Invertase Immobilized on Spacer-Arm Attached Poly(hydroxyethyl methacrylate) Membrane: Preparation and Properties

M. YAKUP ARICA,[1] SERAP ŞENEL,[2] N. GÜRDAL ALAEDDINOĞLU,[3] SÜLEYMAN PATIR,[4] ADIL DENIZLI[2]

[1] Department of Biology, Kırıkkale University, Kırıkkale, Turkey

[2] Department of Chemistry, Hacettepe University, Ankara, Turkey

[3] Department of Biological Sciences, Middle East Technical University, Ankara, Turkey

[4] Department of Science Education, Hacettepe University, Ankara, Turkey

Received 5 March 1999; accepted 28 June 1999

ABSTRACT: Microporous poly(2-hydroxyethyl methacrylate) (pHEMA) membrane was prepared by UV-initiated photopolymerization. The spacer arm (i.e., hexamethylene diamine) was attached covalently and then invertase was immobilized by the condensation reaction of the amino groups of the spacer arm with carboxyl groups of the enzyme in the presence of carbodiimides. The values of the Michael's constant K_m of invertase were significantly larger (ca. 2.5 times) upon immobilization, indicating decreased affinity by the enzyme for its substrate, whereas V_{max} was smaller for the immobilized invertase. Immobilization improved the pH stability of the enzyme as well as its temperature stability. Thermal stability was found to increase with immobilization and at 70°C the half times for the activity decay were 12 min for the free enzyme and 41 min for the immobilized enzyme. The immobilized enzyme activity was found to be quite stable in repeated experiments. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 75: 1685–1692, 2000

Key words: poly(2-hydroxyethyl methacrylate) membrane; spacer arm; covalent bonding; enzyme immobilization; invertase

INTRODUCTION

Immobilization confers additional stability to a variety of enzymes against several forms of denaturation. Enzymes have been immobilized on different shape of supports (i.e., membranes or beads) either by adsorption, entrapment ,or covalent binding.[1–3] Membrane-immobilized enzymes may serve as model systems for enzymes, naturally bound to membranes, or may find practical application in enzyme electrodes and enzyme reactors as less expensive, more stable, and reusable alternatives to free enzymes.[4–6]

Earlier studies showed that poly(2-hydroxyethyl methacrylate), pHEMA, is an attractive enzyme carrier.[7,8] pHEMA is a nontoxic and biocompatible synthetic polymer with adequate mechanical strength for most biotechnological applications. One other advantage is the presence hydroxyl groups that act as attachment sites for bioactive species after activation or introduction of different functional groups to the polymer chain.[9,10] These suggest that pHEMA is a suitable support for enzyme

Correspondence to: M.Yakup Arıca at Department of Biology, Kırıkkale University, 71450 Yahşihan-Kırıkkale, Turkey.

Journal of Applied Polymer Science, Vol. 75, 1685–1692 (2000)

immobilization and bioreactor applications. It has been used in the previous studies to immobilize enzymes and cells after preparation via various techniques including suspension, bulk, and solution polymerization using thermal, chemical ,and γ-irradiation as initiators.[3,7,11] It can readily be put into different shapes.[3, 9]

Invertase is a specific enzyme for converting sucrose to glucose and fructose. A membrane-immobilized invertase can be used in the analytical field for the construction of sucrose biosensors and in the enzyme reactors for hydrolysis of sucrose. The product (sugar mixture) obtained by invertase has the advantage of being colorless compared to the colored version obtained through acid hydrolysis.[12–15]

Immobilization of invertase on natural polymers such as corn grits,[16] gelatin,[17] and various cellulose derivatives[18] has already been achieved, while its immobilization onto hydrogel polymers is limited. The latter has good chemical and mechanical stability, and is not susceptible to microbial attack.[19,20]

In this study, the aim was to immobilize invertase with a significantly higher activity and stability than those described in earlier studies. Invertase was covalently immobilized onto a spacer-arm attached pHEMA membrane. The resultant immobilized invertase system was characterized and its activity retention, catalytic properties, reusability, and stability aspects were compared.

EXPERIMENTAL

Materials

Invertase (β-fructofuranosidase, EC 3.2.1.26, Grade VII from baker's yeast), glucose oxidase (GOD, EC 1.1.3.4, Type II from *Aspergillus niger*), peroxidase (POD, EC 1.11.1.7. Type II from horseradish), bovine serum albumin (BSA), *o*-dianisidine dihydrochloride, sucrose, glucose, and *p*-toluenesulfonic acid were all obtained from the Sigma Chemical Company (St. Louis, USA) and used as received.

2-Hydroxyethyl methacrylate (HEMA) was obtained from Fluka AG (Switzerland), distilled under reduced pressure in the presence of hydroquinone, and stored at 4°C until use. Ethylene glycol dimethacrylate (EGDMA) was obtained from Fluka AG. Inhibitors were removed by alkaline salt extraction (20% NaCl and 5% NaOH), washed twice with distilled water, dried with $CaCl_2$, and stored at 4°C until use. α,-α′-Azoisobutyronitrile (AIBN) was obtained from Fluka AG and used as received. Hexamethylene diamine (HMDA) was obtained from BDH Chemicals Co. (UK). All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany).

Membrane Preparation

The pHEMA membrane was prepared as previously described.[9] The membrane preparation mixture (5 mL) contained 2 mL (HEMA), 20 μL (EGDMA) as crosslinker, 5 mg AIBN as polymerization initiator, and 3 mL 0.1*M* $SnCl_4$. The mixture was then poured into a round glass mold (diameter 9 cm) and exposed to ultraviolet radiation (12 W lamp) for 10 min, while a nitrogen atmosphere was maintained in the mold. The membrane was washed several times with distilled water and cut into circular pieces (diameter 1.0 cm) with a perforator.

Incorporation of HMDA onto pHEMA Membrane

In order to prepare the HMDA-derived membranes, the following procedure was applied. pHEMA membrane pieces (23 g) were transferred into benzene (60 mL) containing HMDA (15 g) and *p*-toluenesulfonic acid (300 mg). The reaction medium was boiled at 80°C in a Dean-Stark apparatus and refluxed for 48 h. At the end of this period, the HMDA-derived membrane pieces were removed and washed several times with methanol and then dried in vacuum oven for 24 h. They were then stored at 4°C until use.

Immobilization of Invertase onto HMDA-Derived pHEMA Membrane

HMDA-derived pHEMA membrane disks (diameter 1 cm) were equilibrated in phosphate buffer (50 m*M*, pH 7.0) and immersed in the same fresh medium containing invertase (2 mg mL^{-1}). The immobilization of invertase was carried out at 4°C for 24 h, while continuously stirring the medium. The HMDA-derived pHEMA membrane containing invertase was washed with acetate buffer (0.1*M*, pH 6.0).

Determination of Immobilization Efficiency

The amount of protein in the enzyme solution and in the wash solutions was determined by using

Coomassie Brilliant Blue as described by Bradford[21] with BSA as a standard.

Activity Assays

The activities of both the free and the immobilized invertase preparations were determined by measuring the amount of glucose liberated from the invertase-catalyzed hydrolysis of sucrose per unit time.

In the determination of the activity of the free enzyme, the reaction medium consisted of acetate buffer (2.5 mL, 50 m*M*, pH 5.0), sucrose (0.1 mL, 300 m*M*). Following a preincubation period (5 min at 35°C), the assay was started by the addition of the enzyme solution (0.1 mL, 10 mg mL^{-1}) and incubation was continued for 5 min. In order to terminate the enzymatic reaction, the reaction medium was then placed in a boiling water bath for 5 min.

The same assay medium was used to determine the activity of the immobilized enzyme. The enzymatic reaction was started by the introduction of ten membrane disks into the assay medium (10 mL) and was carried out at 35°C with shaking in a water bath. After 15 min, the reaction was terminated by removal of the membrane disks from the reaction mixture.

Sucrose hydrolysis performance of the free and immobilized preparation was determined by measuring the glucose content of the medium according to a method described previously.[3] The assay mixture contained GOD (25 mg), POD (6.0 mg), and *o*-dianisidine (13.2 mg) in phosphate buffer (100 mL, 0.1*M*, pH 7.0). An aliquot (2.5 mL) and 0.1 mL of enzymatically hydrolyzed sample were mixed and then incubated in a water bath at 35°C for 30 min. After addition of sulfuric acid (1.5 mL, 30%), absorbance was measured in a UV-visible spectrophotometer (Shimadzu, Model 1601, Tokyo, Japan), at 525 nm.

The activities of the free and the immobilized invertase were expressed in μmol sucrose min^{-1} mg^{-1} of enzyme and μmol sucrose min^{-1} cm^{-2} of enzyme membrane.

These activity assays were carried out over the pH range 4.0–8.0 and temperature range 20–60°C to determine the pH and temperature profiles of the free and the immobilized enzyme. The results of dependence of pH, temperature, storage stability, and repeated run are presented in a normalized form with the highest value of each set being assigned the value of 100% activity.

Determination of the Kinetic Constants

K_m and V_{max} values of the free enzyme were determined by measuring initial rates of the reaction with sucrose (3.0–300 m*M*) in acetate buffer (50 m*M*, pH 5.0) at 35°C. The kinetic parameters of immobilized invertase were determined in a batch system by varying the concentrations of sucrose (3–300 m*M*) in acetate buffer (50 m*M*, pH 5.0). The reaction temperature was 35C. K_m and V_{max} values were calculated from the data obtained after 15 min.

Batch Use of Immobilized Invertase

The retention of the immobilized invertase activity after repeated batch use was tested as described above in Activity Assays. After each run, the enzyme–membrane disks were washed with acetate buffer (50 m*M*, pH 5.0) and reintroduced into fresh medium 10 times successively.

Storage Stability

The activity of the free and the immobilized invertase after storage in acetate buffer (50 m*M*, pH 5.0) at 4°C was measured in a batch operation mode under the experimental conditions given above.

Thermal Stability of Free and Immobilized Enzyme

The thermal stabilities of the free and the immobilized invertase were carried out by measuring the residual activity of the enzyme exposed to three different temperatures (50–70°C) in acetate buffer (50 m*M*, pH 5.0) for 2 h. After every 15 min time interval, a sample was removed and assayed for enzymatic activity as described above. The first-order inactivation rate constants k_i were calculated from

$$\ln A = \ln A_0 - k_i t \quad (1)$$

where A_0 is the initial activity and A is the activity after a time t (min).

Characterization of pHEMA Membranes

Water Content of pHEMA Membranes

The water content of pHEMA membrane was determined in distilled water. Dry membrane pieces were placed into distilled water at 25 ± 0.5°C until they reached a constant weight for about 4 h. Swollen membrane was weighed by an

electronic balance (Shimadzu, Japan, EB.280 ± 1 × 10^{-3} g). The water content of the swollen membranes were calculated by using the following expression:

$$\text{Water content } \% = \{(W_s - W_o)/W_o\} \cdot 100 \quad (2)$$

where W_o and W_s are weights of dry and swollen membrane, respectively.

Fourier Transform IR Spectra

Fourier transform IR (FTIR) spectra of the plain and the HMDA-attached pHEMA membranes were obtained by using a FTIR spectrophotometer (Shimadzu, FTIR 8000 Series, Japan). The pHEMA membrane (0.1 g) and KBr (0.1 g) were thoroughly mixed and the mixture was then pressed in a pellet form, and the spectrum was recorded.

Scanning Electron Microscopy

Scanning electron micrographs of the pHEMA membrane were obtained using a Leitz AMR-1000 (Germany) after coating with gold under vacuum.

RESULTS AND DISCUSSION

Properties of pHEMA Membrane

The water content of the hydrogel membrane used in the present study was about 58% weight base. It should be mentioned that the water content of the membrane did not change after HMDA attachment. The SEM micrograph of the surface of pHEMA membrane is presented elsewhere.[9] The surface structure of pHEMA membrane is very porous, and this should lead to a large external surface area for the immobilization of the proteins.

To identify the HMDA-derived polymeric surface of pHEMA membrane, FTIR spectra of plain and HMDA-derived pHEMA membrane were obtained. As shown in Figure 1, FTIR spectra of both the pHEMA [in Fig. 1(A)] and the HMDA-derived pHEMA [in Figure 1(B)] membranes show a broad band which is characterized by hydrogen-bonded alcohol O—H stretching vibration bands at about 3500 cm^{-1}. The FTIR spectra of HMDA-attached pHEMA shows some different absorption bands from pHEMA. The bands are at 1565, 1550, and 1470 cm^{-1} and the characteristic

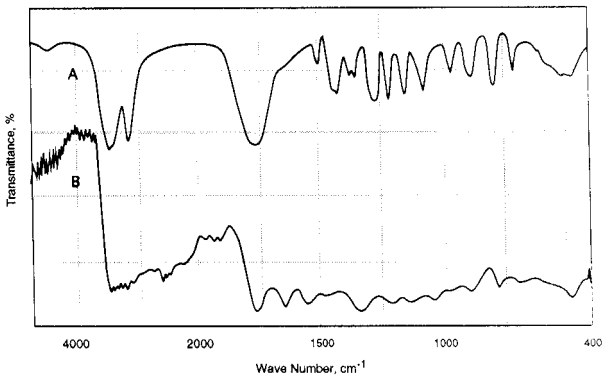

Figure 1 FTIR spectra of membranes: (A) pHEMA; (B) HMDA-pHEMA.

—NH_2, —NH, and —CH_2 bending mode (scissoring), respectively. The bands at 2960, 1720, and 1150 cm^{-1} represent stretching vibration of C—H, C═O and C—N, respectively. On the other hand, hydrogen-bonded alcohol O—H stretching vibration band intensity of plain pHEMA is higher than the same band of HMDA-derived pHEMA membrane. The reason for this is because of the —OH groups lost during the condensation reaction between —OH groups of HEMA and —NH_2 groups of HMDA. The HMDA-derived pHEMA has a shoulder absorption band at around 3415 cm^{-1}, which is characterized by N—H absorption coming from HMDA on pHEMA membrane.

Immobilization of Invertase on pHEMA Membrane

A two-step process was carried out for the covalent immobilization of invertase on the pHEMA membrane. In the first step, hexamethylene diamine (i.e., spacer arm) was covalently attached to the pHEMA membranes through the hydroxy groups. The second step consisted of the condensation reaction of the amino groups of the support with the carboxyl groups of enzyme in the presence of carbodiimides (Fig. 2). The amino or hydrazine group containing supports provide a method of binding enzyme via their carboxyl groups. In this method, the support and condensing agent (in our case carbodiimide) are added simultaneously to the enzyme solution. During the condensation reaction, amide bonds are formed between amino groups of the support and carboxyl groups of the enzyme. The spacer arm constituted by aliphatic chains of 6 carbon atoms has been used to move away immobilized invertase from the support. Invertase also has a large

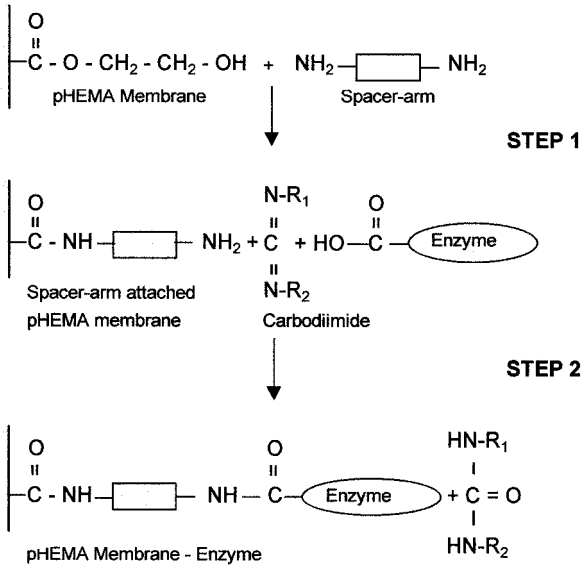

Figure 2 Schematic representation of hexamethylene diamine attachment and invertase immobilization on to pHEMA membrane.

molecular weight about 1200 kDa.[22] The attachment of 6 carbon atoms hydrophobic spacer arm on the membrane surface could a prevent an undesirable side interaction between the large enzyme molecule and support. In this way, all areas of the immobilized invertase could become fully accessible to its substrate sucrose. pHEMA and HMDA–pHEMA membranes were subjected to elemental analysis. The amount of HMDA attached to the membrane was calculated from the data (by considering the nitrogen stoichiometry) to be 0.39 μmol HMDA cm^{-2}. The enzyme loading and the retained enzyme activity after immobilization on the HMDA–pHEMA membrane were 106 μg cm^{-2} (or 8.5 x 10^{-5} (mol cm^{-2}), and 67%, respectively. The binding ratio was about 4500 HMDA molecules per invertase molecule.

Kinetic Constants

Kinetic parameters K_m and the V_{max} for the free and the immobilized invertase were determined for sucrose hydrolysis (Table I). The K_m value for the free enzyme estimated from the Lineweaver–Burk plot was 24 mM, whereas the V_{max} value was calculated as 320 U mg^{-1} protein. The value of apparent K_m for covalently bound invertase was approximately 2.5-fold higher than that of the free enzyme. In a similar study, Kotzelski and Staude[13] reported that the K_m values of covalently bound invertase on the polysulfone membranes were larger—about up to 13 times that of the native invertase. The change in the affinity of the enzyme to its substrate is probably caused by structural changes in the enzyme introduced by the immobilization procedure and by lower accessibility of the substrate to the active site of the immobilized enzyme.[1,3] The V_{max} value of immobilized invertase was found to be 214 U^{-1} bound mg protein (or 23 U per cm^2 membrane) on the pHEMA membrane, and it decreased approximately 33% with respect to free enzyme after covalent immobilization. The V_{max} of invertase immobilized on the porous pHEMA membrane can be affected by external or internal diffusional resistances, which respectively correspond to the transport of the substrate and products from the bulk solution to the outer surface of the enzyme–membrane, and to the internal transport of these species inside the porous system of the membrane. As expected, the K_m and V_{max} values were significantly affected after covalent immobilization of invertase onto the HMDA-attached pHEMA membranes.

Effect of Temperature on the Catalytic Activity

The temperature dependence of the activities of the free and the immobilized invertase were studied in acetate buffer (50 mM, pH 6.0) in the temperature range 20–60°C (Fig. 3). The optimum temperature for the immobilized invertase was at 50°C, being 5°C higher than that of the free enzyme at 45°C. Arrhenius plots in the temperature range from 20°C to optimum appeared linear and the activation energies were found to be 1.54 and 1.87 kcal mol^{-1} for the free and the immobilized

Table I Kinetic Properties of the Free and Immobilized Invertase

Form of Enzyme	K_m (mM)	V_{max} (U mg^{-1} Enzyme)	Recovered Activity (%)	Enzyme Loading (μg cm^{-2} Membrane)	Activity (U cm^{-2} Membrane)
Free invertase	24	320	100	—	—
Immobilized invertase	62	214	67	106	23

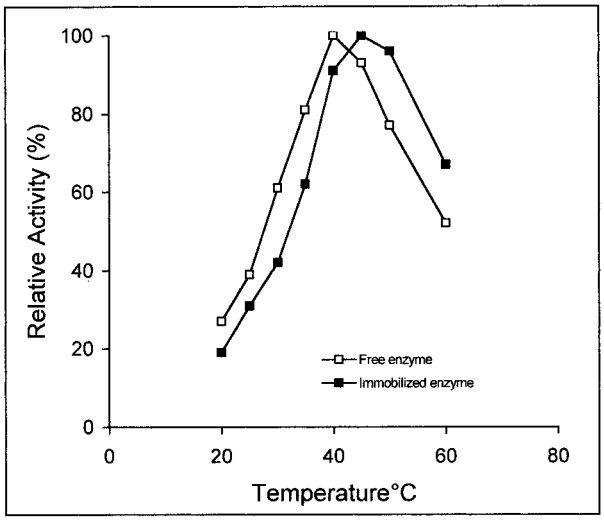

Figure 3 Temperature profiles of the free and the immobilized invertase.

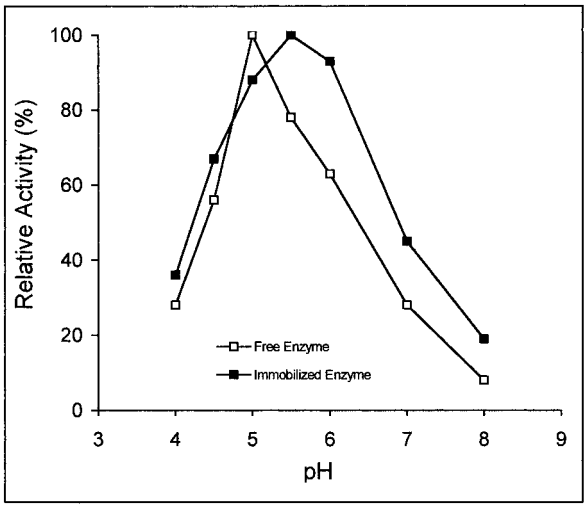

Figure 4 The pH profiles of the free and the immobilized invertase.

invertase, respectively. The increase in optimum temperature and activation energy was caused by the changing physical and chemical properties of the enzyme. The covalent bond formation via carboxyl groups of the immobilized invertase might also reduce the conformational flexibility resulting in higher activation energy for the molecule to reorganize to the proper conformation for the binding to substrate.

Effect of pH on the Activity

The pH effect on the activity of the free and immobilized invertase preparations for sucrose hydrolysis was studied at various pHs at 35°C. The reactions were carried out in acetate and phosphate buffers and the results are presented in Figure 4. Optimal conversion was obtained at pH 5.0 for the free enzyme. The optimum pH of the immobilized enzyme was shifted 0.5 pH unit to alkaline region. This shift is possibly due to the secondary interactions (e.g.. ionic and polar interactions, hydrogen bonding) between the enzyme and the polymeric matrix.[7] Other researchers[9,15,23] have reported similar observations upon immobilization of invertase and other enzymes.

Batch Use of Immobilized Invertase

The operational stability of immobilized enzyme systems is very important economically; an increased stability could make the immobilized enzyme more advantageous than its free counterparts especially if its use as a biosensor or an enzyme reactor is contemplated. The activities of the immobilized invertase was retained its all-initial activity after 10 successive batch reactions mode.

Storage Stability

The free and the immobilized invertase preparations were stored in acetate buffer (50 m*M*, pH 5.0) at 4°C and the activities were measured for a period of 56 days (Fig. 5). The free enzyme lost all its activity within 28 days. Immobilized enzyme lost about 36% of its activity during the storage period.

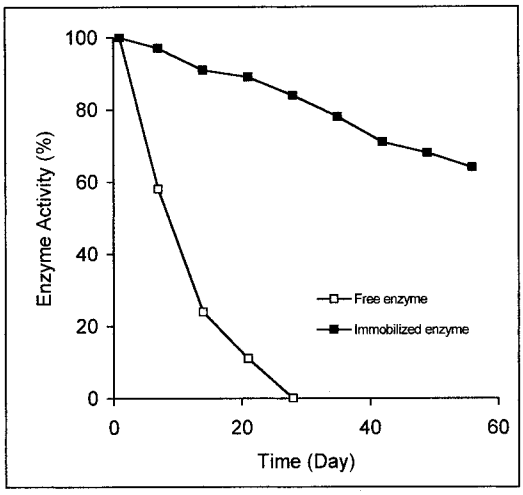

Figure 5 Storage stabilities of the free and the immobilized invertase.

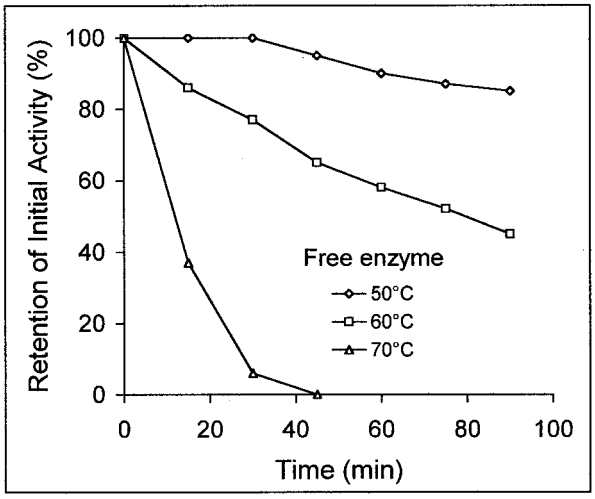

Figure 6 Influence of temperature on the stability of the free invertase.

Thermal Stability

Thermal stability experiments were carried out with the free and the immobilized enzymes, incubated in the absence of substrate at various temperatures. Figure 6 and 7 show the heat inactivation curves between 50 and 70°C for the free and immobilized enzymes, respectively. The immobilized invertase preserved its activity at 50°C and free enzyme retained its initial activity about 90% during a 90 min incubation period. At 60°C the immobilized and the free enzymes retained their activity about to a level of 78 and 45%, respectively. The immobilized form was inactivated at a much slower rate than the native form. After a 90 min treatment at 70°C, the half-lives determined for the free and the immobilized enzymes were about 12 and 41 min, respectively. The thermal inactivation rate constants (k_i) at 70°C were calculated as 9.36 x 10^{-2} min^{-1} for free and 3.21 x 10^{-2} min^{-1} for the immobilized invertase. These results suggest that the thermostability of immobilized invertase increased considerably because of covalent immobilization onto pHEMA membrane. Similar results have been previously reported for various immobilized enzymes.[3,24,25] Ulbrich et al.[25] report that the activity of the immobilized preparation, especially in a covalently bound system, is more resistant than that of the soluble form against heat and denaturing agents.

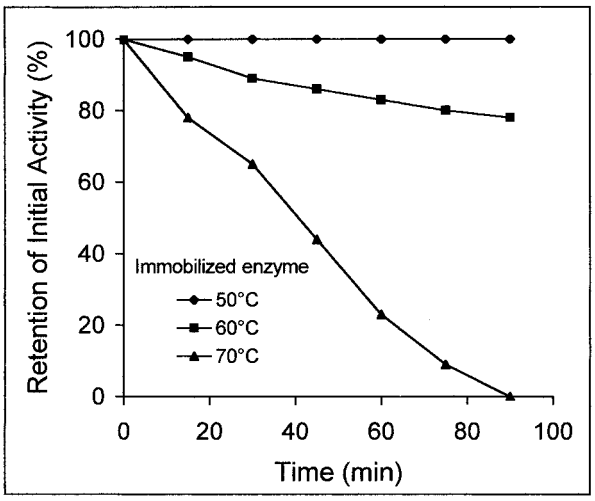

Figure 7 Influence of temperature on the stability of the immobilized invertase.

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