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Preparation and Properties of Immobilized Pig Kidney Aminoacylase and Optical Resolution of *N*-Acyl-DL-Alanine

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Abstract

Aminoacylase (EC 3.5.1.14) was immobilized into DEAE-Sephadex A-25 by ion-exchange absorption for optical resolution of *N*-acyl-DL-alanine. The effects of pH, temperature, and Co^{2+} concentration on the activity of free and immobilized enzymes were investigated along with the operational and the thermal stability of the immobilized enzyme. The immobilized enzyme retained high catalytic activity. The optimum pH and temperature for the hydrolysis of *N*-acyl-L-alanine in the DL-isomer mixture were 8.0 and 65°C, respectively. Co^{2+} was an activator for the immobilized enzyme in a similar role as for the free enzyme. No significant loss of activity was observed for at least 300 h of continuous operation. The yield of L-alanine was about 70% of the theoretical yield. The immobilized aminoacylase column decayed over a very long period of operation, but could be completely reactivated by regeneration.

Index Entries: Aminoacylase; pig kidney; immobilization; optical resolution.

Introduction

Immobilized enzymes have been extensively used for the industrial production of L-amino acids and for industrial and medical analysis. The immobilization of enzymes in insoluble supports has been a topic of active research in enzyme technology for many years *(1)*. Chibata et al. *(2)* previously reported on immobilized aminoacylase from *Aspergillus*. Lee et al. *(3)* recently reported on the covalent immobilization of aminoacylase to

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alginate for L-phenylalanine production, as well as on the immobilization of aminoacylase by encapsulation in poly-L-lysine-stabilized calcium alginate beads *(4,5)*. The immobilized D-aminoacylase of *Alcaligenes faecalis* DA1 for optical resolution of *N*-acyl-DL-amino acids has also been investigated *(6)*. However, previous investigators have largely concentrated on immobilized enzymes from microbiological sources. This article presents the properties of immobilized pig kidney aminoacylase and the optical resolution of *N*-acetyl-DL-alanine.

Materials and Methods

Aminoacylase I was prepared from pig kidney according to the procedure of Birnbaum *(7)* to the step of acetone fractionation. The enzyme proportion was used for the production of immobilized aminoacylase. The preparation of immobilized pig kidney aminoacylase was carried out according to the method previously described by Chibata et al. *(3)*. DEAE-Sephadex A-25 was from Pharmacia, and the other chemicals were local products of analytical grade.

Enzyme concentration was determined as described by Lowry *(8)*. Enzyme activity was determined using the ninhydrin method to monitor the formation of L-alanine from the hydrolysis of *N*-acyl-DL-alanine as previously described by Chibata et al. *(2)*. The assay of native enzyme was carried out as follows. A reaction mixture of 0.3 mL of 0.1*M* phosphate buffer (pH 7.0), 0.3 mL of 0.1*M* *N*-acetyl-DL-alanine (pH 7.0) containing 1.5 m*M* Co^{2+}, and 0.3 mL of aminoacylase solution was incubated at 37°C for 30 min. Then 0.9 mL of 0.1*M* acetate buffer (pH 5.6) and 0.9 mL of ninhydrin solution were added to the reaction mixture. The mixture was heated in boiling water for 15 min, then immediately chilled to room temperature. Next 2.7 mL of 60% ethanol were added to the mixture, and the absorption of the mixture was measured at 570 nm. A standard calibration curve was used to determine the aminoacylase activity, which was expressed in micromoles of L-alanine liberated per hour.

The immobilized enzyme assay was carried out as follows. A reaction mixture of 3.0 mL of 0.1*M* phosphate buffer (pH 7.0), 3 mL of 0.1*M* L-acetyl-DL-alanine (pH 7.0) containing 1.5 m*M* Co^{2+}, 3 mL of water, and 10 mg of the immobilized enzyme was incubated at 37°C with shaking. After reacting for 30 min, 0.3 mL of suspension was used for measuring the quantity of L-alanine liberated per hour.

All absorption measurements were carried out with a Perkin Elmer Lambda Bio U/V spectrometer at 25°C.

Results

Optimal Reaction pH

Figure 1 shows the effects of pH on the activities of free and immobilized enzymes. The optimal pH of the immobilized enzyme was 7.5, which

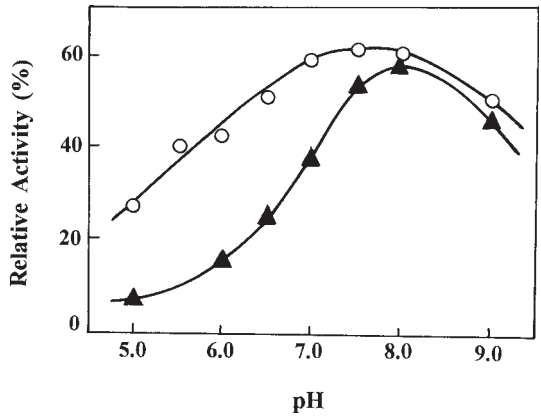

Fig. 1. Effects of pH on the activities of free (▲) and immobilized (○) aminoacylase. The activities of the free and immobilized enzymes were determined by the ninhydrin method under the assay conditions described in Materials and Methods, except for the pH. Final concentrations of the free and immobilized enzymes in the assay system were 0.3 μg/mL and 1 mg/mL, respectively.

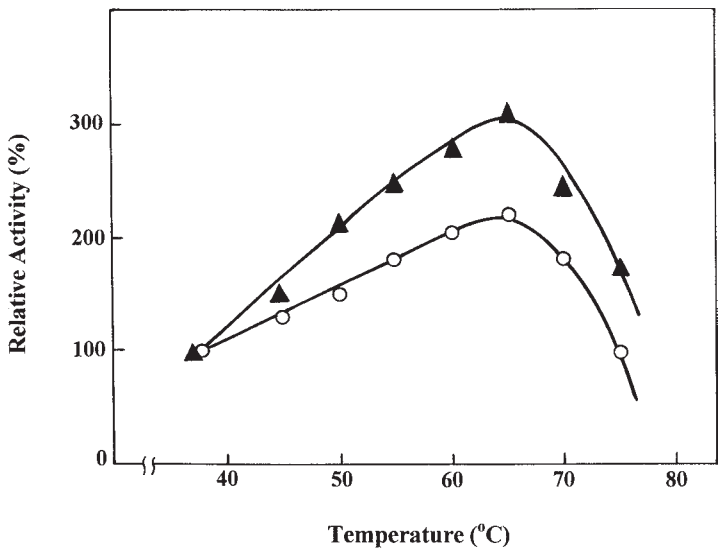

Fig. 2. Effects of temperature on the activity of free (○) and immobilized (▲) aminoacylase. Experimental conditions were as for Fig. 1 expect for the temperatures.

was about 0.5 pH value lower than that of the free enzyme. Moreover, the range of optimal pH for the immobilized enzyme was markedly larger than that of the free enzyme.

Optimal Reaction Temperature and Thermal Stability

Investigation of the temperature dependence of the activities of free and immobilized enzymes (*see* Fig. 2) showed that the optimal reaction

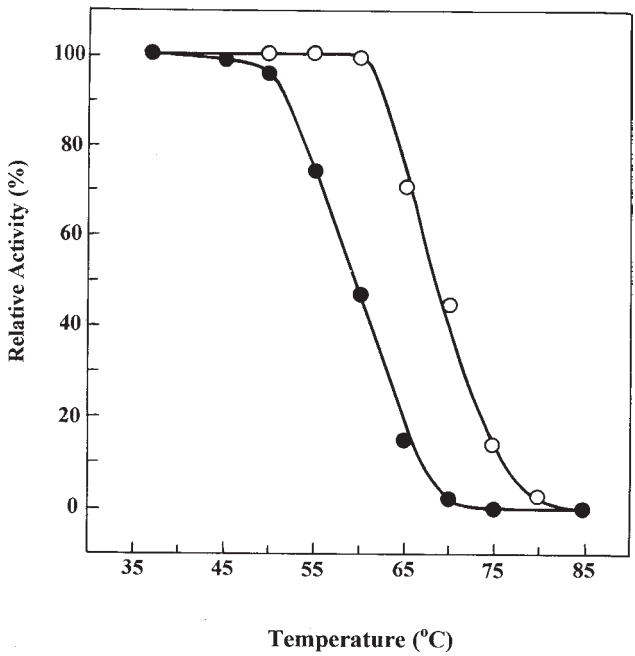

Fig. 3. Thermal stability of free (●) and immobilized (○) enzymes. Experimental conditions were as for Fig. 1. The free and immobilized enzymes were incubated at different temperatures for 10 min before measuring the activity.

temperatures for both free and immobilized enzymes were 65°C. The activity of the free enzyme at the optimal temperature was twice the activity at 37°C, whereas the activity of the immobilized enzyme was three times the activity at 37°C.

The thermal stability of free and immobilized enzymes was studied by treating these enzymes at various temperatures for 10 min, then immediately chilling the enzymes to 4°C. The remaining activities were determined by the ninhydrin method. The results in Fig. 3 show that the free enzyme is nearly completely inactivated after treatment at 65°C, whereas the immobilized enzyme still retains its full original activity, indicating that the thermal stability of the immobilized enzyme is greater than that of the free enzyme. In addition, the kinetic courses of thermal inactivation of free and immobilized enzymes at higher temperatures were also followed. Figures 4 and 5 show the thermal inactivation courses of free and immobilized enzymes at 70°C. The semilogarithmic plots give straight lines, indicating that thermal inactivation of both free and immobilized enzymes are both first-order reactions. The thermal inactivation rate constants can be obtained from the slopes of the straight lines. Table 1 summarizes the thermal inactivation rate constants at 70 and 72°C.

The half-life of the immobilized enzyme at 30°C was 15 d. When stored at 4°C without bacteria, the immobilized enzyme was stable for more than 2 mo without any significant loss of activity.

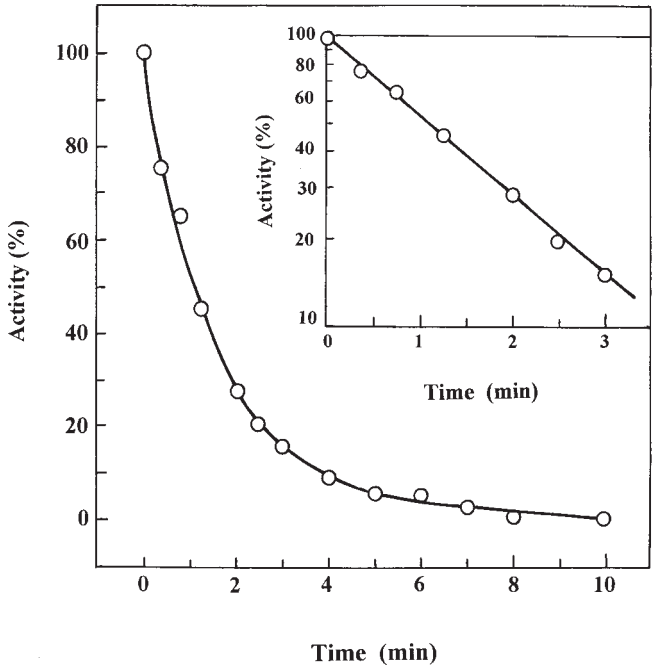

Fig. 4. Kinetic course of thermal inactivation of free aminoacylase at 70°C. Experimental conditions were as for Fig. 1. The inset represents a semilogarithmic plot.

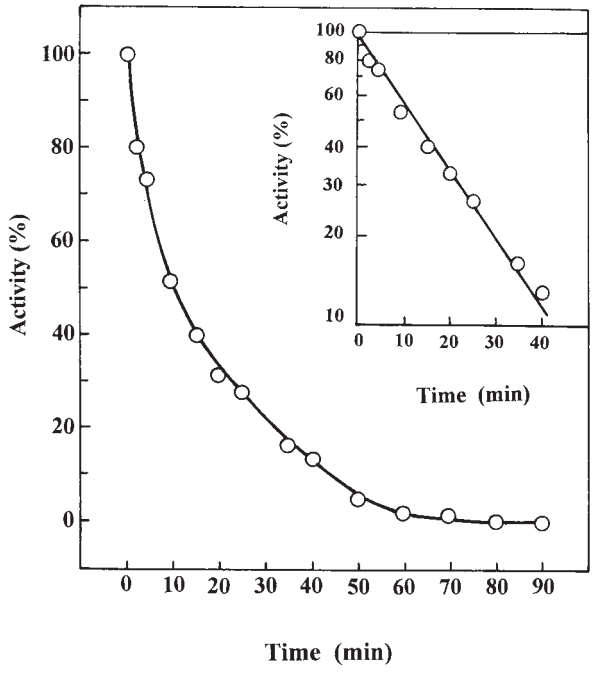

Fig. 5. Kinetic course of thermal inactivation of the immobilized enzyme at 70°C. Experimental conditions were as for Fig. 1. The inset represents a semilogarithmic plot.

Table 1
Apparent Rate Constants
of Thermal Inactivation of Free and Immobilized Aminoacylase

Temperature (°C)	Apparent rate constants of thermal inactivation (S^{-1})	
	Free enzyme	Immobilized enzyme
70	10.5×10^{-3}	0.90×10^{-3}
72	19.0×10^{-3}	2.26×10^{-3}

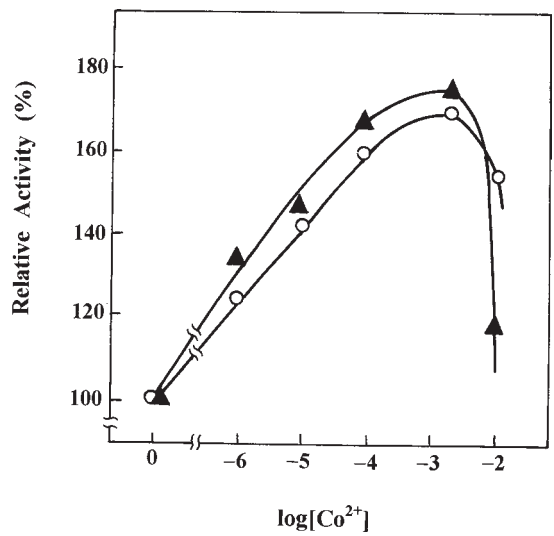

Fig. 6. Effects of Co^{2+} concentration on the activities of free (▲) and immobilized (○) aminoacylase. Experimental conditions were as for Fig. 1 except for the addition of different Co^{2+} concentrations into the reaction system.

Effects of Co^{2+} Concentration on Activity of Free and Immobilized Enzymes

Figure 6 shows the effects of Co^{2+} concentration on the activity of free and immobilized enzymes. The results show that Co^{2+} activated both free and immobilized enzymes. The optimal Co^{2+} concentrations range between 10^{-4} and $10^{-3}M$.

Effects of Denaturants on Activity of Free and Immobilized Enzymes

Sodium dodecyl sulfate (SDS) and urea are protein denaturants commonly used to study the unfolding and inactivation of enzymes. Figure 7 shows the effects of SDS concentration on the activity of free and immobilized enzymes. It can be seen that the extents of inactivation

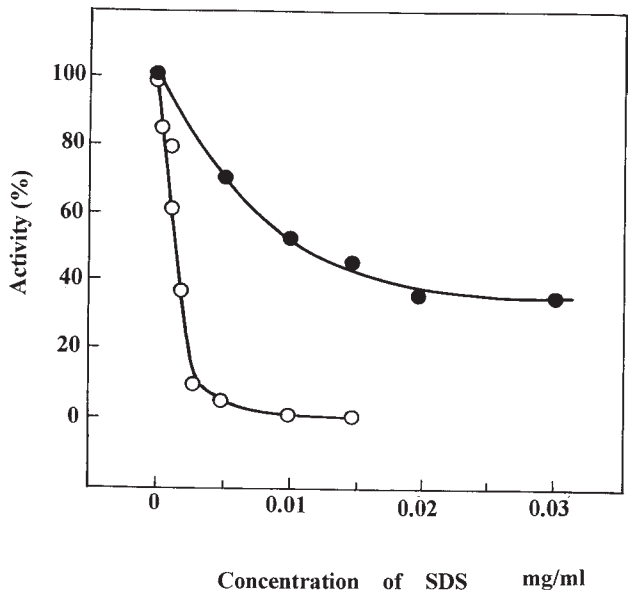

Fig. 7. Effects of SDS concentration on the activities of free (○) and immobilized (●) aminoacylase. Experimental conditions were as for Fig. 1 except that the reaction systems contained different SDS concentrations.

of both free and immobilized enzymes increased with increasing SDS concentrations. However, at low concentrations of SDS, <0.01 mg/L, free enzyme was completely inactivated, but immobilized enzyme still retained most of its original activity. This result suggests that the stability of immobilized enzyme in SDS solution is much greater than that of free enzyme.

The effects of incubation time in 3*M* urea solution on the activity of free and immobilized enzyme (*see* Fig. 8) were similar. For the same incubation time, the extent of inactivation of the free enzyme was greater than that of immobilized enzyme, indicating that the immobilized enzyme has more stability against urea denaturation.

Operational Stability of Immobilized Enzyme

The operational stability of the immobilized aminoacylase was studied by continuously applying 20 mL/h of 0.2*M* *N*-acetyl-DL-alanine solution in 0.1*M* phosphate buffer (pH 7.0) to the enzyme column at 40°C. No significant loss of activity was observed for at least 300 h of continuous operation. After 20 d of continuous operation, the immobilized enzyme still maintained >90% of the original activity (Fig. 9). The L-alanine yield was about 70% of the theoretical yield. After 40 d, the activity of the immobilized enzyme decreased to 60–70% of the original activity, but the immobilized enzyme column could be completely reactivated by regeneration.

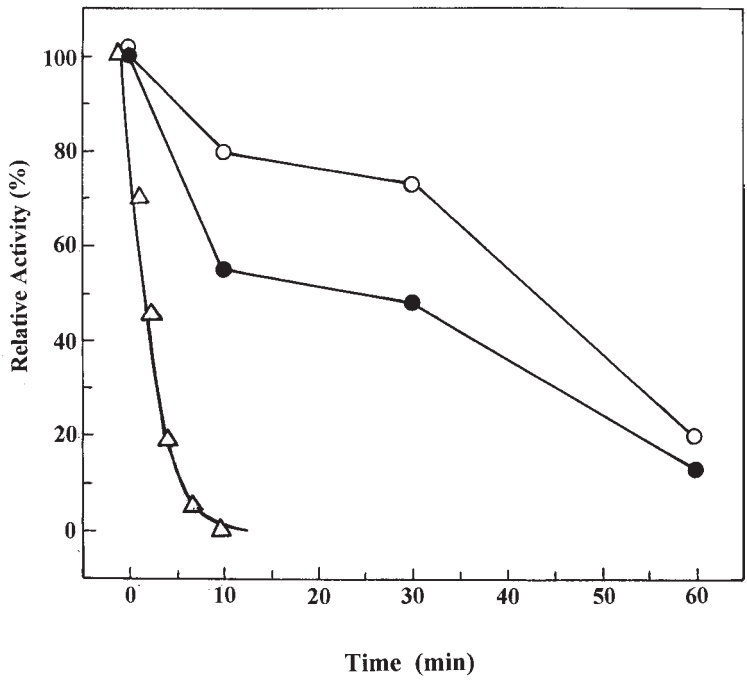

Fig. 8. Effect of incubation time in urea solutions on activity of immobilized aminoacylase. Experimental conditions were as for Fig. 1 except that the reaction mixtures contained urea. The urea concentrations for inactivation of the immobilized enzyme were 1*M* (○) and 3*M* (●). Curve (△) represents the inactivation course of the free enzyme in 3*M* urea.

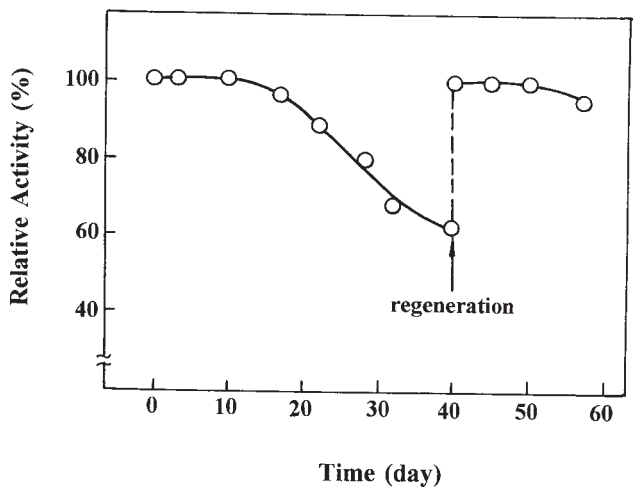

Fig. 9. Stability and regeneration of immobilized aminoacylase column. A solution of 2*M* *N*-acetyl-DL-alanine (pH 7.0, containing 0.1 m*M* Co^{2+}) was continuously applied to the column (13.5 × 1 cm) at 40°C at a flow rate of 20 mL/h for an extended period. The activity was determined by measuring the concentration of L-alanine in the effluent.

Discussion

The present study shows that immobilized pig kidney aminoacylase provides an efficient system for enzyme immobilization. The optimal pH results show that the optimal pH of the immobilized enzyme was greater than that of the free enzyme, indicating that the pH stability of the immobilized enzyme was increased. The effects of Co^{2+} concentration on the activity of the free and immobilized enzymes were identical. Their optimal reaction temperatures were also identical, but the activity of the immobilized enzyme at the optimal temperature was higher than that of the free enzyme. The similarity in the results suggests that the immobilization of the enzyme does not lead to significant conformational changes of the enzyme molecules.

The results from the thermal inactivation kinetics and thermal stability studies show that the immobilized enzyme was more thermally stable than the free enzyme. The immobilized enzyme was also more stable in denaturant solutions than the free enzyme. These two results suggest that the microenvironment of the enzyme molecule in the immobilization system increased the stability of the enzyme molecule conformation.

The operation stability at 40°C of the immobilized enzyme was much better than the storage stability at 30°C, indicating that the presence of the substrate protected the enzyme from denaturation. This preparation can be used in the laboratory-scale equipment for continuous preparation of L-amino acids from *N*-acyl-DL-amino acids.

References

1. Mattiasson, B. (1983), in *Immobilized Cell and Organelles*, vol. 1, Mattiason, B., ed., CRC Press, Boca Raton, FL, pp. 3–5.
2. Chibata, I., Tosa, T., Sato, T., and Mori, T. (1976), *Methods Enzymol.* **44(51),** 746–759.
3. Lee, P. M., Lee, K. H., and Siaw, Y. S. (1993), *J. Chem. Tech. Biotechnol.* **58,** 65–70.
4. Lee, P. M., Lee, K. H., and Siaw, Y. S. (1992), *J. Chem. Tech. Biotechnol.* **54,** 375–382.
5. Lee, P. M., Lee, K. H., and Siaw, Y. S. (1993), *J. Chem. Tech. Biotechnol.* **57,** 27–32.
6. Tsai, Y. C., Lin, C. S., Tseng, T. H., Lee, T., and Wang, Y. J. (1992), *Enzyme Microb. Technol.* **14,** 384–389.
7. Birnbaum, S. M. (1955), *Methods Enzymol.* **2,** 115–119.
8. Lowry, O. H., Rosebrough, N. T., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.