# Retention and Reusability of Trypsin Activity by Covalent Immobilization onto Grafted Polyethylene Plates

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**ABSTRACT:** This study investigated the activity of trypsin that had been covalently immobilized onto acrylic acid (AA)– and methacrylic acid (MAA)–grafted polyethylene (PE) plates—PE–*g*–PAA and PE–*g*–PMAA—using a water-soluble carbodiimide as a coupling agent, as a function of the immobilized amount, the grafted amount, the pH value on immobilization, and the pH value and temperature at the activity measurement. The activity of trypsin immobilized on the PE–*g*–PAA plates at pH 6.0 decreased with an increase in the immobilized amount because of the crowding of trypsin molecules in the vicinity of the surfaces of the grafted PAA layers. The increase in the grafted amount resulted in a decrease in the activity of immobilized trypsin because of a decrease in the diffusivity of BANA molecules caused by the formation of dense grafted PAA layers for the PE–*g*–PAA plates and led to the increased activity because of the increase in the hydrophilicity of the whole grafted layers for the PE–*g*–PMAA plates. The activity of trypsin immobilized on the PE–*g*–PAA and PE–*g*–PMAA plates at pH 6 increased with an increase in the pH value, probably because of the expansion of trypsin-carrying grafted PAA and PMAA chains and the increased diffusivity of *N*α-benzoyl-DL-arginine-nitroanilide hydrochloride molecules in the grafted layers. The optimum temperature of the activity of immobilized trypsin shifted to 50°C from 30°C for native trypsin. Immobilized trypsin was reusable without any denaturation and isolation at temperatures ranging from 20°C to 60°C and pH values ranging from 6 to 10. Trypsin immobilized on a PE–*g*–PAA plate had 95% of the remaining activity in relation to native trypsin at 30°C after preservation in a pH 7.8 buffer at 4°C over 6 months. These results made clear that alkaline and thermal stability, reusability, and storage stability can be much improved by the covalent coupling of trypsin on PE–*g*–PAA and PE–*g*–PMAA plates.
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**Key words:** polyethylene; photografting; immobilization; trypsin; enzyme activity

## INTRODUCTION

Enzymes increasingly are being used as catalysts for chemical reactions and syntheses. Unfortunately, most enzymes are expensive and have limited stability. Consequently, many investigators have attempted to develop various methods for decreasing the expense and increasing the stability of enzymes. Recently, considerable attention has been devoted to developing technologies suitable for using immobilized enzymes in an aqueous medium.[1–4] As easily removable and reusable biocatalysts, immobilized enzymes offer a considerable number of advantages: increased shelf life, operational simplicity, and thermal and pH stabilities.[5–10]

The methods for immobilizing enzymes to an insoluble carrier may be roughly classified in three groups: (1) covalent binding to a carrier, (2) ionic binding and physical adsorption to a carrier, and (3) entrapment in an insoluble crosslinked matrix.[11,12] Of these immobilization methods, covalent binding is one of the most important methods because this method makes it possible to avoid enzyme leakage to a great extent.[13–16] The ionic binding and physical adsorption methods are very easily carried out. However, the enzymes immobilized by these methods tend to be gradually released from the solid supports. For suitable covalent immobilization of enzymes, in general, the supports should have these properties: a hydrophilic nature, an appropriate concentration of chemically active functional groups, resistance to biodegradation, and chemical and mechanical stabilities. In addition, the covalent binding of enzymes onto the water-insoluble supports must be carried out under mild conditions to avoid inactivation of the enzymes used. Covalent immobilization involves the coupling reaction between the functional groups present in the polymer support and the functional groups in the enzymes that are not essential for enzymatic activity.[17–19]

Many researchers have reported on the immobilization of enzymes on polymer matrices prepared by grafting hydrophilic and reactive monomers such as acrylic acid (AA),[6,13,14,20] 2-hydroxyethyl methacrylate,[21,22] and glycidyl methacrylate[23,24] and using the $^{60}$Co γ rays, UV rays, or plasma as an energy source. By careful selection of the polymer support and the

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monomer, it is possible to vary the hydrophobic/hydrophilic nature of the support for enzyme immobilization, which could improve enzyme stability and also enable easier handling and longer storage of the immobilized enzymes.[20–24]

Here, we chose acrylic acid (AA) and methacrylic acid (MAA) grafted onto polyethylene (PE) plates—PE–*g*–PAA and PE–*g*–PMAA—as the supporting materials because PE plates possess mechanical strength, the grafted layers have enough hydrophilicity to immobilize the enzymes, and grafted PAA and PMAA chains are not susceptible to microbial attack.[25] In addition, carboxylic groups affixed to the grafted PAA and PMAA chains react with amino groups in the enzymes by the use of coupling agents under mild conditions, resulting in stable peptide structures.[11–14,24] However, the enzymes undergo more or less conformational changes by the immobilization, which directly influences their catalytic activity. Therefore, it is also necessary to study these changes to better understand the properties and functional responsiveness of the immobilized enzymes.

In this study PE–*g*–PAA and PE–*g*–PMAA plates were prepared by the photografting technique. The proteolytic enzyme trypsin was immobilized onto the grafted PAA and PMAA layers formed on the PE plates by the covalent coupling method, using a water-soluble carbodiimide.[7,26–28] Of the carbodiimides 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMC) is commonly used for the immobilization of enzymes onto PAA and PMAA polymers. Attempts were made to optimize coupling reactions as a function of the amount of immobilized trypsin, the amounts of grafted AA and MAA, and the pH value at the coupling reaction. The activity of the trypsin immobilized on the PE–*g*–PAA and PE–*g*–PMAA plates was measured at different pH values and temperatures and compared with that of native trypsin.

## EXPERIMENTAL

### Materials

A low-density PE plate (thickness, 1.0 mm; density, 0.926 g/cm$^3$) was used as a solid polymer support. The PE plates were washed with water, methanol, and acetone and then dried under reduced pressure. AA and MAA were purified by distillation under reduced pressure. Trypsin (EC 3.4.21.4; from bovine pancreas, sigma type XIII) was chosen as a model enzyme for immobilization because the activity of this enzyme has been studied extensively in aqueous solutions and its amino acid sequence, number of active sites and their locations, and number of amino side groups are fully defined.[29–32] A water-soluble carbodiimide, CMC[7,27] (from Wako Pure Chemical Ind., Tokyo, Japan), was used as a coupling agent for the covalent immobilization. *N*α-Benzoyl-DL-arginine-nitroanilide hydrochloride (BANA) was used as a low molecular substrate for trypsin.[30,32,33]

### Photografting

The photografting was carried out in the manner described in our previous article.[25] The PE plates, which were coated with benzophenone (BP) as a photosensitizer, were immersed in aqueous solutions of AA and MAA monomers at a monomer concentration of 1.0 mol/dm$^3$. Photografting was carried out by applying UV rays emitted from a 400 W high-pressure mercury lamp to the aqueous AA and MAA solutions in which the BP-coated PE plates were immersed at 60°C by the use of a Pyrex glass tube. The amounts of grafted AA and MAA (in $\mu$mol/cm$^2$) were calculated from the weight increase measured from the difference in weights before and after photografting. The grafted layers formed on the PE plates were characterized in detail by surface analysis using electron spectroscopy for chemical analysis, surface wettability, and water absorptivity, as described in our previous articles.[25,34,35]

### Immobilization of trypsin

The PE–*g*–PAA and PE–*g*–PMAA plates with different grafted amounts swollen in the 0.05*M* acetate buffers at the pH values of 5.0 to 7.0 were immersed in the acetate buffers of the same pH values (20 cm$^3$) containing 20 mg of trypsin and 40 mg of CMC at 4°C for 12 to 18 h with moderate stirring.[36–38] Then, the trypsin-immobilized PE–*g*–PAA and PE–*g*–PMAA [trypsin-i-(PE–*g*–PAA) and trypsin-i-(PE-g-PMAA)] plates were washed with the acetate buffers of the same pH values. The amount of immobilized trypsin was spectrophotometrically determined at 290 nm as an isosbestic point of CMC in the acetate buffers used.

### Activity measurements

To start the enzymatic reaction of the immobilized trypsin, the trypsin-i-(PE–*g*–PAA) plates were immersed in 0.1*M* phosphate buffers with pH values ranging from 5.0 to 7.0 and containing 0.025 mol of BANA (0.5 m*M*) at 30°C.[37] The activity of the immobilized trypsin was estimated by spectrophotometrically measuring the concentration of a hydrolysis product of BANA, *p*-nitroaniline, as a function of the reaction time at 410 nm in mmol dm$^{-3}$ mg$^{-1}$ min$^{-1}$.[30,32] The relative activity was expressed as the ratio of the absolute activity of immobilized trypsin to that of free trypsin.

## RESULTS AND DISCUSSION

### Effect of immobilized pH value on activity

The photograftings of AA and MAA onto the PE plates were carried out at 60°C, and the amounts of grafted AA and MAA were adjusted by varying UV irradiation time.[25] Trypsin was covalently immobilized on the PE–*g*–PAA and PE–*g*–PMAA plates by immersing them in acetate buffers with pH values ranging from 5.0 to 7.0 containing trypsin (1.0 mg/cm$^3$) and CMC (2.0 mg/cm$^3$) at 4°C with moderate stirring. Figure 1 shows the change in the amount of immobilized trypsin with immersion time for the PE–*g*–PAA plates whose grafted amount was 3.55 $\mu$mol/cm$^2$. The amount of immobilized trypsin increased with an increase in the reaction time. However, a duration of at least 12 h was required for trypsin to be appreciably immobilized on the PE–*g*–PAA plates. The activities of trypsin immobilized onto the PE–*g*–PAA plate of 3.55 $\mu$mol/cm$^2$ at pH values of 5.0–7.0 were measured at the optimum pH value of pH 7.8 and temperature 30°C for native trypsin.[33,39] The optimum pH value and temperature for immobilized trypsin will be examined later and be shown in Figures 5 and 7, respectively. Figure 2 shows the variation in the relative activity of trypsin immobilized on the PE–*g*–PAA plate of 3.55 $\mu$mol/cm$^2$, which had an average immobilized amount of 0.044 mg/cm$^2$, with the pH value on immobilization. Native trypsin of the same amount as the trypsin immobilized on the PE–*g*–PAA plates was dissolved in a pH 7.8 buffer ($V$ = 40 cm$^3$), and then the specific initial velocity of each native trypsin was determined at 30°C. The relative activity of immobilized trypsin was estimated as the ratio of the specific initial velocity of immobilized trypsin to that of native trypsin. Trypsin immobilized at pH 6.0 had the maximum relative activity value.

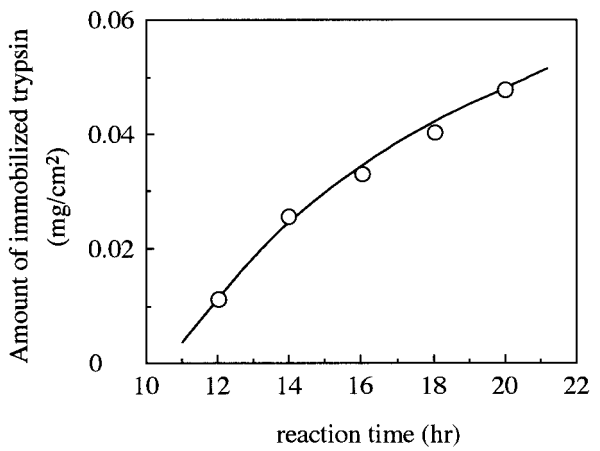


**Figure 1** Change in the amount of trypsin immobilized onto a PE-g-PAA plate of 3.55 $\mu$mol/cm$^2$ with the reaction time in a pH 6.0 buffer containing trypsin (1 mg/cm$^3$) and CMC (2 mg/cm$^3$).

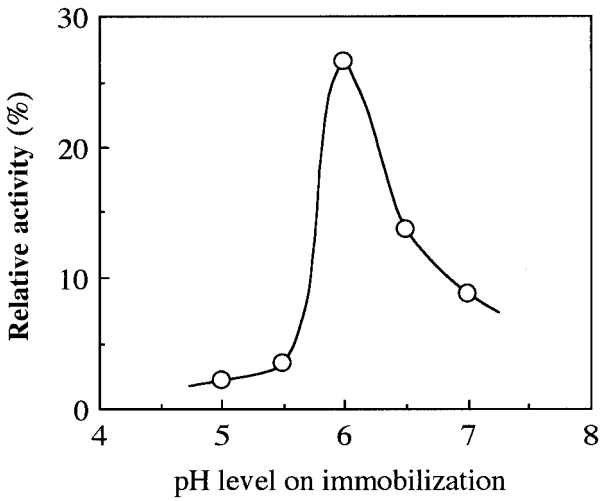


**Figure 2** Variation in the relative activity of trypsin immobilized onto a PE-g-PAA plate of 3.55 $\mu$mol/cm$^2$ with the pH value on immobilization in a pH 7.8 buffer at 30°C. Average immobilized amount = 0.044 mg/cm$^2$, BANA concentration = 0.5 mM.

Because in this study trypsin molecules were covalently immobilized onto grafted PAA chains through peptide bonding between amino groups on trypsin molecules and carboxyl groups affixed to grafted PAA chains, the number of remaining amino groups in a trypsin molecule, which has 15 amino groups including a terminal amino group, decreased via the immobilization reaction, the isoelectric point of immobilized trypsin was lower than that of native trypsin ($pI$ = 10.1).[30] Therefore, the optimum pH value on immobilization of trypsin to the PE–*g*–PAA plates, shown in Figure 2, would be different from that at the activity measurement of trypsin immobilized on the PE–*g*–PAA plates. Many studies on the immobilization of enzymes and the activity measurements of the resulting immobilized enzymes have been reported.[20,28,37,40–44] However, most of these studies were concerned with the pH and temperature dependence of the activity of immobilized enzymes; little was reported on the effect of the pH value on immobilization on the activity of immobilized enzymes.[20] Therefore, in the current study we began by determining the immobilizing conditions in order to discuss the activity of immobilized enzymes.

### Effect of immobilized amount on activity

The activities of trypsin-i-(PE–*g*–PAA) plates of 3.55 $\mu$mol/cm$^2$ with different immobilized amounts, which were prepared by varying the immobilization time in a pH 6.0 acetate buffer containing trypsin and CMC at 4°C, were measured at the optimum pH value of 7.8 and the optimum temperature of 30°C for native trypsin. Figure 3 shows the change in relative activity with the amount immobilized. The relative activity

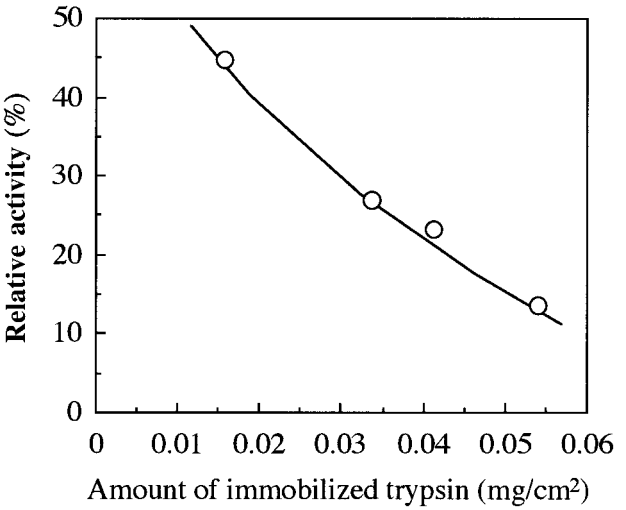


**Figure 3** Change in the relative activity of immobilized trypsin with the immobilized amount for a PE-g-PAA plate of 3.55 $\mu$mol/cm$^3$ in a pH 7.8 buffer at 30°C.

decreased from 45%, at the immobilized amount of 0.015 mg/cm$^2$, to 13%, at 0.053 mg/cm$^2$. Then, the amount of native trypsin equivalent to the immobilized trypsin and 0.020 mmol of BANA were dissolved in 40 cm$^3$ of a pH 7.8 buffer, and the specific initial velocities were determined for native trypsin. The result showed that native trypsin had a constant specific initial velocity of 0.31 mmol mg$^{-1}$ min$^{-1}$ in concentrations ranging from 1.75 to 16.75 $\mu$g/cm$^3$. An increase in the immobilized amount led to the crowding of trypsin molecules in the grafted PAA layers, resulting in spatial restrictions, blocking of active sites, and/or denaturation of enzymes.[2,45] An alternative explanation is that multipoint attachment of trypsin molecules through peptide bonding would decrease their conformational flexibility in the vicinity of the active sites.

### Effect of grafted amount on activity

MAA was also photografted onto PE plates to prepare grafted PE plates with another kind of carboxyl group–carrying grafted polymer chain. Trypsin was immobilized on the PE–*g*–PAA and PE–*g*–PMAA plates with different grafted amounts in order to prepare samples with an almost constant immobilized amount (the average immobilized amounts, 0.030 mg/cm$^2$, for PE–*g*–PAA plates, and 0.041 mg/cm$^2$, for PE–*g*–PMAA plates), and then each specific initial activity of the immobilized samples was measured in a pH 7.8 buffer at 30°C. Figure 4 shows the changes in the specific initial activity with the grafted amount for both PE–*g*–PAA and PE–*g*–PMAA plates. The activity of trypsin immobilized on the PE–*g*–PAA plates decreased with an increase in the grafted amount. In contrast, with the PE–*g*–PMAA plates, the activity gradually increased with an increase in the grafted amount. This difference can be explained by the location and water absorptivity of the grafted layers formed on the substrate surfaces as follows: for the PE–*g*–PAA and PE–*g*–PMAA plates, the intensity ratio O1s/C1s stayed constant above grafted amounts of 15 and 30 $\mu$mol/cm$^2$, respectively. This means the PE surfaces were fully covered with grafted PAA chains than grafted PMAA chains at lower grafted amounts.[25] The results indicate that the location of the photografting of AA was mainly restricted to the surface regions of the PE substrate. Although the amount of absorbed water of both grafted layers formed on the PE substrates increased with the grafted amount, the PE–*g*–PAA plates had greater water absorptivity than the PE–*g*–PMAA plates did. In addition, the $n_{water}$ value, which is the number of water molecules assigned to one monomer segment of the grafted polymer chains and which was calculated from the third equation in Yamada et al.,[46] stayed constant for the PE–*g*–PAA plates over the grafted amount, whereas for the PE–*g*–PMAA plates it increased with an increase in the grafted amount. It can be safely said that although the thickness of the grafted layer formed by the photografting of AA increased with the grafted amount, the hydrophilicity of the whole grafted layer of the PE–*g*–PAA plates depended little on the grafted amount. In addition, because at high grafted amounts trypsin was immobilized on grafted PAA chains in the inside of the grafted layer for the PE–*g*–PAA plates, the diffusion of BANA molecules into the grafted PAA layers was suppressed, resulting in a decrease in the relative activity. Because the PE surfaces were not fully covered with grafted PMAA chains at grafted amounts below 30 $\mu$mol/cm$^2$, the PE–*g*–PMAA plates did not have enough hydrophilicity to retain a high

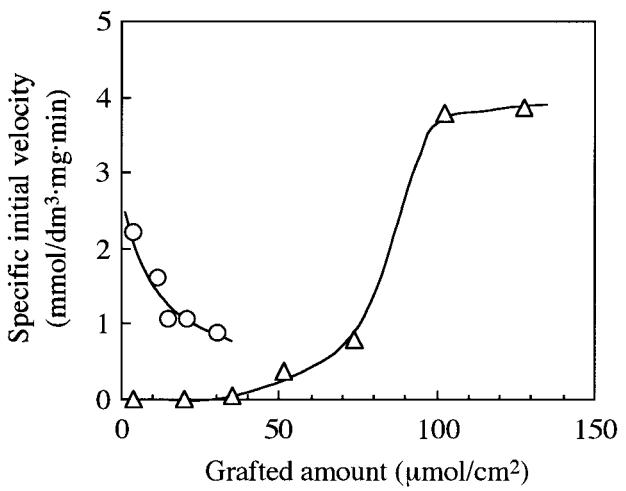


**Figure 4** Changes in the specific initial velocity of immobilized trypsin on the PE-g-PAA (○) and PE-g-PMAA (Δ) plates with the grafted amount in a pH 7.8 buffer at 30°C. Average immobilized amount = 0.030 mg/cm$^2$ for PE-g-PAA and 0.041 mg/cm$^2$ for PE-g-PMAA.

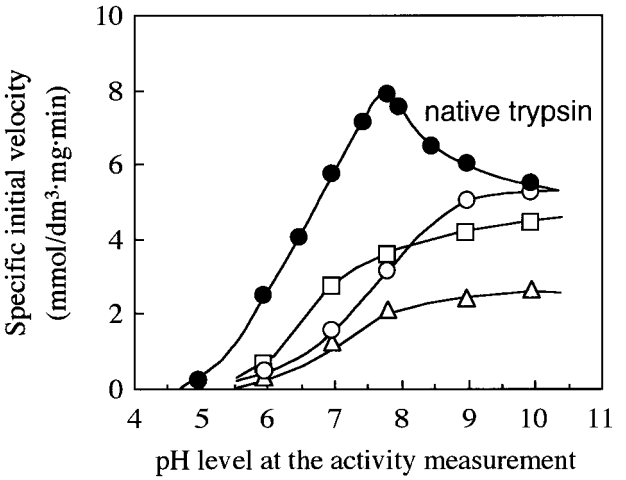


**Figure 5** Variations in the specific initial velocity of native (shaded) and trypsin immobilized on the PE-g-PAA plate of 3.55 $\mu$mol/cm$^2$ (○, Δ) and PE-g-PMAA (□) plate of 103 $\mu$mol/cm$^2$ with the pH value at the activity measurement at 30°C. Immobilized amount(mg/cm$^2$)- ○: 0.014, Δ: 0.035, □: 0.039.

degree of the activity of the immobilized trypsin. With these grafted amounts the PE–*g*–PAA plates had a constant $n_{water}$ value of about 3.8, whereas the $n_{water}$ value of the PE–*g*–PMAA plates was less than 1.0.[25] The increase in the amount of grafted MAA led to enhancement in the water-absorptivity of all the grafted PMAA layers. Therefore, the activity of trypsin immobilized on the PE–*g*–PMAA plates is considered to have increased with an increase in the grafted amount. The activity of trypsin immobilized on PE–*g*–PMAA plates with grafted amounts more than 100 $\mu$mol/cm$^2$ was higher than that immobilized on the PE–*g*–PAA plates. Because internal grafting readily occurs coincident with surface grafting for the photografting of MAA onto the PE plates, the inside of the grafted PMAA layers formed would be rich in PE chains.[25] Consequently, it was difficult for the immobilization of trypsin to proceed to the inside of the grafted layers. In addition, the $n_{water}$ value for the PE–*g*–PMAA plates increased to 3.0 with a grafted amount of 100 $\mu$mol/cm$^2$. In addition to the grafted and immobilized amounts, water absorptivity of the grafted amount also was found to influence the activity of trypsin immobilized on the PE–*g*–PAA and PE–*g*–PMAA plates.

### Effect of pH value at the activity measurement on activity

On the basis of the results shown in Figures 2–4, the activities of trypsin immobilized on PE–*g*–PAA plates of 3.55 $\mu$mol/cm$^2$ and on PE–*g*–PMAA plates of 103 $\mu$mol/cm$^2$ at pH 6.0 were measured in buffers whose pH values ranged from 5.0 to 10.0 at 30°C. Figure 5 shows the variation in the specific initial velocity of trypsin immobilized on the PE–*g*–PAA and PE–*g*–PMAA plates with the pH value at the activity measurements. The specific initial velocity of native trypsin passed the maximum value at pH 7.8. This value was almost in agreement with the experimental values reported in other articles.[30,32,39,42] On the other hand, the specific initial velocities of immobilized trypsin increased with an increase in the pH value irrespective of the amount of grafted AA and the kinds of grafted polymer chains. Trypsin immobilized on the PE–*g*–PAA plate with the lower immobilized amount showed higher activity in pH values ranging from 6 to 10. Trypsin immobilized on the PE–*g*–PAA plate at the immobilized amount of 0.014 mg/cm$^2$ had almost the same specific initial velocity as native trypsin at pH 10.0 and showed 70% of the activity of native trypsin at pH 7.8. The activity of immobilized trypsin would be greatly influenced by the dissociation behavior of the remaining carboxyl groups affixed to the trypsin-carrying grafted PAA and PMAA chains after the immobilization and diffusion of BANA molecules into the grafted PAA and PMAA layers. Because the grafted PAA and PMAA chains could expand more with an increase in pH value as a result of the electrical repulsion between negatively charged carboxyl groups,[47–49] the diffusivity of BANA molecules increased and the crowding of trypsin molecules immobilized on grafted PAA and PMAA chains was reduced in the basic pH region. Although the activity of native trypsin readily decreased because of denaturation by a change in the pH value, the denaturation of trypsin molecules would be considerably restricted by covalent immobilization. It can be assumed that because a trypsin molecule has 15 amino groups including a terminal amino group,[29,30] some amino groups per trypsin molecule would react with carboxyl groups affixed to grafted PAA and PMAA chains.

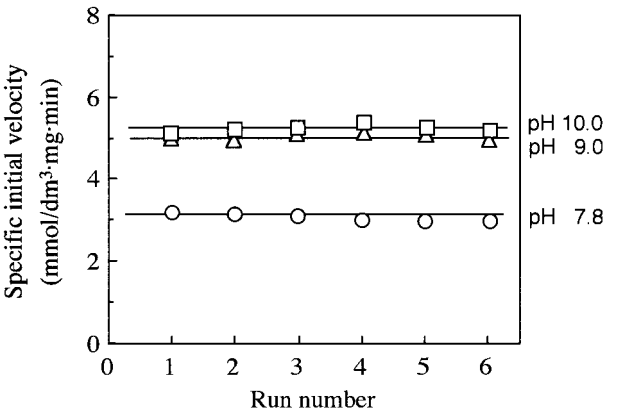


**Figure 6** Reusability of the trypsin immobilized on a PE-g-PAA plate of 3.55 $\mu$mol/cm$^2$ in the buffers of the pH values of 7.8 (○), 9.0 (Δ), and 10.0 (□) at 30°C. Average immobilized amount = 0.035 mg/cm$^2$.

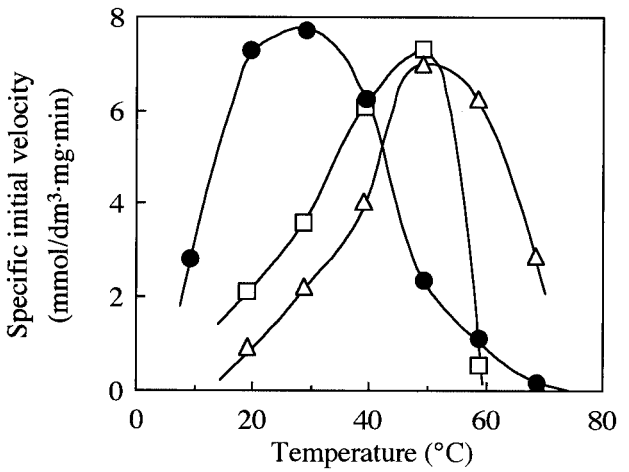


**Figure 7** Variations in the specific initial velocity of native (●) and trypsin immobilized on a PE-g-PAA plate of 3.55 $\mu$mol/cm$^2$ (Δ) and a PE-g-PMAA (□) plate of 103 $\mu$mol/cm$^2$ with the temperature in a pH 7.8 buffer. Immobilized amount (mg/cm$^2$) Δ: 0.035, □: 0.039.

Figure 6 shows the reusability of the remaining hydrolysis reactions for trypsin immobilized on the PE–*g*–PAA plates at different pH values. The activity stayed almost constant, even when the activity measurements were repeated at least 6 times at pH values of 7.8, 9.0, and 10.0. After the activity measurements, the trypsin-i-(PE–*g*–PAA) plates were stored at 4°C in the buffers used as the activity measurements. The activity for trypsin-i-(PE–*g*–PMAA) plates also stayed almost constant over at least six repeated measurements in a manner similar to that for the trypsin-i-(PE–*g*–PAA) plates, as shown in Figure 6. The above results reveal that little leakage and denaturation of trypsin for both grafted PE plates occurred for the repeated-activity measurements and washings.

### Effect of temperature on activity

The measurements of the activities of trypsin immobilized on PE–*g*–PAA and PE–*g*–PMAA plates were repeated at temperatures ranging from 20°C to 70°C in a pH 7.8 buffer. Figure 7 shows the effect of temperature on the specific initial velocities of native and immobilized trypsin. The optimum pH value was shifted to 50°C from 30°C for native trypsin by the immobilization on the PE–*g*–PAA and PE–*g*–PMAA plates, and at 50°C immobilized trypsin showed 90%–95% of the activity in relation to native trypsin at 30°C. The denaturation of native trypsin caused by the temperature increase would result in a considerable decrease in the activity above 30°C. On the other hand, the thermal stability of trypsin is considered to be considerably enhanced by covalent immobilization because the thermal denaturation of trypsin molecules is depressed.[28,37] The prevention of thermal denaturation probably would also come from the multipoint attachment between a trypsin molecule and grafted PAA or PMAA chains. However, above 50°C the activities of immobilized trypsin decreased sharply with an increase in temperature. In particular, the increase in the temperature from 50°C to 60°C caused an abrupt decrease in the activity for the trypsin-i-(PE–*g*–PMAA) plate. Because the PE–*g*–PMAA plate used for the immobilization had a high grafted amount (103 $\mu$mol/cm$^2$), grafted PMAA chains would undergo a considerable thermally conformational change. Figure 8 shows the reusabilities of trypsin immobilized on the PE–*g*–PAA plate at different temperatures in a pH 7.8 buffer. The specific initial velocity stayed constant without any definite loss at temperatures below 60°C. Although the reusabilities of the trypsin activity may come from the unchangeability of the trypsin conformation by the covalent multipoint attachment, the specific initial velocity gradually decreased at 70°C by the repeated-activity measurements because of further thermal denaturation. It was made clear from the above results that the optimum pH value of trypsin was shifted to 50°C from 30°C by the covalent immobilization on the PE–*g*–PAA and PE–*g*–PMAA plates, and immobilized trypsin possessed constant activities without any definite loss at temperatures below 60°C.

### Storage stability of immobilized trypsin

The trypsin-i-(PE–*g*–PAA) plate was preserved in a pH 7.8 buffer at 4°C, and the activity of immobilized trypsin was measured periodically. A trypsin solution was prepared so as to contain the same amount of trypsin as the immobilized trypsin on the PE–*g*–PAA plate in 40 cm$^3$ of a pH 7.8 buffer, and then this solution was stored at 4°C in the same manner as the trypsin-i-(PE–*g*–PAA) plate. The remaining activities of native and immobilized trypsin are shown in Figure

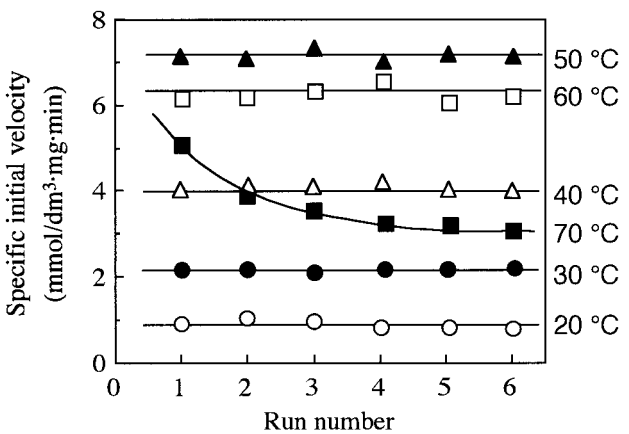


**Figure 8** Reusability of the trypsin immobilized on a PE-g-PAA plate of 3.55 $\mu$mol/cm$^2$ at 20 (○), 30 (●), 40 (Δ), 50 (▲), 60 (□), and 70°C (■) in a pH 7.8 buffer.

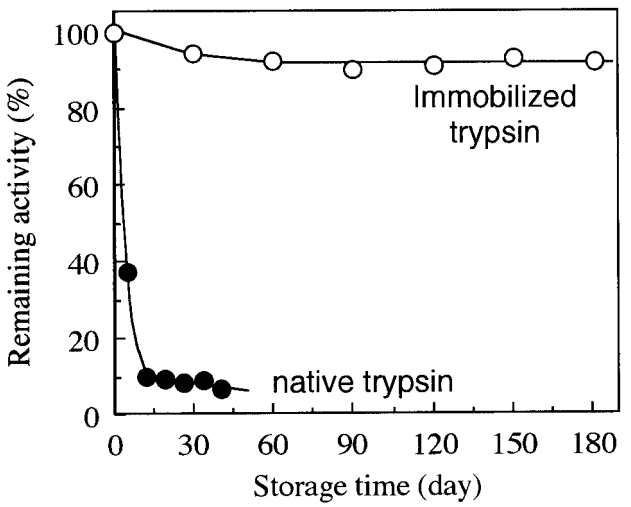


**Figure 9** Stability of native (○) and trypsin immobilized on a PE-g-PAA plate of 3.55 $\mu$mol/cm$^2$ (●) preserved in a pH 7.8 buffer at 4°C. The immobilized amount = 0.014 mg/cm$^2$.

9. The activity of native trypsin sharply decreased, so that after 42 days its remaining activity was only 8%. It is apparent that immobilized trypsin was much more stable than native trypsin, keeping 95% of its original activity over 6 months. These results suggest that with either denaturation of immobilized trypsin or isolation of trypsin molecules from the PE–*g*–PAA plate, markedly little occurred over the preservation period.

## CONCLUSIONS

Grafted polymer chains of the PE–*g*–PAA and PE–*g*–PMAA plates prepared by photografting AA and MAA onto the PE plates were applied to the covalent immobilization of trypsin. The activities of trypsin immobilized on the PE–*g*–PAA and PE–*g*–PMAA plates at pH 6.0 increased with an increase in the pH value at the activity measurements, and its optimum temperature was shifted to 50°C from 30°C for native trypsin because of the enhanced resistance to denaturation by covalent immobilization. With an increase in the grafted amount, the activities of immobilized trypsin increased for the PE–*g*–PMAA plates and decreased for the PE–*g*–PAA plates. These results indicate that both the location of the photografting and the chemical composition and water absorptivity of the grafted layer have a considerable influence on the activities of immobilized trypsin, which remained almost unchanged, even if the batch enzyme reactions were repeated. Trypsin immobilized on the PE–*g*–PAA plates kept 95% of its original activity after storage in a pH 7.8 buffer at 4°C for 6 months. These results made clear that the alkaline resistance and thermal stability of trypsin can be much improved by the covalent coupling of trypsin on PE–*g*–PAA and PE–*g*–PMAA plates.

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