0273-2289/95/5003—0291$06.60

Characterization of Immobilized Catalases and Their Application in Pasteurization of Milk with H_2O_2

EMINE AKERTEK AND LEMAN TARHAN*

Department of Chemistry, Faculty of Education, Dokuz Eylul University Egitim Fak., Kimya Bölümü 35150 Buca-İzmir, Turkey

Received May 23, 1994; Accepted June 27, 1994

ABSTRACT

The catalase (from *Aspergillus niger*) has been immobilized by a chemical method on the pous SiO_2 modified with γ-aminopropyltrietoxysilane, followed with glutaraldehyde and by a physical method in alginate and γ-carrageenan gel. Optimum support:enzyme ratios and pH values were determined for modified SiO_2 in a series of immobilization reactions of catalase in the presence of the crosslinking agent glutaraldehyde, and for alginate and γ-carrageenan in the presence of hemoglobin and bovine serum albimine. pH and temperature-dependent activity variations and the stability properties of immobilized catalase preparations were investigated. Rate constants for H_2O_2 decomposition and catalase deactivation were determined. The decomposition rate of H_2O_2 used in the cold pasteurizatioan of milk were investigated in a discontinuous batch type reactor system. Activity half-lives of immobilized catalase were determined.

Index Entries: Immobilized catalase; modified SiO_2, alginate; γ-carrageenan; cold pasteurization.

INTRODUCTION

The enzymes have drawn great importance in the industrial, medical, and analytical application fields (*1*). Catalase is one of the enzymes having useful applications in the food industry. H_2O_2, which is a substrate for

*Author to whom all correspondence and reprint requests should be addressed.

catalase, is used for cold pasteurization of milk to prevent microbial pollution on its way to a processing center (2). The World Food and Agriculture Organization and United Nations Agriculture Organization permit the addition of H_2O_2 to milk at a rate of 0.05–0.25%, depending on such factors as the number and type of the microorganisms, their ability to resist H_2O_2, and environmental temperature. All of the H_2O_2 remaining in the milk after processing is converted into O_2 and H_2O with catalase. It has been shown that the technique has no important effect on the taste of milk and the amount of nutritional ingredients, such as amino acids, proteins, vitamins, sugars, and fat contents.

Recently there has been extensive studies on the immobilization of catalase to broaden the applications of the cold pasteurization technique (3–5). The main difficulty encountered in reactor applications of immobilized catalase is deactivation of catalase because of the H_2O_2 concentration. The decomposition rate constant of H_2O_2 and deactivation rate constant of immobilized catalase are very important for the application in a milk reactor system.

MATERIALS AND METHODS

The experiments were performed using catalase (from *Aspergillus niger*, EC 1.11.1.6), H_2O_2 (30%), $CaCl_2$, $TiCl_4$, HCl, NaCl, bovin serum albumin, hemoglobin, glutaraldehyde, alginate, γ-carrageenan, SiO_2, trichloroacetic acid, and bis-tris (E. Merck, Darmstadt, Germany). These and all other chemical reagents used were of analytical grade.

Modification of Silica for Covalent Immobilization

Porous SiO_2 (900°A) was silanized with γ-aminopropyltrietoxysilane (6). Silanized SiO_2 (500 mg) was further modified with 50 mL of 4% glutaraldehyde (GDA) solution in 0.1*M* tris buffer (pH 8.5) for 2 h. Modified SiO_2 was washed with tris buffer and water, then dried.

Covalent Immobilization on Modified SiO_2

The immobilization on porous SiO_2 (20 mg), that was silanized and then modified with GDA, was carried out with 500 μL catalase (1 mg/mL) in 50 m*M* phosphate buffer (pH 8.0) and 50 μL GDA (20%) in a total vol of 2 mL by shaking at 25°C for 2 h. Immobilized catalase preparation was washed initially with KCl solution of increasing and then decreasing ionic strengths, prepared in phosphate buffer, and then with doubly distilled water until no absorption at 280 nm could be detected in the effluent.

Entrapment in Alginate Gel

Equal volume of 2% alginate, filtered from sterile membrane (0.2 μm) and catalase (1 mg/mL) in 50 m*M* bis-tris buffer (pH 7.0) having 5 mg BSA/mL (Bovin Serum Albumin) were mixed. The mixture was then dripped into 50 m*M* bris-tris buffer (pH 7.0) with 0.15*M* $CaCl_2$ and 2% GDA. After 2 h, the gel beads were washed with water and bris-tris buffer and kept in the same buffer at 5°C.

The size of the alginate gel beads was controlled by applying a coaxial stream of air (*7*). The average bead diameters of the samples were evaluated as 2.0; 2.4; 2.7; 3.2 mm by means of the microscope; the 2.0-mm beads were employed in the experiments.

Entrapment in γ-Carrageenan Gel

A volume of 0.25 mL of catalase solution (4 mg/mL) in 50 m*M* phosphate buffer was added into 1 mL of γ-carrageenan solution (2.5% w/v) at 30°C. This mixture was dripped into the solution of 2% w/v KCl (pH 7.0) containing 2% GDA (20%) at 10°C and left to harden for 30 min. The gel beads with an average diameter of 1.6 mm were washed with water several times and stored in 2% (w/v) KCl at 5°C.

Determination of the Amount of Immobilized Catalase

This was determined by protein analysis of the washing after immobilization according to the method of Bradford using BSA for the calibration (*8*).

Activity Measurements for Native and Immobilized Catalase

The decomposition reaction of H_2O_2 was initiated by adding 60 μL of catalase (0.01 mg/mL) into 3.5 mL of 10.5 m*M* H_2O_2, prepared in a suitable buffer or milk. The reaction was terminated at the end of the first minute by addition of 3.5 mL of 4% trichloroacetic acid (TCA). The absorption was recorded at 415 nm after addition of 1 mL $TiCl_4$ solution (4 mg/mL, in 5.5*N* HCl) into a 2.5-mL sample that was filtered through a sterile membrane (0.2 μm).

The amount of decomposed H_2O_2 in 1 min was calculated from the calibration curve of H_2O_2 (10–100 μg/mL: extinction coefficient: 0.462 cm^2/μmol) in water and milk (*9*). The activity of native catalase was found to be 2955 mol/min mg in 50 m*M* phosphate buffer at pH 7.0 and 25°C.

Immobilized catalase preparations were removed quickly by vacuum filtration immediately after a 1-min incubation with 10.5 m*M* H_2O_2 prepared in a suitable buffer or in milk. Milk proteins precipitated by the addition of an equal volume of 6% TCA were centrifuged at 5000*g* and

filtered through a sterile membrane (0.2 μm). Absorbance was recorded at 415 nm after addition of 1 mL $TiCl_4$ into a 2.5-mL sample. No leakage of the catalase from the alginate beads under the experimental conditions employed was observed by following the absorption at 280 nm.

RESULTS

A series of immobilizations on modified SiO_2 (20 mg) were carried out with varying amounts of catalase (0.2–1.5 mg) in 50 m*M* phosphate buffer at pH 7.0 in order to determine the support:enzyme ratio that provides the best activity. The pH dependent immobilization studies were also performed in phosphate (pH 7.0–8.5) and borate (pH 9.0) buffers with 0.55 mg catalase for which optimum activity was obtained.

As can be seen from Fig. 1, the optimum immobilization conditions for 20 mg of modified SiO_2 were observed at 0.55 mg catalase and pH 8.0. The activity of immobilized catalase was found to be 2086 μmol/min.g.

In order to determine the optimum conditions for entrapment in alginate gel, a series of immobilization reactions were carried out in varying concentrations of sterile alginate (2.0–5.0% w/v) mixed with an equal volume of catalase (1 mg/mL) prepared in 50 m*M* bris-tris (pH 7.0). Concentrations under 2% alginate resulted in imperfect gel beads (Fig. 1). The most satisfactory activity was observed for a 2% alginate solution. The activity variations of catalase entrapped in alginate gel were investigated in relation to the bead diameter (Fig. 2). The decrease with increasing bead diameter in the activity of catalase entrapped in alginate beads having different sizes indicates some difficulties involved in the diffusion of H_2O_2 and O_2.

A series of entrapment reactions were also carried out with varying amounts of hemoglobin, BSA, and 80 μL of GDA (20%) in a 2% alginate solution (Table 1). The best activity was obtained for the immobilization performed in the presence of 5 mg BSA.

The entrapment of catalase in γ-carrageenan gel beads were obtained in a 2% KCl solution in the absence and presence of 2% GDA at pH 7.0, and the activities were determined as 141.7 and 223.4 μmol/min g, respectively. The γ-carrageenan gel beads obtained in the presence of BSA and hemoglobin were of a defective form. Since these activity values are much lower than those obtained for entrapment in alginate, the use of γ-carrageenan was abandoned.

In the following studies, the catalase preparations immobilized on modified SiO_2 by covalent bonding and in the alginate gel by physical adsorption were designated as immobilized preparations I and II, respectively.

pH—Activity and Stability Properties

The dependent activity variations of pH were investigated at 25°C and at different pH values in 50 m*M* buffers under standard conditions

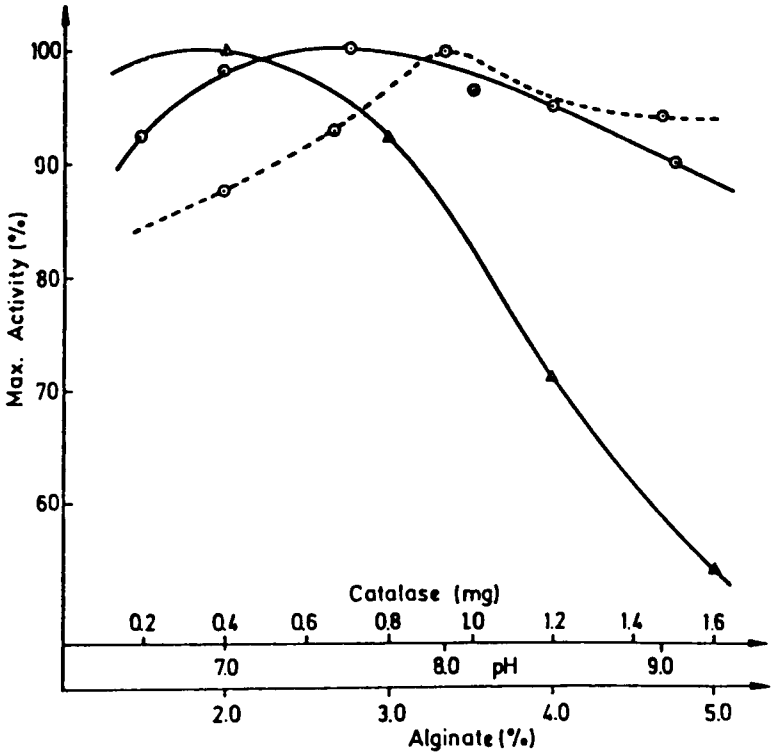

Fig. 1. The activity profiles for immobilization on modified SiO_2 with respect to catalase concentration (—⊙—) and pH (--⊙--) and for entrapment in alginate gel relation to the alginate concentration (-△-).

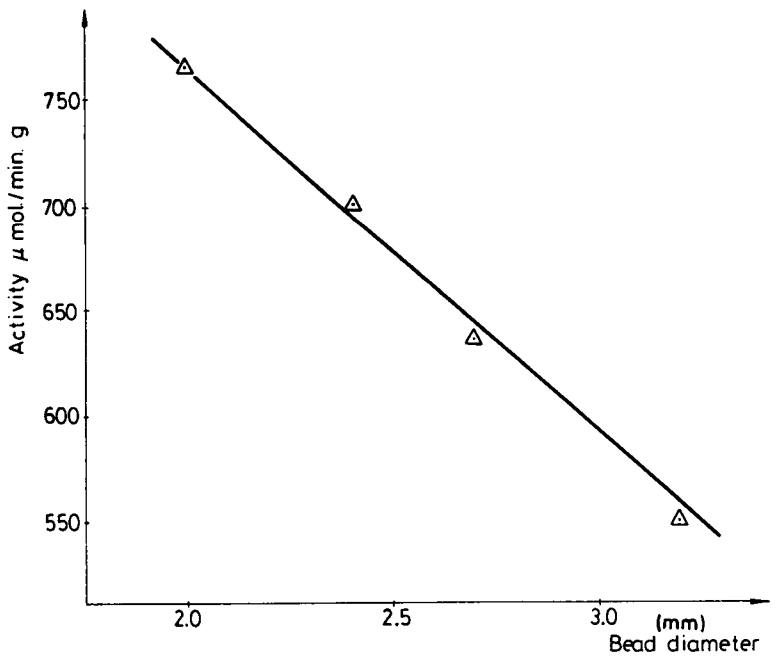

Fig. 2. Bead diameter dependent activity variation of catalase entrapped in alginate gel.

Table 1
Activity Values of Immobilized Catalase
in Alginate Gel Carried Out in Varying Entrapment Conditions

Activity	Hemoglobin, mg				Bovin serum albumin, mg				GDA, μL
	5	10	20	40	4	5	10	30	80
μmol/min.g	383	388	351	172	705	765	760	711	417

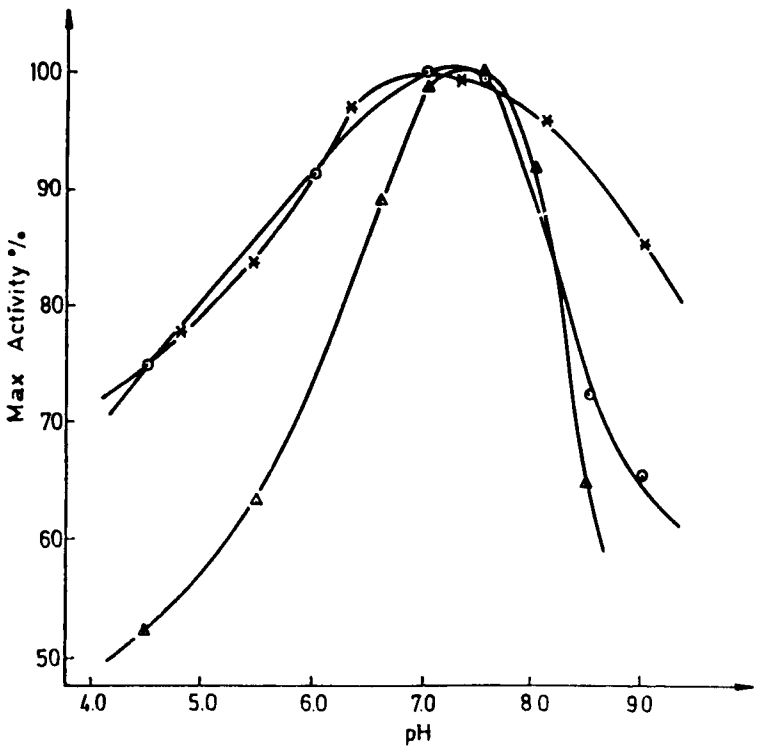

Fig. 3. pH-Dependent activity variations of immobilized preparation I (⊙), preparation II (△), and native catalase (x) at 25°C.

and compared with native catalase. For immobilized preparation I, acetate (pH 4.5), phosphate (pH 6.0–8.5), and borate (pH 9.0) were used as buffers. For preparation II, acetate (pH 4.5), bis-tris (pH 6.5–7.0), and tris (pH 7.5–8.5) buffers were used. It was observed that different types of buffers had no significant effect on the activity and stability of the native catalase.

As can be seen from Fig. 3 native catalase showed maximum activity in the range of pH 6.7–7.0. This range was shifted to pH 7.0–7.5 for the immobilized preparation I, and to pH 7.2–7.5 for the immobilized preparation II.

Retained activity measurements for native and immobilized catalase preparations were made at 25°C and pH 7.0 after incubation in the same buffers at 25°C for 18 h (Fig. 4). Immobilized preparation I showed more stable behavior over the whole pH range, whereas preparation II showed

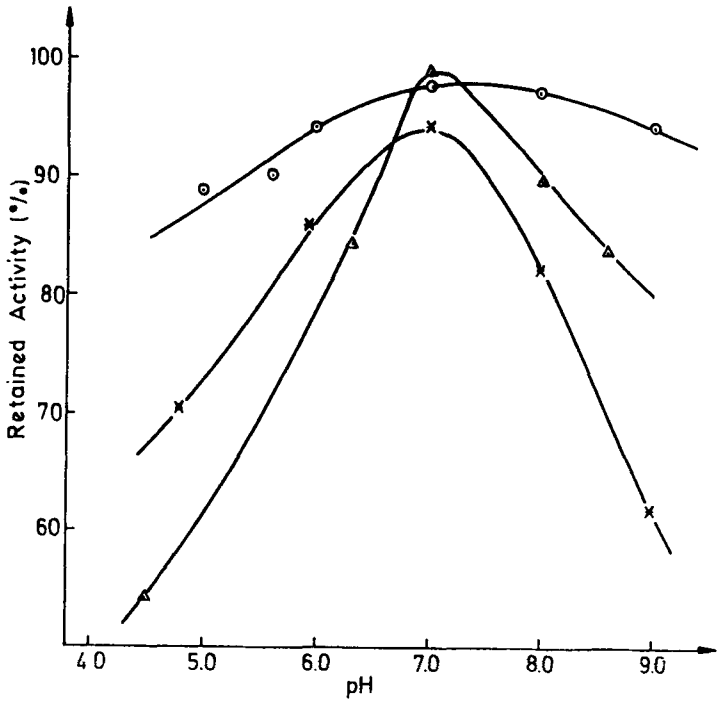

Fig. 4. pH-Stability variations for immobilized preparations I (⊙), preparation II (△), and native catalase (×) incubated at 25°C for 18 h.

better stability only for pH values higher than 6.5 compared to the native catalase.

Temperature—Activity and Stability Properties

Activity variations in relation to the temperature were investigated for native and immobilized catalase on modified SiO_2 in 50 m*M* phosphate buffer pH 7.0, and for the catalase entrapped in alginate gel in bis-tris buffer pH 7.0 (Fig. 5). Immobilized catalase preparations were observed to have a better thermal activity compared to native catalase, except for the range of 25–40°C in which similar activity values were obtained. Thermal stabilities of native and immobilized preparations were investigated by measuring the retained activity at pH 7.0 and 25°C after incubation for 15 h in 25–55°C range in the same buffers. As can be seen from Fig. 6, immobilized preparations showed better stability properties compared to native catalase. The activity values for the catalase entrapped in alginate gel were found to be higher than 100% above 35°C.

Kinetic Parameters

The time dependent decomposition reaction of H_2O_2 with immobilized preparations were investigated in suitable buffers at 25°C in 10 mL

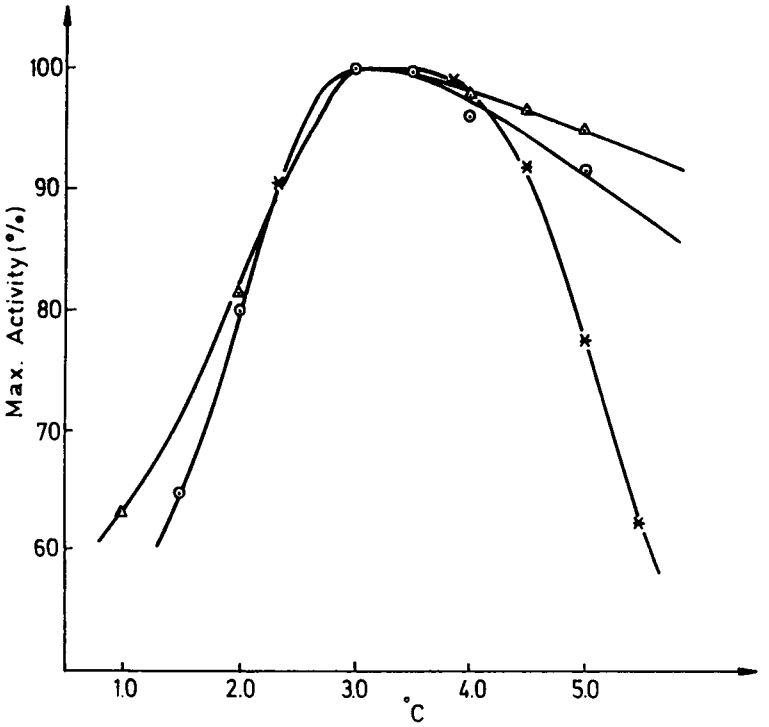

Fig. 5. Temperature-dependent activity variations of immobilized preparation I (⊙), preparation II (△), and native catalase (⁎) at pH 7.0.

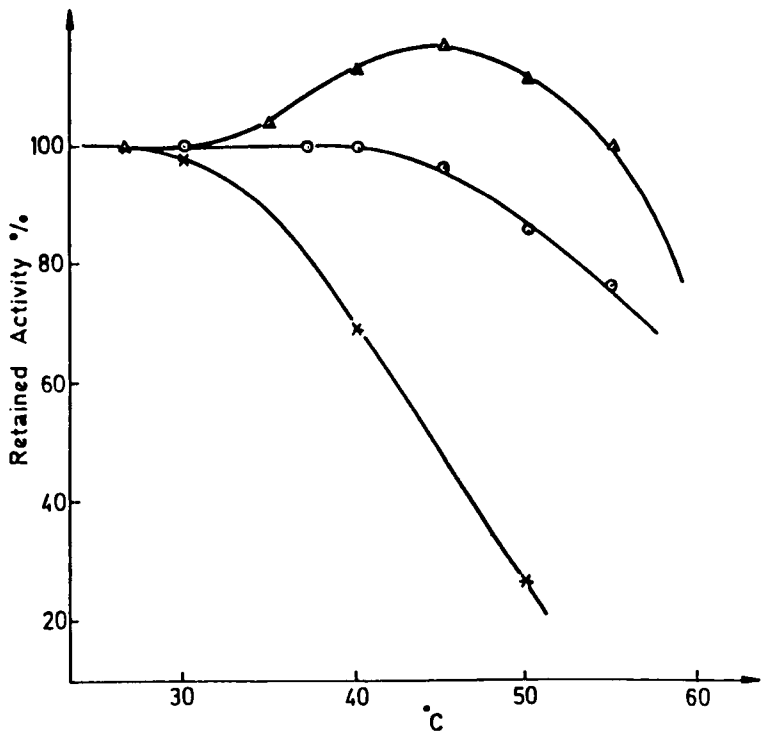

Fig. 6. Thermal stabilities of immobilized preparation I (⊙), preparation II (△), and native catalase (⁎) at pH 7.0.

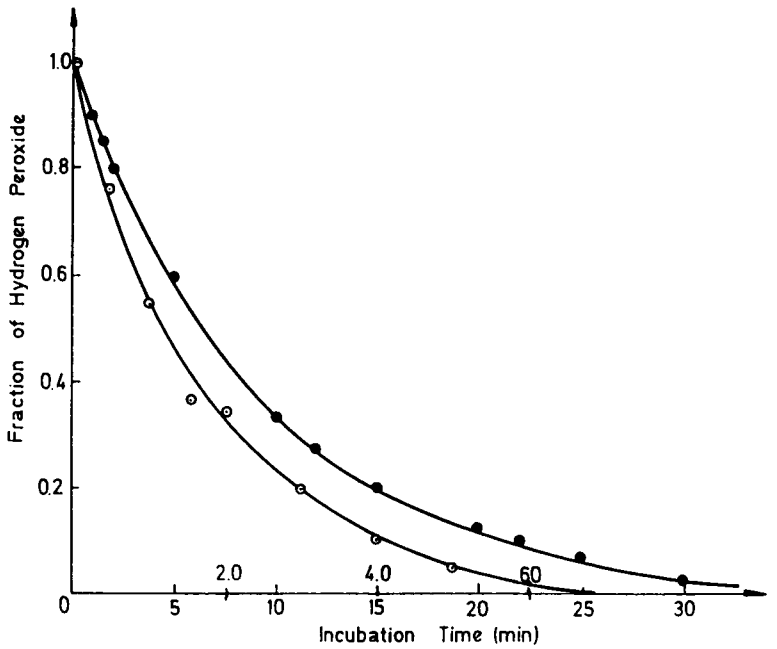

Fig. 7. Decomposition of H_2O_2 by immobilized catalase preparation I (⊙) and preparation II (△) in a batch-type reactor at 25°C, pH 7.0.

Table 2
Apparent Kinetic Constants Obtained for a Discontinuous Batch-Type Reactor

Immobilized preparation	$k_1 \times 10^{-2}\,sn^{-1}$	$k_1' \times 10^5\,L.mol^{-1}\,sn^{-1}$	$k_{d_1}' \times 10^{-2}\,L.mol^{-1}\,sn^{-1}$
I	0.421	7.148	0.102
II	0.074	7.075	0.437

volume (Fig. 7). The decomposition was completed in 6 min for preparation I and 30 min for preparation II. The decomposition of H_2O_2 in the concentration range of 0.1-0.001*M* corresponds to first order reaction kinetics (*10,11*).

$$-\,d\,|H_2O_2|\,/\,dt = k_1'\,|E|\,|H_2O_2|$$

where k_1' = apparent specific rate constant $k_1/\,|E|$, and $|E|$ = the concentration of active immobilized enzyme.

The active catalase concentration of preparations I and II added to the batch type reactor were 5.88×10^{-7} and 1.05×10^{-8} mol/L, respectively, taking the molecular weight of catalase as 240,000. The k_1 and k_1' values given in Table 2 were calculated from Fig. 6. The deactivation of catalase in relation to H_2O_2 concentrations corresponds to first-order reaction kinetics.

$$-\,d\,|E|\,/\,dl = k_{d_1}'\,|H_2O_2|\,|E|$$

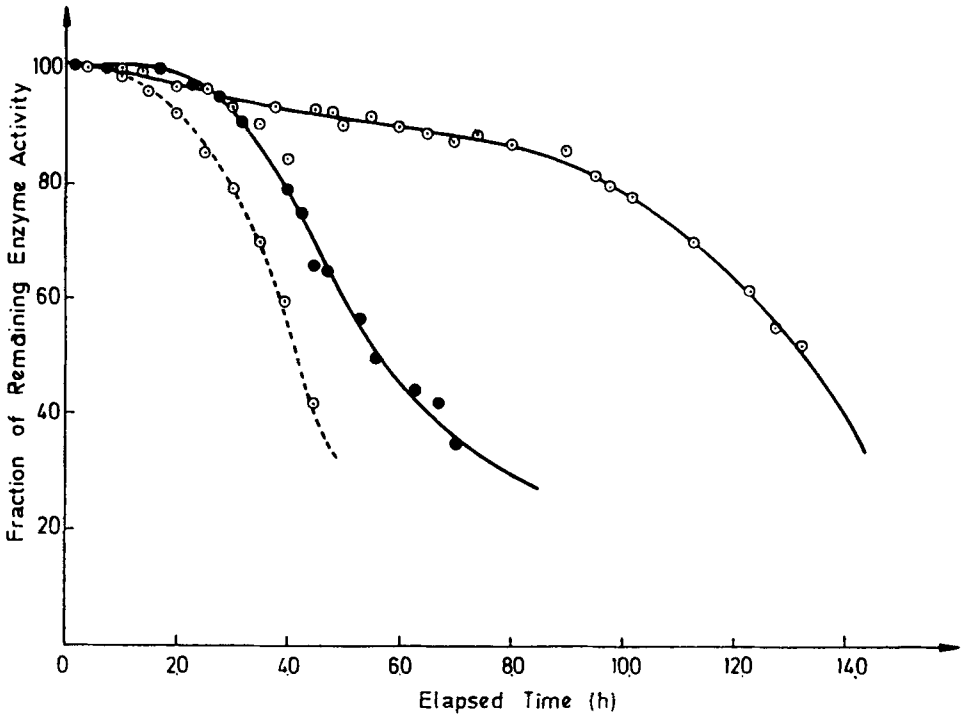

Fig. 8. Deactivation of immobilized catalase in discontinuous batch-type reactor at 30°C, pH 7.0. (H_2O_2) = 10.5 m*M* (—⊙—), 52.5 m*M* (—●—) for preparation I and 10.5 m*M* (--⊙--) for preparation II.

where k'_{d_1} = apparent first order rate constant for catalase deactivation.

The k'_{d_1} values were determined as follows: The complete decomposition of H_2O_2 in the same reactor was followed by repeating the process continuously for preparation I (0.1 g) with 10.5 and 52.5 m*M* H_2O_2 in 50 m*M* phosphate buffer (pH 7.0) and, for preparation II (0.4863 g), with 10.5 m*M* H_2O_2 in 50 m*M* bis-tris buffer (pH 7.0), at 30°C and activity half-lives were determined as 13.4, 5.6, and 4.2 h, respectively (Fig. 8). The k'_{d_1} values of immobilized catalase preparations listed in Table 2 were calculated by using time-dependent activity half-lives in the following equation:

$$k'_{d_1} = 0.693 / (t_{1/2} |H_2O_2|)$$

From the kinetic constants given above, it is apparent that preparation I has more favorable characteristics compared to preparation II.

Decomposition of H_2O_2 in Milk Using a Continuous Batch Type Reactor System

In the reactor system we placed UHT (Ultra High Temperature) pasteurized plain milk, containing 3.5% fat (*12*), and 7.96% fat-free dry components (*13*). Mesophilic and thermophilic bacteria (0/mL) (*14*) were

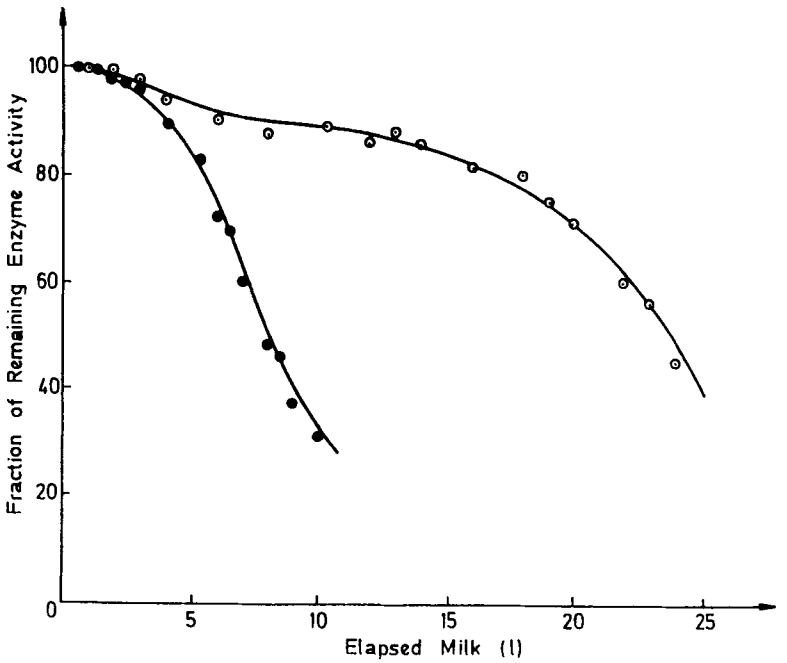

Fig. 9. Activity variations of immobilized preparation I in a discontinuous batch-type reactor at pH 6.72, 30°C and 0.04% (⊙), 0.1%) (●) (H_2O_2) in milk.

used as the test sample. The decomposition of 0.1 and 0.04% H_2O_2 in 150 mL milk with the catalase immobilized on modified SiO_2 was repeated continuously at 30°C until activity half-life was reached (Fig. 9). The rate constants for the decomposition reaction of H_2O_2 in milk at 30°C were found as follows:

$$k_1 = 0.179 \times 10^{-2}\ sn^{-1}$$
$$k_1'' = 1.14 \times 10^{6}\ L.mol^{-1}\ sn^{-1}$$

Cold pasteurized milk, which could be processed free of H_2O_2 with immobilized catalase (1 g), was placed in a discontinuous batch type reactor until the half-life activity was on the order of 300 L for the milk sample containing 0.04% H_2O_2 and on the order of 101.87 L for the milk sample containing 0.1% H_2O_2.

DISCUSSION

The retained activity values of covalently bound catalase on porous SiO_2 modified with γ-aminopropyltriethoxysilane followed by GDA and for the catalase entrapped in alginate gel were 20.17 and 1.54%, respectively (Table 3). As can be seen from Table 3, the retained activity value of preparation II is lower than that of preparation I, although the amount of

Table 3
Same Properties of Immobilized Catalase for Optimum Conditions

Immobilized preparation	The amount of catalase, mg, on the support, g	Activity, μmol/min.g	Active bound catalase, mg	Retained activity, %
I	3.50	2086	0.71	20.17
II	16.82	765	0.26	1.54

entrapped catalase is higher. This is attributed to the interactions between catalase and alginate formed by acidic blocks of β-(1.4)-D-Mannuron and α-(1.4)-L-Guluron, and the steric effects and the problems involved in the diffusion of H_2O_2 into gel.

Optimum pH values were shifted to a more alkaline region for both preparations after the immobilization process. The immobilization of catalase on modified SiO_2, which takes place through the α-NH_2 groups of the *N*-terminal amino acid and the ω-NH_2 groups of lysine residues, and in the case of the immobilization in alginate, the existence of COO^- groups nonchelated with Ca^{2+}, give rise to a polyanionic structure; thus, optimum pHs were shifted to a more alkaline region (*15*).

For the catalase preparations immobilized on hydrophillic supports, better pH-stability properties were obtained for preparation I over the pH range studied, and for preparation II in pH > 7.0 medium, compared to native catalase. A gradual increase in the loss of stability of catalase entrapped in alginate gel, as the pH decreases 4.5–6.5, can be attributed to the change in the pH-dependent charge distribution on catalase and alginate and the resulting interactions among them. Temperature-dependent stability properties for both preparations used were observed to be better than that of native catalase.

The increase in the activity for the catalase entrapped in alginate gel following incubation at 35–50°C for 15 h can be attributed to the decreased steric effects and diffusional problems caused by the structural orientations arising from the temperature changes of support material.

Specific and deactivation rate constants for immobilized catalase preparations in varying H_2O_2 concentrations were found to correspond to first-order reaction kinetics (*16*). Although similar specific rate constants were obtained, the k_{d_1} value for the catalase entrapped in alginate gel was found to be four times greater than that of catalase covalently bound on modified SiO_2. Steric and diffusional effects in alginate gel pellets cause a decrease in the reaction rate and increase the H_2O_2 concentration gradient; thus, enzyme deactivation may occur. Following cold pasteurization in a discontinuous batch-type reactor, using 1 g of catalase immobilized on modified SiO_2, 300 L milk containing 0.04% H_2O_2 can be made ready for packing.

REFERENCES

1. Goldstein, L. and Manecke, G. (1976), *Applied Biochemistry and Bioengineering*, vol. 1, Wingard, L. B., Katchalski Katzir, E., and Goldstein, L., Academic, New York, pp. 23–127.
2. Naguib, Kh. and Hussein, L. (1972), *Milchwissenschaft* **27,** 758–762.
3. Tarhan, L. (1991), *Appl. Biochem. Biotechnol.* **31**(2), 109–117.
4. Tarhan, L. and Uslan, A. H. (1990), *Proc. Biochem.* **25,** 14–18.
5. Schester, A. and Bar-Eli, A. (1979), *Arch. Biochem. Biophys.* **136,** 325–337.
6. Weetal, H. H. and Hersh, L. S. (1970), *Biochim. Biophys. Acta* **206,** 54–62.
7. Smidsrod, O. and Skjak-Braer, G. (1990), *Tibtechnology* **8,** 71–78.
8. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
9. Hicks, G. P. and Updike, S. S. (1966), *Anal. Biochem.* **38**(6), 726–732.
10. Altomare, R. E., Kohler, J., Greenfield, P. F., and Kittrell, J. R. (1974), *Biotech. Bioeng.* **16,** 1659–1667.
11. Tarhan, L. and Telefoncu A. (1990), *Appl. Biochem. Biotechnol.* **26**(1), 45–57.
12. Levowitz, D. J. (1960), *J. Milk Food Technol.* **23,** 69–72.
13. IDF Standard (1982), *21* A.
14. Elmer, H . and Marth, Phe D. (1978), *Standard Methods for the Examinations of Dairy Products,* American Public Health Assoc.
15. Tarhan, L. (1990), *Biomed. Biochim. Acta* **49**(5), 307–316.
16. Wang, S. S., Gallili, G. E., Gilbert, S. G., and Leeder, J. G. (1974), *J. Food Sci.* **39,** 338–350.