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STABILITY OF IMMOBILIZED FROG EPIDERMIS TYROSINASE

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Purified frog epidermis tyrosinase was immobilized on the following supports: CNBr-Sepharose 4B, Enzacryl AA, Enzacryl AH, Enzacryl Polythiolactone, and Enzacryl Polyacetal. The enzyme was active on all supports except when Enzacryl Polyacetal was used. The stability increased on immobilization. Enzacryl AA was the best support assayed. The Enzacryl AA–enzyme complex was 30- to 40-fold more stable to inactivation reaction than soluble enzyme, and maintained its activity when stored and assayed repeatedly. The immobilized enzyme on the other supports was also more stable than the soluble form. The pH–activity profile, thermal stability, storage stability, and the effect of protein concentration on activity of the immobilized enzyme have been studied. The properties observed for the immobilized enzyme were different than those of the soluble enzyme. The main reason for this difference could be due to enzyme modification through tyrosine groups of the enzyme; to conformational changes produced in the union to the matrix; and to microenvironmental differences created by the matrix.

INTRODUCTION

Monophenol monooxygenase (EC 1.14.18.1), also known as tyrosinase (1), is found throughout the animal and plant kingdoms (2). Frog tyrosinase is isolated in an inactive form but can be activated with trypsin (3). The enzyme catalyzes not only hydroxylation of monophenols but also the oxidation of the dihydroxy product to an *o*-quinone, which can undergo further nonenzymatic reactions. The enzyme is inactivated as the reaction proceeds (4).

It is known that immobilized enzymes may exhibit selectively altered chemical and physical properties as compared with soluble enzymes. Mushroom tyrosinase has been immobilized on DEAE-cellulose (5), modified, and subsequently adsorbed on collodion membrane (6) in order to stabilize the enzyme from "reaction inactivation," and to use it for synthesis of L-dopa. Wood and Ingraham (7) suggested that the reaction inactivation phenomenon is due to covalent attachment of the substrate or

products to the enzyme. Shinao et al. (8) demonstrated that inactivation is produced by a "vicinity effect," depending on the tyrosinase concentration present in the reaction.

We believe it is worthwhile to study the nature of this inactivation process. Our investigation is directed toward the use of the immobilization procedure as one approach to determine the major reasons for the process of enhancing the resistance of tyrosinase activity to the inactivation reaction.

MATERIALS AND METHODS

Materials

Trypsin (EC 3.4.4.4, 12,500 BAEE units/mg, type III) was purchased from Sigma Chemical Company (St. Louis, Missouri). Cyanogen bromide-activated Sepharose 4B was obtained from Pharmacia Chemicals (Sweden), and Enzacryl AA, Enzacryl AH, Enzacryl Polythiolactone, and Enzacryl Polyacetal from Koch-Light Laboratories (England). 3,4-Dihydroxy-L-phenylalanine (L-dopa) and all the other reagents were Merck Company analytical grade.

Extraction, Purification, and Activation of the Enzyme

All processes of extraction, purification, and activation of enzyme were carried out as previously published (9).

Immobilization on CNBr–Sepharose 4B

One hundred milligrams of CNBr–Sepharose 4B was placed into a syringe column of 0.9 cm inner diameter. The gel was washed with 125 ml of 0.001 M HCl in order to remove stabilizers. Then the gel was washed by passing 125 ml of 0.1 M phosphate buffer, pH 8, at a rate of 13 ml/h. Twenty milliliters of purified enzyme solution (1 μg/ml) in 0.1 M phosphate buffer, pH 8, was recycled through the column during 3 h at 25°C at a rate of 13 ml/h. Unreacted gel sites were blocked by passing through the column 250 ml of 1 M ethanolamine, pH 8, and finally the gel was washed with 100 ml 0.1 M phosphate buffer, pH 7, and stored in this buffer at 3°–5°C.

Immobilization on Enzacryl AA

One hundred milligrams of Enzacryl AA was placed into a syringe column (0.9 cm inner diameter) and suspended in 2 M HCl at 3°–5°C. Then

a mixture of 10 ml of 2 M HCl and 4 ml of 4% $NaNO_2$ was recycled through the column for 1 h at a rate of 30 ml/h.

The diazo-Enzacryl compound obtained was washed with 10 ml of 0.1 M phosphate buffer, pH 7. A 20 ml solution of enzyme (1 μg/ml) in the same buffer was recycled for up to 48 h. Thereafter, an ice-cold solution of 0.01% phenol in 10% sodium acetate was passed through the column to block unreacted sites. Finally, the gel was washed with 10 ml of 0.5 M phosphate buffer, pH 7, and suspended for later utilization in 0.1 M phosphate buffer, pH 7.

Immobilization on Enzacryl AH

Enzacryl AH (100 mg) was treated with nitrous acid exactly as described for the activation of Enzacryl AA. The Enzacryl azide thus generated was washed with 5 ml of 0.1 M phosphate buffer, pH 7. Twenty milliliters of 1 μg/ml enzyme solution in 0.1 M phosphate buffer, pH 7, was recycled through the column for up to 48 h. The gel was washed and stored in the same form as Enzacryl AA.

Immobilization on Enzacryl Polythiolactone

Enzacryl Polythiolactone (40 mg) was placed into a syringe column (0.9 cm inner diameter) and swollen with 0.1 M phosphate buffer, pH 7. Twenty milliliters of 1 μg/ml enzyme solution in 0.1 M phosphate buffer, pH 7, was recycled for 5 h at 3°–5°C at a rate of 5 ml/h. The gel was washed and stored as in previous procedures.

Immobilization on Enzacryl Polyacetal

One hundred milligrams of Enzacryl Polyacetal was placed into a syringe column of 0.9 cm inner diameter. Ten milliliters of 0.25 M HCl was recycled during 48 h at a rate of 5 ml/h. The gel was washed with 10 ml of 0.1 M phosphate buffer, pH 7. Twenty milliliters of 1 μg/ml enzyme solution in the same buffer was recycled during 15 h at 3°–5°C at the same flow rate. Finally, the gel was washed and stored as previously stated.

Enzymatic Activity Determination

For activity measurements, small columns were made (1.5 mm inner diameter by 4 mm length), each containing an immobilized enzyme derivative in the following quantities: 4 mg of E–Sph, or 2 mg of E–AA,

E–AH, E–PTL, or E–PA.[3] Assays were performed using the following buffers: 0.1 M phosphate, 0.1 M Tris–phosphate, 0.1 M bis–Tris–phosphate, and 0.1 Tes–phosphate, whose pH values ranged from 5 to 9.

For activity determination, a solution of L-dopa (2 mg/ml) in buffer, at constant pH and temperature, was passed through the column, without recycling, at a rate of 4 ml/h. The product formed in this reaction was recorded at 475 nm using a 0.1-ml flow cell in a Perkin–Elmer 402 spectrophotometer. The increase of absorbance per minute was used as the measure of activity. The time necessary to reach an absorbance value one-half of the maximum as the reaction continued, was referred to as "half-life."

After each assay, an enzyme activity control on the effluent was obtained to ascertain that there were no enzyme losses from the column.

Protein Determination

Protein content in solution was measured by the method of Hartree (10) using bovine serum albumin, fraction V (BSA) as a standard specimen.

RESULTS

Immobilized frog epidermis tyrosinase was active on all supports assayed except for Enzacryl Polyacetal. Enzacryl AA was the one that showed the highest activity. In all cases, immobilization was carried out using the same conditions of support weight and protein concentration.

pH–Activity Behavior of Immobilized Tyrosinase

The plot of activity versus pH of immobilized enzyme on different supports as compared with the unmodified enzyme using 0.1 M phosphate buffer as a medium, is shown in Fig. 1. The position of optimum activity was displaced to lower values with all supports assayed, except when E-Sph was used. The magnitude of the displacement was especially dependent on the hydrophilic nature of Enzacryl supports: The more hydrophilic the Enzacryl support, the greater was the displacement to lower values of pH. The results of Fig. 1 were also obtained when the buffer was changed, except when E-Sph was assayed in Tris-phosphate buffer, whose maximum activity was reached at pH 7. This value was the same as the other supports. The pH displacement was not dependent on the ionic strength of any of the buffers used.

[3]Abbreviations: L-dopa, 3,4-dihydroxy-L-phenylalanine; E–Sph, tyrosinase–CNBr Sepharose 4B derivative; E–AA, tyrosinase–Enzacryl AA derivative; E–AH, tyrosinase–Enzacryl AH derivative; E–PTL, tyrosinase–Enzacryl Polythiolactone derivative; E–PA, tyrosinase–Enzacryl Polyacetal derivative.

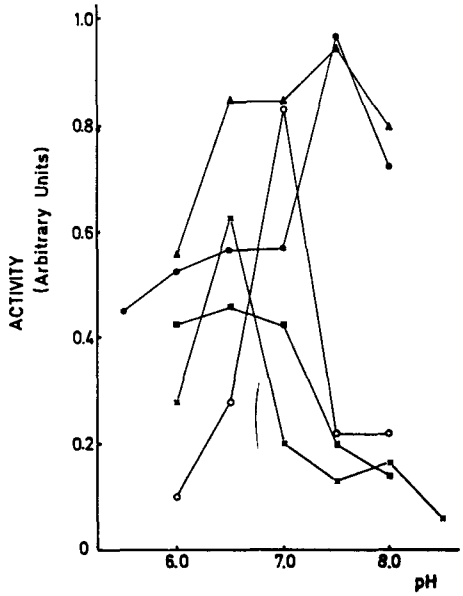

FIG. 1. Relative activity versus pH of soluble and immobilized enzyme using 0.1 M phosphate buffer. One microcolumn of immobilized enzyme was used for each assay to prevent reaction inactivation. The gel was previously stabilized at the pH corresponding to each assay, and assayed as described in Methods. ●, Soluble enzyme; ○, E-AA; ×, E-AH; ■, E-PTL; ▲, E-Sph.

Reaction Inactivation Stability

When an activity assay with soluble enzyme was performed, the enzyme lost activity as the reaction proceeded. Half-lifetimes of this inactivation process were found similar to those previously published (11).

When the substrate solution was passed continuously through the immobilized enzyme column, the absorbance due to product formation increased to a maximum, and then began to fall slightly when E-AA was employed. The inactivation rate was faster when other enzyme–support complexes were utilized.

As a measure of stability versus reaction inactivation, the parameter measured was the time required to decrease to half the maximum absorbance obtained when L-dopa was passed continuously through the column. Table 1 shows the times obtained when both soluble and immobilized enzymes on different supports were assayed at their optimum pH with two different buffers.

The following facts are noteworthy:

1. Immobilization of the enzyme shows a greater stability versus reaction

TABLE 1. **Half-Life Inactivation Times of Tyrosinase**

Enzyme form	Support	Half-life (min)	Buffer	pH
Soluble		4	Phosphate	7.5
		6	Tris	7.5
Immobilized	CNBr-Sepharose 4B	22	Phosphate	7.5
		69	Tris	7
	Enzacryl AA	150	Phosphate	7
		180	Tris	7
	Enzacryl AH	13	Phosphate	6.5
		26	Tris	7
	Enzacryl Polythiolactone	72	Phosphate	6.5
		85	Tris	7
	Enzacryl Polyacetal	0	Phosphate	7
		0	Tris	7

in all cases, except when the enzyme was coupled to Enzacryl Polyacetal.

2. The best stabilizing effect on the enzyme was obtained when Enzacryl AA was used as support (about 30- to 40-fold more stable than soluble enzyme).
3. This stabilization was lower when CNBr-Sepharose and Enzacryl AH were used.
4. Partially inactivated E-PTL could be reactivated by substrate.
5. When Tris-phosphate buffer was used, the times obtained for half-inactivation were always higher than those obtained with phosphate buffer.

When the immobilized enzyme was attached to Sepharose and Enzacryl AH, and assayed discontinuously, like the soluble enzyme it lost its activity as the number of assays increased. This loss of activity was not reversed when buffer was passed through the column and the derivative was quickly stored at 3°–5°C; but when this kind of assay was performed on E-AA, a slight increase in activity was observed. The activity was stabilized over a greater number of assays. When E–PTL was assayed, the activity at first increased, and thereafter a slow fall of activity was observed. Figure 2 shows the relative activity of the immobilized enzyme on different supports when buffer and substrate were passed alternately through the enzyme column. The assays were carried out by passing the buffer (for 15 min) and the substrate (for 5 min) through the column at a rate of 4 ml/h, and repeating this procedure several times.

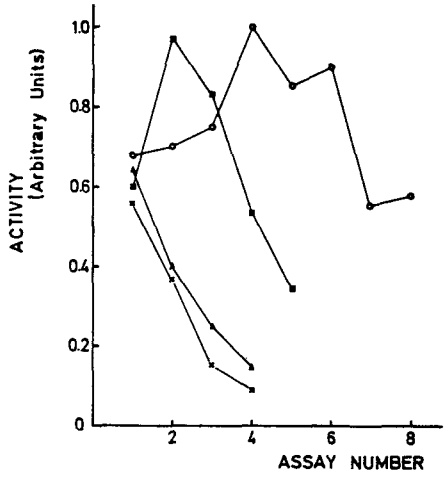

FIG. 2. Variation of the relative activity of immobilized enzyme when an alternative flow of 0.1 M phosphate buffer (pH of optimum activity) for 15 min and substrate for 5 min was passed through the column at a rate of 4 ml/h. ○, E-AA; ×, E-AH; ■, E-PTL; ▲, E-Sph.

Storage Stability

Tyrosinase immobilized on CNBr-Sepharose and Enzacryl AA was stable when stored in phosphate buffer at 3°–5°C for 100 days or more when it had not been previously assayed. When stored under the same conditions, E-AH and E-PTL gradually lost their activities. Only E-AA maintained its activity when stored and assayed repeatedly. The immobilized enzyme on the other supports lost activity when assayed many times and then stored. Loss of activity was faster for E-PTL than for E-AH and fastest for E-Sph. Figure 3 shows the relative activities of immobilized tyrosinase, both assayed and unassayed, versus storage time.

The unassayed immobilized enzyme was more stable than the assayed derivative, when stored under the same conditions. Only E-PTL was more stable after assay, perhaps because it was stabilized by its substrate.

Thermal Stability

Since E-AA had higher stability versus inactivation reaction, its stability was tested versus temperature. Figure 4 shows the relative activity of E-AA with incubation time at 70° and 90°C at pH 7. Although the enzyme was fully active in solution, after immobilization an increase of

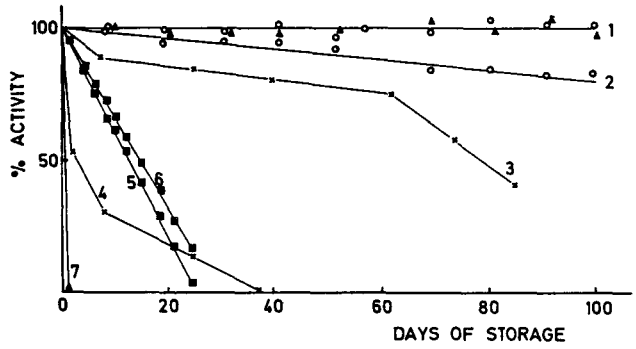

FIG. 3. Residual activity percentage of immobilized enzyme versus storage time. Unassayed enzyme signifies that a new column of unassayed immobilized enzyme was made for each assay, from a pool whose activity was referred to as 100%. Activity measures of assayed enzyme were realized on the same column and the same gel. Once the enzyme was immobilized, its activity was tested and referred to as 100%. Later, it was washed with the appropriate buffer and stored until the next assay, and so on. (1) E-AA and E-Sph both unassayed, (2) E-AA assayed, (3) E-AH unassayed, (4) E-AH assayed, (5) E-PTL unassayed, (6) E-PT; assayed, (7) E-SPH assayed.

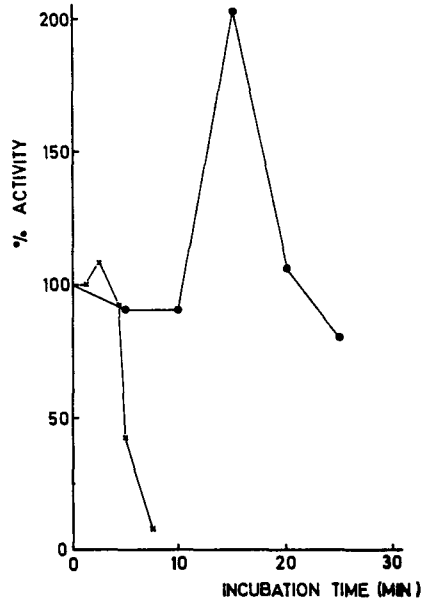

FIG. 4. Plot of activity of immobilized enzyme on Enzacryl AA versus incubation time at two temperatures: (●) 70°C and (×) 90°C.

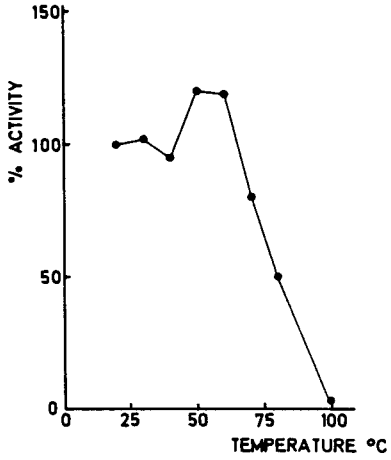

FIG. 5. Behavior of enzyme immobilized on Enzacryl AA incubated for 5 min at several temperatures.

activity was observed at 70°C. Exposure of immobilized enzyme at 90°C resulted in gradual loss of activity.

Figure 5 shows the behavior of E-AA versus temperature after a 5-min incubation period. This plot also shows that immobilized enzyme was thermally activated, reaching a maximum activity at 60°C.

After incubation, 0.1 M phosphate buffer, pH 7, was immediately passed through the column to stabilize the enzyme at 20°C, which was the temperature chosen for activity measurements.

Effect of Protein Concentration

Figure 6 shows the variation of activity of different amounts of immobilized enzyme on Enzacryl AA. Although the amount of immobilized enzyme was not determined, there was a direct relationship between enzyme offered and immobilized on a given amount of gel. As the graph shows, there was a linearity between activity and immobilized enzyme concentration. This indicates that inactivation of enzyme was not observed at increased concentrations. When the concentration of soluble enzyme to be immobilized was increased, a slight loss of permeability of the gel was observed. The assays were more difficult to carry out when higher concentrations of enzyme were immobilized.

DISCUSSION

Water-insoluble supports employed as carriers in this study were cyanogen bromide-activated agarose, and copolymers of acrylamide and

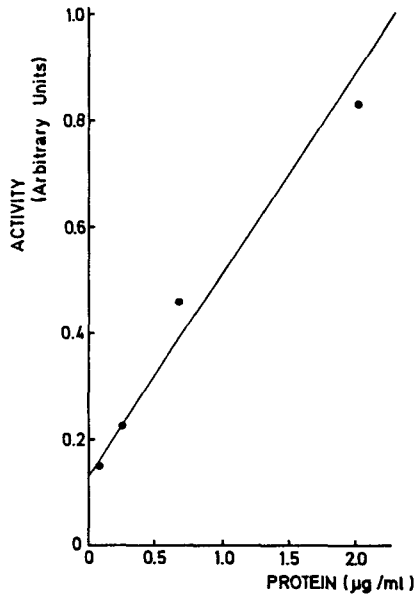

FIG. 6. Variation of the activity of immobilized enzyme on Enzacryl AA versus protein concentration.

various derivatives of acrylamide. These materials are electrically neutral. Thus, the observed changes in enzyme activity could have been produced by chemical modification of enzyme, or by the effect of the microenvironment that was imposed by the matrix on the enzyme (12, 13).

Although the supports were highly hydrophilic, the partially hydrophobic phenyl-containing Enzacryl AA was the best support assayed. The microenvironment which it imposes was responsible for maximum activity, reaction stability, and storage stability.

Just as peroxidase covalently bound to Sepharose (14) exhibits no changes in the pH optimum, there were no remarkable differences among the different buffers used with the immobilized tyrosinase: the pH optimum remained the same. But the nature of the buffer, in close relation with the nature of the support, was of significance in activity. Since soluble enzyme had a good activity in all buffers assayed and immobilized enzymes exhibited wide differences, depending on the nature of buffer, the microenvironment is very important in the activity.

Factors pertaining to the chemically modified tyrosinase that have a profound influence on the reaction stability are local and net charges, conformation changes, and transformation of amino acid residues of the enzyme (15).

In this manner, the conformational changes produced when the enzyme was linked to Sepharose and Enzacryl AH by means of ε-amino groups of lysine, must be more stable to reaction inactivation than the soluble enzyme. But the highly hydrophilic and polar microenvironment, in which the enzyme acts, destabilizes the enzyme. Also, the residues of the gel must be able to unite with the products of enzymatic catalysis, since these gels remain strongly colored after reaction, and the products, immobilized near to the enzyme, could produce a change in local enzyme microenvironment which might involve a loss of activity. On the contrary, in the case of tyrosinase immobilized on Enzacryl AA, there was a chemical modification at the level of tyrosine residues of enzyme and a more hydrophobic microenvironment of the gel that seemed to produce an enzyme complex which did not lose its activity as the reaction proceeded. There were no changes in the color of the gel, and the activity remained constant during storage.

The behavior exhibited when Enzacryl Polythiolactone was used as carrier for enzyme immobilization was different than that observed with E-Sph and E-AH. Enzacryl Polythiolactone is also a hydrophilic matrix. When the enzyme was attached to this gel, the enzyme lost its activity during storage when unassayed. But the enzyme partially recovered its activity progressively as the reaction continued. The reaction reached a maximum, and then declined when it was assayed, in contrast with other gels that lost their activity when repeatedly assayed. The reason for this kind of behavior could be due to the existence of a thiol group close to enzyme location, which could change its microenvironment in such a way that enzymatic behavior would be altered.

When the gel had many linkages through amino groups on the enzyme, i.e., Enzacryl Polyacetal, the conformational changes produced were highly unfavorable and the tyrosinase was not active.

The effect of temperature is likely to be a reflection of two competing phenomena: an increase in enzymatic activity by conformational change due to increasing temperature, and the loss of activity due to thermal denaturation at high temperatures. However, it was apparent from the rate of increase of activity and the temperature of maximum activity, that the linkage of tyrosinase to the Enzacryl AA matrix caused increased thermal stability, as compared with the soluble enzyme (16).

Contrary to the interpretation of Shinao et al. (8) regarding the reaction inactivation mechanism in soluble mushroom tyrosinase, immobilization of increasing amounts of tyrosinase on the same weight of Enzacryl AA showed that there exists a linear correspondence between activity and the amount of immobilized enzyme, over the concentration range assayed.

It was therefore concluded that the stability of the immobilized frog epidermis tyrosinase to inactivation reaction could be due mainly to enzyme modification through tyrosine groups; to conformational change produced in the union to the matrix; and to the microenvironment created by the matrix.

It is possible the Enzacryl AA could create a microenvironment like that of the enzyme in its natural state. This supposition is valid only if the enzymatic activity is maintained *in vivo* for a long time. But, if the opposite effect were desirable *in vivo*, a hydrophilic natural microenvironment close to that of Sepharose, Enzacryl AH, or Enzacryl Polythiolactone could be adequate.

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