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Some Kinetic Parameters and Inactivation of Catalase Immobilized on Modified Polyvinylalcohol

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ABSTRACT

Polyvinylalcohol crosslinked with terephthaldicarboxaldehyde and was modified with 2-amino-4,6-dichloro-s-triazine. Optimum conditions for immobilization of catalase on modified and gelatine-coated modified polyvinylalcohol were investigated. Activity variations with respect to pH, temperature, stability behavior, and K_m(appl) values were investigated for the native and immobilized catalases. Rate constants for H_2O_2 decomposition and for inactivation of immobilized catalase were determined using a discontinuous batch-type reactor. The influence of H_2O_2 concentration on the catalase inactivation was investigated.

Index Entries: Immobilized catalases; polyvinylalcohol; kinetic parameters; batch-type reactors; H_2O_2.

INTRODUCTION

In the field of food storage, use of some additives may be necessary, depending on the conditions employed. In the dairy industry, use is made of H_2O_2 for cold pasterization in order to prevent microbial pollution of milk on its way to processing centers (*1,2*). The World Food and Agriculture organization and United Nations Agriculture Organization permit addition of H_2O_2 to milk at a rate of 0.025–0.05%, on the condition that all of the H_2O_2 remaining in the milk after processing is converted with catalase into O_2 and H_2O. However, direct use of native catalase for

this purpose increases the cost. Various immobilization methods employing physical and chemical approaches have been tried on different supports, in order to render the application of catalase in the dairy industry more economical (*3–7*).

The main difficulty encountered in reactor applications of immobilized catalase is inactivation of catalase because of the H_2O_2 concentration (*8*). It has been determined that inactivation of immobilized catalase by H_2O_2 proceeds in parallel with the decomposition of H_2O_2 (*9*).

MATERIALS AND METHODS

Catalase (from Pilz EC 1.11.1.6), K_2HPO_4, KH_2PO_4, H_3PO_4, H_2O_2 (30%), Na_2CO_3, CH_3COONa, trichloro-*s*-triazin, polyvinylalcohol (PVA), glutaraldehyde (GDA), formaldehyde, and terephthaldicarboxaldehyde were obtained from E. Merck (Darmstadt, FRG). All other chemical materials used were of analytical grade. Adsorption values of H_2O_2 were measured on a UV spectrophotometer (Unicam SP 800).

Preparation of Supports

Crosslinking of polyvinyl alcohol (PVA) was achieved by terephthaldicarboxaldehyde (*10*). PVA (5 g) was dissolved in 200 mL of water at 80–90°C. After cooling, 0.76 g of terephthaldicarboxyaldehyde and 2 mL of conc. HCl were added to the solution and stirred for 3 d. The crosslinked polymer mass was filtered, washed with water and methanol, and dried in a vacuum over at 40°C. This product was ground and fractioned to obtain 0.1- to 0.2-mm particles.

2-Amino-4,6-dichloro-*s*-triazin (AsT) used in the initial modification of crosslinked PVA was obtained by reacting a solution of trichloro-*s*-triazin in toluen/dioxane (20/100) at 15°C with gaseous ammonia while passing through nitrogen gas (*11*).

Crosslinked PVA (3 g) was preswelled at 50°C in a water/acetone mixture(1/1) in which AsT (1.1 g) had been dissolved. Fifteen milliliters of a 15% Na_2CO_3 solution containing 0.6 vol of 1*M* HCl was added at constant temperature to the reaction vessel through a side inlet over a period of 5 min. The pH was adjusted to 7.0 by addition of 2 mL concentrated HCl over a period of 10 min. The modified PVA was washed consecutively with aqueous acetone solutions ranging from cold to hot, in which the acetone concentration was gradually decreased.

Three gelatine was dissolved in 30 mL of 0.1*M* phosphate buffer (pH 8.0) heated to 60°C, and 2-amino-4-chloro-*s*-triazinyl derivatives of crosslinked PVA were added. Two hours later 0.3 mL of formaldehyde was added to the reaction mixture. The mass obtained was dried in an aspirated over drier. Gelatin-coated PVA was ground lightly and fractionated to

obtain 0.20- to 0.25-mm particles. Modified PVA and gelatine-coated modified PVA will be referred to below as preparation I and preparation II.

Immobilization of Catalase

A series of immobilization reactions were conducted with varying amounts of catalase prepared in 50 m*M* phosphate buffer (pH 7.0) in order to determine the support/enzyme ratio that provides the best activity (Fig. 1). Immobilizations were carried out overnight with 30 mg of support and varying amounts of catalase in a total volume of 3 mL at +4°C. To the reaction mixture was added 0.1 mL of GDA for immobilizations carried out with preparation II.

Immobilized catalase products were washed initially with KCl solutions of increasing and then decreasing ionic strengths prepared in phosphate buffer, and then with double-distilled water, until no absorbtion at 280 nm could be detected in the effluent. The amount of bonded catalase was determined by protein analysis of the washing, according to the Lowry method (*12*).

Measurement of Catalase Activity

The activity of catalase was calculated by measuring the period of time necessary for the absorption value at 240 nm of a 10.5 m*M* H_2O_2 solution prepared in 50 m*M* phosphate buffer (pH 7.0) to drop from 0.450 to 0.400 (*13*). Immobilized catalase preparations (30 mg) were incubated in 10-mL substrate solutions for 1 min; at the end of this period the immobilized catalase was removed quickly by vacuum filtration. The activity of immobilized catalase was calculated from the absorbtion value at 240 nm from the unreacted H_2O_2 present in the filtrate. The activity of native catalase was found to be 3712 IU/mg.

RESULTS

In order to find the optimum support/catalase ratios, a series of immobilizations were performed with 30 mg of modified support and varying amount of catalase (0.25–1.5 mg). Depending on the concentration of catalase, the activity first showed an increase up to a certain value and then decreased slowly. From the maximum activity values, optimum ratios for 30-mg modified supports designated I and II have been found to be 0.60 and 0.85 mg catalase, respectively.

Variation of Activity and Stability with pH

Activity variations of immobilized catalase preparations were investigated at 27°C and different pH values in 50 m*M* acetate (pH 4.5), phosphate (pH 5.5, 6.5, 7.0, 7.5, 8.0), and borate (pH 9.0) buffers.

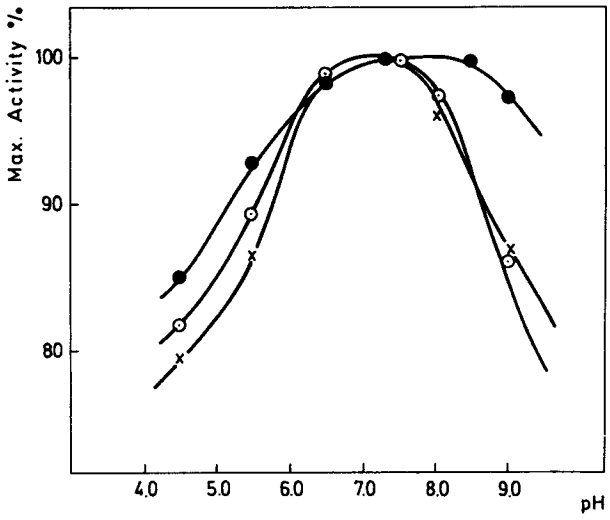

Fig. 1. The pH-activity profiles for the native and immobilized catalases. $|H_2O_2| = 10.5$ m*M*; 27°C. Native catalase (x); preparations I (⊙) and II (●).

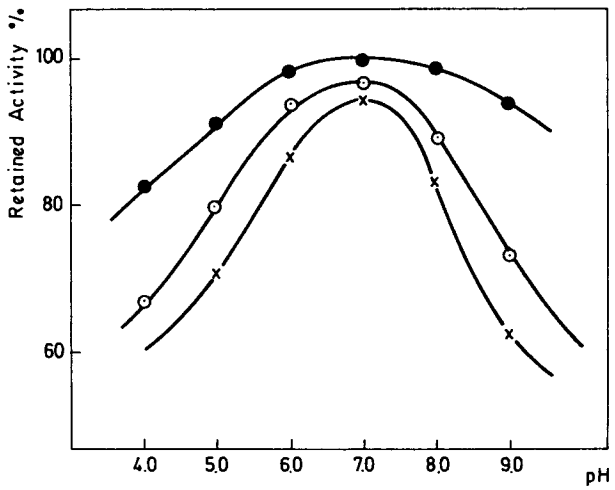

Fig. 2. The pH stability of native and immobilized catalases at 27°C for 18 h. Native catalase (x); preparations I (⊙) and II (●).

As can be seen from Fig. 1, native catalase showed a maximum activity in the pH 6.7–7.5 range. Immobilized catalase, designated I, shows activity variations similar to that of native catalase. With preparation II the optimum pH has shifted to the alkaline region and the bell-shaped curve of pH vs activity has become broader.

Retained activity values of native and immobilized catalase samples after incubation in 50 m*M* buffer of different pH values at 27°C for 18 h are shown as measured at pH 7.0 (Fig. 2).

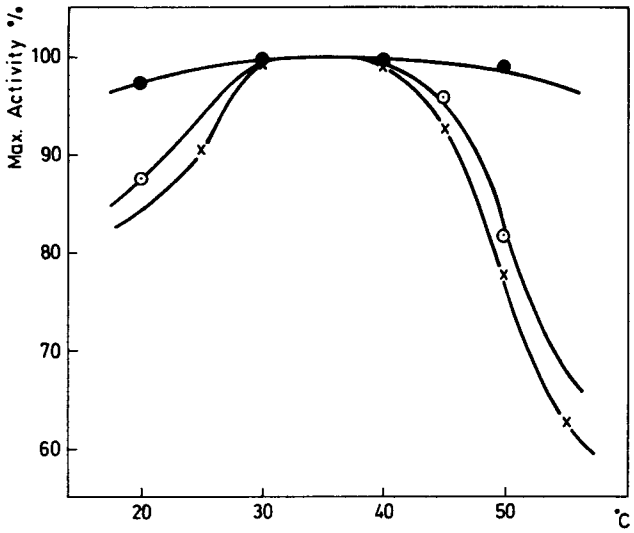

Fig. 3. Temperature-activity profiles for the native and immobilized catalases. $|H_2O_2| = 10.5$ m*M*, pH 7.0. Native catalase (x); preparations I (⊙) and II (●).

Immobilized catalase preparations were found to be more stable than native catalase, preparation I having better stability. The temperature sensitivity of immobilized catalase preparations, especially that of preparation II, was found to be smaller when compared to native catalase.

Activity and Stability Variations with Temperature

Variations of activity in relation to temperature at 20, 30, 40, and 50°C were investigated at pH 7.0 (Fig. 3). The optimum temperature for native catalase was found to be in the 30–37°C range. Thermal stabilities of native and immobilized catalase preparations were investigated by measuring the retained activity after incubation at 20, 30, 40, and 50°C in 50 m*M*. phosphate buffer (pH 7.0) for 15 h. It can be seen from Fig. 4 that the immobilized catalase shows a more stable behavior than the native catalase.

Variation of Activity Depending on H_2O_2 Concentration

Variation was investigated for samples at 2.5, 4.0, 10.5, 30, and 40 m*M* H_2O_2 prepared in 50 m*M* phosphate buffer. The K_m(app) value for native catalase and for preparations I and II were found to be 20, 22, and 40 m*M*, respectively, from the Lineweaver-Burk diagram.

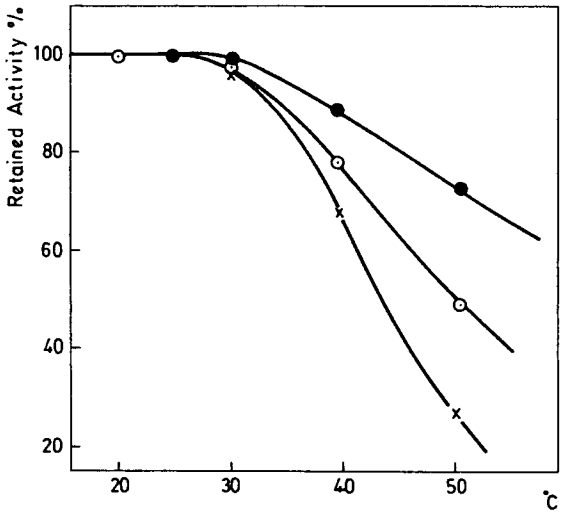

Fig. 4. Thermal stabilities of the native and immobilized catalases at pH 7.0 for 15 h. Native catalase (x); preparations I (⊙) and II (●).

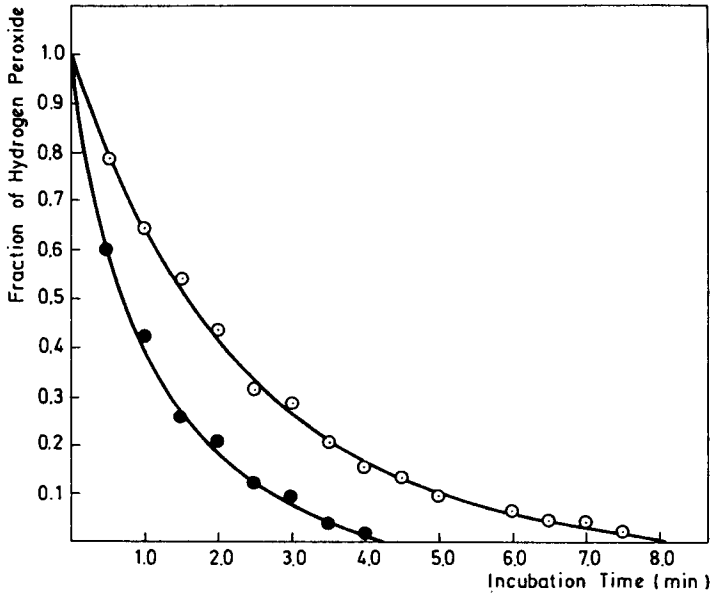

Fig. 5. Hydrogen peroxide decomposition effected by immobilized catalases in a batch-type reactor at 27°C, pH 7.0. Preparations I (⊙) and II (●).

Processing Stability in Discontinuous Batch-Type Reactor

The processing stability of immobilized catalase preparations was investigated in a 20-mL, constant volume, discontinuous batch-type reactor. The reactions of 120 mg of preparation I and preparation II with 10.5 m*M* H_2O_2 in such a reactor were monitored at 30-s intervals (Fig. 5).

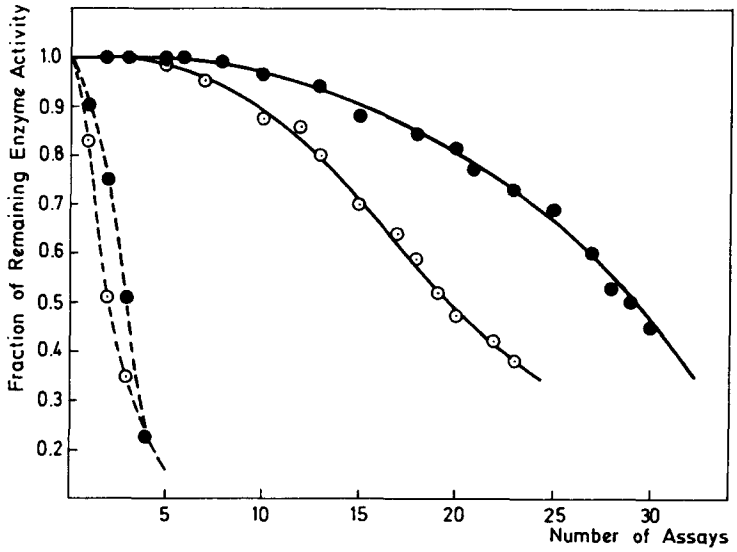

Fig. 6. Effect of H_2O_2 concentration on the inactivation of immobilized catalase, at 27°C, pH 7.0. (H_2O_2) = 10.5 m*M* (——); 100 m*M* (----); preparations I (⊙) and II (●).

The time necessary for the complete decomposition of H_2O_2 in the reactor was determined and the process repeated to determine the effect of H_2O_2 concentrations (10, 5, and 100 m*M*) on the inactivation of catalase. The times necessary for the activity of immobilized catalase preparations I and II to be reduced by half with 10.5 m*M* H_2O_2 were found to be 158 and 121.8 min, respectively, whereas for 100 m*M* H_2O_2 these values were 12.5 and 16 min, respectively (Fig. 6).

DISCUSSION

The structural properties of support materials were changed as a result of the modifications applied to PVA; hence, the amounts of catalase necessary for immobilization under optimum conditions were found to be different for each support. When catalase was bonded to the support in amounts in excess of the optimum support/catalase ratio, it led to a reduction in the activity in consequence of sterical interactions. Some data related to the immobilized catalase preparations are given in Table 1. The amount of active bonded catalase given in Table 1 is the value determined by comparison to the activity of the total bonded catalase that would have been measured in solution.

However, the percentage of retained activity of preparation I in which crosslinking was effected close to the support matrix was found to be low compared to the support, which was coated with gelatine and hence made more hydrophilic in character. This situation is explained by the sterical factors arising as a result of immobilization (*14*).

Table 1
Some Properties of Preparations

Preparation (30 mg)	Measured activity, IU[a]	Bonded catalase, mg	Active bonded catalase, mg	Percent retained activity
I	20.54	0.065	0.0055	8.5
II	35.78	0.074	0.0096	13.1

[a]The amount of H_2O_2, μ mol, converted by 30 mg of preparation in 1 min.

Table 2
Some Optimum Kinetic Parameters of Native and Immobilized Catalase

Material	pH	Temperature, °C	K_m, *mM*
Preparation I	6.8–7.6	28–40	22
Preparation II	7.3–8.3	26–45	40
Native CAT	6.7–7.5	30–37	20

Preparation I shows properties similar to those of native catalase in its pH-dependent activity variation behavior, whereas for preparation II optimum pH value is found to shift toward the alkaline region (Table 2). The total charge acquired through immobilization by preparation I is not much different than that of native catalase. However, as a result of cross-linking between GDA and the W-NH_2 groups of lysine amino acid residues and the α-NH_2 groups of the N-terminal amino acid on the gelatine-coated preparation II and the catalase enzyme, the overall structure gains a polyanionic character and thereby causes the enzymatic reaction in the microenvironment to take place at a pH lower than that in the macroenvironment (*15*). This situation could lead to a shift in the apparent optimum pH value.

K_m, which is an indication of the affinity of the enzyme toward its substrate, in preparation I is quite close to that of native catalase, but is higher in preparation II, which indicates that its affinity toward the substrate is reduced. The observed increases in the K_m values could be a result of diffusion limitations and steric effects (*16*).

In the batch-type stirred reactor, where the processing stability of immobilized catalase samples are investigated, there are no local variations in the rates of the H_2O_2 decomposition and catalase inactivation reactions throughout the process period. Rate constants for H_2O_2 decomposition (k_l) and catalase inactivation with 0.001 and 0.1*M* H_2O_2 are in accord with first-order kinetics (*17*). The k_d' values, which depend on the substrate concentrations, were calculated from the k_l' values, and the activity half-lives observed under the conditions employed in the above reactor are shown in Table 3. Immobilized catalase II has better properties with respect to processing efficiency. It is quite important to have a pre-

Table 3
Observed Kinetic Constants for Preparations

Preparation	Completion time of reaction, min	$k' \times 10^{-2}$ (sec^{-1})	$k'_d \times 10^{-2}$ ($lt \cdot mol^{-1} \cdot sec^{-1}$)
I	8.1	0.731	0.696
II	4.2	1.435	0.902

knowledge of the k_I and k_d values related to immobilized catalase preparations in the reactor applications.

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