Use of Spherical Tannin Resin as a Support for Immobilized Enzyme

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SYNOPSIS

α-Amylase (α-1, 4-glucan 4-glucanohydrolase 3.2.1.1) was used to investigate whether the spherical tannin resins made by the reaction of Mimosa (*Acacia Mollissima*, Wattle) tannin (condensed-type tannin) (MT) with formaldehyde could be used as a support for the immobilization of enzymes. It was found that the optimum pH range of the immobilized α-amylase was wider than that of native α-amylase, and the shelf life of the enzyme was increased by immobilization. Furthermore, the immobilized enzyme could be used repeatedly, and in a continuous enzyme reaction increased a further advantage of the technique. On the basis of these data, it was concluded that this resin could be used as a support for the immobilization of enzymes. © 1992 John Wiley & Sons, Inc.

INTRODUCTION

Tannin has the characteristic properties of bonding to protein and alkaloid by ionic bond, quinone bond, and/or hydrogen bond[1,2] and bonding to heavy metal ions by chelation. To apply these properties, as described in the previous paper,[3] we developed a preparative method for fine, porous, and spherical tannin resins. Our other two reports describe the adsorption capacities for bovine serum albumin[4] and a few heavy-metal ions[5] by this spherical tannin resin, respectively.

Ono et al.[6] and Watanabe et al.[7] have investigated various properties of naringinase and aminoacylase, respectively, to be immobilized by adsorbing to the tannin-aminohexyl cellulose prepared by the reaction of aminohexyl cellulose and cyanogen bromide activated chinese-gallotannin. (This tannin is a hydrolyzable-type tannin.)

In general, it is nevertheless said that enzyme activities are inhibited by tannins. The activity yield of two enzymes in the reports described above are from about 60 to 100% for naringinase[6] and from about 20 to 30% for aminoacylase, respectively. But, since the base material of the tannin-aminohexyl cellulose is cellulose, it has the disadvantage that pressure drops in usage on column are increased;[8] it has been reported that shelves in column need to be established.

The spherical tannin resins prepared by us do not cause pressure drops in usage on column.[3] However, the effects of this tannin resin on the enzyme activity are unknown.

In this study, α-amylase was adsorbed to the spherical tannin resins and immobilized, and some properties of the immobilized enzymes were investigated. The possibility of using the spherical tannin resins as supports for immobilized enzymes was examined.

EXPERIMENTAL

Preparation of the Spherical Tannin Resin

Using the method described in the previous paper,[3] spherical resins were prepared under the following conditions: One mole of formaldehyde was added from a 37% aqueous solution to 1 mol of Mimosa (*Acacia Mollissima*, Wattle) tannin (condensed type) (MT). Deionized water was added down to a 37.5% MT concentration, and then the reaction mixture was resinified in polybutene (54 cP at 60°C)

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Table I Distribution of Resin Grain Size[a]

~16 mesh	16–42 mesh	~42 mesh
1.3%	92.9%	5.8%

[a] Vacuum dried at room temperature and then sieved.

at 60°C for 200 min with stirring at 300 rpm. The prepared spherical resin was filtered, washed five times with toluene, and then refiltered and washed five times with methyl alcohol. The resin was well washed with water, filtered, and then stored in a refrigerator. The grain size distributions and the physical properties of the prepared spherical tannin resin are shown in Tables I and II, respectively.[5] The spherical resin was found to be porous and homogeneous.

Method for Immobilization of the Enzyme

α-Amylase (2050 units/mg solid; *Bacillus* species Type II-A, Sigma Chemical Co. Ltd.) was used. The spherical tannin resins were conditioned in 1/500 mol/L acetate buffer solution at pH 6.0. One milliliter of the enzyme–1/500 mol/L acetate buffer solution, containing 1 mg of α-amylase per milliliter, was added to 1 g of the spherical tannin resin, and the mixture was incubated for 1 h at 23°C. The enzyme was adsorbed and immobilized on the resin. The enzyme–tannin resin complex was washed with 50 mL of 1/400 mol/L acetate buffer solution and deionized water, and then filtered. This wet resin was used.

Enzyme Assays

Enzyme assay was carried out using the modified Robyt–Whelan's method.[10] One gram of soluble starch was dissolved in boiling water, and the solution was made up to 50 mL by addition of cold water. A 50-mL acetate buffer solution of 1/500 mol/L containing 1/500 mol/L $CaCl_2$ and 1/100 mol/L NaCl, were added to the soluble starch solution; 100 mL of the substrate solution were prepared.

A 3-mL substrate solution (1% soluble starch solution) was added either to 1 mL of native α-amylase solution containing 1 μg of α-amylase per milliliter or to 150 mg of the immobilized α-amylase, which contained 28.5 μg of α-amylase. The enzyme was allowed to act for 10 min at 30°C. The reaction solution was then boiled for 10 min to inactivate the residual α-amylase. The amount of reducing sugar produced was determined colorimetrically as described by Somogyi-Nelson, absorbance being determined at 660 nm with a double-beam spectrophotometer (Hitachiseisakusho Co. Ltd., 200-20 type) using maltose solution as the standard. The activity of α-amylase was expressed as units, one unit being defined as the amount of enzyme that produced 1 μmol of maltose per minute from the soluble starch substrate. The enzyme activity of the immobilized α-amylase was assessed on the basis of 1 mg of dried resin.

Determination of the Amount of Immobilized α-Amylase

The amount of α-amylase immobilized on the spherical tannin resin was estimated from the difference between the amounts of α-amylase in the enzyme–buffer solution before and after immobilization. The amounts of α-amylase were determined by the modified method[11] of the standard Lowry protein assay, using bovine serum albumin (BSA) as a standard.

Investigation of the Enzymatic Properties of Native and Immobilized α-Amylase

The following items were investigated:

1. Concentration of enzyme: The changes in the amount of reducing sugar formed with con-

Table II Properties of the Spherical Tannin Resin

Concentration of MT (%)[a]	Specific Surface Area (m^2/g)[b]	Total Pore Volume (cm^3/g)[b]	Average Pore Radius (Å)[b]	Total Pore Volume in Water (cm^3/g)
37.5	139.2	1.77	254.3	1.90

[a] The concentration of MT when the spherical resin were prepared.

[b] Wet resin corresponding to 1 g of the dried resin. Specific surface area: I_2 adsorption method from I_2-*n*-hexane solution (cross-sectional area of I_2: 21.1 $Å^2$). Total pore volume: Liquid substitution method (heating the resin in *n*-hexane, and then drying only the surface of the resin by filtration). Average pore radius: evaluated by $\bar{r} = 2V_g/S$ where V_g is total pore volume and, S is specific surface area.

centration of α-amylase (in the range of 5–100 μg/mL) in the enzyme–buffer solution were measured.
2. Duration of enzyme reaction: The effect of time on the enzyme reaction was investigated in the range of 5–20 min.
3. Temperature of enzyme reaction: The effect of temperature was investigated by varying the temperature of reaction in the range of 30–90°C.
4. pH of substrate solution: The effect of pH was investigated by varying the pH of the 1% soluble starch solution between pH 4.0 and pH 7.0.
5. Concentration of substrate solution: The effect of the concentration of substrate solution was investigated in the range of 0.2–5.0% soluble starch.
6. Heat resistance: The heat-resisting properties of the native and the immobilized enzymes were compared as follows. The enzyme reactions were done at 30°C with either native or immobilized α-amylase, which had been heat-treated for 10 min in the range of temperature of 30–100°C.
7. Stability: The native and immobilized α-amylase were stored at 0–5°C for different periods of time before their activity was determined.
8. Change of activity with repeated usages: The immobilized α-amylase, which had been used in an enzyme reaction, was washed and filtered and then was made to react again with starch. This procedure was repeated, and the successive activities were determined.
9. Persistence of activity during continuous reaction: One gram of the immobilized α-amylase was loaded into a 0.8 × 11 cm column, and then 1% soluble starch solution was passed through the column at a flow rate of 3.8 mL/h. The effluent was collected at different prescribed times and the amounts of reducing sugar present were measured.

RESULTS AND DISCUSSION

Effects of Enzyme Concentration and Reaction Time

In order to investigate kinetically the enzyme activity of the immobilized α-amylase, we measured the changes with time in the amount of reducing sugar formed by 1 mL of the enzyme solution containing the prescribed amount of native α-amylase (Fig. 1). The reaction rate of 1 mL of the enzyme solution containing 100 or 50 μg of the native α-amylase was high, up to 5 min of reaction time, after which it was low. On the other hand, the reaction rate of 1 mL of the enzyme solution containing 10 or 5 μg of native α-amylase was independent of time. Assuming that this property of native α-amylase was the same as that of the immobilized enzyme, the reaction time in the following investigations has been held constant at 10 min.

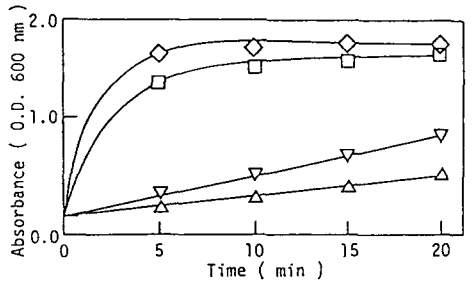

Figure 1 Effect of incubation time on activity. (◇) 100 μg in 1 mL, (□) 50 μg in 1 mL, (▽) 10 μg in 1 mL, (△) 5 μg in 1 mL.

Effect of Temperature on Enzyme Activity

The effect of the temperature of reaction is shown in Figure 2. The optimum temperature was about 60°C for both native and immobilized α-amylase. The enzyme activity was maximum at about 60°C. This was thought to be due to the combination of increasing reaction rate with a rise of temperature in the range below 60°C and the decrease of enzyme activity due to heat denaturation of the enzyme in the range above 60°C. The enzyme activity at 80°C was about 20% of that at 30°C.

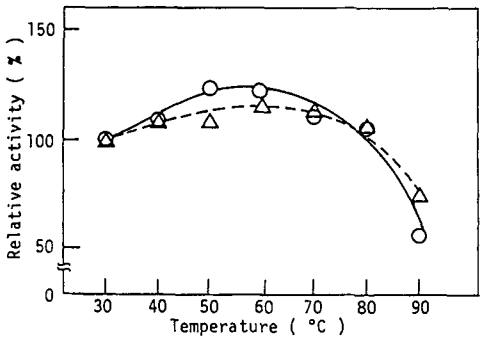

Figure 2 Effect of temperature on the activity of native and immobilized α-amylase. (△) native α-amylase, (○) immobilized α-amylase. Note: The activity obtained at 30°C was taken as 100%.

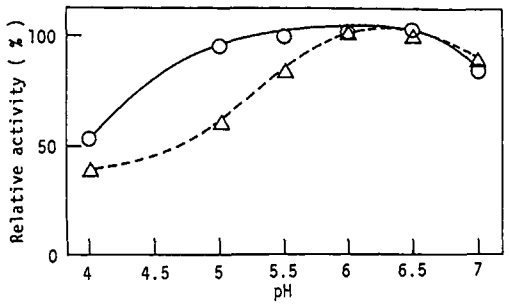

Figure 3 Effect of pH on the activity of native and immobilized α-amylase. Legend and note are the same in Figure 2.

Effect of pH on Enzyme Activity

The effect of the pH on the enzyme activities is shown in Figure 3. The optimum pHs of the native α-amylase were in the range of pH 6.0–6.5, whereas the pH range of the maximum activities of the immobilized enzyme was from pH 5.0 to 6.5; this range was wider than that of the native α-amylase, indicating that immobilization increased the stability of the enzyme, and shifted the optimum pH of the enzyme.

Yield of Enzyme Activity

When 1 mg of α-amylase was added to the spherical tannin resin, the amount of enzyme immobilized was 0.19 mg. On this basis, the yield of enzyme activity at pH 6.0 [that is, the ratio of activity (72.3 U) of the amount of immobilized α-amylase corresponding to 1 mg of α-amylase to the activity (279.6 U) of 1 mg of native α-amylase] was calculated to be 25.9%.

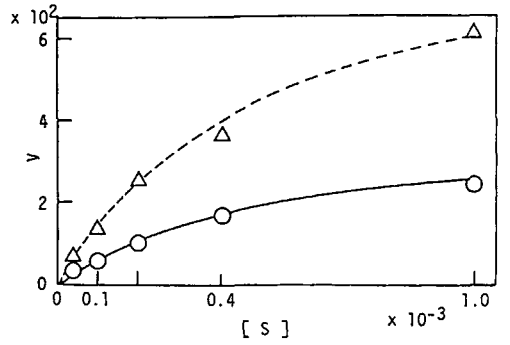

Figure 4 Effect of the concentration of soluble starch on the activity. (△) native α-amylase, (○) immobilized α-amylase. Notes: [S]: Initial concentration of soluble starch (mol/L). V: Activity (mol/min/α-amylase 1 mg). The molecular weight of soluble starch was taken 5.0 × 10^4.

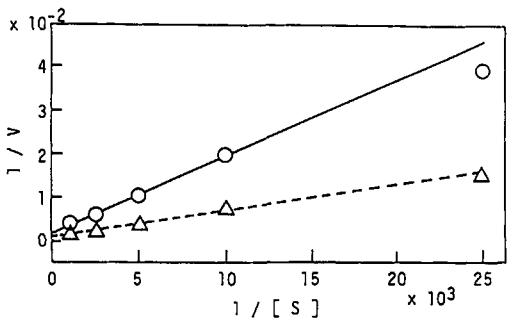

Figure 5 Lineweaver–Burk plots of the effect of concentration of soluble starch on the activity. Legend and note are the same in Figure 4.

Kinetic Constant

The relation between the concentration of substrate solution and the rate of enzyme reaction are shown in Figure 4. The molecular weight of the soluble starch was taken as 5 × 10^4. The enzyme reaction rate reduced with increase in concentration of substrate, the rate tending toward the maximum rate.

The Lineweaver–Burk plots[12] are shown in Figure 5. The maximum reaction rate (V) and the Michaelis constant (K_m) for the both native and immobilized α-amylase, calculated from the values of the Y and X intercepts in the figure, are shown in Table III. When α-amylase was immobilized on the spherical tannin resin, the maximum reaction rate was about 60% of that of native α-amylase, and the K_m constant increased about 2.1 times. That is to say, when the α-amylase was immobilized on the resin, the value of K_m increased by reason of immobilization of the enzyme, since the substrate solution and the reducing sugar formed had to diffuse through the resin.

Heat-Resistant Properties

In general, when an enzyme is immobilized on a support, it is expected that a decrease in its heat resistance is prevented.[13] The heat-resistant temperatures of the native and immobilized α-amylase were investigated (Fig. 6). The expected prevention

Table III Kinetic Constants

α-Amylase	V (×10^2, μmol/min/α-amylase mg)	K_m (×10^{-4} mol/L)
Native	8.89	4.62
Immobilized	5.56	9.88

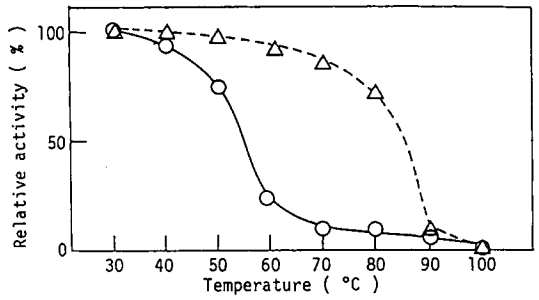

Figure 6 Heat stability. Legend and note are the same in in Figure 2.

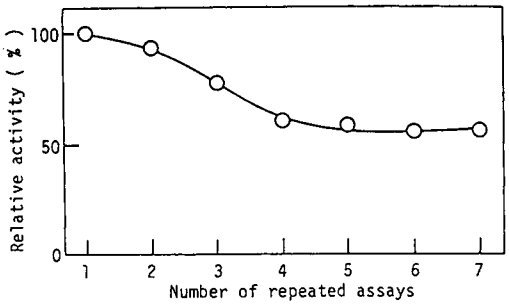

Figure 8 Effect of repeated assay on the activity of immobilized α-amylase. The activity obtained at first assay was taken as 100%.

of a decrease in heat resistance following immobilization did not occur. The influence of heat on the immobilized α-amylase was significantly greater than that on native α-amylase. While the detailed reasons for this are not certain, it may be thought that the change in the porousness of the tannin resin by heating interfered with the diffusion of the substrate through the resin. Since the tannins are bound to the proteins, it may be also thought that the supporting spherical tannin resin acts as a repressor of the enzyme activities.[14]

Stability during Storage

In general, if an enzyme is in solution, it is not stable during storage, and its activity is gradually reduced. Even in the dried state, enzymes are usually preferably kept in a refrigerator. The stability during storage of the α-amylase was investigated (Fig. 7). While the native α-amylase in solution completely lost its activity after 3 days, the immobilized α-amylase retained about 50% of its initial activity even after 30 days. Presumably this is due to the fact that the α-amylase is immobilized and in a concentrated state, and the movement of the immobilized enzyme is limited.

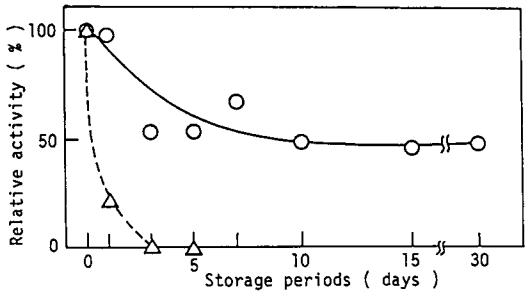

Figure 7 Storage stability. Legends are the same in Figure 2.

Activity during Repeated Use

In general, when enzyme reactions occur in solution, even if the active enzyme remains after the reaction, the desired constituent is removed and the residual enzyme is deactivated. Therefore, it is very important for the effectual use of an enzyme that it can be used repeatedly. We investigated the ability of the α-amylase immobilized on the spherical tannin resin to be used repeatedly. The results are shown in Figure 8. At the initial stage, the enzyme activity was decreased. However, after that it was independent on repeat number, i.e., 50% of its initial activity.

Activity during a Continuous Enzyme Reaction

It is important for the effectual use of an enzyme, as the means for the mass production of the desired product, that the enzyme reaction is continued. One of the problems in continuous enzyme reactions is the stability of the enzyme immobilized on the support. The stability during continuous reaction of the enzyme immobilized on tannin were investigated (Fig. 9). The enzyme activity decreased gradually

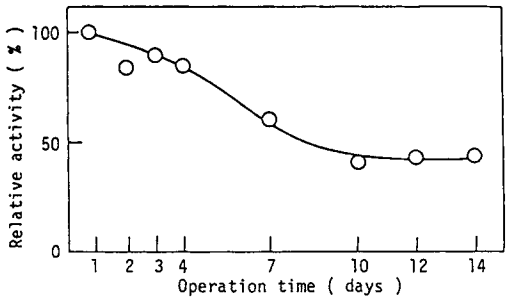

Figure 9 Continuous reaction for hydrolysis of starch with a column of α-amylase immobilized on the resin. The activity obtained on the first day was taken as 100%.

with time during continuous usage, but, even after continuous usage for 2 weeks, about 50% of the initial activity remained. No loss of the resin layer due to pressure during that period was detected by chromatography.

Comparison with Other Supports

The activity yield of the α-amylase immobilized to this resin was 25.9%. On the other hand, the yields of enzyme activities in the studies by Ono et al.[6] and Watanabe et al.[7] were from about 60 to 100% for naringinase and from about 20 to 30% for aminoacylase, respectively. But, the value of our study was greater than 2.2% of α-amylase immobilized to sephalose 4B[15] and was smaller than 85.8% of the enzyme immobilized to chitin.[16] It was thought that the properties of the α-amylase immobilized to this tannin resin were comparable to those of the enzyme immobilized onto various supports.[17-19]

CONCLUSION

The spherical tannin resins prepared by the reaction between Mimosa (*Acacia Mollissima,* Wattle) tannin and formaldehyde were examined for the possibility of use as a support to immobilize enzymes, by using α-amylase as the test enzyme.

The range of optimum pH was extended and the stability during storage was increased (except for a decrease of heat resistance) compared with the native α-amylase. Furthermore, while the maximum reaction rate and the Michaelis constant changed in the usual manner, the capability for repeated and continuous usage of the immobilized enzyme were improved, and the various normal properties of the enzyme were not significantly impaired. It was thought that the properties of the α-amylase immobilized to this tannin resin were comparable to those of the enzyme immobilized onto various supports.[17-19]

On the basis of these results, it may be concluded that this spherical tannin resin can be used for the support and immobilization of enzymes.

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