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Influence of the microenvironment on the activity of enzymes immobilized on Teflon membranes grafted by γ-radiation

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Abstract

The effect of the microenvironment and immobilization method on the activity of immobilized β-galactosidase was investigated. Immobilization was done on Teflon membranes grafted with different acrylic monomers by γ-radiation and activated by two different coupling agents through the functional groups of the grafted monomers. 2-Hydroxyethyl methacrylate (HEMA) and methacrylic acid (MAA) were grafted on the membrane, and 1,6-hexamethylenediamine (HMDA) was used as a spacer. Glutaraldehyde or cyanuric chloride were used as coupling agents to bind the enzyme to the membrane. Four different catalytic membranes were obtained using the same solid support. Direct comparison between the isothermal behaviour of the biocatalyst in its free and immobilized form was carried out. In particular the dependence of the isothermal activity on the temperature and pH was studied and the kinetic parameters determined. The influence of the microenvironment on the observed activity of the four membranes was evidenced and discussed. The way of improving the yield of these catalytic membranes is discussed also.

Keywords: Immobilized enzymes; Biocatalytic membranes; β-Galactosidase; γ-Radiation; Microenvironmental

1. Introduction

Immobilized enzymes offer considerable advantages such as facility of removal and reuse, increased shelf life and thermal stability. For these reasons, studies on immobilization techniques, as well as on employment of catalytic membranes in industrial processes, have been rapidly increasing in recent years [1–3].

Polymeric materials are generally used as support matrices for immobilization, since they have various functional groups and can be easily modified chemically [4–6], or by irradiation [7–17]. The physico-chemical properties of the polymeric support, therefore, directly affect the choice of a suitable coupling procedure for immobilization and, consequently, the biochemical

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and biophysical behaviour of the immobilized enzymes. Among the various methods available for enzyme immobilization, covalent binding is particularly important, since it leads to preparation of stable enzyme derivatives [18–21].

In previous works [22,23], we reported a double grafting technique by which β-galactosidase was successfully immobilized by entrapment on Teflon membranes first grafted with methacrylic acid (MAA), and then with 2-hydroxyethyl methacrylate (HEMA).

In this work, we investigate the changes produced by the immobilization method on the microenvironment in which the immobilized biocatalyst operates and, hence, the effect on the enzyme catalytic activity. The catalytic behaviour of four different membranes will be discussed. They were prepared using the same polymeric matrix but with different grafted monomers, coupling agents, immobilization methods, and in the presence and absence of the spacer. On three membranes, the enzyme is covalently bound, while on the fourth membrane, it is entrapped. A direct comparison will also be made between the activity of the free and the immobilized form of the biocatalyst. The catalytic membranes will be characterized under isothermal conditions in order to obtain indications for the construction of new catalytic membranes that could usefully be employed in non-isothermal bioreactors. In these bioreactors, the enzyme reaction rate was found to increase proportionally to the temperature difference applied across the membrane. This increase depends on the enzyme and immobilization method and was found to be some 20 to 50% when a temperature difference of 1°C is applied across the catalytic membrane [24–30].

2. Materials and methods

Teflon [polytetrafluoroethylene (PTFE)] membranes of the 450 type manufactured by the Gelman Instrument (Ann Arbor, MI, USA) have been used as solid support for the grafting process. Membranes, constituted by a Teflon film supported on one side by a polypropylene net, had a thickness of 150 μm and were endowed with anatomizing irregular channels of 0.450 μm in nominal diameter. The nominal pore diameter is the one of the smallest particles which the membrane is able to retain.

HEMA and MAA monomers were used for grafting. Ferrous ammonium sulphate (FAS) was used as inhibitor for the formation of MAA homopolymers, since the radiation grafting was performed without oxygen using the mutual technique.

1,6-Hexamethylenediamine (HMDA) was used as a spacer. Glutaraldehyde (GLU) or cyanuric chloride were used as coupling agents to bind the enzyme to the activated membranes.

β-Galactosidase (EC. 3.2.1.23) from *Aspergillus Oryzae* was used as catalyst. The enzyme was chosen in view of the employment of these catalytic membranes in the process of lactose hydrolysis in milk. The β-galactosidase activity was assessed by the GOD-Perid method for the glucose determination (Boehringer, Mannheim, Germany).

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

2.1. Catalytic membrane preparation

Membrane grafting was performed by irradiation with γ-rays. The irradiation source was caesium 137 in a γ-cell 1000 Elite by Nordion International (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}.

Four membrane types have been prepared, each one different for the grafting technique, for the monomer and the coupling agent used. For this reason, each membrane type will be identified by a number. The grafting procedure and the methodology for membrane activation will

be indicated. A 10 mg/ml β-galactosidase solution was used for the immobilization process. The solution was prepared dissolving the enzyme in 0.1 M phosphate buffer solution, pH 6.5.

2.1.1. Membrane no. 1

These membranes were obtained by grafting MAA on the Teflon and using HMDA as a spacer. β-Galactosidase was coupled to the activated membrane by means of glutaraldehyde. The resulting catalytic grafted membranesi (Fig. 1a) were Teflon/MAA/HMDA/GLU/enzyme.

The experimental conditions used during the grafting were: 10% (v/v) MAA concentration; 0.1% (w/v) FAS concentration; 10 h of irradiation time. These conditions were found to be optimal in a preliminary work. At the end of the grafting procedure the membranes were washed under running tap water to remove the unbound monomers and the homopolymer produced. The grafted membranes thus obtained were divided into two lots, the first to estimate the grafting percent, the second used to bind the enzyme. The latter was immersed in a 10% (w/v) HMDA aqueous solution, then washed with water before a further treatment with 2.5% (v/v) glutaraldehyde aqueous solution. Both processes were carried out at room temperature for 1 h. The enzyme immobilization was done by immersing the preactivated membrane in the enzyme solution at 4°C for 16 h. In consequence the enzyme was bound to the carboxylic group of the poly-MAA (PMAA) branches via glutaraldehyde and HMDA.

2.1.2. Membrane no. 2

These membranes were prepared by performing a second grafting before the attachment of

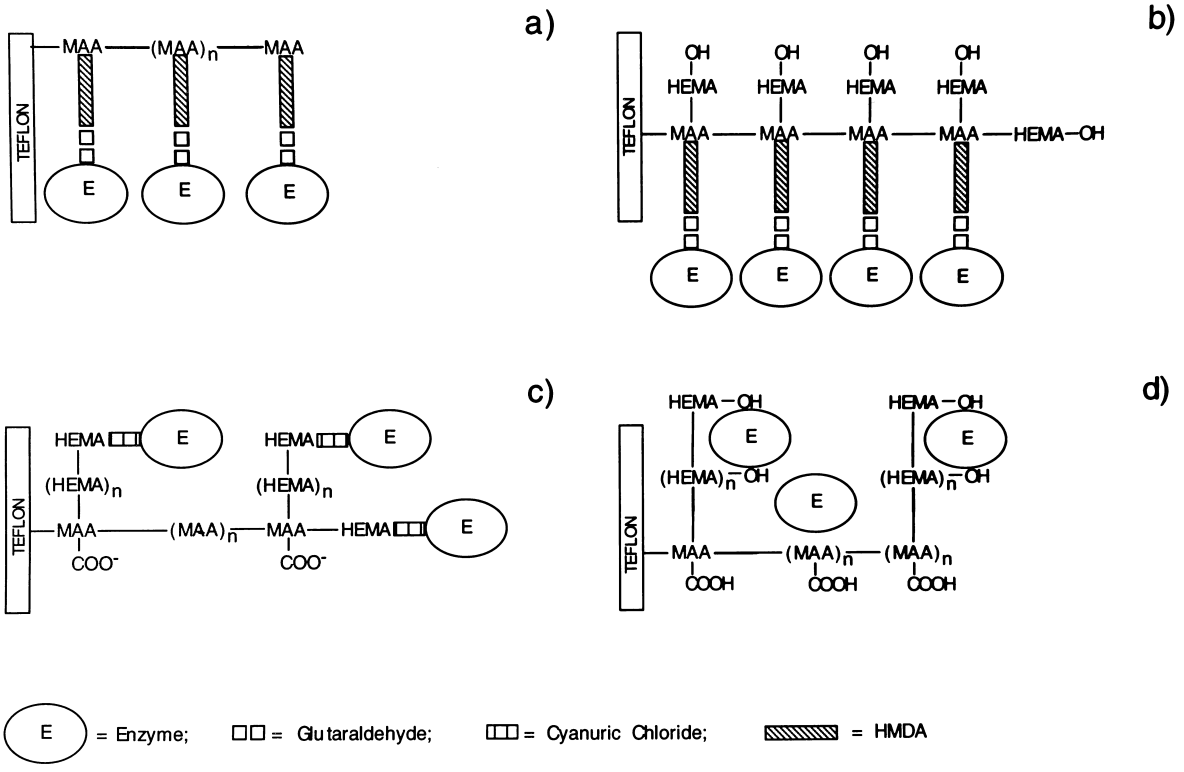

Fig. 1. Hypothetical picture of the location of the immobilized β-galactosidase in the four membrane types: (a) membrane no. 1; (b) membrane no. 2; (c) membrane no. 3; (d) membrane no. 4.

the spacer and the subsequent coupling of the enzyme. The resulting catalytic grafted membranes (Fig. 1b) were Teflon/MAA/HEMA/HMDA/GLU/enzyme.

The first grafting was performed in the same way as for membrane no. 1, while the second grafting by subsequent irradiation for 16 h into the γ-cell of a 10% (v/v) HEMA concentration. The enzyme immobilization was performed according to the procedure described for membrane no. 1. In consequence the enzyme was bound to the carboxylic group of the PMAA branches via glutaraldehyde and HMDA.

2.1.3. Membrane no. 3

These membranes were prepared using the double grafting technique (first grafting with MAA and second grafting with HEMA), but using cyanuric chloride as coupling agent. After the two grafting procedures performed under the conditions described above, the membranes were immersed for 10 min in a 0.2 N NaOH aqueous solution. After this step, the membranes were interposed between two paper filters to remove the NaOH excess from the membrane surfaces. At this point the membranes were immersed in 2.5% (w/v) cyanuric chloride/acetone solution for 20 min at room temperature. The cyanuric chloride excess was then removed by washing the membranes first with an acetone/water solution and subsequently with pure water. Then, enzyme immobilization was performed following the procedure described above. The resulting catalytic grafted membranes (Fig. 1c) were Teflon/MAA/HEMA/cyanuric chloride/enzyme. Enzyme immobilization was the same as previously described. In consequence, the enzyme was bound to the hydroxyl group of the HEMA branches via cyanuric chloride.

2.1.4. Membrane no. 4

These membranes were prepared using only the double grafting technique, with the enzyme dissolved directly in the HEMA solution used during the second grafting. The experimental conditions were the same used for membrane no. 2.

The resulting catalytic grafted membranes (Fig. 1d) thus were Teflon/MAA/HEMA/enzyme. In consequence, the enzyme was entrapped between PMAA and poly-HEMA (PHEMA) grafted branched chains.

2.2. Determination of the grafting degree

As for the percent 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, the grafting by the formula:

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

2.3. Determination of catalytic membrane activity

For the determination of the activity of the catalytic membranes these were put in 20 ml of a well stirred 0.1 M buffer phosphate solution, at the predetermined pH and temperature, containing lactose at 200 mM concentration. Enzyme activity was determined by sampling, at regular time intervals, the solution in contact with the membrane and measuring the glucose concentration by the GOD-Perid test. The test uses a coupled enzyme reaction by which, according to the scheme:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconate} + H_2O_2$$

$$H_2O_2 + \text{ABTS} \xrightarrow{\text{POD}} \text{coloured complex} + H_2O$$

a coloured solution is obtained. The glucose concentration, proportional to the intensity of the solution colour, is spectrophotometrically determined.

Membrane activity, expressed as μmol min^{-1}, is given by the slope of a linear plot of the glucose production as a function of time. In the study of the activity as a function of the pH, we used 0.1 M NaCl–HCl 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.

2.4. Determination of the time stability of the catalytic membrane

The time stability of the biocatalytic membranes was assessed by analyzing their activity under the same experimental conditions every day. After three days, during which the membranes lost some activity, a stable condition was reached remaining unchanged for over two months. Only these stabilized membranes were used in the comparative experiments reported in the following. When not in use the membranes were stored at 4°C in 0.1 M buffer phosphate solution, pH 6.5.

2.5. Treatment of experimental data

Every experimental point reported in the figures represents the average value of four experiments performed under the same conditions. The experimental errors did not exceed 6%. The duration of each experiment was 30 min, but only the initial reaction rates were accounted for in the construction of the figures.

3. Results and discussion

Before discussing the results, it is relevant to know the microenvironment in which the biocatalyst operates, since it directly affects the enzyme activity.

Two different immobilization methods were used: covalent binding for membranes nos. 1, 2 and 3, and entrapment for membrane no. 4.

The enzyme is bound to membrane no. 1 through the carboxylic groups of PMAA branches. This circumstance makes the micro environment around the enzyme neutral. The biocatalyst is bound to membrane no. 2 through the carboxylic groups of the PMAA branches in presence of the hydroxylic groups of PHEMA branches, making the enzyme microenvironment alkaline. In the case of membrane no. 3, the enzyme is attached to the hydroxyl groups of the PHEMA branches in the presence of the –COOH groups of the PHEMA branches, making the microenvironment in which the enzyme is operating acid. Finally in the case of membrane no. 4, the enzyme is entrapped within the –OH groups of the PHEMA branches and the –COOH groups of the PMAA. These circumstances make the enzyme microenvironment neutral.

These considerations must be taken in account while discussing the results, especially when the pH dependence of catalytic membrane activity is examined. This is only one of the aspects of the influence of the microenvironment on the enzyme activity. In general, immobilization may change the kinetics and other properties of the enzyme, usually leading to a decrease of the enzyme specific activity. The changes of the enzymatic properties are considered to be caused by several factors, such as conformational effects, steric effects, partitioning effects and mass transfer effects.

3.1. Grafting values

The average values of the MAA grafting percent were $8 \pm 2\%$ for all membrane types. The average values of the HEMA grafting percent were $26 \pm 3\%$ for all membrane types.

3.2. Temperature dependence of catalytic membrane activity

The temperature dependence of enzyme activity is represented by a bell-shaped curve with

an activity optimum. The curve profile for the immobilized enzyme can be broader, narrower or equal to the one of the free enzyme, while the activity optimum generally shifts towards higher temperatures upon immobilization. This means a higher resistance to thermal inactivation of the protein since the structure of the catalytic site is strengthened by the immobilization procedure which creates strong bonds between the macromolecule and the carrier. When the position of the maximum remains unchanged, it is possible to 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.

Fig. 2 reports the temperature dependence of β-galactosidase activity for the four types of catalytic membranes, prepared using covalent immobilization (Fig. 2a,b,c) or entrapment immobilization (Fig. 2d).

The temperature profile remains almost unchanged; except for the optimum activity position that is shifted towards higher temperatures for all the membranes. This shift is more evident in the case of enzyme bound to the carboxylic groups of PMAA grafted branches on membranes no. 1 and no. 2. The same behaviour is observed for the enzyme entrapped in membrane no. 4. The enzyme immobilized through the hydroxyl groups of the PHEMA branches on the membrane no. 3 shows a less appreciable shift of the optimum temperature.

Defining ‘optimal temperature range’ the range in which the relative activity is comprised

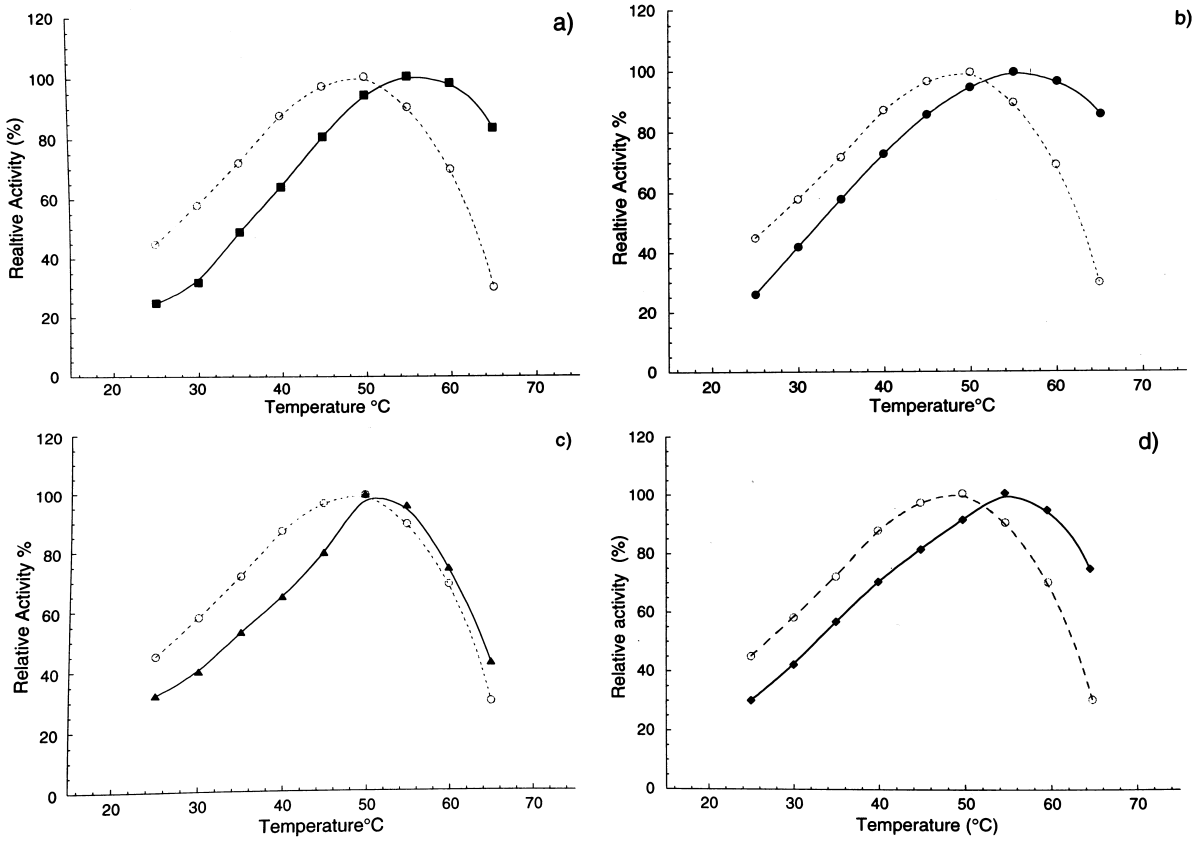

Fig. 2. Relative activity for the free and immobilized form of the β-galactosidase in the four membrane types as a function of temperature: (a) membrane no. 1: (■) = immobilized form, (○) = free enzyme; (b) membrane no. 2: (●) = immobilized form, (○) = free enzyme; (c) membrane no. 3: (▲) = immobilized form, (○) = free enzyme; (d) membrane no. 4: (♦) = immobilized form, (○) = free enzyme.

between 95 and 100%, it is possible to see that this range occurs between 45 and 51°C for the free enzyme, between 50 and 62°C for membranes no. 1 and no. 2, between 50.5 and 53.5°C for membrane no. 3, and between 54 and 58°C for membrane no. 4. From these observations it is possible to deduce that membranes no. 1 and no. 2 are more promising for industrial processes requiring high temperatures, followed by membranes no. 4 and no. 3, respectively.

Similar conclusions can be deduced by considering the results of Fig. 3 where the percent of the enzyme inactivation is reported as a function of the temperature. Again, it clearly appears that at high temperatures the catalytic membrane activity follows the order: membrane no. 2 > membrane no. 1 > membrane no. 4 > membrane no. 3 > free enzyme. For example, at 65°C, the catalytic power of the membrane is 12% reduced for membrane no. 2; 15% for membrane no. 1; 28% for membrane no. 4; 57% for membrane no. 3 and 70% for the free form of the enzyme. These data clearly indicate the influence of the microenvironment on the enzyme activity, membranes no. 1 and no. 2 ensuring more protection to the enzyme structure and, hence, to the enzyme activity.

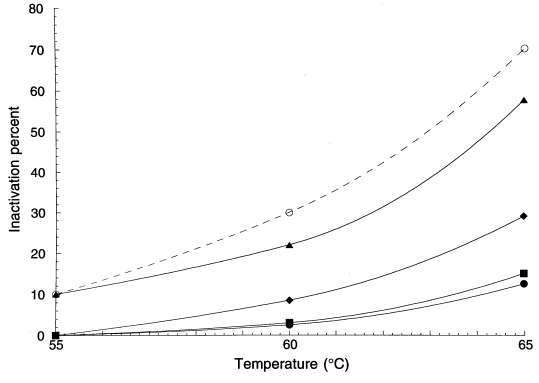

Fig. 3. Percent of enzyme inactivation as a function of temperature for the free and immobilized form of β-galactosidase in the four membrane types: free form: (○); membrane no. 1: (■); membrane no. 2: (●); membrane no. 3: (▲); membrane no. 4: (◆).

3.3. pH dependence of catalytic membrane activity

It is well known that the pH of the aqueous media in which the biocatalyst operates plays a relevant role on enzyme activity. This role is more evident when the enzyme is immobilized, since the support itself may change the pH value around the catalytic site, thus determining differences in the behaviour between the free and bound states of the catalyst. This effect is known as partitioning effect, directly related to the chemical nature of the supporting material which induces electrostatic or hydrophobic interactions between the matrix and the molecular species dissolved in the solutions. These interactions alter the microenvironment in which the enzyme actually operates. Because of this effect, different concentrations of charged species, as H^+ or OH^-, exist in the microenvironment of the immobilized enzyme. As a consequence, the local pH around the catalytic site results different from the pH of the bulk solution. Thus, the pH profile of the activity of the immobilized enzyme compared to the one of the free form is displaced towards more alkaline or acid pH values for negatively or positively charged matrices [31].

Keeping these considerations in mind, we investigated the activity of free and immobilized β-galactosidase in the pH range between 2 and 7. The results are reported in Fig. 4 where the relative activity of each of the four catalytic membranes is reported as a function of pH of the bulk solution. For comparison, the relative activity of the free enzyme is also shown. Temperature was kept constant at 30°C. Optimal activity was found at pH 4.5 for the free enzyme, while a displacement towards a more acid pH value was observed for the three membranes in which the catalyst is covalently bound to the solid support. The entrapped enzyme, instead, did not exhibit relevant changes in the position of the maximal activity. The behaviour of membranes no. 1 and no. 2 may be attributed to the formation of Schiff's bases. We have no

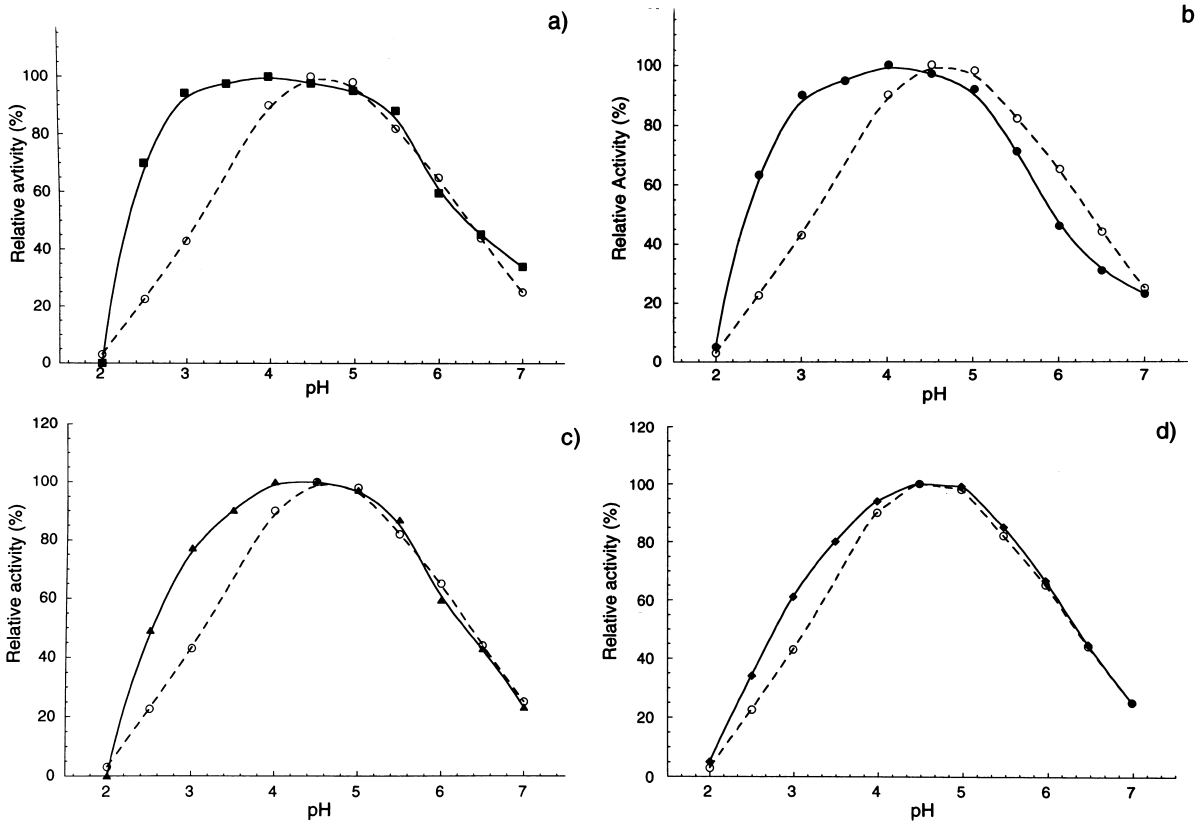

Fig. 4. Relative activity as a function of pH for the free and immobilized form of the β-galactosidase in the four membrane types: (a) membrane no. 1: (■) = immobilized form, (○) = free enzyme; (b) membrane no. 2: (●) = immobilized form, (○) = free enzyme; (c) membrane no. 3: (▲) = immobilized form, (○) = free enzyme; (d) membrane no. 4: (♦) = immobilized form, (○) = free enzyme.

explanation for the behaviour of membrane no. 3 for which a shift towards the alkaline side should be expected owing to the absence of the formation of Schiff's bases and the concomitant presence of the negatively charged carboxylic groups of the PMAA branches. The behaviour of membrane no. 4 appears reasonable since the simultaneous presence of carboxylic groups of the PMAA and hydroxyl groups of PHEMA makes practically the microenvironment around the enzyme neutral. In consequence, the pH profile for the free and immobilized form of the enzyme results almost similar.

Defining 'optimal pH range' the range at which the relative activity is comprised between 95 and 100%, it occurs between 4.5 and 4.9 for the free enzymes; between 3.9 and 4.1 for membrane no. 1; between 3.7 and 4.3 for membrane no. 2; between 3.95 and 4.65 for membrane no. 3; and between 4.45 and 4.95 for membrane no. 4.

From the results of Fig. 4 other considerations regarding the role of the microenvironment on enzyme activity can be deduced. While at high pH values, the immobilized and free forms of the enzyme do not exhibit appreciable differences in the values of the relative activity, marked differences exist at low pH values where the relative activity of the immobilized form results always higher than that of the free form. This means that at more acid solutions the immobilized form is more protected than the free form. For example: at pH 2.5, the relative activity of the free form is 20%; 75% for mem-

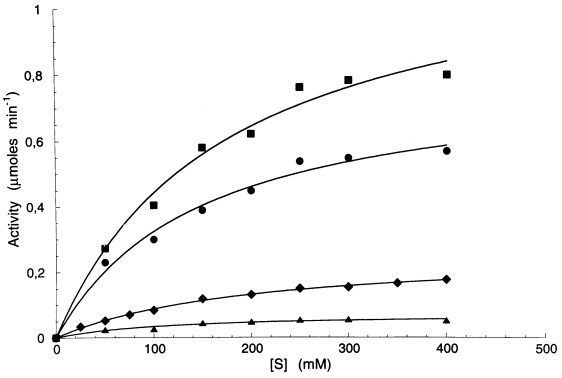

Fig. 5. Absolute activity as a function of the initial substrate concentration for β-galactosidase in the four membrane types. Symbols: (■) = membrane no. 1; (●) = membrane no. 2; (▲) = membrane no. 3; (♦) = membrane no. 4.

brane no. 1; 65% for membrane no. 2; 50% for membrane no. 3; and 35% for membrane no. 4. This is a useful indication for the preparation of catalytic membranes to be employed in industrial biotechnological processes.

3.4. Kinetic parameters

When a biocatalyst is immobilized the kinetic parameters K_m and V_{max} undergo variations with respect to the corresponding parameters of the free form, revealing an affinity change for the substrate. These variations can be attributed to several factors such as protein conformational changes induced by the attachment to the support, steric hindrances and diffusional effects. These factors, which may operate simultaneously or separately, alter in any case the microenvironment around the enzyme. This determines that the apparent K_m value of the immobilized form may decrease [32,33] or increase [34,35]. A decrease in the K_m value leads to a faster reaction rate, whereas an increase of the K_m implies the use of a higher substrate concentration in order to get the same reaction rate observed for the free enzyme. The apparent K_m certainly decreases if the electric charges on the support and substrate are opposite. The opposite occurs if the support and the substrate have electric charges of the same sign. This is a classical example of how the microenvironment affects the reaction rate of an enzymatic process.

Also, the V_{max} values are affected by the immobilization process. In general similar values of V_{max} have been found for the free and the immobilized form of the enzyme, even if increases [36] or decreases [37] have also been reported.

To determine the microenvironmental influences on the kinetic parameters of β-galactosidase immobilized on the four types of membranes the activity of each catalytic membrane was studied as a function of substrate concentration. The pH and temperature of the solutions were 6.5 and 30°C, respectively. The results are reported in Fig. 5. Since the same initial enzyme concentration was used to load the four types of membranes, the difference shown in figure indicate that the yield of immobilization is different. In particular, the absolute activity follows the order: membrane no. 1 > membrane no. 2 > membrane no. 4 > membrane no. 3. Membrane no. 1 and membrane no. 2 should theoretically have the same number of initial free radicals to which the MAA attachment occurs; hence, the same number of MAA chains. In this respect, their activities should have been the same. However, the presence of branches due to PHEMA creates steric hindrances to the enzyme

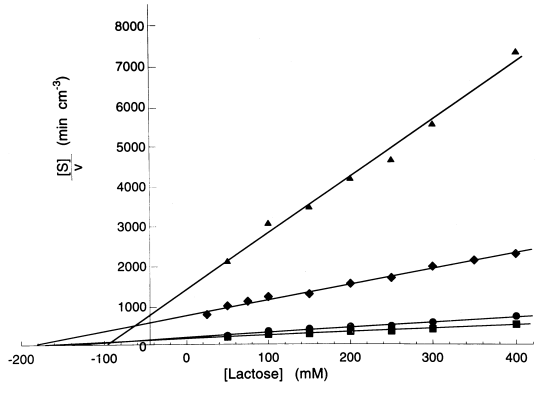

Fig. 6. Hanes plot for β-galactosidase in the four membrane types. Symbols: (■) = membrane no. 1; (●) = membrane no. 2; (▲) = membrane no. 3; (♦) = membrane no. 4.

immobilization process (thus, limiting the amount of the immobilized enzyme) and to the diffusive approach (or removal) of substrate (or of products) towards (or from) the catalytic site. This justifies the higher activity of membrane no. 1.

Membrane no. 2 and membrane no. 3, instead, have the same grafting history, thus their activities should be the same. On the contrary, the results in the figure show for membrane no. 3 an activity one order lower than that of membrane no. 2. This different behaviour can be attributed to the presence of the HDMA as spacer on membrane 2. The spacer, indeed, keeps the enzyme further away from the membrane, thus reducing the interactions with the support.

The behaviour of membrane no. 4, which is intermediate between membranes no. 1 and no. 2 and membrane no. 3, is justified by the presence of the dense polymeric net constituted by PHEMA and PMAA branches, which results in a close barrier entrapping the enzyme and restricting the diffusional rates of substrate and products.

A Hanes plot of the results of Fig. 5 allows the calculation of the apparent kinetic parameters for β-galactosidase immobilized on the four membrane types. This was done in Fig. 6. The apparent values of K_m and V_{max} are reported in Table 1, together with the ones relative to the free enzyme. Surprisingly, the most active membranes exhibit a lower affinity for the substrate. This is a clear indication of the fact that:

Table 1
Kinetic parameters

Enzyme system	K_m^{app} (mM)	υ_{max}^{app} (μmol min^{-1})
Free enzyme	21.4	3.2
Membrane no. 1	171.2	1.20
Membrane no. 2	140.0	0.79
Membrane no. 3	97.6	0.074
Membrane no. 4	191.3	0.526

Apparent K_m and V_{max} values for the free and immobilized form of the β-galactosidase in the four membrane types.
The values for the free enzyme were calculated by us in a previous work and are real values.

(i) the immobilization technique affects the amount of immobilized enzyme; (ii) the microenvironment influences the rate of the enzyme reaction and the affinity for the substrate.

4. Conclusion

The aim of this work, focused on the study of the influence of the microenvironment on the activity of immobilized enzymes, has been reached. pH and temperature profiles of the relative activity were different for the four membranes and between these and the free form of the enzyme. Membranes no. 1 and no. 2 were more suitable for practical applications because they offer more protection to the enzyme activity at high temperature and at alkaline pH values.

It has also been shown that the kinetic parameters are influenced by the microenvironmental properties in which the enzyme operates, while the absolute activity depends on the immobilization method.

The axiom ‘more activity more affinity’ in our case does not hold good. The more active membranes, indeed, exhibited smaller affinity for the substrate.

The lower affinity for the substrate shown by the immobilized form with respect to the free enzyme can be overcome by employing the catalytic membranes in bioreactors operating under non-isothermal conditions. Under these conditions, we observed not only an enzyme activity increase, but also an increase of its affinity for the substrate [30–35]. The K_m values for the catalytic membranes operating under non-isothermal conditions were, indeed, lower than the values of the same membranes operating in isothermal bioreactors. These results were obtained with catalytic hydrophilic membrane coupled to a hydrophobic teflon membrane. The same results, together with an efficiency increase, were recently obtained with a non-isothermal bioreactor employing a Teflon grafted membrane [38], not only hydrophobic but also

catalytic. If the technology of the non-isothermal bioreactors is applied to the catalytic membranes prepared for this research, we expect an improvement of their enzymatic yield together with an increase of the bioreactor efficiency. Experiments in this direction are in progress in our laboratory.

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