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Characterization of Trypsin Immobilized on Acrylic Acid Grafted Polyethylene Plates for Reusability

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The activity of trypsin covalently immobilized onto acrylic acid (AA)-grafted PE (PE-g-PAA) plates with a water-soluble carbodiimide as a coupling agent was investigated as a function of the immobilized amount, pH levels on immobilization and at the activity measurement, and temperature. The activity of trypsin immobilized at pH 6.0 decreased with an increase in the immobilized amount due to the crowding of trypsin molecules in the vicinity of the surfaces of the grafted layers and increased with an increase in the pH level probably due to the expansion of the trypsin-carrying grafted PAA chains and the increased diffusivity of BANA molecules in the grafted layers. The optimum temperature of the activity for immobilized trypsin was shifted to 50 °C from 30 °C for native trypsin. The immobilized trypsin showed 92 % of the activity in relation to native trypsin at 30 °C and retained more than 90% of the activity after the immobilization in a pH 7.8 buffer solution at 4 °C over a period of 6 months. These results suggest that immobilized trypsin is repeatedly usable without denaturation and isolation over the period.
Key words: polyethylene, photografting, acrylic acid, enzymatic activity, immobilization

1. INTRODUCTION

Considerable attention has been devoted to the development of technologies suitable for using immobilized enzymes under aqueous conditions. Immobilized enzymes offer considerable advantages as easily removable and reusable biocatalysts that possess increased shelf life, operational simplicity, and thermal and pH stabilities. The methods for immobilization of enzymes to an insoluble carrier may be roughly classified into the following three groups: 1) covalent binding to a carrier, 2) ionic binding and physical adsorption to a carrier, and 3) entrapping in the crosslinked matrices. Among these methods, the covalent binding is the most important method because leakage of the enzyme can be avoided to a great extent [1, 2]. To be suitable for the covalent binding of enzymes, in general, the supports should display the following 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 water-insoluble supports must be carried out under mild conditions to avoid inactivation of the enzymes [3-5].

A large variety of grafted copolymers have been used as supporting materials for the immobilization of enzymes. Here, we have chosen acrylic acid (AA)-grafted polyethylene (PE-g-PAA) plates as a supporting material [6] because the PE plate possess mechanical strength and the grafted PAA layers have hydrophilicity enough to immobilize enzymes and grafted PAA chains are not susceptible to microbial attack. In addition, carboxylic groups affixed to the grafted PAA chains are known to react with amino groups in the enzymes under mild conditions, resulting in stable peptide bonds.

In this study, the PE-g-PAA plates were prepared by the photografting technique and the proteolytic enzyme, trypsin, was immobilized onto the grafted PAA layers formed on the PE plates by the covalent coupling method using a water-soluble carbodiimide. Attempts were made to optimize coupling conditions as a function of the grafted amount, the immobilized amount, and the pH level on immobilization. The activity of immobilized trypsin was measured at different pH levels and temperatures and compared with that of native trypsin.

2. EXPERIMENTAL

2.1 Photografting

A PE plate (thickness, 1.0 mm: density, 0.926 g/cm^3) was used as a polymeric support for enzyme immobilization. The photografting of AA onto the PE plates was carried out by the same method described previously in detail [6, 7].

2.2 Immobilization of trypsin

Trypsin (EC 3. 4. 21. 3) from bovine pancreas was chosen as an immobilizing enzyme because the following parameters are clear: the absolute molecular weight, the conformation, the amino acid sequence, the number of active sites and their location, and the number of amino side groups [8]. The used trypsin was reported by the manufacturer to have a specific activity of 10,400 U/mg-solid.

The PE-g-PAA plates (25 × 25 mm) with different grafted amounts were immersed in the acetate buffers (I = 0.05 M, V = 20 cm^3) at the pH levels of 5.0 to 7.0 containing 20 mg of trypsin and 40 mg of 1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimide metho-*p*-toluene sulfonate (CMC) for 12 to 18 hrs with a moderate stirring [9-11]. After the immobilization, the trypsin-immobilized PE-g-PAA (trypsin-i-(PE-g-PAA)) plates were washed with the acetate buffers at the same pH levels, and then preserved in the phosphate buffers of pH 6.0 to 10.0 at 4 °C. The amount of immobilized trypsin

was spectrophotometrically determined at 290 nm as an isosbestic point of trypsin in the acetate buffers.

2.3 Activity measurements

The phosphate buffers of the pH levels of 6.0 to 10.0 (I = 0.1 M, V = 40 cm^3) containing 0.02 mmol of Nα-benzoil-DL-arginine-*p*-nitoroanilide hydrochloride (BANA) were incubated at 30 °C, and then the trypsin-i-(PE-g-PAA) plates were immersed in these buffers to start the enzymatic reaction of the immobilized trypsin [8]. The activity of immobilized trypsin was estimated by spectrophotometrically measuring the initial rate of formation of *p*-nitroaniline as a hydrolysis product of BANA as a function of the reaction time at 410 nm in mmol/dm^3·mg·min [12]. The relative activity was expressed as the ratio of the absolute activity of the immobilized trypsin to that of native trypsin.

3. RESULTS and DISCUSSION

3.1 The effect of the pH level on immobilization on the activity

The grafted amount was adjusted by varying the irradiation time for the photografting of AA onto the PE plates at 60 °C. A PE-g-PAA plate with the grafted amount of 3.55 μmol/cm^2-PE plate was immersed in the buffers of the pH levels of 5.0 to 7.0 containing CMC and trypsin with a moderate stirring at 4 °C to covalently immobilize trypsin onto the PE-g-PAA plate. Figure 1 shows the amount of immobilized trypsin with the reaction time. The amount of immobilized trypsin increased with an increase in the reaction time. A time of at least 12 hrs was required for trypsin to be immobilized onto the PE-g-PAA plates. The activity of trypsin immobilized onto the PE-g-PAA plate of 3.55 μmol/cm^2-PE plate at the pH levels of 5.0 to 7.0 was measured at the optimum pH level of 7.8 for native trypsin. (The determination of the optimum pH level of native trypsin will be described in Figure 5.) Figure 2 shows the variation in the relative activity with the pH level on immobilization (the average immobilized amount = 0.0443 mg/cm^2). Native trypsin of the same amount as the trypsin immobilized onto the PE-g-PAA plate was dissolved in a pH 7.8 buffer (V = 40 cm^3), and then their specific initial velocities were determined at 30 °C. The relative activities were expressed as the ratio of the specific initial velocity of the immobilized trypsin

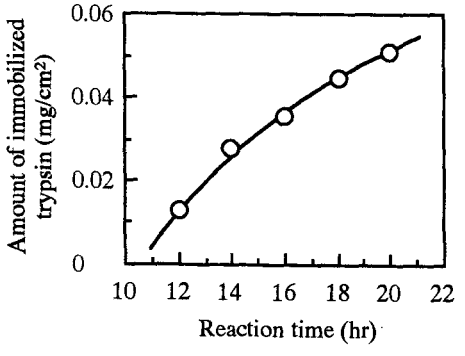

Figure 1 Change in the amount of trypsin immobilized onto a PE-g-PAA plate of 3.55 mmol/g-PE plate with the reaction time in a pH 6.0 buffer containing trypsin (1 mg/cm^3) and CMC (2 mg/cm^3).

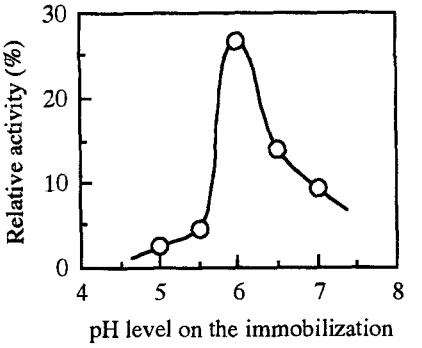

Figure 2 Variation in the relative activity of trypsin immobilized onto a PE-g-PAA plate of 3.55 mmol/g-PE with the pH level on immobilization (Average immobilized amount = 0.044 mg/cm^2).

to that of native trypsin. The relative activity passed the maximum value at pH 6.0 on immobilization. Since trypsin molecules are covalently immobilized onto grafted AA chains through the peptide bonding between amino groups on trypsin molecules and carboxyl groups affixed to grafted PAA chains, the number of amino groups in a trypsin molecule decrease by the immobilization and the isoelectric point of the immobilized trypsin is lower than that of free trypsin. Therefore, the optimum pH level on immobilization of trypsin as shown in Figure 1 would be different from that for the enzymatic activity.

3.2 The effect of the grafted amount on the activity

The immobilization of trypsin was carried out onto PE-g-PAA plates with different grafted amounts so as to prepare the samples with an almost constant immobilized amount (the average immobilized amount = 0.0297 mg/cm^2). Figure 3 shows the change in the relative activity with the grafted amount. The activity of immobilized trypsin decreased with an increase in the grafted amount. Here, the increase in the grafted amount leads to the increase in the thickness of the grafted layer. Therefore, when trypsin is immobilized onto grafted PAA chains in the inside of the grafted layer at a high grafted amount, the relative activity is considered to apparently decrease mainly due to the decrease in the

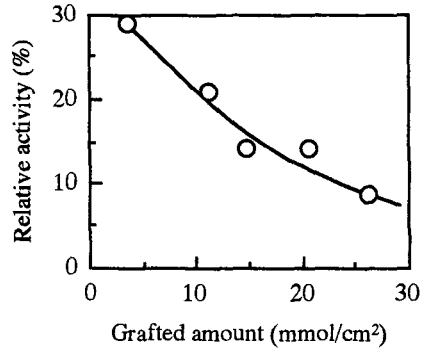

Figure 3 Change in the relative activity of immobilized trypsin with the grafted amount of the PE-g-PAA plates (Average immobilized amount = 0.0 30 mg/cm^2).

diffusivity of BANA molecules into the grafted layers.

3.3 The effect of the immobilized amount on the activity

The activities of the trypsin-i-(PE-g-PAA) plates of 3.50 μmol/cm^2 with different immobilized amounts were measured at the optimum pH level of 7.8 for native trypsin. Figure 4 shows the change in the relative activity with the immobilized amount. The relative activity increased with a decrease in the immobilized amount and went up to 45 % at the immobilized amount of 0.015 mg/cm^2. The amounts of native trypsin equivalent to the immobilized trypsin and 0.020 mmol of BANA were dissolved in a pH 7.8 buffer of 40 cm^3, and then the specific initial velocities were calculated from the activity measurements. Native trypsin had a constant specific initial velocity of 0.31 (μmol/mg·min) in the concentration ranging from 1.75 to 16.75 μg/cm^3. The increase in the immobilized amount leads to crowding of trypsin molecules in the grafted layers, resulting in spatial restrictions, blocking active sites and/or denaturing proteins. Alternatively, multiple point attachment of trypsin molecules through the peptide bonds would decrease their conformational flexibility at the active sites, thereby inhibiting the ability of trypsin to adapt to binding of the substrate.

3.4 The effect of pH level at the activity measurement on the activity

The activity of trypsin immobilized onto the PE-g-PAA plates of 3-4 mmol/cm^2 at pH 6.0 was measured in the pH 5.0 to 10.0 region at 30 °C. Figure 5 shows the variations in the specific initial velocity of the immobilized trypsin with the pH level at the activity measurements. The specific initial velocity of native trypsin passed the maximum value at pH 7.8. This pH level was in good agreement with the experimental value reported in the articles [12-14]. On the other hand, the specific initial velocities of the immobilized trypsin increased with an increase in the pH level. The decrease in the immobilized amount led to the activity irrespective of the pH level. Trypsin immobilized onto the PE-g-PAA plate with 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 enzymatic activity in relation to native trypsin at pH 7.8. The activity of immobilized trypsin would be

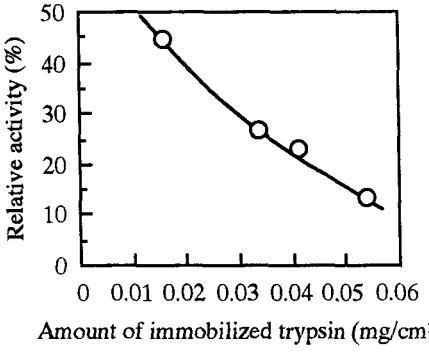

Figure 4 Change in the relative activity of immobilized trypsin with the immobilized amount using a PE-g-PAA plate of 3.5 μmol/cm^2-PE.

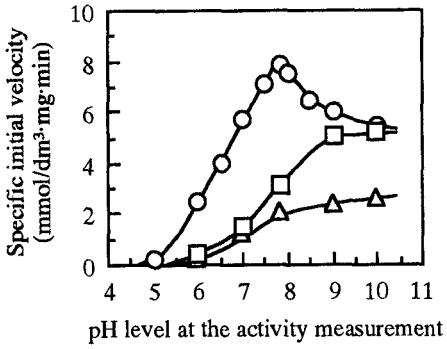

Figure 5 Variations in the specific initial velocity of native (○) and immobilized trypsin (△,□) with the pH level on the activity measurement.
Immobilized amount (mg/cm^2) = △:0.035, □:0.014

attributed to the influence of the dissociation behavior of the remaining carboxyl groups affixed to the trypsin-carrying grafted PAA chains and the diffusivity of BANA molecules into the grafted layers. Since the grafted PAA chains can more expand with an increase in the pH level due to the electrical repulsion between negatively charged carboxyl groups, the diffusivity of BANA molecules increase and the crowdedness of trypsin molecules immobilized onto grafted PAA chains reduce in the basic pH region. The activity of native trypsin is readily decreased due to the denaturation by alterations in the pH level. The denaturation of trypsin molecules would be considerably restricted by the covalent immobilization. In addition, since a trypsin molecule has 15 amino groups including a terminal amino group, it can be assumed that some amino groups per trypsin molecule react with carboxyl groups affixed to the grafted PAA chains. In addition, the activity was kept almost constant irrespective of the pH level, even if the activity measurements were repeated at least 6 times at the pH levels ranging 6.0 to 10.0. After the activity measurements, trypsin-i-(PE-g-PAA) plates were stored at 4 °C in the buffers used at the activity measurements. It was found that little leakage and denaturation of immobilized trypsin occurred during the repeated activity measurement, washing, and storage.

3.5 The effect of temperature on the activity

The activity of the immobilized trypsin was

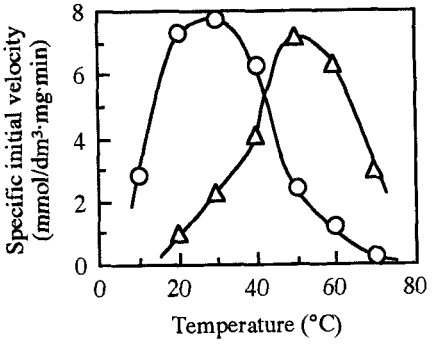

Figure 6 Variations in the specific initial velocity of native (○) and immobilized trypsin (△) with the temperature.
Immobilized amount = 0.035 (mg/cm^2)

repeatedly measured at the temperature ranging 20 to 70 °C in a pH 7.8 buffer. Figure 6 shows the effect of temperature on the relative activity of the native and immobilized trypsin. The optimum pH level for the immobilized trypsin was shifted to 50 °C from 30 °C for native trypsin and the immobilized trypsin showed 95 % of the enzymatic activity in relation to native trypsin at 30 °C. The increase in the optimum temperature for the immobilized trypsin can be attributed to the prevention of autodigestion and thermal denaturation probably due to the multipoint attachment between a trypsin molecule and grafted PAA chains. Figure 7 shows the effect of repeated use on the residual hydrolysis reactions for immobilized trypsin at different temperatures. The specific initial velocity of the immobilized trypsin was kept constant without any definite loss, even if the activity measurements were repeated at least 6 times at the temperatures ranging 20 to 60 °C. This may be presumably due to the unchangeability of the trypsin conformation by multibinding. However, the specific initial velocity at 70 °C gradually decreased by the repeated activity measurements because of some thermal denaturation.

3.6 Storage stability of immobilized trypsin

The trypsin-i-(PE-g-PAA) plate was in a pH 7.8 buffer at 4 °C, and the activity of the immobilized trypsin was measured periodically. The residual activity

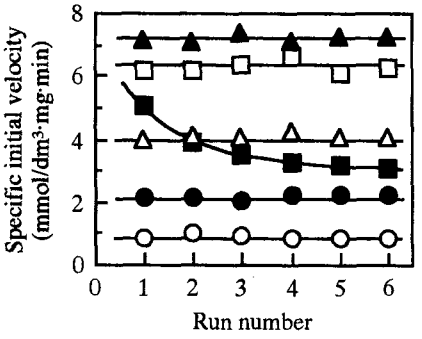

Figure 7 Reusability of the trypsin immobilized onto a PE-g-PAA plate of 3.61 mmol/cm² at 20 (○), 30 (●), 40 (△), 50 (▲), 60 (□), and 70 (■) °C. Immobilized amount=0.034 (mg/cm²)

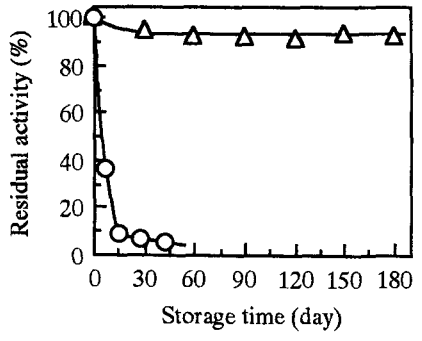

Figure 8 Stability of native (○) and immobilized trypsin (△) preserved in a pH 7.8 buffer at 4 °C : Grafted amount=3.50 (mmol/cm²), Immobilized amount=0.014 (mg/cm²).

of the native and immobilized trypsin is shown in Figure 8. It is apparent that the immobilized trypsin is much more stable than free trypsin and kept 95% of its original activity over a period of 6 months. This result suggests that the both denaturation of immobilized trypsin and isolation of trypsin molecules from the PE-g-PAA plate little occur markedly over the period.

CONCLUSIONS

Grafted PAA chains of the PE-g-PAA plates prepared by the photografting of AA onto the PE plates were applied to covalent immobilization of trypsin. The activity of trypsin immobilized at pH 6.0 increased with an increase in the pH level at the activity measurements and its optimum temperature was shifted to 50 °C from 30 °C for native trypsin because of the prevention of autodigestion and denaturation by covalent immobilization. The activity of immobilized trypsin maintained almost unchanged, even if the batch enzyme reaction was repeatedly carried out. The immobilized trypsin was kept 95% of its original activity in a pH 7.8 buffer at 4 °C over a period of 6 month. It is made clear from the above results that the alkaline-resistance and thermal stability of trypsin can be mach improved by the covalent coupling of trypsin onto the PE-g-PAA plates.

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