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Urease immobilisation on chemically grafted nylon membranes Part 1: Isothermal characterisation

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Abstract

Urease–HMDA–poly(BMA)–nylon membranes have been prepared by grafting butyl methacrylate (BMA) monomers on nylon sheets. Hexamethylenediamine (HMDA) and glutaraldehyde have been used as spacer and coupling agent, respectively. The catalytic activity of the membrane has been characterised as a function of pH, temperature, and urea concentration. The activity of the free enzyme has also been studied for comparison. The results indicated good enzyme-binding capacity of the pre-treated membrane and a shift of the optimum pH and temperature. These membranes represent an useful system to be used in biotechnological processes occurring under acidic conditions and high temperatures. A three-dimensional (3D) model of the free enzyme has been built by computer simulation with the aim of understanding the enzyme-membrane interaction and the role of β-mercaptoethanol on improving the stability of the catalytic membranes. The use of the catalytic membranes in biosensors or bioreactors operating under non-isothermal conditions has been described as an useful tool to overcome the increased K_m value of the immobilised enzyme and the diffusion limitation problems due to the immobilisation procedure.

Keywords: Urease; Urea; Catalytic membranes; Grafted nylon membranes; Isothermal bioreactors; Urease 3D structure

1. Introduction

Employment of free enzymes in biotechnological processes is limited owing to their cost and to the impossibility of recovering and separating them from the reaction medium after the catalytic process. To overcome these difficulties, biotechnologists have immobilised the enzymes, thus obtaining catalysts which are easily separable from the reaction medium. Moreover, they are endowed with good properties such as resistance to temperature and denaturants, as well as a stability longer than their free counterpart [1].

The enzyme immobilisation on natural or synthetic membranes has found interesting applications in

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biosensors [2,3] or membrane bioreactors [4,5]. One technique employed for the immobilisation is grafting, by which inert supports become available for enzyme attachment. Grafting is carried out chemically or by γ-radiation.

However, one disadvantage of the immobilisation process is the lower specific activity of the enzyme derivatives with respect to that exhibited by the same amount of free catalyst. Some years ago, we sought to overcome this drawback by employing the catalytic membrane in bioreactors [6–16] or biosensors [17,18] operating under non-isothermal conditions. Indeed, when catalytic membranes separate two substrate solutions kept at different temperatures, an increase in enzyme activity with respect to that under comparable isothermal conditions is observed. This increase, ranging from 10 to 30% for a temperature difference of 1°C across the membrane, depends on the enzyme and immobilisation method, and is proportional to the transmembrane temperature difference. A crucial requisite for the occurrence of these effects is the presence of a hydrophobic membrane, which ensures the existence of the process of thermodialysis [19,20]. The latter is a process by which a differential transport of solvent and solute across a porous hydrophobic membrane separating two solutions at different temperatures occurs. The process of thermodialysis has been recognised as responsible for the efficiency increase of the yield of non-isothermal bioreactors.

The aim of this work is the construction of nylon grafted membranes, loaded with urease (EC 3.5.1.5) from jack beans and suitable for non-isothermal bioreactors. Urease occupies a unique place in enzymology, in that it has been the first enzyme to be crystallised [21]. Its immobilised form has found broad applications [22,23], such as blood detoxification in artificial kidneys, the removal of urea from beverages and foods in food industry, and the reduction of urea content in effluent treatment in agriculture.

The present study has been undertaken with the aim of applying the new technology of non-isothermal bioreactors to environmental problems and, in particular, to waste water treatment. Urease has been chosen as a model enzyme. Moreover, to better understand the behaviour of the immobilised enzyme, a three-dimensional (3D) structure of the macromolecule was built and discussed.

2. Apparatus, materials and methods

2.1. Materials

Nylon hydrolon membranes by Pall (Pall Italia, Milano, Italy) were used as solid support to be grafted. These hydrophobic membranes, 150 μm thick, have a nominal pore size of 0.2 μm. Pore size is defined as the diameter of the smallest particles that the membrane retains, since in the membrane there are no "classical" pores but irregular cavities, crossing the entire membrane thickness, formed by the interstices between the nylon fibers.

Butyl methacrylate (BMA) was used as a hydrophobic monomer to be grafted on the membrane. Potassium persulfate was used as initiator of the grafting process. Hexamethylenediamine (HMDA) and glutaraldehyde (Glu) were used as spacer and coupling agent, respectively. The presence of the spacer was required to minimise the effect of the electric charges of the nylon support on the macromolecule structure and on the microenvironment in which the enzyme operates.

Type III urease (EC 3.5.1.5) from *Jack beans* was used as catalyst. Urease hydrolyses urea into ammonia and carbon dioxide.

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

2.2. The bioreactor

The apparatus consisted of two cylindrical half-cells (Fig. 1), filled with the working solution containing the substrate and separated by the catalytic membrane. Solutions were recirculated in each half-cell by means of peristaltic pumps through hydraulic circuits starting and ending in the common cylinder C. Each half-cell was thermostatted at the required temperature by circulation in external jackets of water coming from a thermostatic bath. Thermocouples, placed at 1.5 mm from each of the membrane surfaces, were used to measure the temperatures inside each half-cell.

2.3. Methods

2.3.1. Catalytic membrane preparation

Grafting was obtained by interaction for 30 min at 60°C of the membrane with a 0.172 M BMA alcoholic

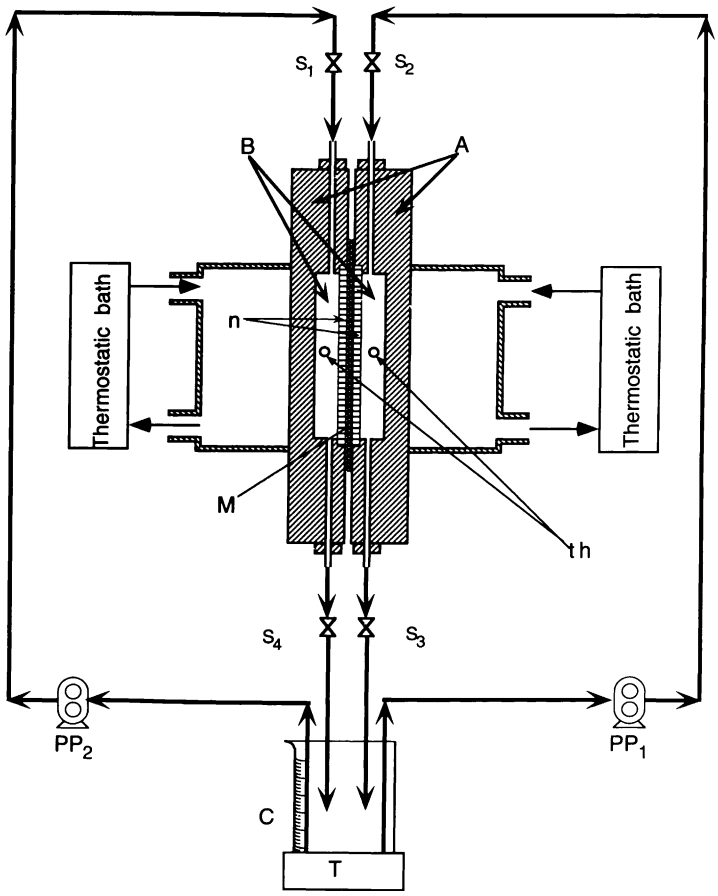

Fig. 1. Schematic (not to scale) representation of the bioreactor. Symbols — A: half-cells; B: internal working volumes; C: external working volume; M: membrane; n: supporting nets; th: thermocouples; S_i: stopclocks; T: thermostatic magnetic stirrer; PP_i: peristaltic pumps.

solution (water:ethanol, 0.913:1) containing potassium persulfate at a concentration of 0.037 M. After washing with acetone, the grafted membranes were dried to estimate the grafting percentage (X, %), which was calculated through the formula

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

where G_A and G_B were original and grafted membrane weights, respectively. All membranes used in the following experiments had a grafting percentage value of $13.5 \pm 2\%$. A second step was performed by reacting the grafted membranes for 30 min at 60°C with 50% (v/v) HMDA aqueous solution. After several washes with running water to remove the unreacted amine, the membranes were dried and then immersed in 2.5% (v/v) glutaraldehyde aqueous solutions for 60 min at room temperature. Enzyme immobilisation was obtained by immersion, for 16 h at 4°C, of the preactivated membranes in 0.1 M phosphate buffer, pH 6.5, containing urease (1 mg/ml).

Coupling of urease to glutaraldehyde is performed through the amine groups of the enzyme, thus forming amide bonds. Shiff-base formation between amine and aldehyde groups should also be considered. The –SH groups of urease may crosslink with glutaraldehyde after amine groups have been used. It is important to notice that $–NH_2$ groups are more susceptible than –SH groups to react with glutaraldehyde [24].

The overall procedure of grafting, membrane activation and enzyme immobilisation is schematically represented in Fig. 2.

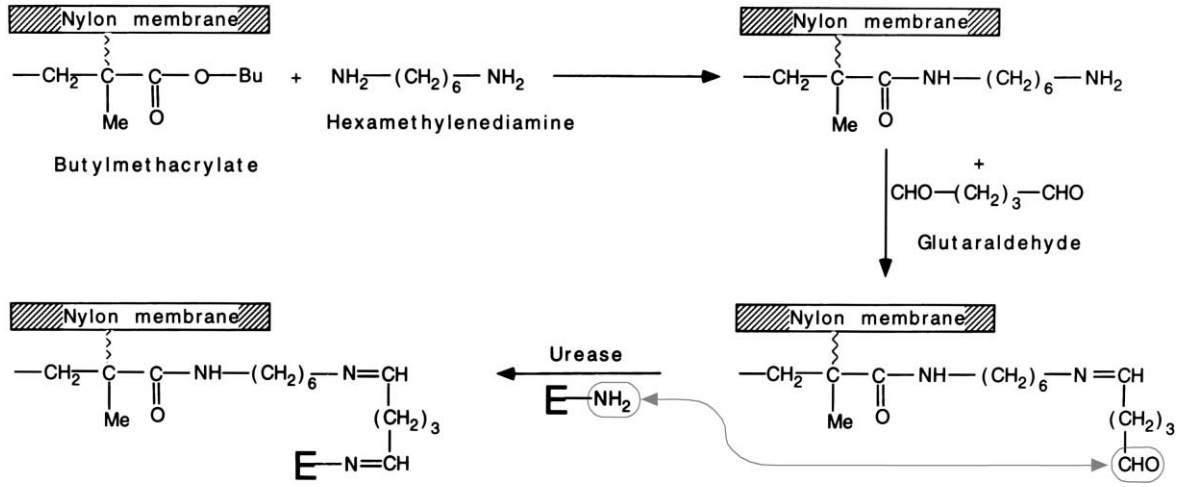

Fig. 2. Schematic representation of grafting process, membrane activation and urease immobilisation.

2.3.2. Determination of the membrane activity

Membrane activity was determined by sampling, at regular time intervals into cylinder C in Fig. 1, the solution interacting with the catalytic membrane, and measuring the ammonia concentration resulting from the reaction of urease with the urea. Ammonia concentration was measured by means of phenol-hypochlorite method [25]. In this way, a blue coloured solution is obtained. The ammonia concentration, proportional to the intensity of the solution colour, is spectrophotometrically determined at 625 nm. Membrane activity, indicated also as enzyme reaction rate, is expressed as μmol min^{-1} and is given by the slope of the linear plot of the ammonia production as a function of time.

Each experiment lasted 18 min, during which the rate of ammonia production resulted constant.

2.3.3. Membrane stability and treatment of the experimental data

Urease contains several –SH groups per molecule. The presence of these groups affects the structure of the enzyme and, consequently, its activity. Sulfydryl groups at the active site may be in the ionised form, which is prone to oxidation. Normally, inside the cell the reducing medium and the presence of other sulfydryl-containing molecules protect these groups on urease. When exposed to a more oxidising medium, as that one of our experiments, several reactions of such groups are possible: (i) they may form disulfide bonds (–S–S–); (ii) they can be partially and reversibly oxidised to sulfinic acid (–SOH); (iii) they can be irreversibly oxidised to sulfonic acid (–SOOH). Each of these reactions affects the enzyme structure and produces a loss of activity in the course of time [26]. To prevent these oxidation effects, a solution of sulfydryl containing reagents, such as glutathione, cysteine, β-mercaptoethanol, is required. In our present case, we used a 14 mM β-mercaptoethanol solution. At this concentration, enzyme oxidation is prevented conferring time stability to the catalytic membrane. In the absence of β-mercaptoethanol, the activity of the catalytic membrane was lost in one day.

The time stability of the biocatalytic membranes was assessed by measuring every day their activity under the same experimental conditions, i.e. at 30°C and using a 15 mM urea concentration in 0.1 M citrate buffer. After few days, during which the membrane lost part of its activity, probably owing to some enzyme release, a stable condition was reached which remained unchanged for more than one month. Only these stabilised membranes have been used in the following experiments.

When not used, the membranes were stored at 4°C in 0.1 M citrate buffer.

All points reported in the figures are the average of five independent experiments performed under the same conditions. The experimental errors never exceeded 4.5%.

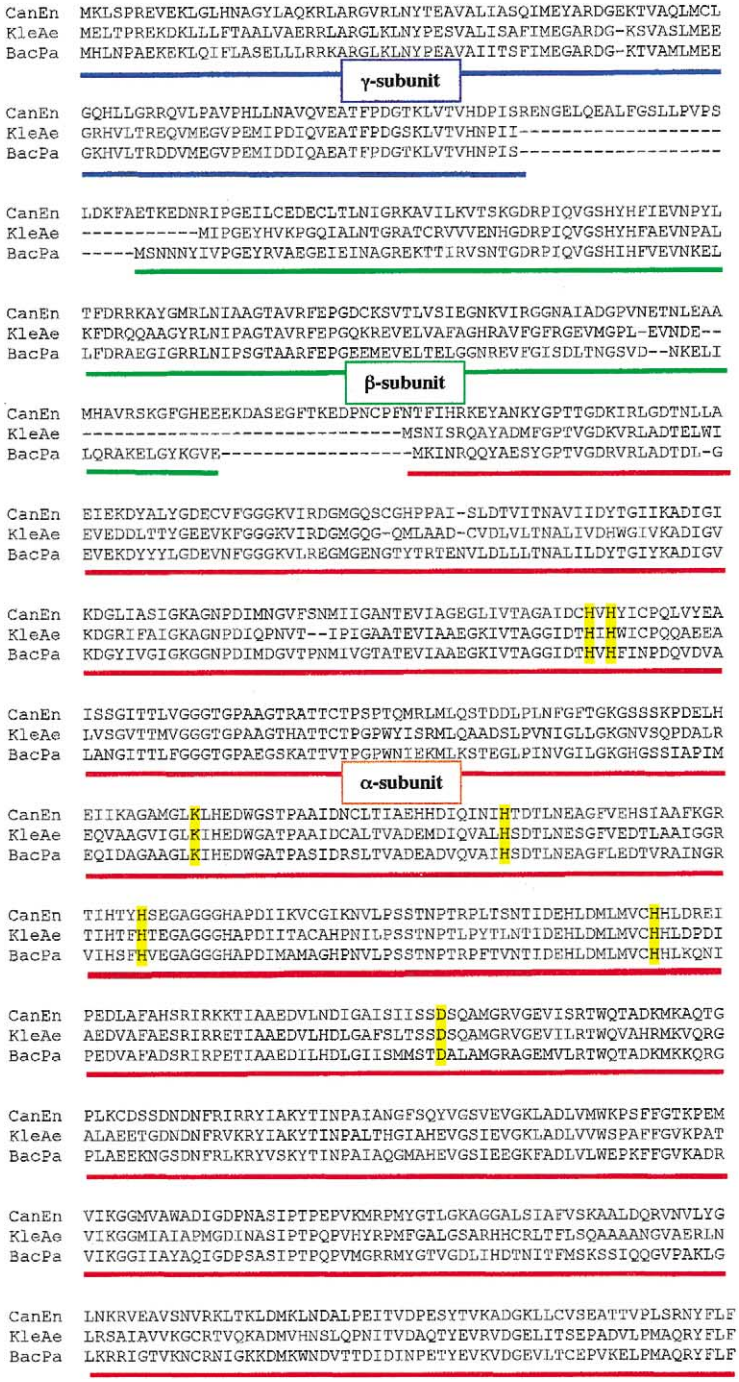

Fig. 3. Sequence alignment of the jack bean urease with the bacterial counterparts with the highest homology as computed using the BLAST program. The sequence of the jack bean urease is taken from the UREA_CANEN (CanEn) in the SwissProt data base. The aligned bacterial ureases are from *Klebsiella aerogenes* (KleAe, UREI_KLEAE and 2KAU in the SwissProt and PDB data base, respectively) and from *Bacillus pasteurii* (BacPa, UREI_BACPA and 2UBP). The three sequences of the corresponding subunits in the bacterial crystals as well as the residues in the active site are highlighted.

2.3.4. Modelling procedure

The 3D structure of jack bean urease was computed using an homology building strategy. The sequence (UREA_CANEN in the SwissProt data base) was aligned with the structures in the PDB data base using BLAST [27] and CLUSTALW [28]. Two templates were found with sequence homology to the target higher than 50%: the ureases from *Bacillus pasteurii* and *Klebsiella aerogenes*. 3D modelling was then performed using MODELLER [29]. The quality of the 3D structure of the enzyme was checked with PROCHECK [30] (biotech.embl-ebi.ac.uk:8400). Multimeric aggregates were built using the EBI MSD data base [31]. Prediction of secondary structure was performed with the PHD program [32]. Prediction of the cysteine bonding state was done with a neural-network based program suited to predict the tendency of a cysteine residue to be or not to be in a disulfide bridge [33]. Solvent accessibility was computed with the DSSP program [34]. Model structures are visualised using RASMOL [35].

3. Results and discussion

3.1. The 3D enzyme structure

Several structures of urease from bacteria are present in the PDB data base. Urease from bacteria comprises three subunits (α, β and γ) of different size. The active site contains two nickel ions and is structurally located within the subunit. However, the jack bean urease, the first nickel containing metalloenzyme identified, still lacks a well resolved crystal structure. Here, we present a model of the jack bean urease computed with a procedure based on homology building. Following this method, two templates were found with homology search on the data base of well-known structures.

Sequence alignment is shown in Fig. 3. Noticeably, the ureases from bacteria comprise three subunits which align with a different region of the jack bean urease. However, only 3% of the residues of the jack bean urease is without a counterpart in the crystals. Residues involved in the nickel coordination at the protein active sites are also highlighted.

The 3D structure of jack bean urease (UREA_CANEN in the SwissProt data base) is shown in Fig. 4. Following building by homology, the protein comprises three domains corresponding to the γ, β and α subunits of the bacterial counterparts, respectively, from the N to the C terminus of the sequence. The two nickel ions in the protein active site, embedded in the largest C-terminus domain, are shown in Fig. 5 from which it results that the active site architecture is similar to that of the two bacterial templates. The secondary structure of the protein is characterised by 24.4% of α helices and 20.2% of β strands. The protein can therefore be defined of mixed type [36]. Two short loops (corresponding to 3% of the protein residues) do not have a counterpart in the published crystal structures of the templates. These loops are, however, modelled by the Monte Carlo simulated annealing procedure of MODELLER and are highlighted in green colour in Fig. 4. Modelling produces a short α helix (from residue in position 111 to that in 115) in the first loop (comprising residues from 101 to 125). Residues contained in the 253–270 segment are in a coil structural type. These secondary structure types are also confirmed when secondary structure prediction is performed with PHD [32]. For this reason, the 3D structure of this small portion of the protein was not further refined. Coming back to Fig. 5, the residues correspond to those coordinating the two nickel ions: His (H) 407, His (H) 409, Lys (K) 490, Asp (D) 633 for Ni II and His (H) 519, His (H) 545 and Lys (K) 490 for Ni I.

Jack bean urease (product number U7752), although active as a monomer, is found in the form of multimeric aggregates, mainly trimers and hexamers.

Given the crystal symmetry of the templates, we were able to model only the trimer form of the enzyme (Fig. 6). Using this aggregate, we computed all the possible binding sites accessible to the solvent and to the interaction with the activated membrane. Data are listed in Table 1, where the exposed Lys and Arg residues are evaluated both for the monomer and trimeric enzymes. It appears that the possible membrane binding residues (Arg and Lys) are not very much changed upon aggregation of monomers into trimers (20 and 21% of the total accessible surface area, in monomers and trimers, respectively). More interesting, it is evident from Fig. 6 that in the trimers the Cys residues exposed cluster mainly on one side of the structures with only six Cys residues not accessible as compared to the monomer. These exposed

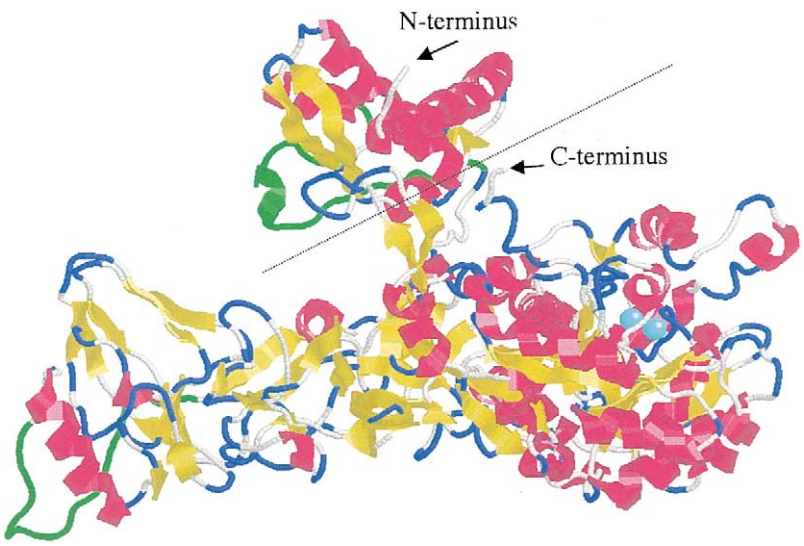

Fig. 4. Three-dimensional (3D) structure of jack bean ureas (UREA_CANEN in the SwissProt data base). The three domains are named after alignment with the subunits of the bacteria templates used for homology building.

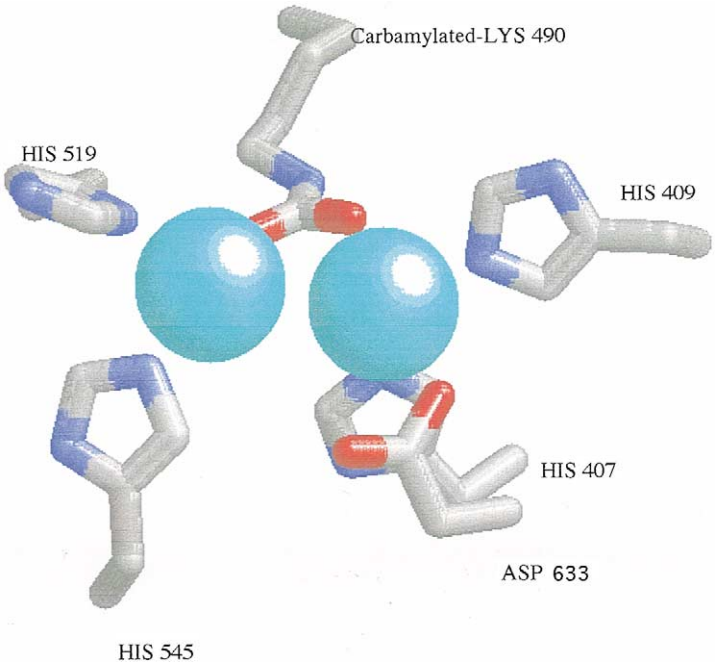

Fig. 5. Active site of the jack bean urease. The nickel ions and the coordinating residues are shown.

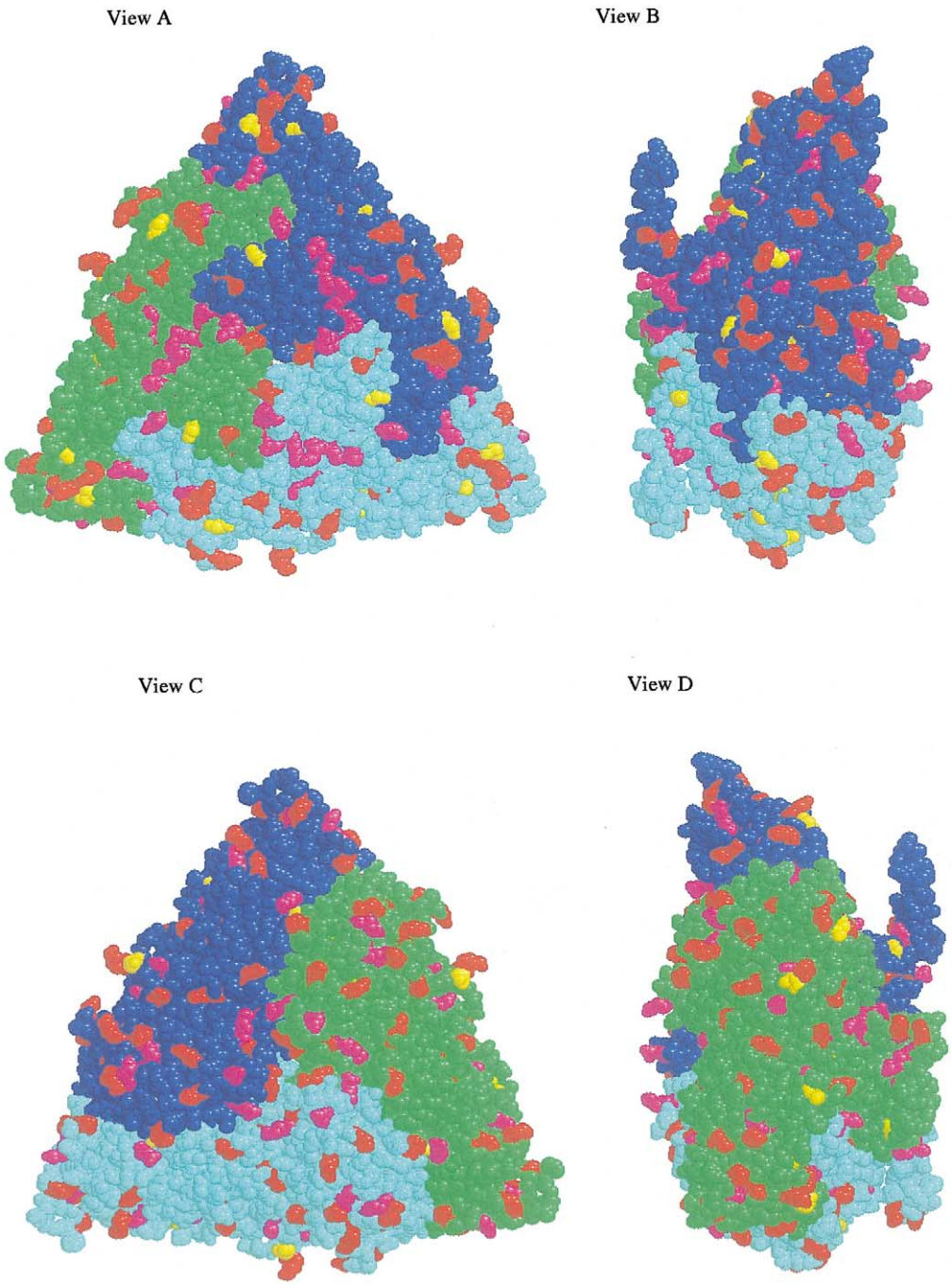

Fig. 6. The trimer of the jack bean urease. The three monomers partecipating into the trimer are shown in green, blue and cyan, respectively. Four different views of the trimer are shown, by rotating the aggregate of 90° with respect to the vertical axis. Exposed Lys, Arg and Cys residues are in red, magenta and yellow, respectively. Interestingly, view C is characterised by the lowest density of exposed cysteines.

Table 1
Number of Lys, Arg and Cys exposed to solvent in the monomer and trimeric enzyme[a]

	Accessibility (Å^2)
Monomer	
Total	35467
38/50 LYS[b]	4051
23/37 ARG[b]	3100
8/15 CYS[b]	496
Trimer	
Total	77523
90/150 LYS[b]	9959
48/111 ARG[b]	6051
18/45 CYS[b]	1185

[a] Solvent accessibility of selected residues in jack bean urease.
[b] Exposed over total number of residue type in the sequence of jack bean urease.

residues are at the basis of the stabilising interaction with β-mercaptoethanol. Moreover, disulfide bridges are not found in the templates and are also absent from the jack bean urease. This was confirmed also upon prediction of the cysteine bonding state of the jack bean urease with a neural network based predictor [33].

3.2. Concentration dependence of membrane activity

The catalytic activity of urease–HMDA–poly (BMA)–nylon membranes has been studied as a function of substrate concentration in the range 0–120 mM. Substrate concentrations were obtained by dissolving an appropriate urea amount in 0.1 M citrate buffer solution. The experimental temperature was 25°C. In Fig. 7, the ammonia production as a function of time is reported for different substrate concentrations. The ammonia production was linear during the experiment and increased with the increase of concentration. The membrane catalytic activity at each substrate concentration was obtained from the slope of each straight line. These activities are reported in Fig. 8a, together with the catalytic activity of the free enzyme. Fig. 8b shows the Hanes plots from which the values of the kinetic parameters K_m and V_{max} of the enzyme reaction for the free and immobilised urease were calculated. These values are reported in Table 2, together with the analogous values found for other urease derivatives and urease sources. Inspection of this table shows that the value of the apparent K_m of the immobilised enzyme is higher than the corresponding value

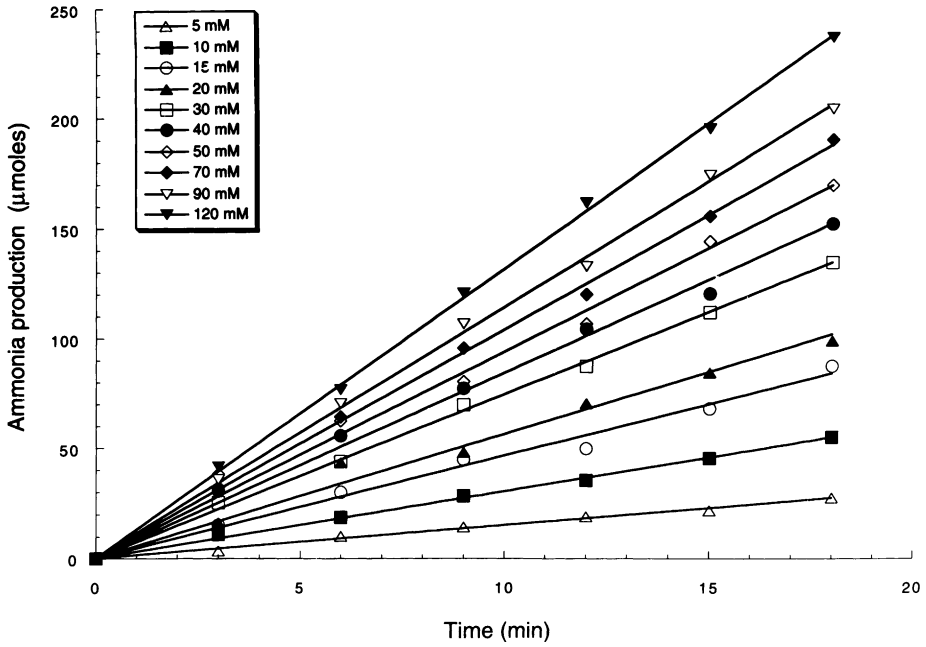

Fig. 7. Ammonia production as a function of time for different substrate concentrations.

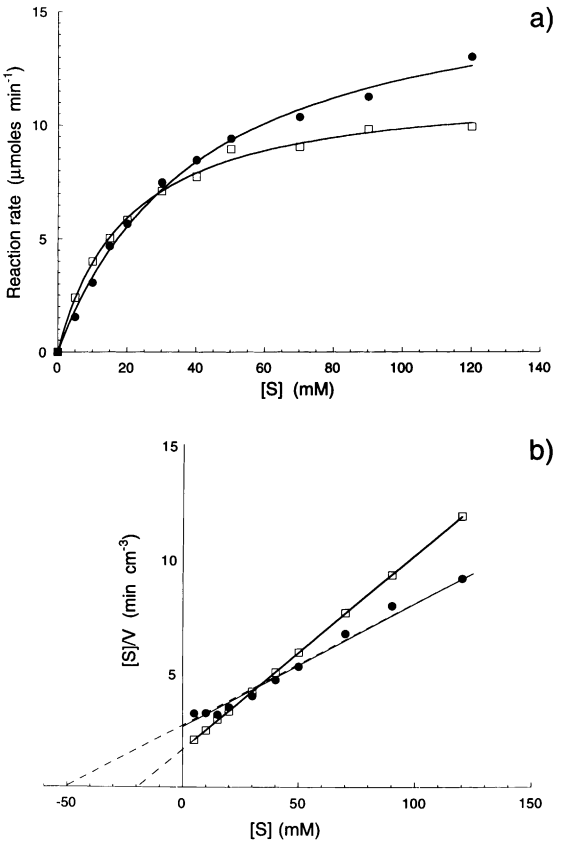

Fig. 8. (a) Catalytic activity of the free (□) and immobilised (●) urease as a function of substrate concentration. (b) Hanes plots for free (□) and immobilised (●) urease.

of the free counterpart, independently of the carrier and the immobilisation method used. The increase of the apparent K_m after immobilisation clearly indicates an apparent lower affinity of the immobilised enzyme for its substrate compared to that of the free enzyme. This result may be attributed to changes in enzyme structure induced by the interaction of the macromolecule with the support. Alternatively, the increased diffusional resistance encountered by both the substrate in its approach to the catalytic site and by the reaction product leaving from the same place could be responsible for the higher values of K_m. Another cause may be related to the changes induced by the immobilisation process in the microenvironment in which the enzyme operates. Similar results were obtained by us with β-galactosidase and penicillin G acylase immobilised on teflon or nylon membranes, grafted with different monomers [14,15].

3.3. pH dependence of membrane activity

The pH dependence of the immobilised enzyme activity is characteristic of the nature of enzyme, immobilisation method used and of the carrier used. The support can change the pH value around the catalytic site, thus determining a different catalytic behaviour of the free and bound state of the catalyst. This effect, known as partitioning effect, is directly related to the nature of the support (and of grafted monomers) which induces electrostatic or hydrophobic interactions between the matrix and the low molecular weight species

Table 2
K_m and V_{max} values for free and immobilised urease as found by different authors (last column) using different systems

Carrier	Immobilised urease		Free urease		Reference
	K_m (mM)	V_{max} (mmol min^{-1})	K_m (mM)	V_{max} (mmol min^{-1})	
BMA–nylon membrane	50.00	19.23	19.5	11.96	This paper
Acrylamide-grafted PET	4.50[a]	98.0[a,b]	2.82[a]	32.3[a,b]	[24]
Polyaniline	11.74	2.4×10^{-3}	8.7	2.8×10^{-3}	[37]
Polyvinyl alcohol	7.1×10^{-4}	2.4×10^{-5}	2.7×10^{-5}	3.4×10^{-2}	[38]
Gelatin–poly(HEMA) copolymer	11.00	Not available	3.23	Not available	[39]
Methacrylic acid–acrylamide–PET	3.71[a]	72.11[a,b]	2.82[a]	32.3[a,b]	[40]
Vermiculite	4.17[c]	1.45[d]	3.12[c]	16[d]	[41]

[a] Urease from sword beans.
[b] V_{max} is defined as mM s^{-1} (g protein)$^{-1}$.
[c] K_m is expressed in %.
[d] V_{max} is expressed as mmol min^{-1} (mg enzyme)$^{-1}$.

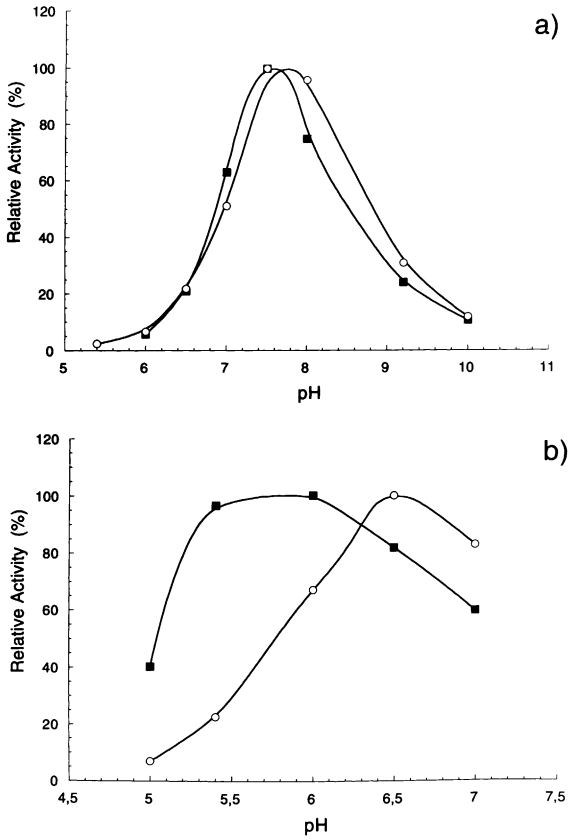

Fig. 9. (a) Activity of free (○) and immobilised (■) urease as a function of pH. Experimental conditions: 15 mM urea in 0.1 M phosphate buffer; temperature was 25°C. (b) Activity of free (○) and immobilised (■) urease as a function of pH. Experimental conditions: 15 mM urea in 0.1 M citrate buffer; temperature was 25°C.

present in solution. In particular, partitioning effects cause differential concentrations of charged species (e.g. hydrogen and hydroxyl ions) in the microenvironment of the immobilised enzymes. Thus, the pH profile of the immobilised enzyme is displaced toward more alkaline or acidic pH values for negatively or positively charged matrices.

In Fig. 9, the reaction rate of urea hydrolysis by the free and immobilised urease as a function of pH is reported. The results are expressed as relative activity. Fig. 9a refers to experiments performed in 0.1 M phosphate buffer solutions, while Fig. 9b refers to results obtained in 0.1 M citrate buffer solutions. In both cases, the urea concentration was 15 mM and the temperature was kept at 25°C. From these figures, it clearly emerges that the optimum pH values depend on the buffer system for both free and immobilised form of urease. This behaviour is not surprising since it is well-known that the type of buffer not only affects the activity and stability of an enzyme, but also the thermodynamics of reaction. This is particularly true when formation of an intermediate product occurs first, which then leads to the desired final product. Indeed, this is the case for hydrolysis of urea by urease, which has been shown to proceed via an ammonium carbamate, which then gives the desired product. A buffer-mediated proton transfer is thus responsible for the conversion of carbamate to final products, so that the nature of buffer plays a key role. Probably the proton transfer is influenced by the presence of the charged groups on nylon membrane, and this can justify the different size of the split of the pH optimum between the free and immobilised enzyme when the two buffer systems are used. In Table 3, the optimum pH values for the two systems are reported, together with the corresponding values of other urease derivatives. Inspection of Table 3 shows that, in general, no differences have been found between the optimum pH values of the immobilised and free urease. Only two exceptions appear: our system and the one with urease from sword beans immobilised on acrylamide–PET (poly(ethylene terephthalate)) supports.

Calling "optimum pH range", the range in which the relative enzyme activity is higher than 95%, these ranges in phosphate buffer are comprised between 7.5 and 8.0, and between 7.3 and 7.8 for the free and immobilised enzyme, respectively. In the presence of citrate buffer, "the optimum pH range" occurs between 6.3 and 6.7 for the free urease and between 5.4 and 6.1 for the immobilised counterpart. The conclusion is that our membranes can be usefully employed when the urea hydrolysis has to be performed at pH values more acidic that the ones at which the optimum pH of the free enzyme occurs.

3.4. Temperature dependence of membrane activity

In Fig. 10a, the activity of the catalytic membrane as a function of temperature is reported. Experiments have been performed in 0.1 M citrate buffer. The activity of the free urease has also been reported in order to allow a direct comparison between the temperature dependence of the free and immobilised

Table 3
Optimum pH values in phosphate buffer for free and immobilised urease as found by different authors (last column) using different systems

Carrier	pH optimum		Reference
	Immobilised urease	Free urease	
BMA–nylon membrane	7.5	8.0	This paper
	5.7[a]	6.5[a]	This paper
Acrylamide-grafted PET	7.0[b]	8.5[b]	[24]
Gelatin–poly(HEMA) copolymer	7.5	7.5	[39]
Chitosan membrane	7.0	7.0	[42]
Nylon tube	7.0	7.0	[43]
Nylon	6.5	6.5	[44]

[a] Citrate buffer.
[b] Urease from sword beans.

form. The free urease shows an optimum temperature at about 67°C, while the optimum temperature of the immobilised urease was shifted to about 80°C. This means that the immobilisation procedures preserve the active structure of the macromolecule at temperatures higher than those denaturating the free form, independently of the values of thermal stability. The same behaviour has also been found by other authors with urease immobilised on different supports, as one can see in Table 4. Two remarkable differences are evident in this table. The first one concerns the low optimum temperature of the free urease found by Anita et al. These authors studied urease of type IV from Sigma, and found a shift of 40°C in the optimum temperature when the enzyme was immobilised on vermiculite [41] or nylon tubes [42]. The second difference is shown by urease from soybeans, which exhibits low optimum temperatures, 40 and 45°C, for the free and immobilised enzyme, respectively.

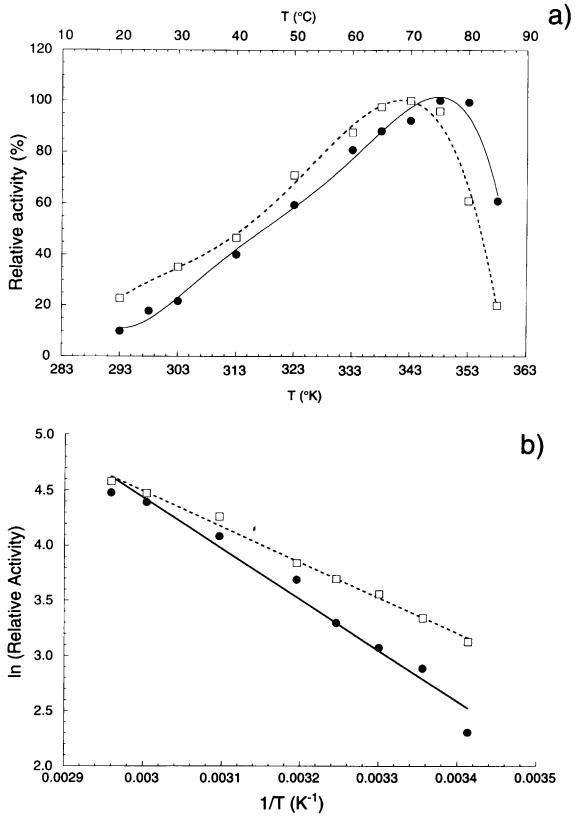

Fig. 10. (a) Temperature dependence of the activity of free (□) and immobilised (●) urease in citrate buffer; (b) Arrhenius plot of the activity of free (□) and immobilised (●) urease.

Calling "optimum temperature range", the range in which the relative enzyme activity is higher than 95%, this range occurs between 62 and 73°C for the free enzyme and between 68 and 80°C for the immobilised urease. Considering that at low temperatures, a small difference in the enzyme activity is found between the free and the immobilised form, one can conclude that our membranes can be usefully employed in biotechnological processes occurring at large temperature ranges.

In Fig. 10b, the results of Fig. 10a are presented as Arrhenius plots, relatively to the temperature range from 15°C to the optimum temperatures. The plots for both forms of enzyme result quite linear and the calculated values of the activation energy were equal to 5.9 and 9.1 kcal mol^{-1} for the free and immobilised urease, respectively. These values are reported in Table 5, where the values obtained by other authors with other urease derivatives are also listed. The

Table 4
Optimum temperature values for free and immobilised urease as found by different authors (last column) using different systems

Carrier	Temperature optimum (°C)		Reference
	Immobilised urease	Free urease	
BMA–nylon membrane	75	70	This paper
Gelatin–poly(HEMA) copolymer	75	60	[39]
Vermiculite	65[a]	25[a]	[41]
Chitosan membrane	75	65	[42]
Nylon tube	50	60	[43]
Nylon tubes	65[a]	25[a]	[44]
Acrylamide	45[b]	40[b]	[49]

[a] Urease type IV by Sigma.
[b] Urease from soybeans.

Table 5
Activation energy values for free and immobilised urease as found by different authors (last column) using different systems

Carrier	E_a (kcal mol^{-1})		Reference
	Immobilised urease	Free urease	
BMA–nylon membrane	9.10	5.90	This paper
Acrylamide-grafted PET	14.14	11.76	[24]
Poly(vinyl alcohol)	4.95	3.70	[38]
Methacrylic acid–acrylamide–PET	4.13	11.70	[40]
Chitosan membrane	7.37	5.31	[42]

activation energy values for the immobilised form are higher than those of the free form. This suggests that the immobilisation procedures introduce changes on the structure of the urease macromolecule, which make more difficult the enzyme-catalysed reaction, and that the reaction is diffusion controlled. A way to overcome the diffusion limitation problems has been recently suggested by us by using the catalytic membranes in bioreactors operating under non-isothermal conditions. In this way, the process of thermodialysis adds to the isothermal diffusion process, with the result of reducing the apparent K_m of the immobilised enzyme.

3.5. Operational stability of the membrane

One of the advantages of immobilised enzymes is their reusability. Fig. 11 illustrates the operational stability of the immobilised urease in phosphate buffer (●), in citrate buffer (□), and in citrate buffer containing 14 mM β-mercaptoethanol (◆). The activity of immobilised urease in phosphate buffer decreased sharply, although the immobilised enzyme maintained an approximately constant activity during four experiments. Other authors have also found the same behaviour in phosphate buffer [37,45], although using different supports. The reason for this sudden decrease

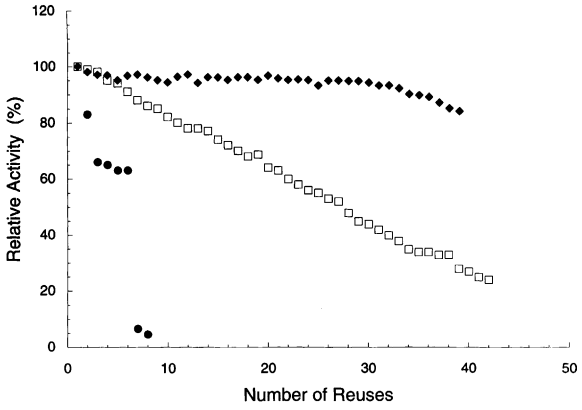

Fig. 11. Operational stability of immobilised urease in phosphate buffer (●), in citrate buffer (□) and in citrate buffer with 14 mM β-mercaptoethanol (◆).

is that phosphate is an inhibitor of urease [46,47]. In fact, the enzyme –SH groups may form thiophosphate of the type $–SPO_3^-$, which can be considered as catalytic intermediates. Thus, the enzyme is easily inactivated by compounds reacting with –SH groups [48].

When citrate buffer was used without β-mercaptoethanol, the activity decreased with a rate of 10% per day. Each day corresponds to two reuses. This behaviour may be attributed to the oxidation process of the large number of –SH groups present in urease. By contrast, addition of β-mercaptoethanol in citrate buffer kept the activity quite constant during the 35 experiments carried out in about 1 month. This confirms the protecting action of compounds endowed with sulfydryl groups.

4. Conclusions

This work shows that urease can be successfully immobilised by means of glutaraldehyde on pre-activated HMDA–poly(BMA)–nylon membranes. This reaction occurs through the interaction of the exposed arginine and lysine residues found in the 3D structures of the enzyme. Apart from enzyme-binding capacity and the shift of pH and temperature optimum, the operational stability of our catalytic membrane for over a month is of great relevance if the system is to be used for biotechnological processes or biomedical purposes. This stability is equivalent or superior to that of other systems previously described [37,45].

The elaborated 3D enzyme model helped us to understand the β-mercaptoethanol role in stabilising the enzyme, since 40% of the cysteine residues is accessible to the reagent.

The increase of the apparent K_m value for the immobilised urease with respect to the value of the free enzyme is a disadvantage which can be easily overcome by using the catalytic membranes in a membrane reactor operating under non-isothermal conditions. Results relative to this new technology, using the same membrane type studied in this work, are reported in a separate paper [50].

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