ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 21 (2003) 239–252

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Influence of the spacer length on the activity of enzymes immobilised on nylon/polyGMA membranes Part 1. Isothermal conditions

A. De Maio [a,b], M.M. El-Masry [a,1], M. Portaccio [a,b], N. Diano [a], S. Di Martino [a], A. Mattei [b,2], U. Bencivenga [a], D.G. Mita [a,b,*]

[a] *Institute of Genetics and Biophysics of CNR, Via Guglielmo Marconi 12, 80125 Naples, Italy*
[b] *Department of Experimental Medicine, Second University of Naples, Via S. M. di Costantinopoli 16, 80138 Naples, Italy*

Received 29 May 2002; received in revised form 23 September 2002; accepted 23 September 2002

Abstract

β-Galactosidase was immobilized on nylon/poly(glycidyl methacrylate) membranes through spacers of different length: hexamethylenediamine, ethylenediamine or hydrazine. The effect of the spacer length on the catalytic behavior of the three membranes was studied in isothermal bioreactors. The behavior of the soluble and insoluble enzymes was compared to know the effects of the immobilization process and of the spacer length.

The enzyme derivatives in comparison with the soluble enzyme exhibited shifts of the optimum pH values towards more acidic solutions. These shifts were found decreasing with the spacer length; while an opposite trend was observed when the optimum temperature values were considered. Also the values of the apparent K_m were found to decrease with the spacer length.

All these results indicated that a soluble enzyme could be considered as an enzyme immobilized on a solid support through a spacer of infinite length.

Keywords: β-Galactosidase; Chemical grafting; Biocatalytic membranes; Nylon membranes; Isothermal bioreactors

1. Introduction

Immobilized catalyst, enzymes or whole cells, are successfully employed in an increasing number of industrial processes [1–3]. Typical examples are the optical resolution of racemic aminoacids by aminoacylase [4], the development of an enzymatic process by means of immobilized bacterial cells for manufacturing acrylamide [5], the lactose hydrolysis in milk by β-galactosidase [6], the waste water treatment by urease [7,8].

The success encountered by the employment of immobilized enzymes in biotechnological processes stimulated the interest of the researchers towards the basic research addressed to improve the performance of the biocatalytic membranes. To this aim, the study of the interactions between the enzyme and the support became of fundamental relevance.

It is well known that the immobilization process affects the enzyme activity in respect to that of the soluble counterpart. The physico-chemical nature of the

* Corresponding author. Tel.: +39-81-2395887; fax: +39-81-2395887.
E-mail address: mita@iigb.na.cnr.it (D.G. Mita).
[1] Permanent address: Department of Polymers and Pigments, National Research Center, Dokki, Cairo, Egypt.
[2] Permanent address: Dipartimento di Scienze Chirurgiche ed Anestesiologiche, Seconda Università di Napoli, Napoli, Italia.

1381-1177/02/$ – see front matter
PII: S1381-1177(02)00229-1

carrier as well as the immobilization methods are the main causes of the observed differences. The carrier nature acts mainly through the "partitioning effect" [9] which is responsible of the changes in the chemical composition of the microenvironment in which the immobilized enzyme is operating in respect to that of the bulk solution. The immobilization method acts through the nature of the binding forces or through the type and the position of the aminoacidic residues involved in the enzyme attachment. Covalent coupling is the most convenient immobilization technique since its allows high accessibility and reusability of the bound enzyme [10]. Covalent attachment, in addition, offer the advantage that no relevant leaking of enzyme takes place in repeated uses since the binding forces are stronger than those occurring in the absorption or ionic binding.

When the support and the immobilization method are the same, one more subtle cause which can affect the activity of the immobilized enzyme is the length of the spacer between the catalyst and the activated support.

In this paper, we will discuss the results obtained with catalytic membranes prepared by using the same support (a nylon/polyGMA membrane), the same immobilization method (condensation by means of glutaraldehyde as bifunctional binding agent), but spacers of different length. Comparison with the behavior of the soluble enzyme will be performed to evidenziate the effects induced by the immobilization process, with particular reference to the spacer length. All experiments were carried out under isothermal conditions.

In a separate paper [11], we have reported the behavior in a non-isothermal bioreactor of the membranes characterized in this paper. The advantage of using non-isothermal bioreactors in biotechnological processes has been demonstrated [12–21].

2. Apparatus, materials and methods

2.1. The bioreactor

The bioreactor consisted of two cylindrical half-cells, 2.5 mm in depth and 35 mm in diameter, filled with the working solution and separated by the catalytic membrane. Substrate solutions were recirculated in each half-cell at a rate of 3.5 mL min^{-1} by means of a peristaltic pump through hydraulic circuits starting and ending in a common container. In this way, the whole solution reacted with the two faces of the catalytic membrane and the catalytic power of both faces was averaged. Thermocouples, placed at 1.5 mm from each of the membrane surfaces, were used to measure the temperatures inside each half-cell. The temperature in each half-cell was programmed by means of circulation in external jackets of water coming from a thermostatic baths. A 3D picture, not to scale, of the core of the bioreactor is represented in Fig. 1.

2.2. Materials

As solid support to be grafted nylon Hydrolon membranes, a gift of Pall Italia (Pall Italia srl, Milano, Italy) were used. These membranes, 150 μm in thickness, are hydrophobic and have a nominal pore diameter of 0.2 μm. Pore size is the measure of the diameter of the smallest particles that the membrane retains, since the membrane has no "classical pores", but irregular and interconnected cavities crossing the membrane thickness. Every kind of transmembrane matter transport, such as water or solute fluxes driven by concentration, pressure, temperature or electrical gradients, takes place through these cavities.

All chemicals, including the enzyme, were purchased from Sigma (Sigma-Aldrich srl, Milano, Italy) and used without further purification. Glycidyl methacrylate (GMA) was used as monomer to be grafted. Hexamethylenediamine, ethylenediamine or hydrazine were separately employed as spacer between the grafted membrane and the enzyme. A 2.5% glutaraldehyde aqueous solution was also used as bifunctional coupling agent for covalently binding the enzyme to the activated membranes.

The enzyme employed was a β-galactosidase (EC 3.2.1.23) from *Aspergillus oryzae*. β-Galactosidase has been used in view of the employment of these membranes in lactose hydrolysis in milk or in the treatment of the waste waters coming from dairy industry.

2.3. Methods

2.3.1. Preparation of the catalytic membranes

The preparation of the catalytic membranes was carried out in two steps: (a) grafting copolymerization, and (b) enzyme immobilization.

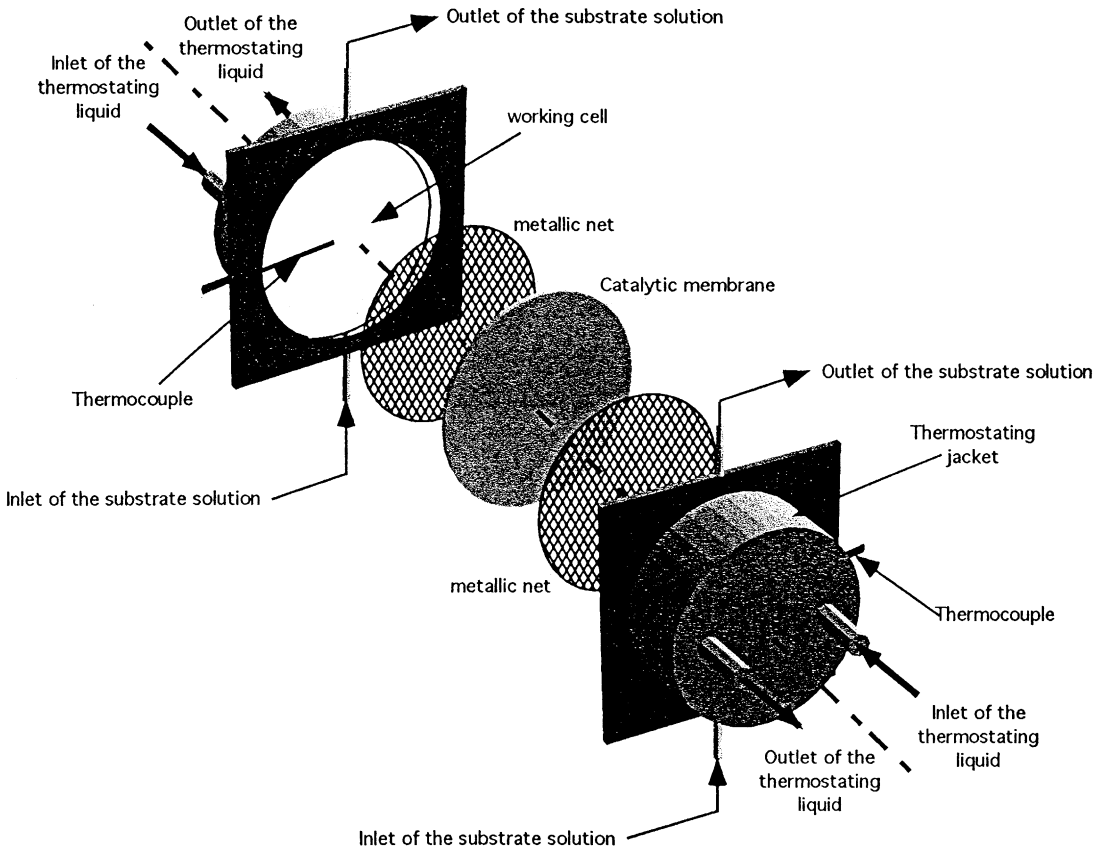

Fig. 1. A 3D picture, not to scale, of the core of the bioreactor. The hydraulic circuits, through which the substrate solutions are recirculated and the common cylinder have been omitted.

2.3.1.1. Grafting copolymerization. Grafting copolymerization was carried out by using as initiating system $K_2S_2O_8/Na_2S_2O_3$ in the ratio 1:1. The membranes were immersed, for 1 h at 40 °C, in a reaction vessel filled with a 1:1 water/ethanol solution containing 0.3 M GMA, 0.008 M $K_2S_2O_8$ and $Na_2S_2O_3$ and in the presence of 0.004% (w/v) copper acetate. Later on, the membranes were treated with methylethyl ketone to remove the produced homopolymer, then dried at 40 °C until a constant weight was measured. At this point, a nylon/polyGMA membrane was obtained, The grafting percentage (X, %) was determined by the difference between membrane masses before, G_B, and after, G_A, the grafting by means of the formula:

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

The GMA grafting occurred also into the membrane pores.

2.3.1.2. Enzyme immobilization. Hexamethylenediamine, ethylenediamine or hydrazine were used as spacers of different length. To this aim the membranes were treated, for 45 min at room temperature, with a 1% (v/v) spacer solution in 0.1 M sodium carbonate buffer, pH 9. After washing with running tap water to remove the unreacted amines, the membranes were treated for 90 min at room temperature in a 2.5% (v/v) glutaraldehyde aqueous solution. At the end of this treatment each membrane type was at first washed at room temperature with double distilled water, after with 0.1 M phosphate buffer solution, pH 6.5, finally treated for 16 h at 4 °C with the same buffer solution

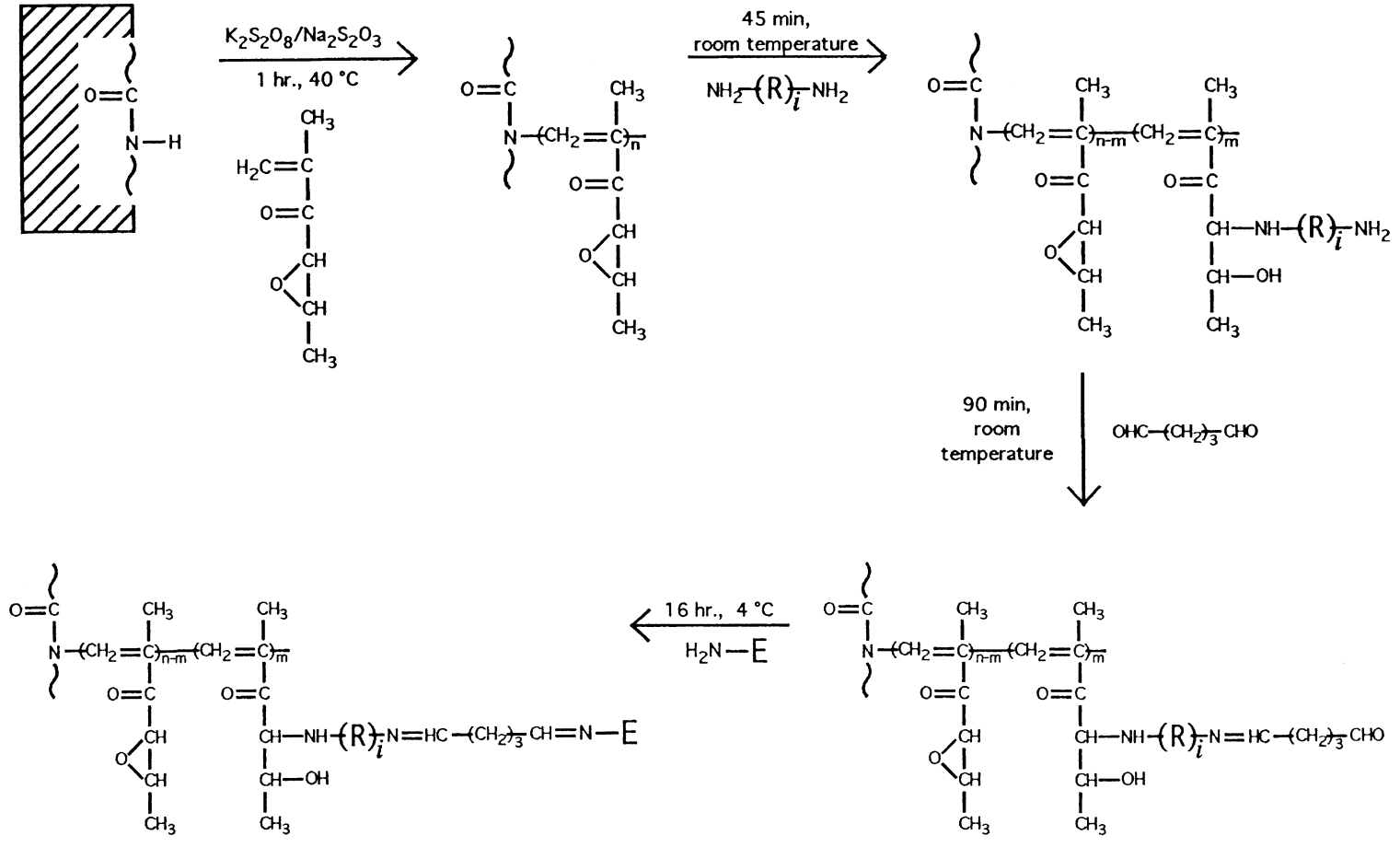

Fig. 2. Schematic sequence of grafting and enzyme immobilization processes to obtain membranes M_0, M_4 and M_6. R is CH_2. E-NH_2 indicates the enzyme with its terminal NH_2 group.

containing β-galactosidase at a concentration of 3 mg/mL. When this step was over, the membranes were washed with the 0.1 M phosphate buffer solution, pH 6.5, to remove the unbound enzymes. These conditions were found to be optimal in preliminary experiments aimed to obtain membrane types with comparable grafting degree, hydrophobicity and amount of immobilized enzyme.

The steps for the preparation of the catalytic membranes are reported in Fig. 2, were the spacer has been indicated with NH_2–$(R)_i$–NH_2, R being the CH_2 group. When *i* is equal to 6, hexamethylenediamine is the spacer and the corresponding catalytic membrane henceford will be named M_6; when *i* is 4, ethylenediamine is the spacer and the corresponding catalytic membrane will be named M_4, when *i* is 0 the spacer is hydrazine and the corresponding catalytic membrane will be named M_0.

2.3.2. Determination of membrane activity and stability

β-Galactosidase hydrolyses lactose to glucose and galactose. Enzyme activity was determined by sampling at regular time intervals in the common container the solution in contact with the two catalytic surfaces of the membrane and by measuring the glucose concentration by the GOD-Perid test. The test uses a coupled enzyme reaction by which a colored solution is obtained. The glucose concentration, proportional to the color solution intensity, is spectrophotometrically determined at 570 nm.

Membrane activity, expressed as $\mu\text{moles}\,\text{min}^{-1}$, is given by the angular coefficient of the straight line interpolating the experimental point of the glucose production (μmoles) as a function of time (min).

Time stability of the biocatalytic membranes was assessed by analysing every day their activity under the same experimental conditions, i.e. 0.2 M lactose in 0.1 M phosphate buffer solution, pH 6.5 and $T = 25\,^{\circ}\text{C}$. After 3/4 days, during which the membranes lost some activity, a stable condition was reached, remaining unchanged for over 2 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.

2.3.3. Experimental data treatment

Every experimental point reported in the figures represents the average value of five experiments performed under the same conditions. Each experiment lasted 30 min, but only the initial reaction rates were accounted for in the construction of the figures. The duration of each experiment, the composition of our solutions, and the hydrophobic nature of the membrane excluded the occurrence of membrane fouling. In any case, to avoid fouling due to membrane reuse, a cleaning 0.1 M phosphate buffer solution was circulated for 60 min through the bioreactor and the membrane between two subsequent experiments. Effects due to concentration polarization, even if present, have not been taken in account for considering the hydrophobic nature of the membrane.

3. Results

To compare the behavior of membranes M_0, M_4 and M_6, it is important to know the amount of enzyme immobilized on each membrane. This has been done by measuring the enzyme activity in the initial solution used for the immobilization, the residual activity of this solution after the immobilization process and the activity in all the solutions used to wash the membrane. Since at constant substrate concentration the activity is proportional to the enzyme concentration, it is easy to evaluate the amount of immobilized enzyme through a calibration curve of the catalytic activity of the free enzyme as a function of its concentration.

To this aim the equation: $a = b - c - \sum_{i=1}^{n} d_i$, has been used. "*a*" is the amount of immobilized enzyme, "*b*" and "*c*" are the amount of enzyme in the initial and final solution used for immobilization, respectively, and d_i the amount of enzyme found in the *i*th washing, *n* being the number of washings. The washing ends when d_n becomes zero. The result of this procedure gives the amount of enzyme immobilized on each membrane. In Table 1, the amount of immobilized enzyme as well as the absolute and relative catalytic activities for each of the three membrane types are reported. Absolute membrane activity has been defined as the activity for the total membrane surface (two surfaces of 35 cm^2 each one), while the specific activity as the activity for milligram of immobilized protein. In Table 1, the grafting percentages,

Table 1
Physical and biochemical properties of the catalytic membranes

Membrane type	Grafting degree (%)	Immobilized enzyme (mg)	Absolute activity (μmoles min^{-1})	Specific activity (μmoles min^{-1} mg^{-1})	Activity retention (%)
M_0	13.96	2.8	0.12	0.043	2.5
M_4	14.03	3.1	0.41	0.132	7.2
M_6	13.86	2.9	1.99	0.686	38.0

The activity retention has been calculated considering that 1 mg of soluble enzyme in a 0.2 M lactose concentration in 0.1 M phosphate buffer, pH 6.5 and $T = 25\,^{\circ}C$, gives a catalytic activity of 1.82 μmoles min^{-1}.

measured according to Eq. (1), are also listed for each of the three membranes.

Inspection of the data in Table 1 shows the strong dependence of the activity of the immobilized enzyme on the spacer length since the physical parameters, such as grafting degree and amount of immobilized enzyme, are practically the same for all the three membrane types. The high value of the activity retention of membrane M_6, three times in respect to that of membrane M_4 and 14 times higher in comparison to membrane M_0, induces to consider a soluble enzyme as an enzyme immobilized on a support through a spacer of infinite length. Under these conditions the interactions between the enzyme and the support, indeed, vanish.

3.1. Kinetic parameters

When a biocatalyst is immobilized, the kinetic parameters K_m and V_{max} undergo variations in comparison with the corresponding parameters of the soluble enzyme. To indicate that the kinetic parameters are changed they are indicated as $K_{m,app.}$ and $V_{max,app.}$. These variations are attributed to several factors such as: (i) the changes in the protein conformation induced by the interactions between the support and the enzyme; (ii) the immobilization methods which, in the case of covalent attachment, can involve different aminoacidic residues; (iii) the steric hindrances and the diffusional effects introduced by the grafted monomers or by the spacer. These factors may operate simultaneously or separately. Consequently the apparent $K_{m,app.}$ value may increase [22,23] or decrease [24,25] in comparison with that of the soluble enzyme. A decrease of the $K_{m,app.}$ value leads to a faster reaction rate, whereas an increase of the $K_{m,app.}$ implies the use of a higher substrate concentration in order to get the same reaction rate observed for the free enzyme. The $K_{m,app.}$ increases if, for example, the electric charges on support and substrate are of the same sign. The contrary occurs if the support and the substrate have opposite electric charges.

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 or decreases have also been reported. Nevertheless it is difficult to compare the values of the $V_{max,app.}$ with the ones of the soluble enzymes, as the reaction rates are proportional to the amount of enzymes, and in the case of immobilization, even if we know the amount of immobilized enzymes, we don't know the percentage of active enzymes.

To determine the kinetic parameters for β-galactosidase immobilized on M_0, M_4 and M_6, the activity of the catalytic membranes was studied as a function of substrate concentration. The pH and temperature of the solutions were 6.5 and 25 °C, respectively. The results, reported in Fig. 3a, show that: (i) each of the three membrane types exhibited a Michaelis–Menten behavior; (ii) at each lactose concentration investigated the reaction rate of membrane M_0 was lower than that of membrane M_2 which, in turn, had a reaction rate lower than that of membrane M_6. The latter observation confirms the conclusion derived from Table 1: more far the enzyme is from the support, more high is its reaction rate. This means that the enzyme affinity towards the substrate increases with to the spacer length, by assuming that greater apparent affinities toward the substrate must correspond at higher reaction rate, under equal substrate concentrations.

To verify this hypothesis, in Fig. 3b, the experimental points of Fig. 3a have been reported in form of Hanes plots. The $K_{m,app.}$ and $V_{max,app.}$ values, calculated from Fig. 3b, are listed in Table 2, together with

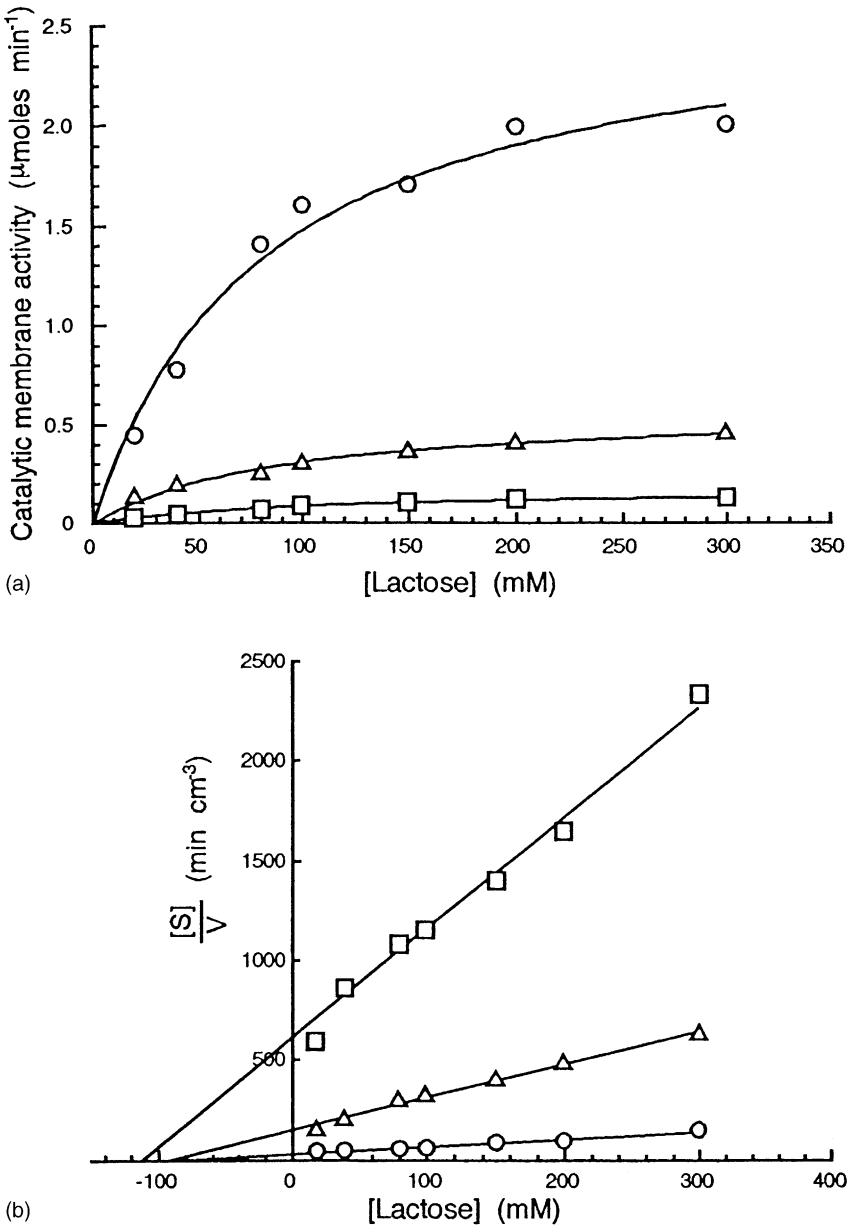

Fig. 3. (a) Catalytic membrane activity as a function of substrate concentration. (b) Hanes plots of the experimental points in Fig. 3a. (○) Membrane M_6; (△) membrane M_4; (□) membrane M_0.

the ones relative to the free enzyme [21]. Different values of $K_{m,app.}$ and $V_{max,app.}$ have been found. The affinity of the enzyme towards the substrate is smaller for the immobilized enzymes in comparison with that found for the soluble one. Moreover, the affinity towards the substrate for the β-galactosidase immobilized on membrane M_6 is higher than that of the enzyme immobilized on M_2, the latter being, in turn, higher than that of the enzyme immobilized on M_0. Practically, the apparent affinity towards the substrate

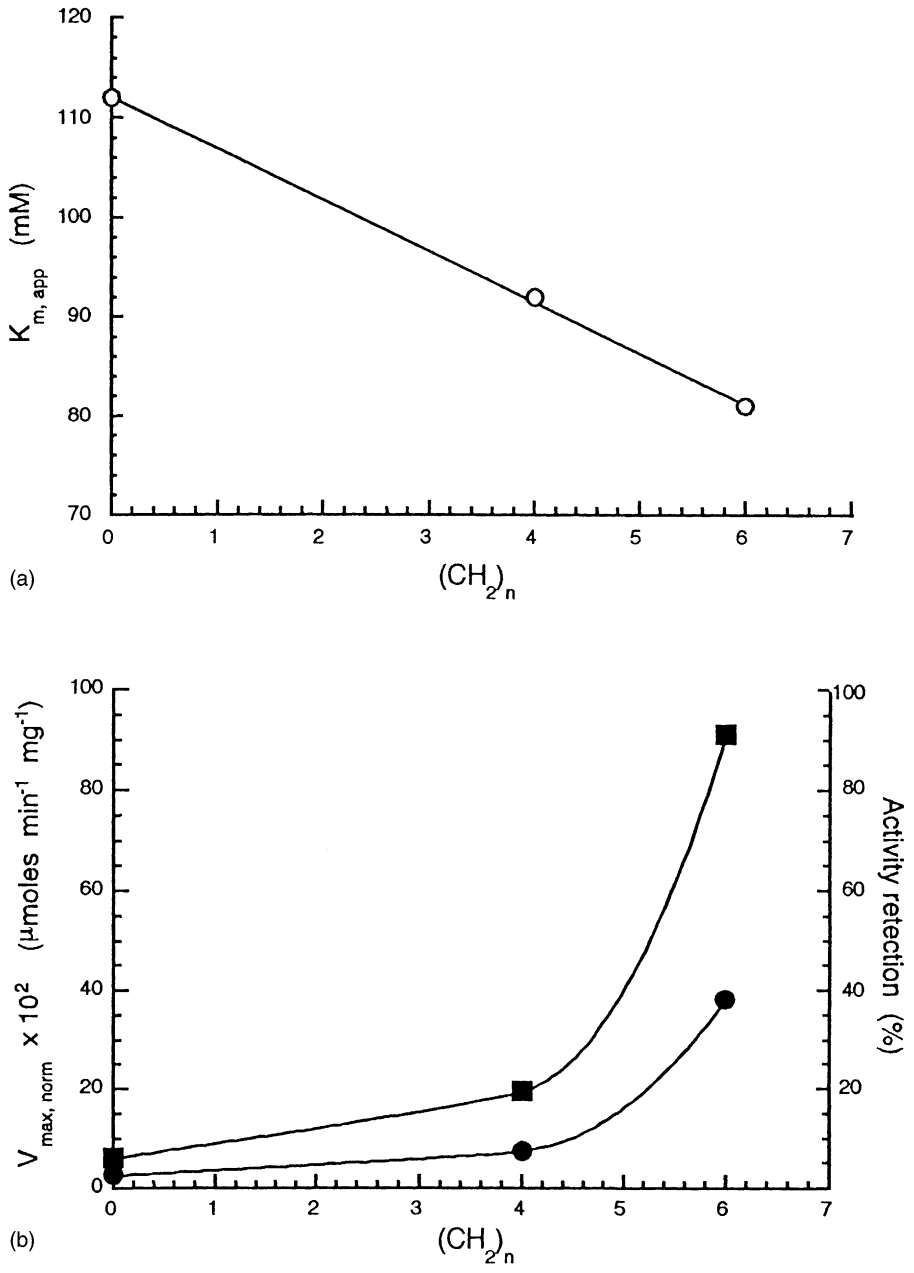

Fig. 4. (a) $K_{m,app.}$ as a function of the number of CH_2 groups in the spacer chain. (b) Normalized maximum reaction rates (●, left scale) and activity retention (■, right scale) as a function of the number of CH_2 groups in the spacer chain.

linearly increases with the increase of the spacer length, as it can be seen in Fig. 4a, where the $K_{m,app.}$ values are reported as a function of the number of CH_2 groups in the spacer chain. The analytical equation representing the experimental data of Fig. 4a is: $y = 110.57 - 4.07x$, where y is the value of the $K_{m,app.}$ and x the number of (CH_2) groups in the spacer chain. The coefficient R is equal to 0.99.

Table 2
Kinetic parameters

Enzyme status	$K_{m,app.}$ (mM)	V_{max} (μmoles min^{-1})
Soluble	21.40	3.20
Immobilized on M_0	81.0	0.18
Immobilized on M_4	92.0	0.59
Immobilized on M_6	112.0	2.67

The values of the kinetics parameters relative to the soluble enzyme have been taken from [21].

The $V_{max,app.}$ values increase exponentially with the increase of the spacer length (Fig. 4b). A similar behavior was found in the case of the activity retention, how it appears in the same Fig. 4b. The increase of the reaction rate with the number of CH_2 groups in the spacer chain agrees with the increase of the enzyme affinity with this parameter.

All these results indicate that the presence of the spacer in somewhat reduces the modification on the enzyme structure induced by the interactions between the electric charges present on the nylon membranes and the surface charges exposed by the enzyme. This protective effect increases with the spacer length according to the Coulomb's law.

3.2. Effect of pH

The pH-activity profile of an immobilized enzyme is characteristic of the enzyme, immobilization method and carrier used. The support, indeed, can change the pH value around the catalytic site, thus determining appreciable differences in the catalytic behavior of the soluble and insoluble form of the catalyst. This effect, known as partitioning effect, is directly related to the nature of support (and grafted monomers) which induce electrostatic or hydrophobic interactions between the matrix and the low molecular weight species present in bulk solution. Partitioning effect, indeed, causes in the microenvironment in which the immobilized enzymes are operating concentration changes of the charged species (e.g. hydrogen and hydroxyl ions) in respect to the bulk solution.

To know the effect of the spacer length on the partitioning effect we have investigated the activity of the β-galactosidase, in the free and immobilized forms, as a function of pH in the range between 2.5 and 6.5.

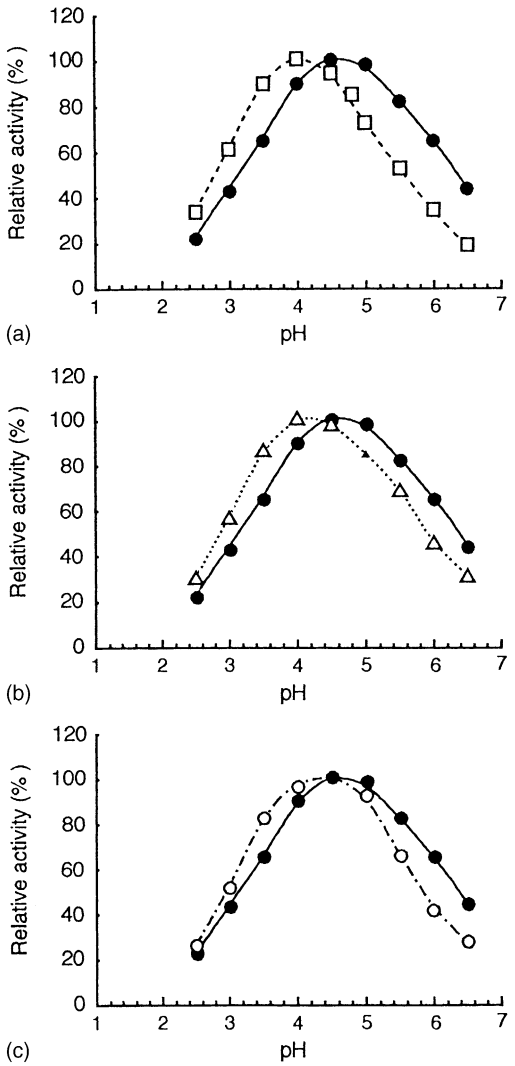

Fig. 5. Relative activity as a function of pH for membrane M_0 (a), membrane M_4 (b) and membrane M_6 (c). (●) Soluble enzyme; (○) membrane M_6; (△) membrane M_4; (□) membrane M_0.

We used 0.1 M NaCl–HCl buffer solution for pH 2.5; 0.1 M citrate buffer solution in the pH range from 3 to 5, and 0.1 M phosphate buffer solution in the pH range from 5 to 6.5. The results of this investigation are reported in Fig. 5a–c, where the relative activities of membranes M_0, M_4 and M_6 are, respectively, reported as a function of pH. In the same figures the relative activity of the soluble β-galactosidase is also reported, to allow comparison. Inspection of the

figures shows different positions of the optimum pH between the free and insoluble forms of β-galactosidase, with a shift of the optimum position towards more acidic solutions in the case of the enzyme derivatives. Indeed, the position of the optimum pH for the free enzyme occurs at pH 4.6; while this position occurs at pH 4.0 for the enzyme immobilized on M_0, at pH 4.2 for the enzyme immobilized on M_2 and at pH 4.4 for the enzyme immobilized on M_6. It is interesting to observe (Fig. 6a) that the values of the optimum pH positions for the enzyme derivatives linearly increase with the increase of the spacer length. The analytical expression interpolating the experimental points of Fig. 6a is $y = 3.92 + 0.08x$, where

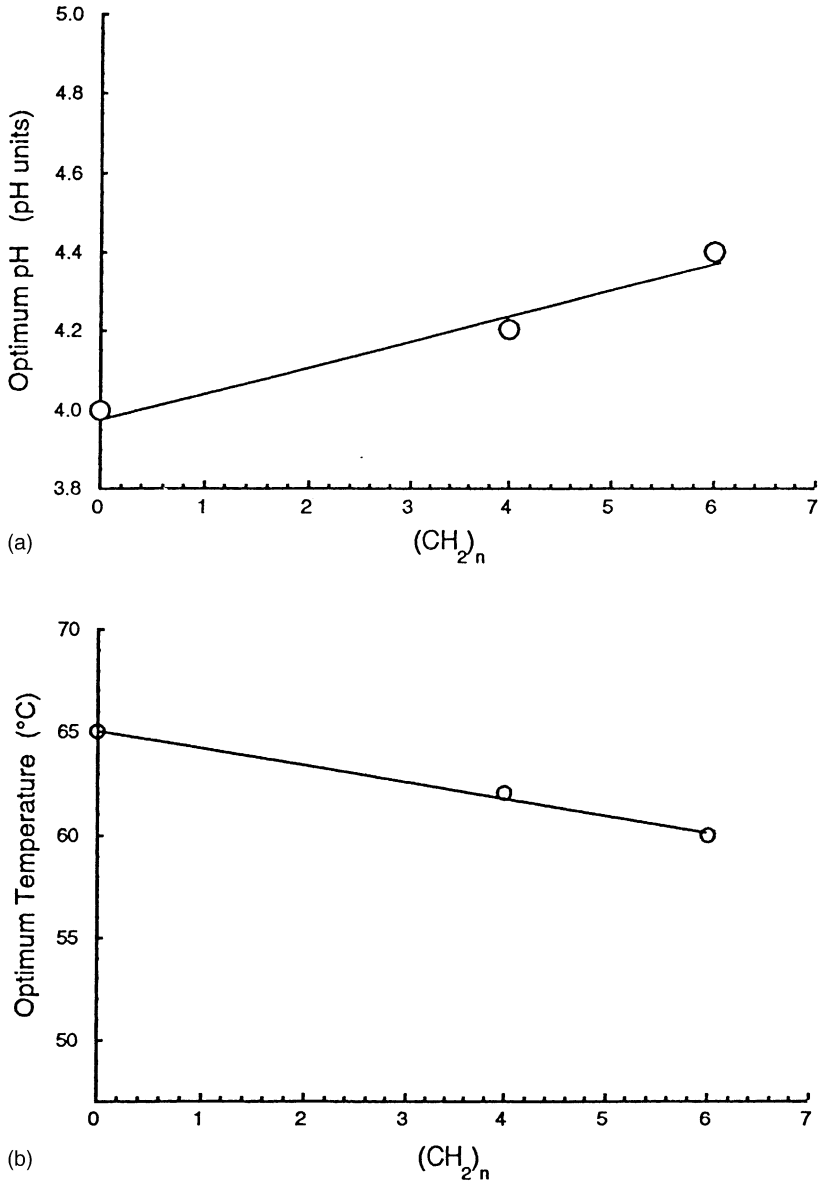

Fig. 6. Optimum pH (a) and optimum temperature (b) as a function of the number of CH_2 groups in the spacer chain.

y is the value of the position of the optimum pH and *x* the number of (CH_2) groups in the spacer chain. The coefficient *R* is equal to 0.99. Once again, the behavior represented in Fig. 6a suggests that the free enzyme can be considered as an enzyme immobilized on a solid support through a spacer of infinite length, in a position in which the partitioning effects induced by the carrier are zero. On the contrary, the more short is the spacer, the more effective is the partitioning effect. In other words, the hexamethylenediamine, being enough long and keeping the enzyme enough far from the electric field of the nylon membrane, creates the conditions by which the microenvironment around the catalytic site of the immobilized enzyme is quite similar to that around the free form.

By defining "optimum pH range" the range in which the relative enzyme activity is higher than 90% from Fig. 5a-c, it is possible to see that this range occurs between 4.20 and 5.08 for the free enzyme; between 3.69 and 4.44 for the enzyme immobilized on M_0, between 3.80 and 4.61 for the enzyme immobilized on M_4, and between 3.87 and 4.84 for the enzyme immobilized on M_6. When the optimum pH range is considered, it is possible to conclude that the extent of the optimum pH range is little affected by the spacer length.

3.3. Temperature dependence

The isothermal characterization of membrane activity is one of the principal parameters required to know how the immobilization procedure and the spacer length affect the enzyme activity. Generally, enzymatic derivatives show optimum temperatures shifted towards higher temperatures than those of the soluble counterpart.

In Fig. 7, the relative activities of the three membrane types as a function of temperature are reported, together with that of the free enzyme, for comparison. Fig. 7a refers to membrane M_0, Fig. 7b to membrane M_4, and Fig. 7c to membrane M_6. All membranes exhibit a shift of the optimum activity towards higher temperatures in comparison to the position of the soluble enzyme, evidencing in this way that the immobilization procedure protects the enzyme structure. The optimum temperature for the free enzyme occurs at about 48 °C, while for membrane M_0 at about 65 °C, for membrane M_4 at about 62 °C, and for membrane

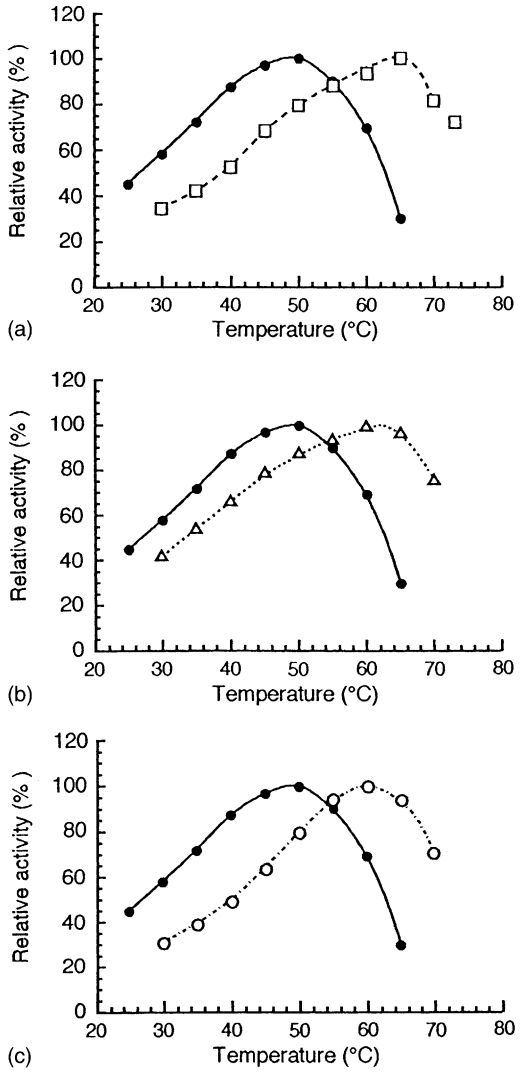

Fig. 7. Relative activity as a function of temperature for membrane M_0 (a), membrane M_4 (b) and membrane M_6 (c). (●) Soluble enzyme; (○) membrane M_6; (△) membrane M_4; (□) membrane M_0.

M_6 at about 60 °C. In Fig. 6b, the value of the optimum temperature of the three membrane types as a function of the spacer length are reported. Results in Fig. 6b show that the values of the optimum temperature of the immobilized enzymes linearly decrease with the increase of the spacer length. Once again, the more far is the enzyme from the carrier, i.e. the more long is the spacer, the more the optimum temperature of the immobilized enzyme approaches that

of the soluble counterpart. This means that the immobilization process, besides strengthening the enzyme structure, gives to the macromolecule a protective effect against the heat denaturation. This protective effect decrease with the increase of the spacer length. The analytical expression representing the linear relationship between the optimum temperature value (y) and the number of CH_2 groups (x) in the spacer chain is given by: $y = 65.07 - 0.82x$, with $R = 0.99$.

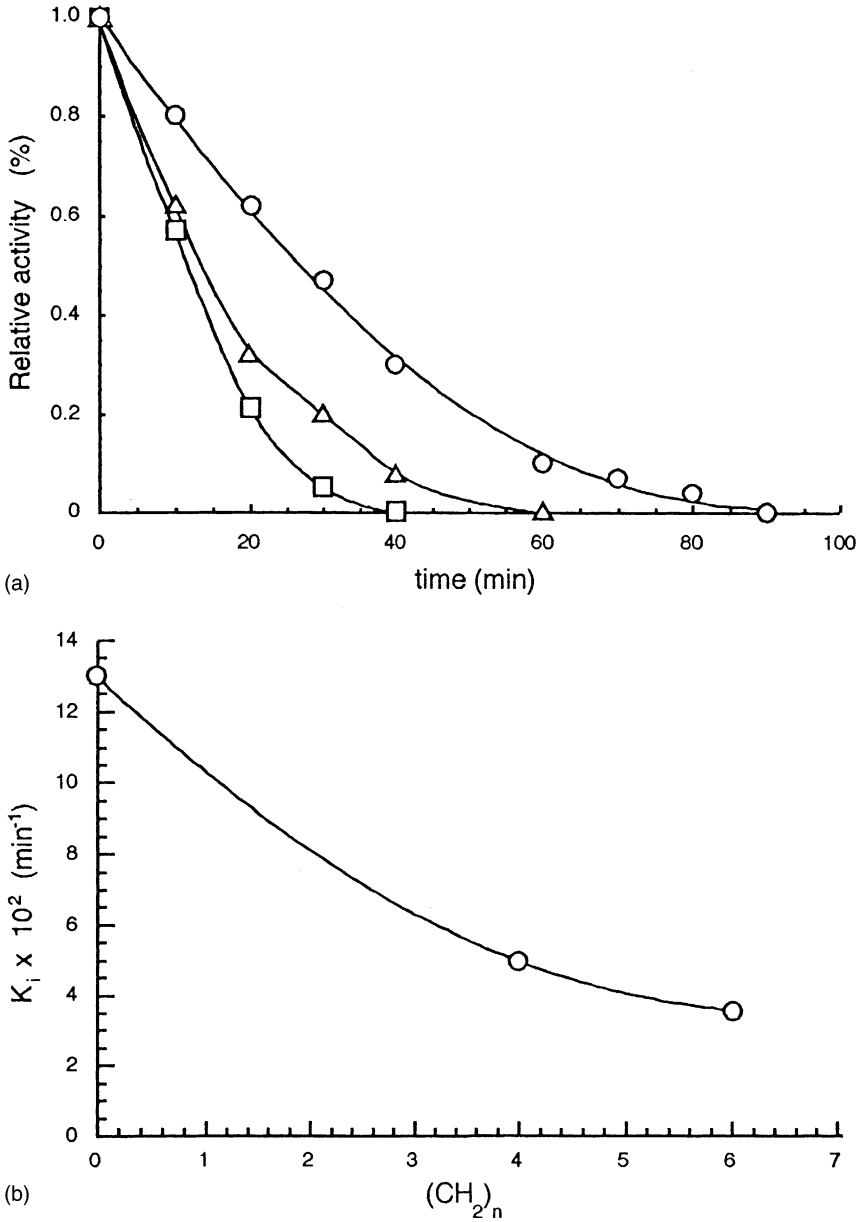

Fig. 8. (a) Thermal inactivation of the membrane: relative activity as a function of the incubation time at 60 °C. (○) Membrane M_6; (△) membrane M_4; (□) membrane M_0. (b) Thermal inactivation constants as a function of the number of CH_2 groups in the spacer chain.

Calling "optimum temperature range" the range in which the relative enzyme activity is higher than 90%, it is possible to see that this range is between 41.5 and 54.9 °C for the free enzyme; between 56.3 and 68.8 °C for the enzyme immobilized on M_0; between 52.1 and 66.9 °C for the enzyme immobilized on M_4, between 53.6 and 66.3 °C for the enzyme immobilized on M_6. The simultaneous existence of a large optimum temperature range and of the shift of the optimum temperature position suggest the use of our membranes in processes requiring high working temperatures.

3.4. Thermal stability

In view of industrial applications it is important to know the thermal stability of a catalytic membrane. To measure the thermal stability of the three membrane types we have adopted the following procedure. Catalytic membranes of large surface were prepared according to the methodology described in Section 2.3.1. Then the membranes were cut in several pieces. The catalytic activity of each piece was then measured under standard conditions, i.e. 0.2 M lactose in 0.1 M phosphate buffer solution, pH 6.5 and $T = 25$ °C, before the heat treatment and after incubation at 60 °C for the required time. Calling A_0 the initial membrane activity and A the activity measured at the end of the thermal treatment, the ratio A/A_0 gives directly the thermal deactivation of the membrane. In Fig. 8a, the A/A_0 ratio is reported as a function of time for each of the three membrane types used in this experimentation. Through the expression $\log A = \log A_0 - K_i t$ it is possible to calculate directly the thermoinactivation constant K_i (min^{-1}) according to the indications reported in [26]. The values of this constant, reported in Fig. 8b as a function of the spacer length, show a decrease with the increase of the spacer length. These results, together with those of Fig. 7, confirm that membrane M_6 is the most indicated for processes requiring high temperatures.

4. Conclusions

All the results above reported have shown the influence of the spacer length on the isothermal performance of a catalytic membrane, when the same enzyme is attached to the same support through spacers of different length.

The optimum pH values of the insoluble enzymes in comparison with that of the soluble counterpart exhibit shifts proportional to the spacer length, whereas an opposite trend is observed when the values of the optimum temperature are considered. In both cases, increasing the spacer length the values relative to the immobilized enzymes approach to that of the free enzyme, indicating in this way that a soluble enzyme can be considered an enzyme immobilized on a solid support through a spacer of infinite length. This conclusion agrees also with the behavior of the $K_{m,app.}$ values, which linearly decrease with the increase of the spacer length, approaching to the value of the free enzyme.

If attention is paid to the applied aspects, membrane M_6, i.e. the membrane obtained with the longest spacer used in this research, appears the most resistant to high temperatures, acidic solutions, and thermal inactivation.

Acknowledgements

This work was partially supported by the Target Project "Biotechnology" of CNR, by MURST (ex 40% founds), by MURST/CNR L. 27/12/1997 no. 449, and by "Regione Campania".

References

[1] A. Tanaka, T. Toya, T. Kobayashi, Industrial Application of Immobilized Biocatalysts, Marcel Dekker, New York, 1993.
[2] H.W. Blanch, D.S. Clark, Applied Biocatalysis, Marcel Dekker, New York, 1991.
[3] A. Liese, K. Seelbach, C. Wandrey, Industrial Biotransformations, Wiley-VCH, Weinheim, 2000.
[4] T. Sato, T. Tosa, in: A. Tanaka, T. Toya, T. Kobayashi (Eds.), Industrial Application of Immobilized Biocatalysts, Marcel Dekker, New York, 1993, p. 3.
[5] Y. Ashina, M. Suto, in: A. Tanaka, T. Toya, T. Kobayashi (Eds.), Industrial Application of Immobilized Biocatalysts, Marcel Dekker, New York, 1993, p. 91.
[6] Y. Honda, M. Kako, K. Abiko, Y. Sogo, in: A. Tanaka, T. Toya, T. Kobayashi (Eds.), Industrial Application of Immobilized Biocatalysts, Marcel Dekker, New York, 1993, p. 209.
[7] H. El-Sherif, P.L. Martelli, R. Casadio, U. Bencivenga, D.G. Mita, J. Mol. Catal. B: Enzym. 14 (2001) 15.

[8] H. El-Sherif, A. De Maio, S. Di Martino, E. Zito, S. Rossi, P. Canciglia, F.S. Gaeta, D.G. Mita, J. Mol. Catal. B: Enzym. 14 (2001) 31.
[9] T. Atkinson, M.D. Scawen, P.M. Hammond, in: H.J. Rehm, G. Reed (Eds.), Biotechnology, VCH, Weinheim, 1987, p. 279.
[10] H.H. Weetall, in: R.A. Messing (Ed.), Immobilized Enzyme for Industrial Reaction, Academic Press, New York, 1995, p. 99.
[11] A. De Maio, M.M. El-Masry, P. De Luca, V. Grano, S. Rossi, N. Pagliuca, F.S. Gaeta, M. Portaccio, D.G. Mita, Influence of the spacer length on the activity of enzymes immobilized on nylon/polyGMA membranes. Part 2. Non-isothermal conditions, J. Mol. Catal. B: Enzym., 21 (2003) 253–265.
[12] S. Stellato, M. Portaccio, S. Rossi, U. Bencivenga, G. La Sala, G. Mazza, F.S. Gaeta, D.G. Mita, J. Membr. Sci. 129 (1997) 175.
[13] F. Febbraio, M. Portaccio, S. Stellato, S. Rossi, U. Bencivenga, R. Nucci, M. Rossi, F.S. Gaeta, D.G. Mita, Biotechnol. Bioeng. 59 (1998) 108.
[14] M.S. Mohy Eldin, A. De Maio, S. Di Martino, M. Portaccio, S. Stellato, U. Bencivenga, S. Rossi, M. Santucci, P. Canciglia, F.S. Gaeta, D.G. Mita, J. Membr. Sci. 146 (1998) 237.
[15] M.S. Mohy Eldin, A. De Maio, S. Di Martino, N. Diano, V. Grano, N. Pagliuca, S. Rossi, U. Bencivenga, F.S. Gaeta, D.G. Mita, J. Membr. Sci. 47 (1999) 1.
[16] M.S. Mohy Eldin, M. Santucci, S. Rossi, U. Bencivenga, P. Canciglia, F.S. Gaeta, J. Tramper, A.E.M. Janssen, C.G.P.H. Schroen, D.G. Mita, J. Mol. Catal. B: Enzym. 8 (2000) 221.
[17] M.M. El-Masry, A. De Maio, S. Di Martino, U. Bencivenga, S. Rossi, B.A. Manzo, N. Pagliuca, P. Canciglia, M. Portaccio, F.S. Gaeta, D.G. Mita, J. Mol. Catal. B: Enzym. 9 (2000) 231.
[18] N. Diano, M.M. El-Masry, M. Portaccio, M. Santucci, A. De Maio, V. Grano, U. Bencivenga, F.S. Gaeta, D.G. Mita, J. Mol. Catal. B: Enzym. 11 (2000) 97.
[19] C.G.P.H. Schroen, M.S. Mohy Eldin, A.E.M. Janssen, D.G. Mita, J. Tramper, J. Mol. Catal. B: Enzym. 15 (2001) 163–172.
[20] M.M. El-Masry, A. De Maio, M. Portaccio, S. Di Martino, U. Bencivenga, S. Rossi, F.S. Gaeta, D.G. Mita, Enzyme Microb. Technol. 28 (2001) 773–784.
[21] A. De Maio, M.M. El-Masry, Z.H. El-Latif, M. Portaccio, U. Bencivenga, D.G. Mita, J. Mol. Catal. B: Enzym. 16 (2001) 191–204.
[22] S.W. May, N.N. Li, Biochem. Biophys. Res. Commun. 47 (1972) 1178.
[23] W.E. Horndy, M.D. Lilly, E.M. Crock, Biochem. J. 107 (1968) 668.
[24] R. Kleine, P. Spangerberg, C. Fleming, Hoppe-Seyler's Z. Physiol. Chem. 357 (1976) 629.
[25] T. Sato, T. Mori, I. Chibata, Arch. Biochem. Biophys. 147 (1971) 788.
[26] T. Hayashi, Y. Ikada, J. Appl. Polym. Sci. 42 (1991) 85.