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PREPARATION AND CHARACTERIZATION OF PEPSIN IMMOBILIZED ON POLYMERIC SUPPORTS

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ABSTRACT

Immobilization of pepsin on crosslinked resinous materials SRF (salicylic acid–resorcinol–formaldehyde), Amberlite IRA-400, and poly(vinyl alcohol) is reported. Enzyme concentration, pH of the coupling medium, and nature and concentration of crosslinking agents were optimized for the better retention of activity of immobilized pepsin. The immobilized systems were characterized through pH, thermal, and storage stabilities. Michaelis constant (K_m) and maximum reaction velocity (V_m) for the free and immobilized enzymes were calculated from Lineweaver–Burk plots. Effect of temperature on enzyme activity was studied, and the thermoinactivation constant (K_{ti}) and energy of activation (E_a) for free and immobilized enzymes were also calculated. The immobilized pepsin was used in a continuous fluidized bed reactor for the study of clotting of skimmed milk. Rate of coagulation was considerably high for the treated milk sample at 50°C and pH 6–6.2.

INTRODUCTION

Conventional methods of milk coagulation for the production of cheese are batch processes and involve expensive nonreusable enzymes. This can be overcome by using immobilized proteases. Of the many proteases investigated, pepsin and rennin showed better retention of activity, stability, and eventual curd-forming

capacity [1]. Various types of supports such as alumina, titania, iron oxide, Teflon, stainless steel [2], Sepharose-4B [3], derivatives of amino alkyl glass [4], cellulose [5], and Duolite [6] have been used for the immobilization of pepsin. Glass derivatives are extensively used for immobilization, but they are high cost materials. In this paper we report on some cheaper polymeric materials such as poly(vinyl alcohol), salicylic acid-resorcinol-formaldehyde (SRF) and Amberlite IRA-400 resins for pepsin immobilization. The major advantage of these polymeric materials is that they can be prepared in such desirable polymorphic forms as films, beads, particles, and powder with different porosities and surface areas. The immobilized pepsin was further used for the study of secondary phase coagulation of milk, which has not been thoroughly investigated in the past.

MATERIALS AND METHODS

Pepsin (E.C. 3.4.23.1) from porcine stomach mucosa of strength 3380 units/mg solid and tyrosine were obtained from Sigma Chemical Co., USA.

The support material, salicylic acid-resorcinol-formaldehyde (SRF) resin of 60–100 mesh size, was synthesized in our laboratory as discussed earlier [7]. The other two supports, poly(vinyl alcohol) of ~74,800–78,200 molecular weight and 98.99 mol% hydrolysis, and Amberlite IRA-400, were obtained from Loba (India) and Rohm and Hass Co. (USA), respectively. All the other reagents used were of Analytical Grade. Double distilled deionized water was used throughout the work. Fresh skim milk of pH 6–6.7 was procured from Baroda Dairy Plant, Vadodara, India.

Immobilization of Pepsin

Activation of the polymeric supports SRF, Amberlite IRA-400 and poly(vinyl alcohol) (PVA) was done through the hydroxyl group by using *p*-benzoquinone (PBQ) [8], *p*-tolyl sulfonyl chloride (PTS) [9], and cyanuric chloride [10]. During activation of the supports through cyanuric chloride ~0.2–0.4 g of the polymeric materials were treated with 1 g cyanuric chloride in 10 cm^3 dioxane at room temperature for 10 minutes and then with 25 cm^3 water and 25 cm^3 of 20% acetic acid for 5 minutes. The activated product was washed with water, acetone, and water before immobilization of the pepsin.

In the process of activation through benzoquinone polymeric supports, SRF, PVA, and Amberlite IRA-400 (~0.2–0.4 g) were stirred with 0.05 g benzoquinone in 10 cm^3 of 20% ethanol and 0.1 $mol \cdot L^{-1}$ phosphate buffer of pH 8 at room temperature for 2 hours. The activated product was washed with ethanol, ethanol-water (until the filtrate was free from benzoquinone), 1 $mol \cdot L^{-1}$ NaCl, and water before immobilization.

For the activation of the supports through *p*-tolylsulfonyl chloride, ~0.2–0.4 g polymer was activated for 1 hour using 0.1 g *p*-toluene sulfonyl chloride in 10 cm^3 dry dioxane at room temperature. Pyridine (1 cm^3) was added dropwise over 1 minute. The support was washed with dry acetone, acetone-water (1:3), and water before immobilization of pepsin.

The activated supports were incubated with 4 cm^3 of 0.5 mg cm^{-3} pepsin at 277 K for 18 hours. A low temperature shaker bath (INSREF) was used for the reaction. The protein content was determined using the Lowry assay procedure [11], and the activity of the coupled protein was measured by using hemoglobin substrate [12].

The effect of type and concentration of crosslinking agents and enzyme concentration on the extent of coupled protein and its retention of activity was studied through appropriate experiments.

The Michaelis constant (K_m) and maximum reaction velocity (V_m) were calculated from Lineweaver-Burk plots using varying amounts of hemoglobin substrate.

The pH stability studies were carried out for free and immobilized enzyme by determining the enzyme activity at various pH values.

The thermal stability of the free and immobilized pepsins was evaluated by measuring the residual activity of the system exposed to various temperatures for 30 minutes. The energy of activation (E_a) and the thermoinactivation constant (K_{ti}) were calculated by using

$$\ln A = \ln A_0 - K_{ti}T$$

where K_{ti} is the first-order inactivation rate constant, A_0 is the initial activity, and A is the activity after T minutes of temperature effect [13].

The storage stability of the free and immobilized pepsins was determined by storing the enzyme at 4°C for various time intervals and measuring the residual activity periodically.

Clotting of Milk

The schematic diagram of the fluidized bed reactor (0.6 × 10 cm) used for the study of milk clotting is illustrated in Fig. 1. A porous glass frit was placed at the bottom of the reactor. A compact column of pepsin immobilized on SRF was prepared by using 1 g of the material. The flow velocities were maintained at 2

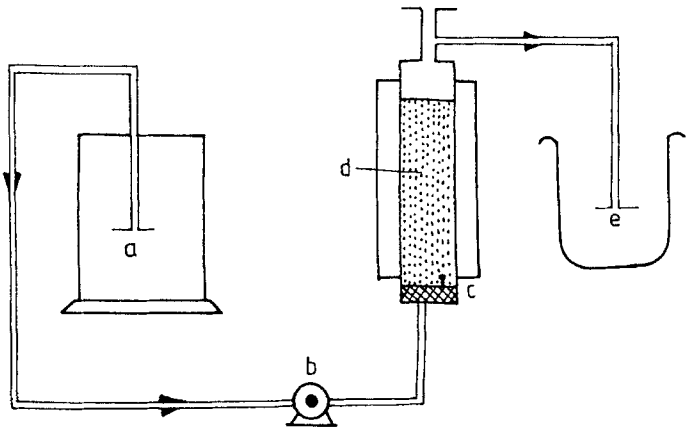

FIG. 1. Schematic diagram of the fluidized bed reactor: (a) milk reservoir, (b) peristaltic pump, (c) porous glass disk, (d) suspended IME, and (e) treated milk.

$cm^3 \cdot min^{-1}$. The space time of the reactor was (W/F) 0.5 $g \cdot cm^{-3} \cdot min^{-1}$. The reactor was maintained at 15°C by circulating water through the outer jacket. A low temperature shaker bath (INSREF) was used for the reaction. Two other supports, poly(vinyl alcohol) and Amberlite IRA-400, exhibited comparatively inferior properties in terms of retention of activity and hence were not used for the study of milk clotting. The enzymatic treatment of milk was carried out at 15°C for 30 minutes. In the secondary phase of clotting the temperature was raised to 30–50°C and the time required for coagulation was measured. To test the practical applicability of the method, the dilution of treated milk with fresh skim milk was done in a 1:2 (v/v) ratio prior to the clotting studies.

To study the effect of pH on milk clotting the pH of the milk was adjusted with 2 $mol \cdot L^{-1}$ phosphoric acid and 1 $mol \cdot L^{-1}$ sodium hydroxide prior to treatment. The absence of enzyme in the secondary phase of clotting was confirmed by measuring the enzyme activity in the washings of the reactor bed and in simulated milk ultrafiltrate (supernatent liquid of the salted-out milk).

RESULTS AND DISCUSSION

Characteristic properties of the synthesized salicylic acid–resorcinol–formaldehyde (SRF) resinous material were reported earlier [7].

Activation of Support

Among the various enzyme immobilization techniques, covalent coupling of the enzyme to the support is the most popular due to the high accessibility and reusability of the bound enzyme [14]. For this paper we prepared covalently bound pepsin–polymeric support systems through different activation procedures as discussed earlier. The results obtained are given in Table 1. It was observed that SRF shows maximum coupling and retention of pepsin activity on *p*-tolylsulfonyl chloride as well as *p*-benzoquinone activation. The other two supports, Amberlite IRA-400 and PVA, showed the highest retention of pepsin activity only on *p*-benzoquinone activation. Hence, further studies were carried out by activating all the supports with *p*-benzoquinone. This activity may be due to the quinone moieties generated during the activation process, for they may have a higher affinity toward the enzyme under study. A similar observation was made by Ponnuchamy et al. [15].

Effect of pH of Coupling Medium

Immobilization of pepsin on activated supports was carried out in a coupling media of pH 1–8. From the results given in Fig. 2 it is seen that maximum coupling of the protein and its retention of activity takes place at pH 1–2 for all the systems under study.

Effect of Enzyme Concentration

The effect of enzyme concentration on the extent of immobilization was studied by using 4 cm^3 of 0.25–4.0 $mg \cdot cm^{-3}$ enzyme. It was observed that all the supports show increased protein coupled but decreased percent retention of activity

TABLE 1. Immobilization of Pepsin on Polymeric Supports[a]

	SRF resin		Poly(vinyl alcohol)		Amberlite IRA-400	
Method of activation	Immobilized protein coupled, $mg \cdot g^{-1}$	Retention of activity, %	Immobilized protein coupled, $mg \cdot g^{-1}$	Retention of activity, %	Immobilized protein coupled, $mg \cdot g^{-1}$	Retention of activity, %
Cynuric chloride	9.0	3.3	8.4	7.9	8.5	3.5
p-Benzoquinone	8.5	80.0	9.4	45.0	8.8	70.4
p-Tolyl sulfonyl chloride	8.5	100.0	9.7	34.5	3.65	53.0

[a]Four cubic centimeters of 0.5 $mg \cdot cm^{-3}$ pepsin, 0.2 g support, 277 K temperature, and 18 hours of coupling time.

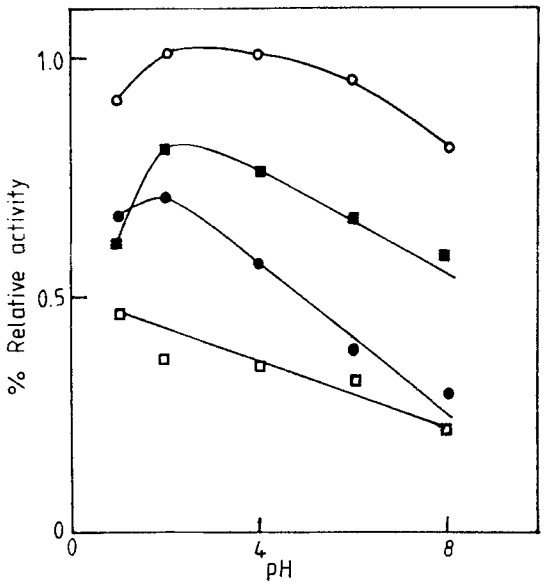

FIG. 2. Effect of pH of the medium on immobilization: (○) pepsin-SRF/PTS, (■) pepsin-SRF/PBQ, (●) pepsin-IRA/PBQ, and (□) pepsin-PVA/PBQ.

with increased enzyme concentration (Figs. 3A and 3B). The decrease in activity can be attributed to the coupling of the enzyme to the support through active sites and to the denaturation of the free and partially coupled enzyme under the action of the activator. Similar results were reported earlier [16].

Effect of Coupling Time

The optimum time required for the immobilization of pepsin on the supports was determined by measuring the amount of protein coupled on the support at different time intervals ranging from 30 minutes to 16 hours reaction time. From the results illustrated in Fig. 4 it is evident that the rate of immobilization is fast at the beginning and almost quantitative coupling takes place within 2 hours for SRF and IRA-400, whereas in the case of PVA the time required for quantitative coupling is less than 1 hour. The retention of activity shown by the three supports is considerably different. The SRF support shows the highest retention of pepsin activity (100%) on activation through PTS. SRF activated with PBQ shows 80% retention of pepsin activity, and Amberlite IRA-400 and poly(vinyl alcohol) show 70 and 45% pepsin activity on immobilization.

Characterization of the Immobilized Systems

pH Dependence of Activity

The pH activity profiles of the free and immobilized pepsins, shown in Fig. 5, indicate that free enzyme activity decreases with increasing pH of the substrate. The pepsin immobilized on SRF through PTS and PBQ activation shows improved pH

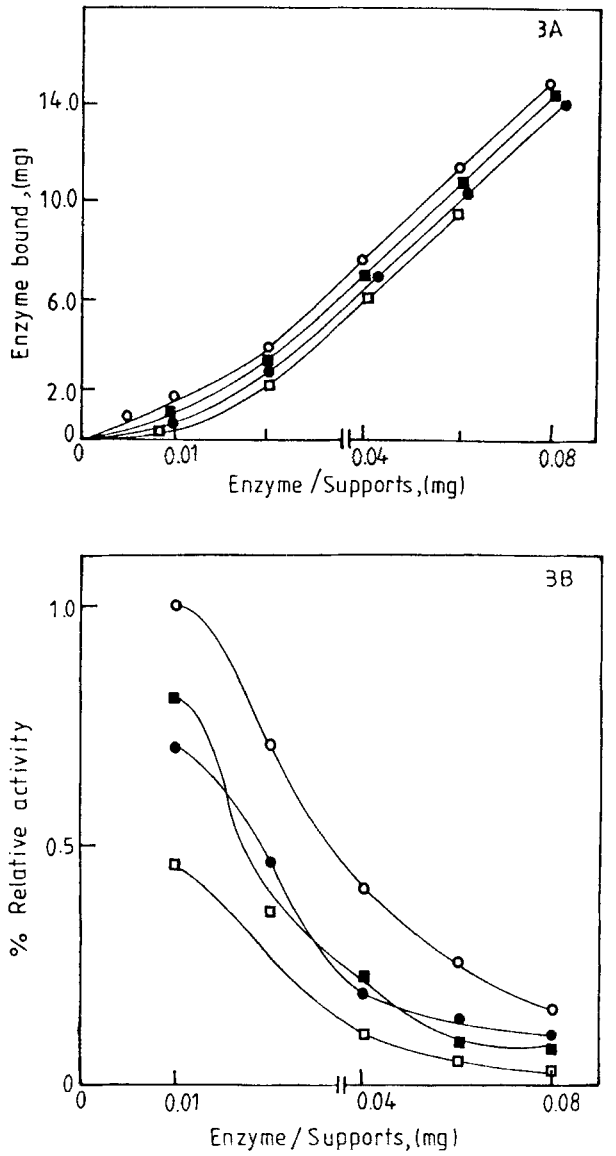

FIG. 3. Effect of enzyme concentration on immobilization. (3A) Protein coupled, (3B) percent relative activity: (○) pepsin-SRF/PTS, (■) pepsin-SRF/PBQ, (●) pepsin-IRA/PBQ, and (□) pepsin-PVA/PBQ.

stability over that of free enzyme, with very little change in its activity over the pH range studied. This is due to diffusional limitations [17].

Temperature–Activity Profile and Thermal Stability

The temperature effect study of immobilized enzyme is one of the most important criteria. The effect of temperature on the activity of free and immobilized pepsins was studied at 30 to 70°C. From the results presented in Fig. 6, it is seen

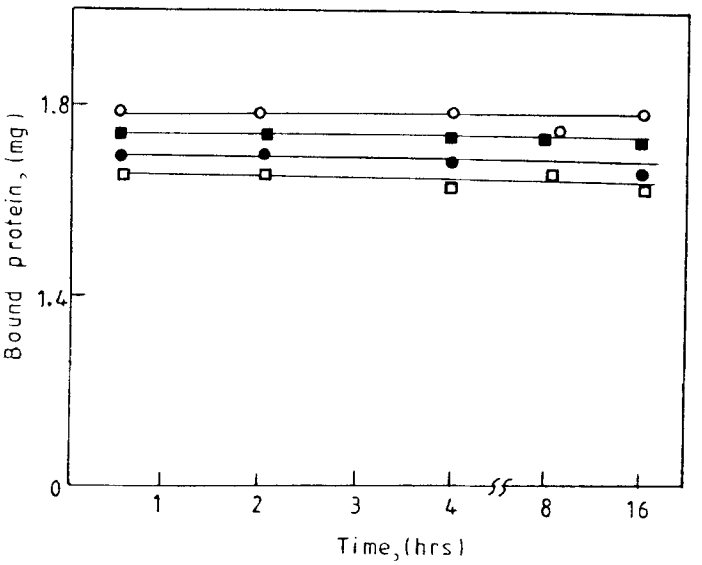

FIG. 4. Effect of coupling time on immobilization: (○) pepsin-SRF/PTS, (■) pepsin-SRF/PBQ, (●) pepsin-IRA/PBQ, and (□) pepsin-PVA/PBQ.

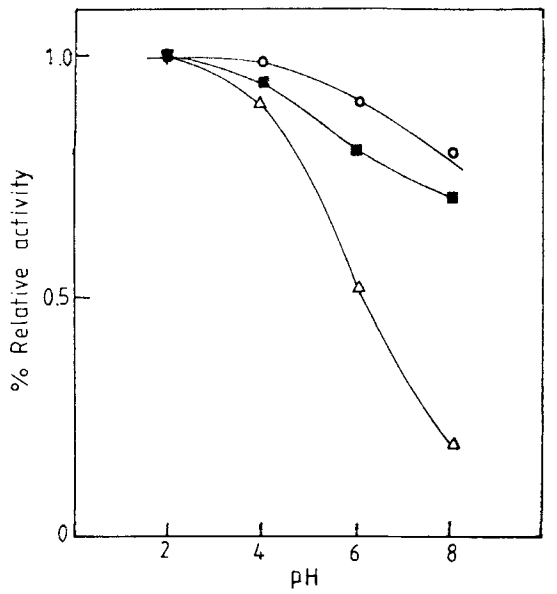

FIG. 5. pH profile for free and immobilized enzyme: (○) pepsin-SRF/PTS, (■) pepsin-SRF/PBQ, and (△) free pepsin.

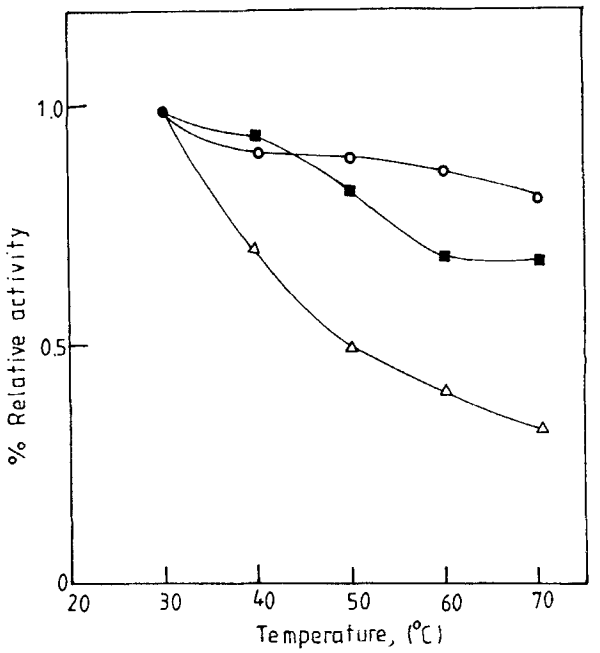

FIG. 6. Effect of temperature on the activity of free and immobilized enzyme: (○) pepsin-SRF/PTS, (■) pepsin-SRF/PBQ, and (△) free enzyme.

that the pepsin-SRF systems show better retention of activity at higher temperatures compared with the free enzyme. This may be due to the higher resistance of a covalently bound system against heat and denaturing agents than does the free form [18]. The activation energy (E_a) for the free and immobilized systems was calculated from the results obtained by using the Arrhenius equation and a plot of log V_{max} vs $1/T$. The pepsin-SRF system requires -1.37 and -1.85 kcal/mol activation energy for PTS and PBQ activation, respectively. The free enzyme requires -2.7 kcal/mol activation energy. The decrease in E_a for the immobilized system also confirms pore diffusion control of the process rather than kinetic control. To study the thermal deactivation of the free and immobilized systems, they were heated at 45°C for longer time periods. The results are given in Fig. 7. It is observed that the free enzyme loses more than 35% of its activity after 60 minutes incubation whereas the immobilized enzyme retains its activity over 3 hours of incubation. The thermoinactivation constant (K_{ti}) was calculated from the results to be -1.18 and -6.49 for free and immobilized enzymes, respectively.

Determination of Michaelis Constant (K_m) and Maximum Reaction Velocity (V_m)

The enzymatic kinetics was studied by carrying out the reactions with different concentrations of substrate hemoglobin. The concentration of hemoglobin was varied from 1×10^2 to 6×10^2 mg. The Michaelis constant (K_m) and the reaction velocity (V_m) were calculated from Lineweaver-Burk plots of $1/V$ vs $1/S$ where V

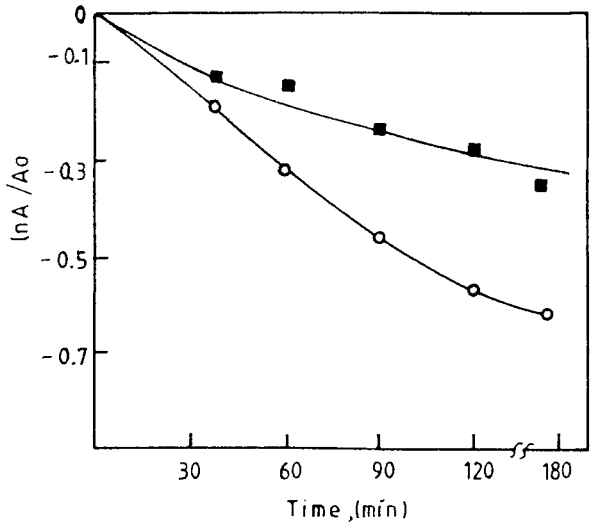

FIG. 7. Kinetics of thermal inactivation at 45°C for soluble and immobilized enzyme through hemoglobin hydrolysis at pH 2: (■) pepsin-SRF/PBQ and (○) free enzyme.

= velocity of the reaction and S = substrate concentration. The results obtained are given in Table 2.

K_m and V_m were calculated by extrapolating the Lineweaver-Burk plots. An intercept on the Y-axis corresponds to $1/V_m$ and an intercept on the X-axis corresponds to $-1/K_m$. K_m was observed to be smaller for the immobilized enzyme due to strong electrostatic interactions between the support and the protein chain [5].

Storage Stability

All the immobilized pepsin systems showed 50% or above retention of activity even after 3 months of moist storage at low temperature. Retention of activity was determined with respect to the activity of enzyme just after immobilization using hemoglobin substrate.

TABLE 2. Michaelis Constant (k_m) and Maximum Reaction Velocity (V_m) for Native and Immobilized Pepsin[a]

Systems	K_m, $\mu g \cdot cm^{-3} \cdot min^{-1}$	V_m, $\mu g \cdot cm^{-3} \cdot min^{-1}$
Pepsin-SRF/PTS	0.76	–
Pepsin-SRF/PBQ	0.75	2.38
Pepsin-PVA/PBQ	2.01	1.30
Pepsin	0.76	1.36

[a]Hemoglobin hydrolysis at pH 2, temperature 37°C for 30 minutes.

Immobilized Enzyme Reactor

Retardation of the clotting process at a lower temperature of the secondary phase is a well-studied phenomenon and forms the basis for continuous coagulation of skim milk with immobilized enzyme [1]. Accordingly, we studied the effect of temperature on the coagulation of skim milk treated with the pepsin immobilized on SRF. The results obtained in a study of the effect of varying the temperature of the secondary phase are given in Fig. 8. A considerable decrease in clotting time was observed with increasing temperature of the treated milk. The clotting time shows a pH dependence at lower temperatures, but at a higher temperature (50°C) it becomes pH independent. On the other hand, the clotting time required for untreated milk was high, and even at higher temperatures it was pH dependent. The sharp decrease in clotting time for the treated milk can be attributed to the strong attractive forces between the micelles at higher temperatures, resulting in coagulation. The higher potential of pepsin-treated micelles at higher temperatures results in lowering the clotting time [19]. The decay of immobilized enzyme activity at lower pH values for the pepsin immobilized on controlled pore glass is reported in earlier studies [20]. This may be due to the adsorption of milk casein on the glass, resulting into a lower clotting activity at a lower pH. In our study the treatment of milk at a

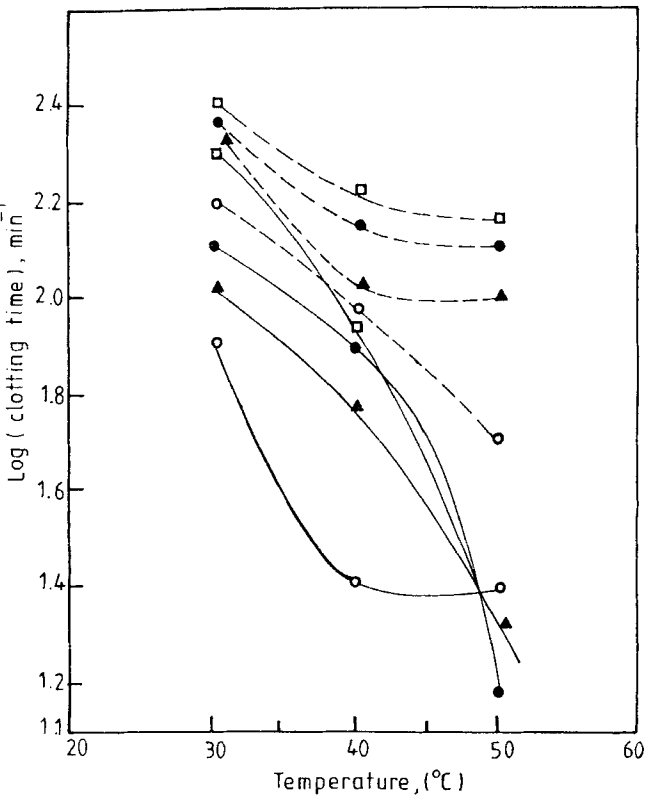

FIG. 8. Effect of temperature on secondary phase of milk clotting: (—) treated milk and (--) untreated milk at pH 5.80 (○), 6.0 (▲), 6.2 (●), and 6.4 (□).

lower pH resulted in greater clotting activity; this may be due to the reduced adsorption of milk proteins on the support. Moreover, the difference between the clotting times for untreated and treated milks continues to decrease as the pH value increases at different temperatures. The activity measured at predetermined time intervals indicates no leakage of the enzyme from the reactor. This was further confirmed by mixing the whey of the clotted milk with an equal volume of fresh skim milk of the same pH.

CONCLUSION

Among the polymeric supports studied, SRF resin give a better performance for the immobilization of pepsin. In the fluidized bed reactor studies, clotting of milk was observed to be less dependent on the pH of the milk. The deactivation of the reactor and the adsorption of casein were negligible. The immobilized pepsin showed better thermal, storage, and pH stability than the free pepsin.

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