Characterization of the Activity of β-Galactosidase Immobilized on Teflon Membranes Preactivated with Different Monomers by γ-Irradiation

M. S. MOHY ELDIN,[1,*] U. BENCIVENGA,[1] M. PORTACCIO,[1,2] S. STELLATO,[1] S. ROSSI,[1] M. SANTUCCI,[1] P. CANCIGLIA,[1,†] D. CASTAGNOLO,[3] F. S. GAETA,[1] D. G. MITA[1,2]

[1] International Institute of Genetics and Biophysics of CNR, via Guglielmo Marconi, 12, 80125 Naples, Italy

[2] Department of Human Physiology and Integrated Biological Functions of the Second University of Naples, via S. M. di Costantinopoli, 16, 80138 Naples, Italy

[3] Mars Center, via Comunale Tavernola, 80144 Naples, Italy

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ABSTRACT: The activity of β-galactosidase, immobilized by grafting technique on Teflon membranes preactivated with four different monomers, has been characterized from the biochemical and biophysical points of view. The monomers used were acrylic acid or acrylamide, or methacrylic acid and 2-hydroxyethyl methacrylate. When 2-hydroxyethyl methacrylate was used in the second grafting, the first three monomers have been used in the first modification step. The behavior of the free and immobilized enzyme has been analyzed as a function of temperature and pH. For each catalytic membrane, we have found general equations relating the absolute enzyme activity to pH and temperature. From these expressions, the experimental conditions giving the best yield of each catalytic membrane have been calculated. The kinetic parameters for the four membranes have also been determined. The advantages of using these membranes in nonisothermal bioreactors are also indicated. © 1998 John Wiley & Sons, Inc. J Appl Polym Sci 68: 613–623, 1998

Key words: radiation grafting; polytetrafluoroethylene; immobilized enzymes; β-galactosidase; acrylic acid; acrylamide; methacrylic acid; 2-hydroxyethyl methacrylate

INTRODUCTION

Immobilized enzymes are widely used in biotechnological processes spanning from the food industry to ecology, and from fine chemical production to the construction of biosensors.

With only the exception of the enzyme, the most important contributing component to the performance of a biocatalytic membrane is the carrier. At present, synthetic carriers constitute the largest number of supports available for catalyst immobilization, owing to their resistance against microbial attack and their capability to be copolymerized with different monomers. Modifications of preformed polymers are easily done chemically[1–3] or by irradiations[4–17] using grafting techniques.

In a previous work,[18] we reported a technique by which we successfully immobilized β-galactosidase on premodified Teflon membrane using γ-irradiation grafting. What we had obtained was a Teflon [polytetrafluoroethylene (PTFE)]

Correspondence to: D. G. Mita (mita@iigbna.iigb.na.cnr.it).
* *Permanent address:* Department of Polymers and Pigments, National Research Center, Dokki, Cairo, Egypt.
† *Permanent address:* Institute of General Physiology of the University of Messina, Salita Sperone, 36, Messina, Italy.

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membrane on which, in the presence of γ-irradiation, acrylic acid (AA), 2-hydroxyethyl methacrylate (HEMA), and the enzyme were attached in sequence. The best performance of this catalytic membrane was found as a function of the most relevant experimental parameters affecting its behavior.

In the present work, we want to characterize, from a biochemical and biophysical point of view, the behavior of the β-galactosidase immobilized on Teflon membranes differently premodified. The activity of an immobilized enzyme is affected by the immobilization process that induces changes in the spatial structure of the protein or in the microenvironment in which the immobilized enzyme operates. To understand how the microenvironment affects the enzyme activity, we used in this research four different monomers, having different chemical and physical properties. Additional factors affecting enzyme activity are the pH and temperature. For this reason, dependence of the enzyme performance on these factors is also studied and discussed here in the following for each of the catalytic membranes, premodified with different monomers.

EXPERIMENTAL

Materials

Membranes used as a solid support on which to perform the grafting process were PTFE membranes of the type TF-450 manufactured by Gelman Instrument Company (Ann Arbor, MI). Characteristics of the membranes are reported elsewhere.[18]

The monomers used for grafting were HEMA and AA, or acrylamide (AM) or methacrylic acid (MAA). Ferrous ammonium sulfate (FAS) was used during the premodification step as inhibitor for the formation of homopolymers.

The enzyme used was a β-galactosidase (EC 3.2.1.23) from *Aspergillus oryzae*.

All chemical products, including the enzyme, were purchased from Sigma Chemical Company (St. Louis, MO) and used without any further purification.

Catalytic Membrane Preparation

Membrane grafting was performed by irradiation with γ-rays. The irradiation source was cesium 137 in a γ-cell 1000 Elite by Nordion International, Inc. (Kanata, ON, Canada). The average dose rate in the core of the radiation chamber (central dose rate) was of 2.35×10^4 rad h^{-1}.

Double-Grafted Membranes

First Grafting: Modification of PTFE Membrane by AA or AM or MAA Grafting

First grafting was performed by irradiating in the γ-cell the PTFE membranes with a FAS solution of AA or AM or MA monomers, thus obtaining a PTFE-AA or a PTFE-AM, or a PTFE-MAA membrane. Experimental conditions used are specified according to the following scheme: a = 10% of monomer concentration (v/v); b = 0.1% FAS concentration (w/v); and $c = t_1$ = 10 h of irradiation time. Only in the case of the PTFE-AM membrane the b parameter was equal to 5% FAS concentration (w/v).

Second Grafting: Immobilization of β-Galactosidase by HEMA Radiation Grafting

Second grafting was performed by subsequent irradiation in the γ-cell a solution of HEMA and enzyme in which the previously grafted membranes were immersed. Experimental conditions used are specified according to the following scheme: d = 10% HEMA concentration (v/v); e = 45 mg mL^{-1} enzyme concentration; and $f = t_2$ = 16 h of irradiation time.

Monografted Membranes

These membranes were directly obtained following the procedure used for second grafting [i.e., under the conditions defined by: d = 10% HEMA concentration (v/v); e = 45 mg mL^{-1} enzyme concentration; and $f = t$ = 16 h of irradiation time].

Determination of Grafting Degree

As for the percentage of grafting degree, we adopted the classical definition for this parameter. The degree of grafting (X, %) was determined by the difference between membrane masses before, G_B, and after, G_A, grafting by the formula

$$X(\%) = \frac{G_A - G_B}{G_B} \times 100 \qquad (1)$$

Determination of Swelling Degree

The swelling degree (i.e., the membrane hydrophilicity) was calculated as the weight difference

between the water-swollen and dry membrane divided by the weight of the dry membrane.

Determination of the Activity of the Catalytic Membrane

β-Galactosidase hydrolyzes lactose in glucose and galactose. The methodology used for determining the enzyme activity has been described elsewhere.[18] For activity determination, catalytic membranes were put in 20 mL of a well-stirred 200 m*M* lactose in 0.1*M* buffer solution, at the desired pH and temperature. Glucose production was measured in the course of time, and activity of the catalytic membrane was calculated as previously described.[18] In the study of the activity as a function of the pH, we used 0.1*M* NaCl buffer solution for pH 2, 0.1*M* citrate buffer solution for the 3–5 pH range, and 0.1*M* phosphate buffer solution for the 6–8 pH range.

Determination of Time Stability of the Catalytic Membrane

Time stability of the biocatalytic membranes was assessed by analyzing their activity every day under the same experimental conditions. After 3 days, during which the membranes lost some activity, a stable condition was reached that remained unchanged for more than 2 months. Only these stabilized membranes have been used in the comparative experiments reported herein. When not used, the membranes were stored at 4°C in 0.1*M* phosphate buffer (pH 6.5).

Treatment of Experimental Data

Every experimental point reported in the figures represents the average of three experiments performed under the same conditions. The experimental errors did not exceed 6%. For each of the three experiments, the procedures in the various steps were performed according to the following methodology. Thirty-two Teflon membrane disks (2.5 cm in diameter) were weighed and put in the solution for the first grafting. After irradiation, the membrane disks were repeatedly washed under vigorous stirring in abundant double distilled water to remove the adherent homopolymers. Then, eight disks were taken for determining the first degree of grafting, and the remaining were used for the second grafting. At the end of this operation, the disks were washed in 0.1*M* phosphate buffer solution (pH 6.5), then separated in three groups of eight membranes each for determining the second grafting degree, the swelling degree, and catalytic activity, respectively.

RESULTS AND DISCUSSION

Effect of Temperature

In studying the dependence of enzyme activity on temperature, a bell-shaped curve, with an optimum in activity, is obtained. The curve for the immobilized enzyme can be broader, narrower, or equal to one of the free enzymes, whereas optimum activity generally presents a shift toward higher temperatures upon immobilization. This means a higher resistance to enzyme thermal deactivation since the structure of the catalytic site is strengthened by the immobilization procedure that created strong bounds between the macromolecule and the carrier. When the maximum position remains unaltered, we can conclude that the structure of the active site and the microenvironment in which it is operating are the same in the free and bound forms.

In Figure 1, we report the temperature dependence of the β-galactosidase activity for the four types of catalytic membranes used. Temperature dependence of the activity of free enzyme is also reported as the reference curve. In all cases, an approximately bell-shaped curve is found, the temperature profile remains almost unchanged; what changes is the optimum activity position that is shifted toward higher temperatures in the case of immobilized enzyme. The only exception occurs for the Teflon-AA membrane [Fig. 1(b)] for which the optimum activity temperature is coincident with that of the free enzyme. All of these experiments were performed at pH 6.5. We chose this pH value because it corresponds to that of the pH of milk. We want to use these new catalytic membranes in processes dealing with lactose hydrolysis in this food.

Effect of pH

It is well known that the pH plays a relevant role on enzyme activity. This role is clearly evidenced when the activities of soluble and immobilized enzymes are studied. In the latter case, the support itself can change the pH value around the catalytic site, thus determining different catalytic performances between the free and bound states of

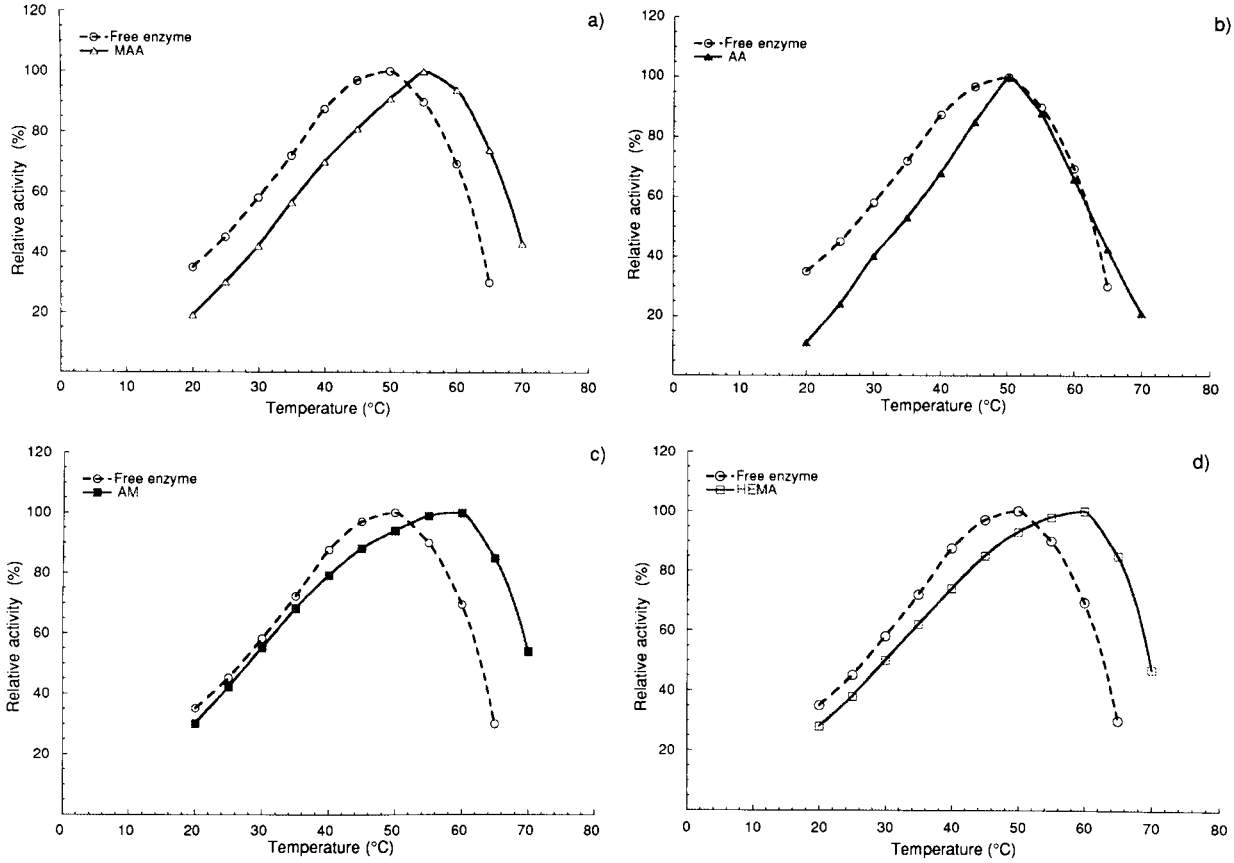

Figure 1 Relative activity of the free enzyme and catalytic membrane as a function of temperature for the systems: (a) Teflon-MAA; (b) Teflon-AA; (c) Teflon-AM; and (d) HEMA directly grafted on Teflon. The pH of the solution was 6.5.

the catalyst. This effect is known as the partitioning effect. Of course, if the results obtained for the two forms of the enzyme are coincident, the conclusion can be drawn that the catalyst operates under the same environmental conditions.

We have investigated the activity of β-galactosidase in the free and immobilized states as a function of pH in the range between 2 and 8. The results of this investigation are reported in Figure 2, where the relative activities of each of the four catalytic membranes are reported as a function of pH. In the figure, the relative activity of the free enzyme is also reported as the reference curve. Temperature was kept constant at 30°C in all of the experiments. Optimal activity was obtained at pH 4.5 for the free enzyme and for the system Teflon-MAA. A shift of about one-half pH unit toward more alkaline pH values was found for the Teflon-AA and Teflon-AM systems. On the contrary, a shift of about one-half pH unit toward more acid pH values was found for the HEMA directly grafted on Teflon. The latter result is unexpected, considering the neutral nature of HEMA. This behavior can be attributed to secondary interactions between the enzyme and the modified polymeric matrix.

We want to discuss now the causes of the observed shifts. As we have previously described, the more relevant factor influencing enzyme activity is the partitioning effect, directly related to the chemical nature of the support material (in this case the grafted monomers) that induces electrostatic or hydrophobic interactions between the matrix and the low molecular weight species present in the solution. These interactions lead to alterations in the microenvironment in which the enzyme actually operates. In particular, the partitioning effects cause different concentrations of charged species, as hydrogen ions or hydroxyl groups, in the microenvironment of the immobilized enzyme. Generally, the pH profile is displaced toward more alkaline or acid pH values for negatively or positively charged matrices.[19] In our case, the AM, AA, and MAA branches behave as

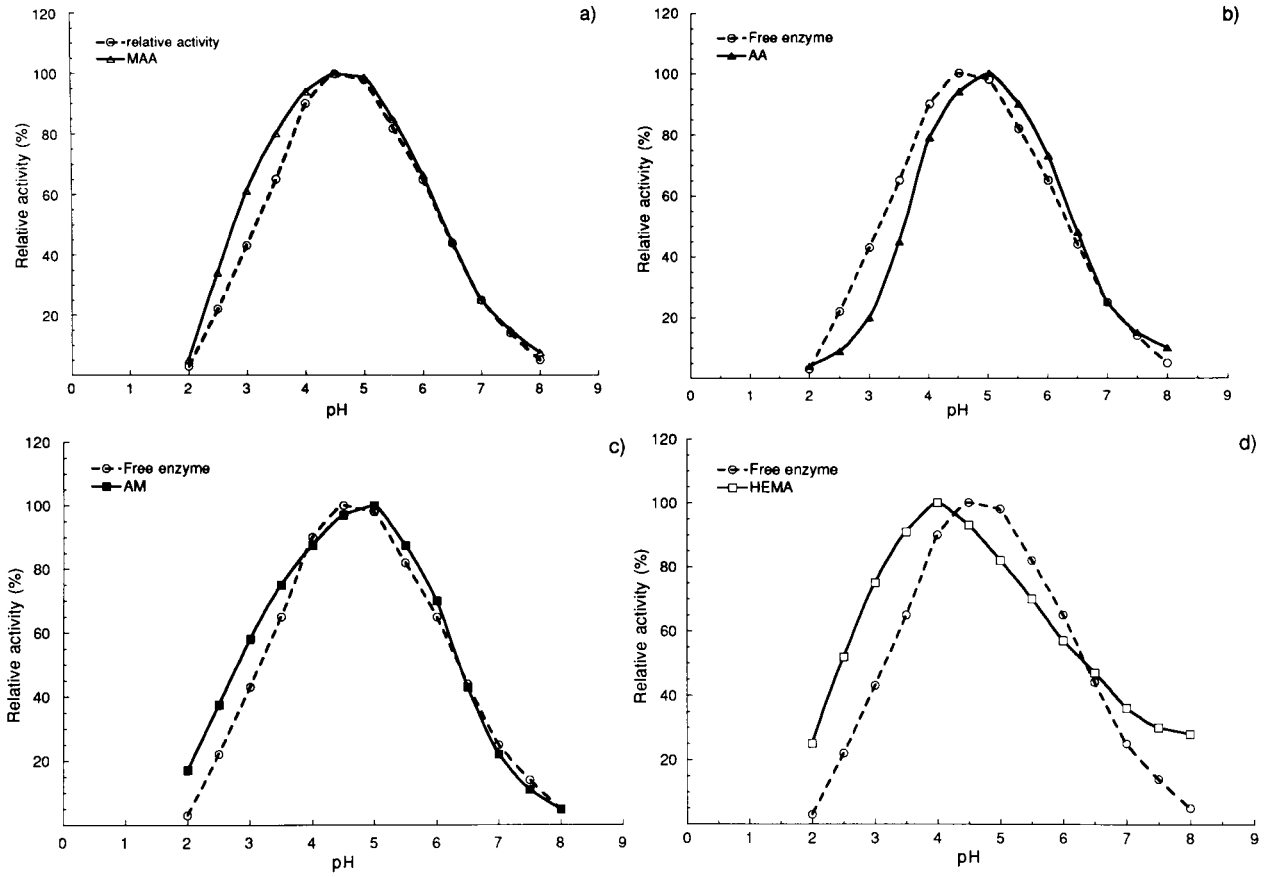

Figure 2 Relative activity of the free enzyme and catalytic membrane as a function of pH for the systems: (a) Teflon-MAA; (b) Teflon-AA; (c) Teflon-AM; and HEMA directly grafted on (d) Teflon. The temperature of the solutions was 30°C.

negatively charged in the solution thus inducing the maximum pH activity shift toward more alkaline pH values. The negative charge is determined naturally by the two lone pair of electrons on the nitrogen atom of the amine group in the case of AM or by the loss of H^+ ions from the functional carboxylic groups, in the case of AA and MAA. The pH shift and the value of this shift depend on the nature of the charge and on the density of the charges, which in our case is directly proportional to the density of the grafted monomers. Because the grafting percentage is different for the various monomers (see Table I), the shift will also be different. The grafting percentage of MAA is so low that practically no significant changes in the enzymatic microenvironment are induced. For

Table I Grafting and Swelling Degree

Membrane System	Degree of First Grafting (%)	Degree of Second Grafting (%)	Degree of Swelling (%)
Teflon-MAA	8	26	50
Teflon-AA	30	83	300
Teflon-AM	41	69	160
HEMA directly grafted on Teflon	None	10	10

Experimental conditions used are reported in the text.

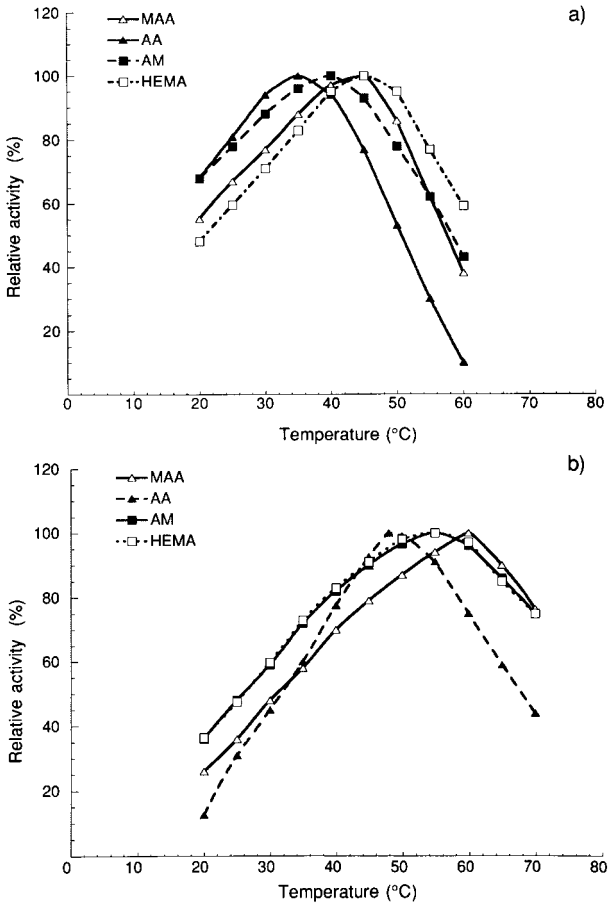

Figure 3 Relative activity of the catalytic membrane as a function of temperature for the systems: (△) Teflon-MAA; (▲) Teflon-AA; (■) Teflon-AM; and (□) HEMA directly grafted on Teflon. The pH of the solution was 3.5 in the experiments reported in (a) and 5.0 in the experiments reported in (b).

this reason, optimum pH is coincident for free and immobilized enzymes.

As for the change of pH activity profile, the cases in which the pH activity curve becomes broader can be explained by the increased resistance of the enzyme to pH changes upon immobilization; the opposite holds true when the curve profile becomes more narrow.

Synergetic Dependence on Temperature and pH

As previously described, we have found that the maximum of the relative activity of the catalytic membrane is a function of temperature and pH. Now, we want to see if there is some graphical form relating simultaneously the relative maximum activity of each catalytic membrane to temperature and pH values at which this maximum occurs.

Thus, we have extended our experimentation at two other pH values, studying the catalytic behavior of the grafted membranes as a function of temperature. In Figure 3(a), the results of experiments performed at pH 3.5 are reported for the four membrane systems. Membrane activity has been expressed as relative activity. Comparison between these results and the ones reported in Figure 1 shows that the maximum activity is shifted toward lower temperatures when the solution pH is 3.5.

In Figure 3(b), the results of experiments performed at pH 5.0 are reported for the four membrane systems. The membrane activity has been expressed as relative activity. Also, in this case, the comparison between these results and the ones reported in Figures 1 and 3(a) shows that the maximum of relative activity is shifted when the solution pH is changed. Also, the curve profiles are altered when the pH is changed.

All of the above results clearly indicate that there is a correlation between the position of the maximum of the relative activity and the corresponding values of pH and temperature. To identify this correlation in Figure 4, we reported the values of the temperatures at which the maxima of the relative activities occur as a function of corresponding pH values. Points are deduced from the experiments reported in Figures 1 and 3. We are aware that the three experimental points are too few to draw a curve; for this reason, the curves in the figures were drawn by the computer, imposing the best fit between the experimental data. The quadratic correlation coefficient between the three experimental points and the fitted curve is 0.95 ± 0.02 for each membrane system. Notwithstanding that the experimental data are few, it is possible to write a mathematical equation satisfying the experimental points reported in the figures. This expression is a parabolic equation of the type

$$T = M_2(\mathrm{pH})^2 + M_1(\mathrm{pH}) + M_0 \qquad (2)$$

The parabolic profile, of course, is more evident in Figure 4(a). Obviously, any membrane system is characterized by its own M_i values. The M_i values for the four membrane systems are reported in Table II. The use of this equation allows us to plan the best experimental conditions for obtaining the higher yield of the enzyme reaction when one of the two parameters (temperature or

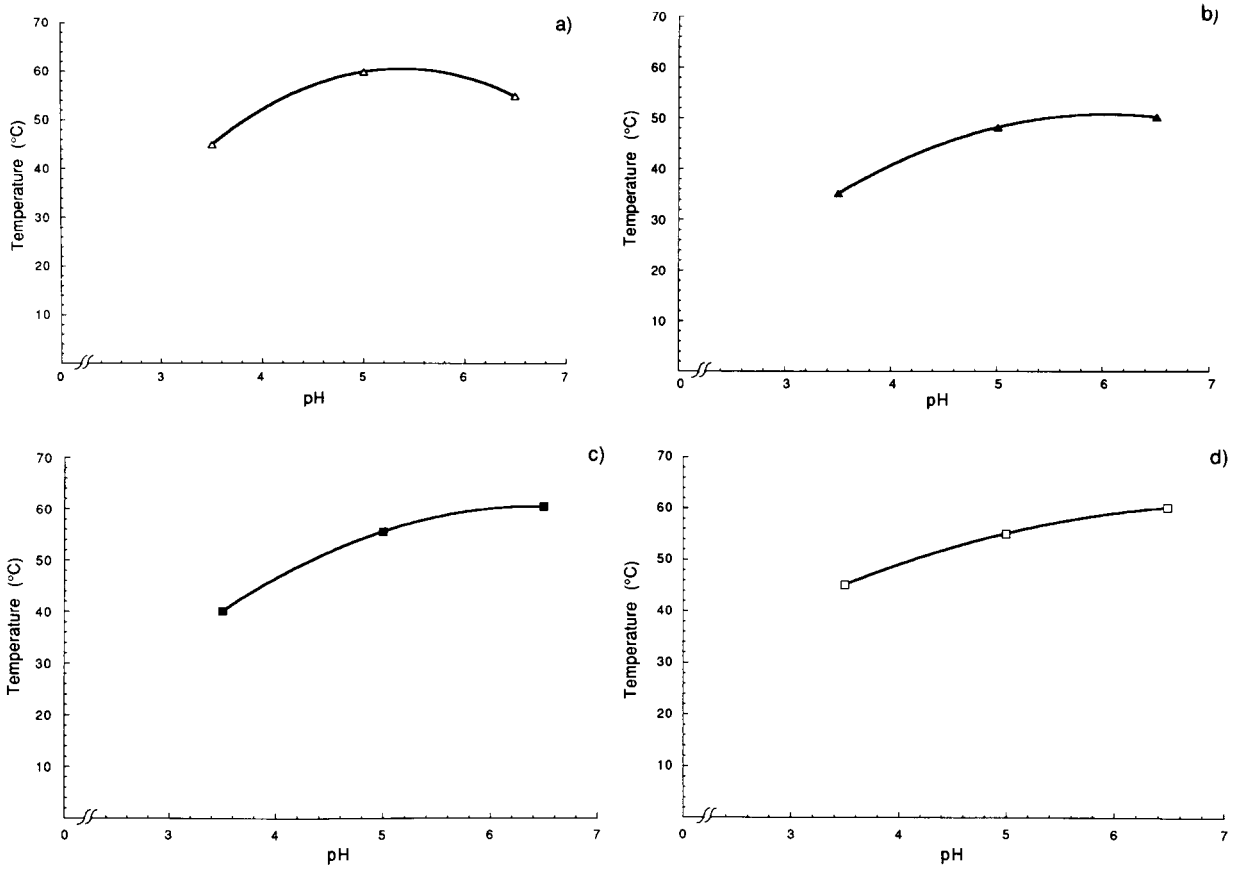

Figure 4 Temperature of the relative maximum of the catalytic membrane activity as a function of the corresponding pH value for the systems: (a) Teflon-MAA; (b) Teflon-AA; (c) Teflon-AM; and (d) HEMA directly grafted on Teflon.

pH) are fixed. This means that, if we are using, for example, a Teflon-MAA membrane and the solution to be treated has a pH value of 6.0, the temperature for obtaining the best yield of the process must be 59°C. This value can be deduced by Figure 4(a), as well as by solving the parabolic equation with the appropriate values for the M_i coefficients. Vice versa for obtaining the best yield we must use a solution at pH 3.7 with a Teflon-MAA membrane, a solution at pH 3.9 with a Teflon-AA membrane, a solution at pH 4.5 with a Teflon-AM membrane, and a solution at pH 5.0 with a Teflon membrane directly grafted with HEMA if we are compelled to work at a temperature of 48°C.

Table II Numerical Values of the Coefficients of Eq. (1)

Membrane System	M_0 (°C)	M_1 (°C)	M_2 (°C)
Teflon-MAA	−67.77	47.77	−4.44
Teflon-AA	−38.11	29.44	−2.44
Teflon-AM	−37.00	30.17	−2.33
HEMA directly grafted on Teflon	2.22	16.11	−1.11

Coefficients of the parabolic equation relating the temperature of the maximum of the relative activity to the corresponding pH value.

A larger number of experimental results than at present available would be desirable to pinpoint the position of the absolute maxima of membrane activity in our systems. However, on the basis of the data that are in our hands, a function $A(T, \text{pH})$ of the absolute activity may be constructed for each immobilized enzyme system. To this aim, polynomial fitting is done for each group of measurements conducted keeping constant one of the two variables, whereas the other varies within the adopted range. The $A(\text{pH})$ and $A(T)$ curves obtained in this way can be fitted by a polynomial form that, in each case, cannot be of a higher degree than the total number of the cor-

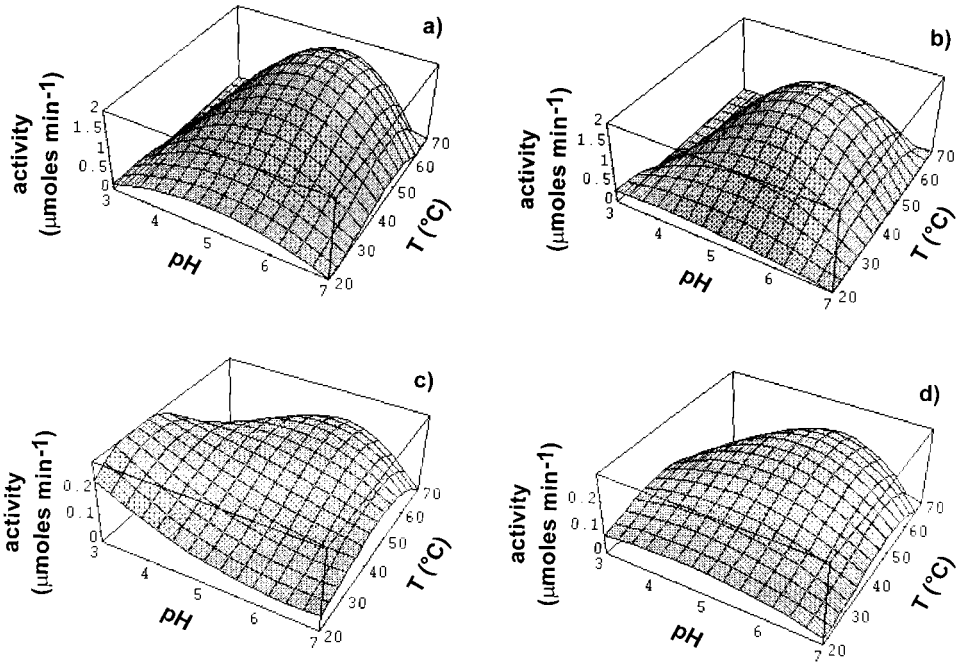

Figure 5 Catalytic membrane activity as a function of temperature and pH for the systems: (a) Teflon-MAA; (b) Teflon-AA; (c) Teflon-AM; and (d) HEMA directly grafted on Teflon.

responding independent experimental data minus one. Thus, we could fit the pH dependence with a second order and the T dependence with a higher best-fit polynomial. These curves were then arranged into a three-dimensional mesh over a Cartesian axis system (A, pH, T). The resulting three-dimensional surfaces are reported in Figure 5. Each of the surfaces can be represented by an equation of the general form

$$A(T, \text{pH}) = [a + b(\text{pH}) + c(\text{pH})^2](d + eT + fT^2 + gT^3) \quad (3)$$

Table III T_{Optimum} and $\text{pH}_{\text{Optimum}}$ for the Four Catalytic Membranes

Membrane System	T_{Optimum} (°C)	$\text{pH}_{\text{Optimum}}$
Teflon-MAA	58.6	5.2
Teflon-AA	52.2	5.2
Teflon-AM	56.1	4.9
HEMA directly grafted on Teflon	56.4	5.0

pH and temperature couples giving the best efficiency for each catalytic membrane. Values have been calculated by means of eq. (3).

from which, by a double partial differentiation with respect pH or T, one gets the couple of values of the independent variables pH and T yielding the maximum membrane activity in the range of experimental conditions used. These values are reported in Table III.

Kinetic Parameters

For using immobilized enzymes in bioreactors or in biosensors, it is very important to know the apparent kinetic parameters resulting from the immobilization process. In general, these parameters undergo variations indicating an affinity change for the substrate. These variations can be attributed to protein conformational changes, steric hindrances, and partitioning and diffusion effects. All of these factors may occur simultaneously or separately. As a consequence, the affinity between enzyme and substrate may be modified by immobilization. This modification can result in a decrease[20,21] or increase[22,23] of the apparent K_m value. A decrease in the K_m value of an immobilized enzyme leads to a faster reaction rate than its free counterpart, whereas an increase of K_m implies the use of a higher substrate concentration to achieve the same rate of the reaction ob-

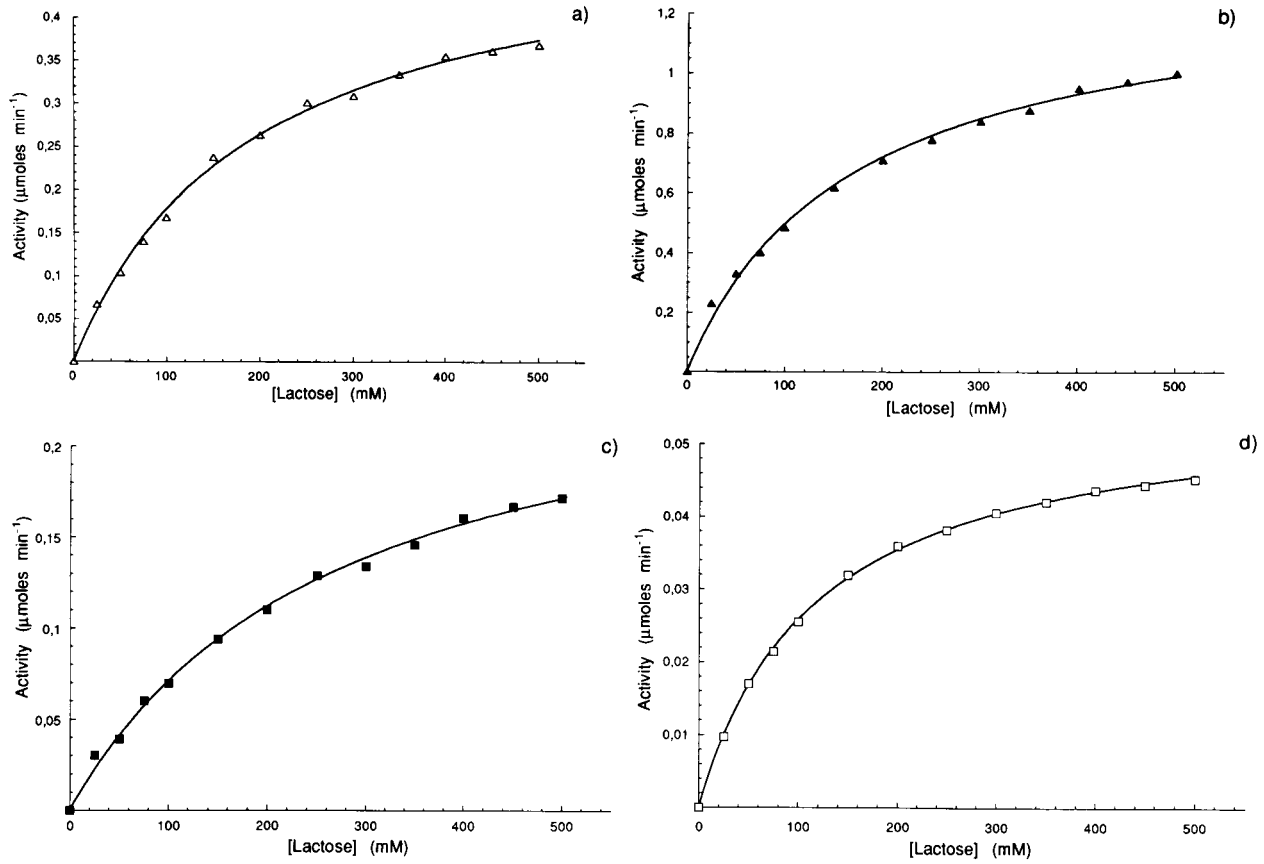

Figure 6 Catalytic membrane activity as a function of initial substrate concentration for the systems: (a) Teflon-MAA; (b) Teflon-AA; (c) Teflon-AM; and (d) HEMA directly grafted on Teflon.

tained with the free enzyme. The apparent K_m certainly decreases if the charges on the support and the substrate are opposite. The conformational changes of the proteic molecule and steric hindrances usually lead to an increase in the K_m values, due to a decrease in the affinity between the enzyme and the substrate.

Also, the V_m values are affected by the immobilization procedure. In general, the values of V_m obtained for the immobilized enzymes are almost the same of that for the free enzyme, also if increases[24] or decreases[25] are reported.

To know the apparent values of K_m and V_m of the β-galactosidase immobilized on the four types of membranes used, we have studied the activity of each catalytic membrane as a function of substrate concentration in the range from 25 to 500 m*M*. The pH and temperature of the solutions 6.5 and 30°C, respectively. The results of this investigation are illustrated in Figure 6, where we reported the catalytic activity of each of the four membranes used as a function of initial substrate concentration. From the figure, it is evident how each membrane exhibits different values of enzyme activity at the same substrate concentration. Having used the same initial enzyme concentration to load the membranes, these results indicate that the yield of immobilization is different for the four membrane types. In particular, we observe that the absolute activity of the membranes follows the order: AA $\gg$ MAA $>$ AM $>$ HEMA. None of the parameters reported in Table I seems to justify this behavior.

From the results reported in Figure 6, we have calculated the K_m and V_m values for the β-galactosidase immobilized on each of the four membrane types. These values are reported in Table IV, where it is possible to see for all of the membrane systems used how the apparent values of K_m of the immobilized enzymes are higher than that of the free enzyme.

The remarkable decrease of the enzyme affinity

Table IV Kinetics Parameters

System	K_m (mM)	V_m (μmol min^{-1})
Free enzyme	21.4	3.2
Teflon-MAA	190.5	0.53
Teflon-AA	145.8	1.20
Teflon-AM	236.9	0.27
HEMA directly grafted on Teflon	120.0	0.06

K_m and V_m values are reported for the free and immobilized form of β-galactosidase. The V_m value for the free enzyme is expressed in μmol min^{-1} for mg of enzyme. The units of V_m for immobilized enzyme are expressed in μmol min^{-1}, because we do not know the amount of enzyme bound to the membrane.

for the substrate when the β-galactosidase is immobilized on our modified Teflon membranes points out to some limitations about the possibility of using these membranes in industrial processes. In these processes, the reduction of the affinity of an immobilized enzyme is compensated for by the possibility of reusing the membrane. We have a technology that gives an added advantage in using immobilized enzymes in bioreactors. We have demonstrated[26] that the value of the apparent K_m for an immobilized enzyme operating under a nonisothermal condition is lower than that of the enzyme immobilized under isothermal conditions. This circumstance results in an efficiency increase of the yield of the enzyme reaction, making available for industrial processes also membranes endowed with low isothermal activity.

CONCLUSIONS

The aim of characterizing each membrane system biochemically and biophysically has been reached. Also, the operational properties of the membranes have been defined. The time stability of each membrane is good, because after an initial phase in which a small loss of enzyme activity is observed, the catalytic activity of the membranes remains unchanged more than 2 months.

More interesting for the practical use of our membranes are eqs. (2) and (3). Equation (1) allows us to choose the correlated parameter in the pH and temperature couple giving the highest membrane efficiency when the other parameter is imposed. Equation (3), instead, allows us to define for each membrane system the pH and temperature couple giving the best yield of each catalytic membrane.

With respect to the possibility of using these catalytic membranes in bioreactors operating under nonisothermal conditions,[26–31] the aim of simplifying the membrane system used until now seems reached. In our previous works, indeed, the membrane system used in nonisothermal bioreactors was a catalytic membrane, of natural or synthetic origin, coupled with a Teflon membranes. The advantage in using nonisothermal bioreactors is due to the fact that these ones have an increased efficiency in respect to that of the same bioreactors operating under comparable isothermal conditions. The catalytic Teflon membranes obtained in this research must be tested in the bioreactors before deciding if they are useful or not for working under nonisothermal conditions. If reference is done to the swelling degree, the following order is expected to be found in the performance of the nonisothermal bioreactor: HEMA $\gg$ MAA $>$ AM $>$ AA. This indication follows from the consideration that, for the occurrence of the process of thermodialysis,[32–36] which is the principal cause responsible for the efficiency increase of a nonisothermal bioreactor, hydrophobic membranes must be used. Experiments in this direction are in our laboratory.

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