201. Interaction of Enzyme with Polymer Matrix in Immobilized Enzymes

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Summary

Thermolysin was immobilized by radiation polymerization of hydroxyalkyl acrylate and tetradecaethylene glycol dimethacrylate monomers at low temperatures in the presence of the enzyme, and the degree of interaction of the enzyme with the polymer matrix was studied by measuring the thermal stability of the immobilized enzyme. The thermal stability was affected by the molecular structure of the monomer; the thermal stability of the immobilized enzyme from hydrophilic monofunctional monomers in the wet state was higher than that from hydrophobic bifunctional monomers. The thermal stability in polymers formed from hydroxyalkyl acrylates decreased with an increase in the number of methylene units in the monomer, owing to a change of the state of the enzyme trapped in the porous polymer matrix. The enzyme molecule trapped in a hydrophilic porous polymer matrix appeared to be stabilized by interaction with the polymer chains.

Introduction. – Immobilized enzymes with their properties of reusability and ease of handling have found wide application in biochemistry over the past decade. Few nucleic-acid-modifying enzymes, such as polymerases, ligases, restriction endonucleases, and kinases, all of which are used extensively in contemporary biochemistry, especially in the evolving methodology of gene cloning, have been reported as immobilized and functional. Reviews have recently been published on the different methods of immobilization of enzymes on various organic and inorganic carriers [1–4]. Enzymes are usually stabilized by immobilization, for example immobilized proteases are often more stable owing to protection from autolysis. The properties of immobilized proteases and kinetic studies of their deactivation have been reported [5–12]. However, the interaction of the enzyme with the polymer matrix in immobilized enzymes has not been studied.

In this work thermolysin was immobilized by radiation polymerization of various monomers in the presence of the enzyme and the interaction between enzyme and polymer matrix was studied by measuring the thermal stability of the immobilized enzyme.

Experimental. – *Materials.* Thermolysin (*Bacillus thermoproteolyticus rokk.*, 100 units/mg) was obtained from *Sigma Chemical Co. Ltd.* Hydroxyalkyl acrylates, 2-hydroxyethyl acrylate (HEA), and tetradecaethylene glycol dimethacrylate (14 G) were obtained from *Shin Nakamura Chemical Co. Ltd.* Hammarstein casein and trichloroacetic acid were obtained from *Merck* and *Kanto Chemical Co. Ltd.*, respectively.

Immobilization. The enzyme solution (1.0 ml), containing the enzyme (1.0 mg/ml), $CaCl_2$ (5 mM), and monomer were dissolved in 0.1M Tris buffer solution (pH 7.5) was placed in a glass tube (20 cm in length and 0.8 cm in diameter). The tube was cooled to $-78°$ and irradiated with an irradiation dose of 1.0 Mrad by γ-rays from ^{60}Co-source. The irradiation temperature was kept at $-78°$ by immersing the tube in a *Dewar* flask filled with dry ice/MeOH. After irradiation, immobilized enzyme composites obtained by polymerization were cut into thin pellets form (0.5 mm in thickness) at r.t.

Degree of Hydration. The hydrophilicity of the polymer matrix was evaluated by measurement of the degree of hydration, which was determined as the ratio of the weight of water to the weight of polymer at swelling equilibrium at 25° in H_2O.

Enzyme Activity. The enzyme activity (%) of the immobilized enzyme was determined by comparison of the reaction of the immobilized enzyme with casein with the reaction of an equivalent amount of native enzyme. The enzyme reaction was carried out using 0.5% casein solution at 50° for 30 min. After the reaction, 10% trichloroacetic acid solution was added to the reaction solution and unreacted casein was removed by filtration. The absorbance of the filtrate was measured with a spectrophotometer at 280 nm.

Thermal Stability. The thermal stability of the immobilized enzyme was determined by measuring the enzyme activities after heating in the presence or absence of the buffer solution at various temperatures for 30 min.

Results and Discussion. – *Effect of the Polymer Matrix on Thermal Stability.* The enzyme was immobilized by radiation polymerization of HEA and 14G, and the effect of the polymer matrix on the thermal stability of the immobilized enzyme was examined. The relationship between residual enzyme activity and temperature is shown in *Figure 1*.

The thermal stability of the enzyme was increased by immobilization and the thermal stability of the immobilized enzyme in the dry state was higher than that in the wet state. In the dry state the thermal stability of the immobilized enzyme from 14G was higher than that from HEA. The polymer matrix of the immobilized enzyme from hydrophilic monofunctional monomers such as HEA was a soft gel with a porous structure. On the other hand, the polymer matrix from hydrophobic bifunctional monomers such as 14G was rigid with a porous structure. The formation of the porous structure (pore size: 0.1–5 μm in diameter) in the polymer matrix took place when the ice produced in the polymerized composites after irradiation, melted, owing to the rise in temperature of the tube. The structure of the pore formed by irradiation at temperatures below 0° was of continuous or cylindrical and that at temperatures above 0° was discontinuous or spherical, and was dependent on the nature of the ice formation in the monomer mixture system, in which the enzymes were trapped on or near the surface of the polymer matrix. The degrees of hydration of the polymers of HEA and 14G were 0.45 and 0.25, respectively, indicating that the hydrophilicity of the polymer matrix from HEA is higher than that from 14G. Therefore, the porous polymer matrix from HEA can swell in water so that the enzymes become surrounded by polymer matrix and strongly interact with it. The excess motion (mobility) of the enzyme molecule caused by heating,

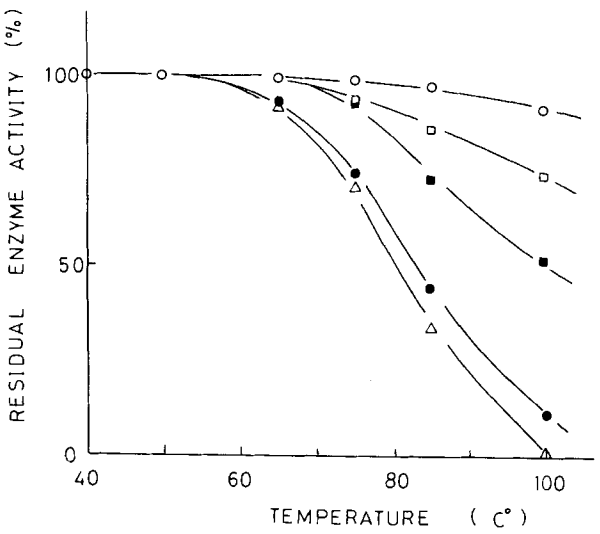

Fig. 1. *Relationship between residual enzyme activity and temperature.* Dry state: ○ 14G (90%), □ HEA (90%); Wet state: ● 14G (90%), ■ HEA (90%); Native enzymes: △.

leading to thermal denaturation would thus be reduced. However, the polymer matrix from 14G is cross-linked by radiation polymerization of the two vinyl groups, so that the polymer matrix does not swell, easily although the polymer chains possess ether groups. In such a rigid polymer matrix the enzyme molecule is firmly trapped and it cannot interact with the polymer matrix. Furthermore, it is proposed that the enzymes located on the rigid polymer matrix interact with each other and undergo autolysis. The thermal stability of 14G in the wet state was low and was slightly higher than or comparable with that of the native enzyme. This result indicated that the environment of the enzyme trapped in the polymer matrix differs considerably between the polymer matrices from monofunctional hydrophilic and bifunctional hydrophobic monomers. In the dry state of the immobilized enzyme, the thermal stability in 14G was higher than that in HEA as seen in *Figure 1*. This high thermal stability in the dry state on the 14G polymer matrix is due to the fact that the enzyme is more firmly trapped.

Effect of the Molecular Structure of the Monomers on the Thermal Stability. The enzyme was immobilized using hydroxyalkyl acrylate monomers, and the relationship between molecular structure and thermal stability was studied *(Fig. 2)*. The thermal stability decreased with increasing the number (n) of methylene (CH_2) units in the monomers. The form of the immobilized enzyme varied with the number of CH_2-units; the immobilized enzyme from monomers with small n-values ($n \leq 3$) had a bulk form and the immobilized enzyme from those with large n-values ($n \geq 4$) had a particle form (particle size: 100–200 μm). This change in the form

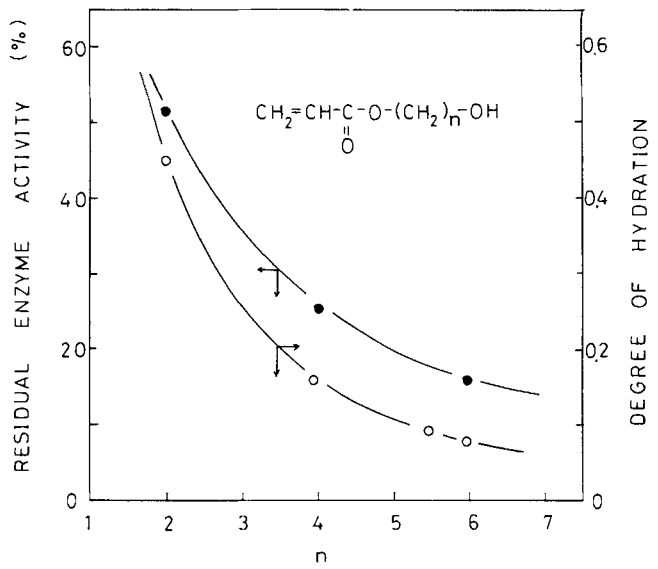

Fig. 2. *Relationship between monomer structure and degree hydration* (○) *and residual enzyme activity* (●) *after heating to 100° in the wet state.* Monomer: hydroxyalkyl acrylate; Monomer concentration: 90%.

of the immobilized enzyme with n was related to the hydrophilicity of the monomers *(Fig. 2)*. The degree of hydration of the polymer decreased with increasing n. The formation of particles in hydrophobic monomers was due to the radiation polymerization of the particular monomer phase dispersed in the monomer mixture solution, in which the enzyme molecules were trapped on its surface. The enzyme molecules trapped on the surface of the particles interact with each other, resulting in autolysis. Furthermore, the enzyme molecules located on the surface are not adequately protected by the polymer matrix, so that they can be deactivated by thermal denaturation.

The immobilized enzyme in particle form can react with a substrate owing to its surface trapping, in contrast with the bulk form where the enzyme molecules are trapped in the polymer matrix. However, the enzyme activity (60–70%) of the immobilized enzymes in bulk form was higher than that (about 40%) in particle form, because the trapping yield of the enzyme in the formation of the particles was lower than that in the formation of the bulk form.

Effect of the Monomer Concentration on the Thermal Stability. The thermal stability of the immobilized enzyme varied also with hydrophilic monomer concentration as shown in *Figure 3*. The thermal stability appeared to increase slightly with an increase in HEA concentration. As monomer concentration increases, the pore size in the porous polymer matrix decreases and the polymer matrix apparently becomes more rigid, so that the enzymes are more firmly trapped in it. The enzyme

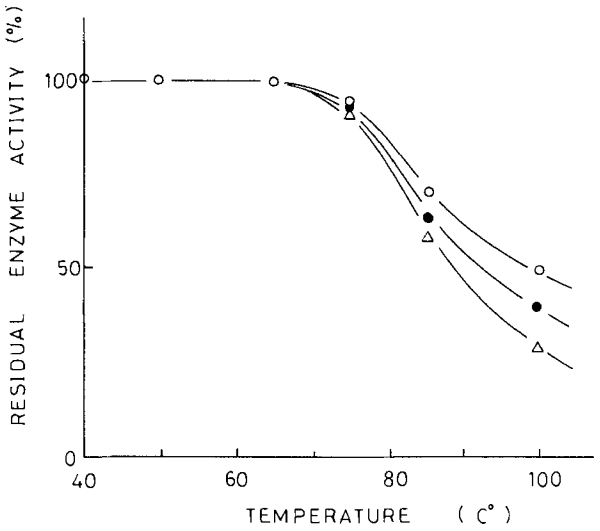

Fig. 3. *Relationship between residual enzyme activity and temperature in immobilized enzyme from various HEA concentrations.* HEA concentration: △: 30%, ●: 60%, ○: 90%.

molecule which is more firmly trapped in the polymer matrix from high monomer concentrations interacts more strongly with the polymer chains of the polymer matrix when the latter swells. It is proposed that this interaction leads to an increase in the thermal stability of the immobilized enzyme.

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