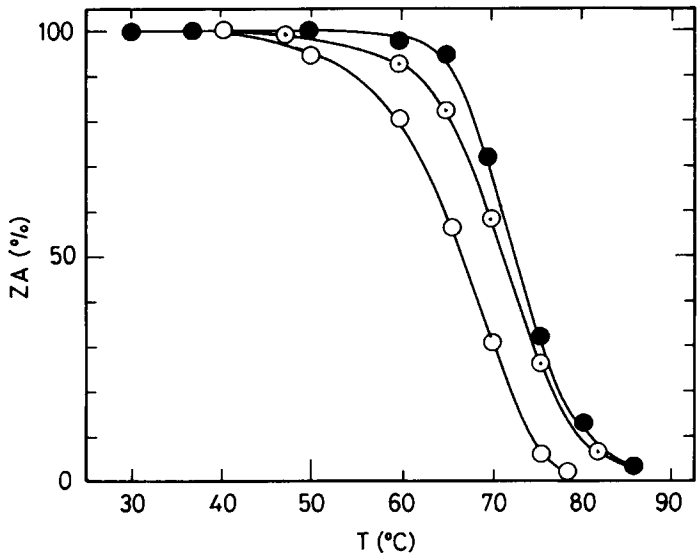


Fig. 9. Effect of the heat treatment at the given temperature and pH 8.0 for 1 h on the residual activity (ZA) of BAEE hydrolysis at pH 8.0 and 37.0°C: (○) free papain; (⊙) EAA-3-G(3)-papain; (●) EAA-3-papain.

summarizes the results, which show that the immobilized proteases even without spacer are still active in hydrolysis toward the low molecular weight substrates, but practically inactive or 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 enzymes because of steric hindrance caused by the enzyme immobilization and the large size of the macromolecular substrate.

In addition, it is apparent in Table III that an optimum spacer length exists for all of the immobilized proteases toward the hydrolysis of BAEE, a low molecular weight substrate. The highest activity was obtained with tri- or tetraglycine ($n = 3$–4) as the spacer for both cases. On the other hand, the enzymatic activity toward the high molecular weight substrate increased as the spacer became longer, at least in the length range examined. This fact indicates that the addition of spacer to the carrier surface probably reduces the steric interference with the substrate binding process, especially toward high molecular weight substrates.

## Thermal Stability

The thermal stability of immobilized enzymes is one of the most important criteria of their application. As is well known, the activity of immobilized enzymes, especially in covalently bound systems, is more resistant against heat and denaturing agents than for the soluble form.[20] The effects of temperature on the stability of the immobilized papain in PBS are shown in Figure 9. The immobilized papains, with and without spacer, are more stable than the free papain at higher temperatures. The immobilized papain at 70°C after 60 min exhibits activity around three times higher than that of the free one. The kinetic

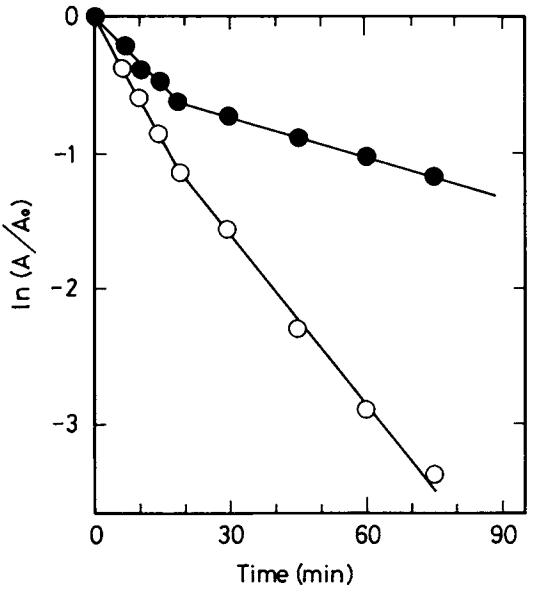


Fig. 10. Kinetics of temperature inactivation at 75°C of BAEE hydrolysis at pH 8.0 and 37.0°C: (○) free papain; (●) EAA-3–papain

curve of thermal inactivation of the immobilized preparations at 75°C reveals a two-stage process characterized by the following constants; $k_1 = 3.20 \times 10^{-2}$ $min^{-1}$ and $k_2 = 0.90 \times 10^{-2}$ $min^{-1}$ (Fig. 10). The free papain loses 90% of its initial activity at 75°C for 45 min.

The immobilized papain onto EAA fibers with spacer is slightly less stable than that without spacer (Fig. 9). This result suggests that the immobilization of papain onto EAA fibers without spacer stabilized the papain molecule due to the multipoint attachment of the papain molecule to the polymer carrier through reduction in molecular mobility compared to the case of that with spacer.

## Effect of Denaturants

When the inactivation was carried out in the presence of denaturants such as urea or detergent, the inactivation kinetics changed as a result of the concentration of the denaturant. Figure 11 gives the inactivation curves for the thermal inactivation of immobilized papain at 70°C in the presence of different urea concentrations. Urea up to 3 *M* does not affect the thermal inactivation, whereas higher concentrations accelerate the inactivation. Interestingly, at urea concentrations greater than 6 *M*, the inactivation progress appears as a straight line in the semilogarithmic plot. A very similar inactivation behavior was observed when immobilized papain was inactivated at 70°C in the presence of SDS, a detergent. The destabilizing influence of SDS started even at concentrations of 0.1% detergent, but agreed with that described for urea qualitatively. The inactivation caused by the combined denaturing actions of temperature and denaturant proved also to be irreversible. In contrast, the inactivation of immobilized papain by urea at 25°C was found to be reversible.

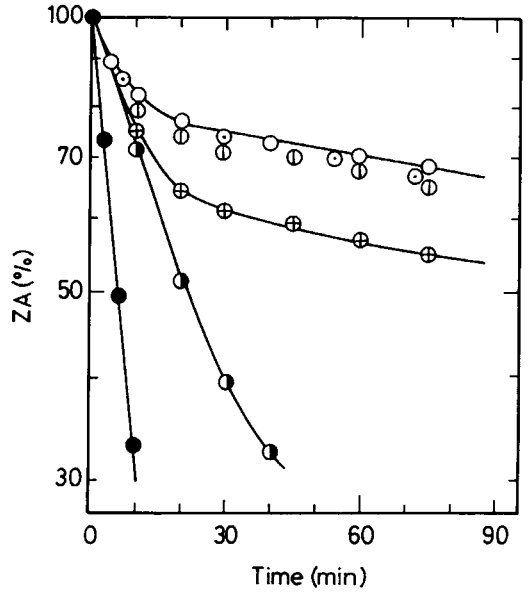


Fig. 11. Thermal inactivation of EAA-3-papain at 70°C in the presence of urea. Hydrolysis; BAEE, pH 8.0, and 37.0°C: (○) without urea; (⊙) 1.0 *M* urea; (◑) 3.0 *M* urea; (⊕) 4.5 *M* urea; (◑) 6.0 *M* urea; (●) 8.0 *M* urea.

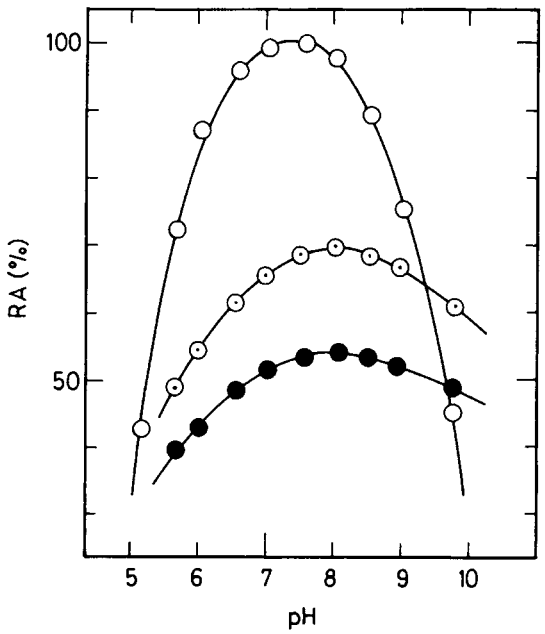


Fig. 12. Effect of pH of the reaction medium on the relative activities of BAEE hydrolysis at 37.0°C: (○) free papain; (⊙) EAA-3-G(3)-papain; (●) EAA-3-papain.

### pH Effect

The pH effect on the activity of the immobilized and free papain for BAEE hydrolysis was studied in PBS at 37.0°C in various pH region, and is presented in Figure 12. The immobilized papain has the same pH optimum as the free one (pH 8.0), but the pH profile is considerably widened due to diffusional limitations.[21] Immobilized papain displays a greater stability at higher pH values.

### Durability for Repeated Use

The durability of the immobilized papain is also very important in use, because it is subjected to repeated hydrolysis reactions. Figure 13 illustrates the effect of repeated use on the residual activity of BAEE hydrolysis by the immobilized papain. The activity is seen to be retained without any definite loss, 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 was equivalent to the original one within experimental error in each case, suggesting that no leakage of the immobilized papain occurred under repeated washing. This high stability is in marked contrast with the rather poor durability of the papain which was immobilized by ionic adsorbance on the EAA fibers (see Fig. 13).[22]

### Storage Stability

Aqueous suspensions of the immobilized papain could be stored at 4°C for 6 months without a significant loss of activity, whereas the corresponding free

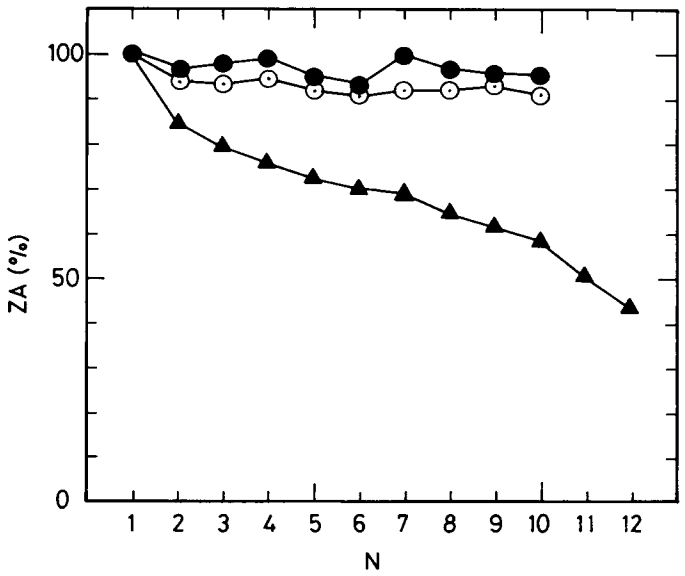


Fig. 13. Effect of the repeated use on the residual activity (ZA) of BAEE hydrolysis at pH 8.0 and 37.0°C: (●) EAA-3-papain; (⊙) EAA-3-G(3)-papain; (▲) EAA-3/papain (ionic adsorption).

TABLE IV
Residual Activity (ZA) After Lyophilization (6 months)

| Sample code | SC (wt %) | ZA (%) (BAEE) |
|---|---|---|
| Papain | — | 70 |
| EAA-2-papain | 0.13 | 92 |
| EAA-3-papain | 0.23 | 90 |

papain lost more than 30% of its initial activity under the same conditions. 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 EAA fibers. However, it is often pointed out that lyophilization of enzymes directly from the water suspensions is normally accompanied by loss of the enzymatic activity. Table IV gives the enzymatic activity retained after lyophilization of the immobilized and free proteases. Very high residual activities are observed for the immobilized proteases for BAEE hydrolysis. It is of interest to point out that there is a similarity between the thermal and storage stabilities to lyophilization. These findings can be accommodated in a general framework by considering the state of the covalent fixation between the carrier material and the enzyme molecules.

To examine the enzymatic stability in the continuous reaction system under a rather drastic condition, effects of the storage in PBS of pH 7.4 at 37.0°C were studied for the immobilized papain. The residual activity at BAEE hydrolysis is given in Figure 14. It is apparent that the immobilized papain is

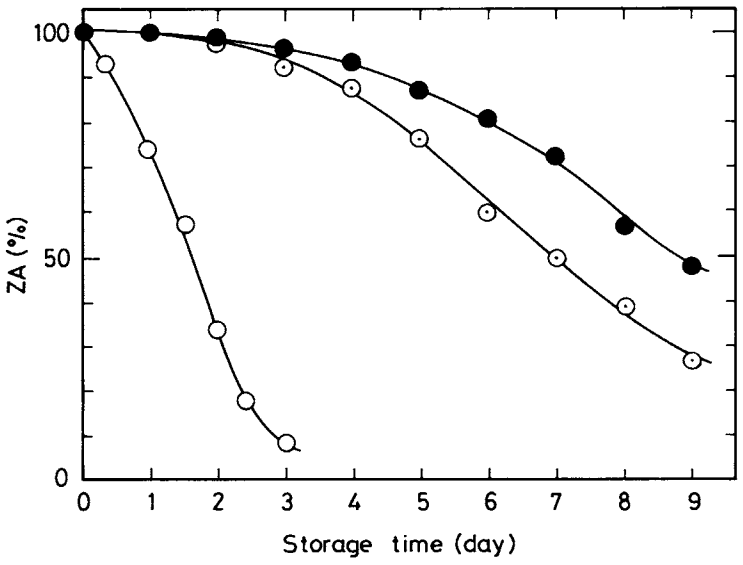


Fig. 14. Effect of the storage in PBS at pH 7.4 and 37.0°C on the residual activity (ZA) of BAEE hydrolysis at pH 8.0 and 37.0°C: (○) free papain; (⊙) EAA-3-G(3)-papain; (●) EAA-3-papain.

much more stable than the free one. Again, the immobilized papain without spacer shows a more stable activity than that with spacer in spite of the initial lower activity. Similar behavior is observed in the case of chymotrypsin.

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