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Preparation Of Catalytic Materials By Trypsin Immobilisation On Carboxylic Textile And Carbon Matrixes Activated By Dicyclohexylcarbodiimide (DCC)

N.Bouhaine
Faculté des Sciences,
Département de Chimie,
Université Badji Mokhtar, 23000 (ALGÉRIE)
E-mail: bouhainen@yahoo.fr

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A.Djerourou[1], C.Innocent[2], P.Seta[2]
[1]Faculté des Sciences, Département de Chimie, Université Badji Mokhtar, 23000, (ALGÉRIE)
[2]Institut Européen des Membranes (IEM), CNRS-ENSCM-UMII, UMR 5635, 1919 Route de Mende, 34293 Montpellier, Cedex 05 (FRANCE)

ABSTRACT

New catalytic materials have been prepared by immobilisation of a protease, the trypsin, on a carboxylic cation exchange textile and carbon tube matrixes. In both cases the catalytic activity decreases in time like the soluble enzyme. Then, it takes a value nearly constant beyond 20 or 30 days and the materials keeps until 40% of its activity. In the case of the soluble enzyme this activity was losted continually during time. The Michaelis constants, V_m and K_m of the immobilized enzyme were lower than that of the soluble enzyme due to the less accessibility of the substrate to the enzyme active sites.

KEYWORDS

Trypsin;
Covalent grafting;
Cotton;
Carboxylic textile.

INTRODUCTION

Membrane techniques are being more and more attractive because they are faster, more efficient and more economic than conventional processes. These advantages are especially important for biotechnology (pharmaceutical or food) or medical industries. However, in these processes the membrane is only a separator, and to improve more these processes it would be interesting to use the membrane to achieve other functions as it is the case of the natural membranes that makes, beyond the selective permeability, conversion of energy and chemical synthesis. The main objective of this approach is to make the membranes "more intelligent". The application of the chemical reactivity concept at the level of a membrane is a new artificial membrane objective. A technological improvement can be carried out for exam-

ple by the reduction of the number of elementary stages of a transformation chain or treatment of a product, or by the coupling of several properties at the level of a membrane process. The development of a new membrane of filtration that would bring the possibility to transform chemically a substrate during the phase of permeation would be considered like a considerable advanced in the membrane technology. Biologic catalysts such as enzymes are good candidates for the development of such chemically reactive membranes.

Enzymes immobilization was for a long time the topic of a large number of tentatives. However, one of the difficulties met in this field is the conservation of the enzyme activity which is generally quickly losted. Even though the kinetics of enzymatic reaction is not affected distinctly by the confinement of enzymes in matrixes, diffusion parameters do not change considerably with the composition of these matrixes. The loss of activity is owed mainly to immobilization conditions that mislead the denaturising of enzymes. Desorption of the enzyme is among the most critical factors that limited the development of enzyme immobilization systems for a big scale application. The realization of a "good catalytic membrane" passes therefore by the elaboration of a non denaturing immobilization method, that means capable to preserve the efficiency of the biocatalyst. In this work, we propose to study the efficiency of a covalent grafting of trypsin on two different kinds of matrixes, a carboxylic fibrous textile and a carbon microporous solid tube, using dicyclohexylcarbodiimide (DCC) as an activating agent.

Cotton used for the confection of clothes not being expensive and very available, has been used with success in the immobilization of enzymes or micro-organisms and fermentation processes[1-4]. Lately, a cellulose matrix (hydrophilic cotton) covered by polyethyleneimine has been used as a support for the immobilization of glucose oxidase[5], urease[6,7], and invertase[8] by ionic adsorption followed by reticulation with glutaraldehyde. Cellulose fibbers can be activated with a variety of reagents such as CNBr, sulfonyl chlorides, and periodates to form covalent bonding with the enzyme[9,10]. However, activation of cellulose by CNBr gives a slightly reactive cyclic imidocarbonates, with a labile bond which provokes the release of the coupled catalyst[10-16].

Ion exchange membranes are used in electromembrane processes (electrodialysis) and the chemical structure of their matrix is not very different of the ion exchange textiles. These membranes have been associated to a textile in a coupled process (electrodeionisation)[11] and the association of the textile and the enzyme have been already used in the industry of textiles[12]. The immobilization of enzymes on the textile can be developed for the realization of new functionalized textile which will not only used in clothing industry but also as catalytic materials. The porosity of the textile associated to enzymatic catalysis can give a filtration catalytic membrane. Ion exchange textiles have been already used in our laboratory for the immobilization of enzymes such as glucose oxydase and urease by means of a biotin-avidin immobilization method[13,14].

The modification of an electrode surface by electro-chemical polymerization of a monomer such as pyrrole and its derivatives, thiophenes, aniline, phenol is currently a method used[15]. The big interest of this method resides in the possibility to control directly by colometry the quantity of polymer electro generated on the surface of the electrode and therefore the thickness of the deposit. The modification of an electrode surface by entities such as redox mediating agents, chemical catalysts or photosensibilisators constitute a sophisticated approach, extensively used to develop electro-chemical applications, in the domains of synthesis, analysis and the conversion of solar energy. The use of a porous electrode can lead to the realization of a porous material susceptible to be used like a membrane material.

Grafting mechanisms

The ion exchange textile used to achieve our works was a cellulose textile containing carboxylic groups. Its exchange capacity was 2-5 meq/g or 300-450 g/cm^3). The COOH functions served as precursors for trypsin grafting after activation by DCC. The textile support was immersed in a 0.1 M DCC

FULL PAPER

solution prepared in a mixture of methanol and chloroform (v/v) under nitrogen vacuum at 40°C during 6 hours. Then, the textile was firstly rinsed with methanol to remove the unreacted DCC and then with the Tris-HCl buffer. It was then immersed in the enzyme solution (4ml of tris-HCl buffer containing 1mg of trypsin).

The immobilisation mechanism occurs as following:

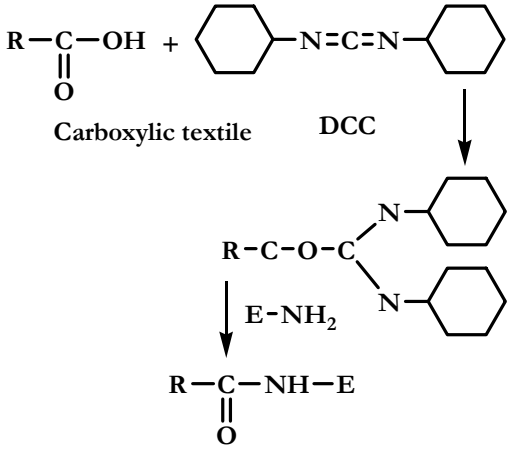

Figure 1: Immobilisation mechanism on the carboxylic textile by DCC activation

- we obtain a cationic dimmer which must lose two protons; it is the first step of polymerization.
- we obtain a dimmer species which is a monocation radical. A supplementary oxydization step and the loss of two protons are necessary then to succeed to the same dimmer species that in the first case.

The dimmer is a driver of electricity and it can be oxidized all over again by transfer of electrons. It can react with another molecule of pyrrole monomer or with its oxidized form; it occurs a chain of reactions that succeeds to the formation of polypyrrole in thin layer on the electrode.

The functionalisation of the carbon tube was realized as follows: we prepared a monomer solution of cyanoethylpyrrole 1M in CH_3CN, $NaClO_4$ O.1 M in an electro-chemical cell with three electrodes, and imposed an electric potential of +0.9 V/CSE. After having formed a deposit of cyano-ethyl-pyrrole polymer, we transformed the CN functions of the polymeric material to COOH functions by immersing this last in a solution of HCl 0.1 N. The enzyme was then immobilized as described before.

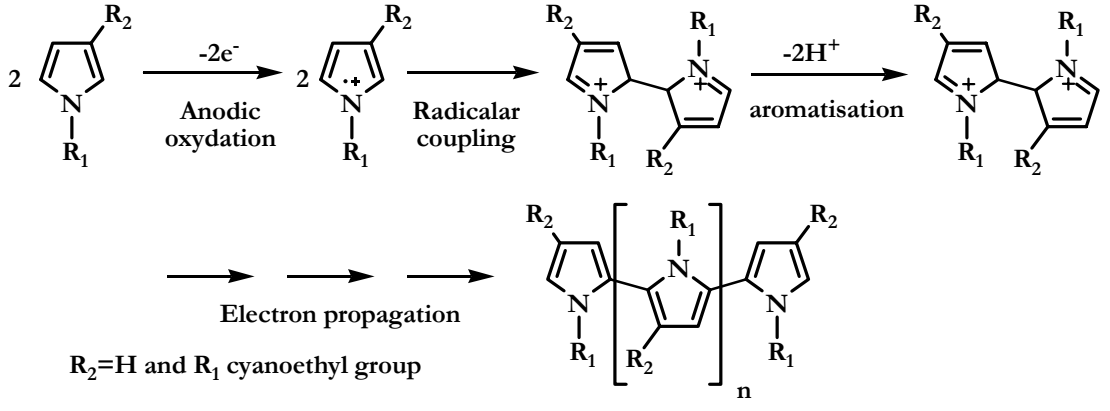

Figure 2: Electropolymerisation mechanism of pyrrole

In the case of enzyme immobilisation on the carbon matrix, the first step was electropolymerisation started by the oxidation of the pyrrole which leads to the formation of a radical cation. This radical cation can react with another one. Two cases can be present:

- if the radical cation reacts with a monomer molecule, we obtain a cationic dimmer which must lose two protons; it is the first step of polymerization.
- we obtain a dimmer species which is a monocation radical. A supplementary oxydization step and the loss of two protons are necessary then to succeed to the same dimmer species that in the first case.

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In the case of our study we tried to immobilize the trypsin on a tube of carbon by chemical grafting. We have electropolymerized the cyano ethyl pyrrole on the tube of carbon. Then, the CN functions have been transformed in COOH in an acidic medium, the obtained material was functionalized according to the mechanism of figure 1.

FULL PAPER

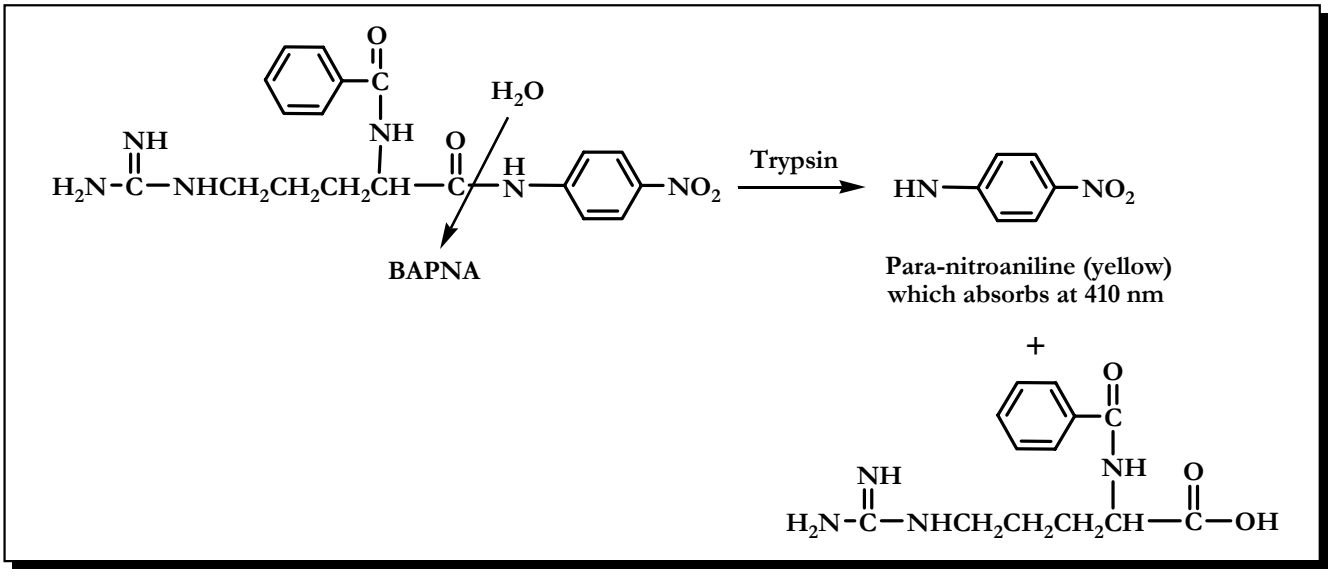
EXPERIMENTAL

Dicyclohexylcarbodiimide (DCC), sodium hydroxyde, sodium perchlorate, cyanoethylepyrrole were commercial products from Sigma Aldrich (St Louis, MO). Dimethylsulfoxide (DMSO) was a Mallinckrodts product (Kentucky, USA).

The Trypsin used was a Sigma product (St Louis, MO). The buffer used for the preparation of the trypsin solutions was tris-HCl, containing 0.02 M of $CaCl_2$, $2H_2O$ (pH 5-12) prepared in the laboratory. Distilled water has been used for the preparation of solutions.

Activity assay

The activity of the trypsine was measured using the N-benzoyl-DL-arginin-p-nitroanilide (BAPNA) Fluka product) as substrate. The specific reactions implied in the coupling mechanism are schematized like follows:



The experimental protocol of determination of trypsin activity by BAPNA is described in the technical paper provided by Sigma[16].

43.5 mgs of BAPNA have been dissolved in 2 ml of dimethylsulfoxide (DMSO) then the volume is completed to 100 ml with tris-HCl buffer. A volume of 25 µl of trypsin solution is mixed then with 4 ml of a freshly prepared BAPNA solution. The absorbance of the mixture is followed during time (during 10 minutes) at the wavelength of 410 nm at which the p-nitroaniline product presents a maximum of absorption.

The activity was calculated by the following formula:

$$\frac{\text{Absorbance } \textit{du} \text{ produit} \times 1000 \times \text{volume } \textit{du mélange réactionnel}}{10\text{mn} \times 8800 \times \text{mg protéine } \textit{dans le mélange}}$$

where 8800 is the extinction coefficient of p-nitroaniline at 410 nm (M^{-1}/cm).

A unit of activity of trypsin will produce a change of the absorbance at 410 nm of 0.001 per minute at pH 7.5 and 25°C with BAPNA as substrate.

The activity of the immobilized enzyme has been determined immediately by difference between the initial activity of a given enzyme quantity in solution and those of the washing solutions.

The reaction products obtained by the immobilized trypsin on the support were analyzed continuously using the experimental set-up of figure 3. The solution of BAPNA is put in continuous circulation in a tube containing the biomaterial. The out-put of the reaction product is sent to the Uv-Visible spectrophotometer to be analyzed continuously, and then reinserted again in the circuit. Solutions circulation is assured by a peristaltic pump. After 10 minutes of reaction the circulation of the different solutions was stopped.

The UV-Visible spectrophotometer used was KONTRON, Uvikon 940.

FULL PAPER

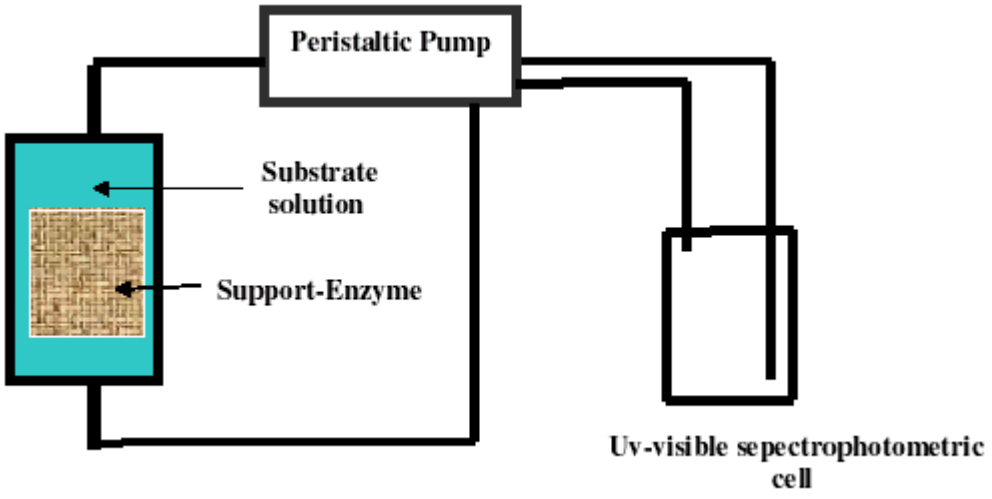


Figure 3: Experimental set-up

After the calculation the activity of the enzyme, we can convert it in concentration of products transformed knowing that 1 unit of activity of the enzyme catalyses the hydrolysis of 1 μmol of substrate per minute at a temperature of 25°C and a pH of 7.5.

Infra-red spectra (FTIR) have been performed using a Nicolet 710 spectrophotometer. The different pH of the solutions has been measured with a Metler Toledo MP 120 pH meter.

RESULTS AND DISCUSSION

Figure 4 shows a clear difference between the two spectra of the textile confirming the grafting of DCC. The peak which appears at about 3000cm^{-1} is related to the C-N- functions, the one at 1250cm^{-1} owed to the presence of C-O-C bound, as well as the one at 850cm^{-1} relative to the R-N-R bound confirms the mechanism well described on figure 1.

The optimal pH of trypsin activity has been determined by preparing different enzyme solutions in

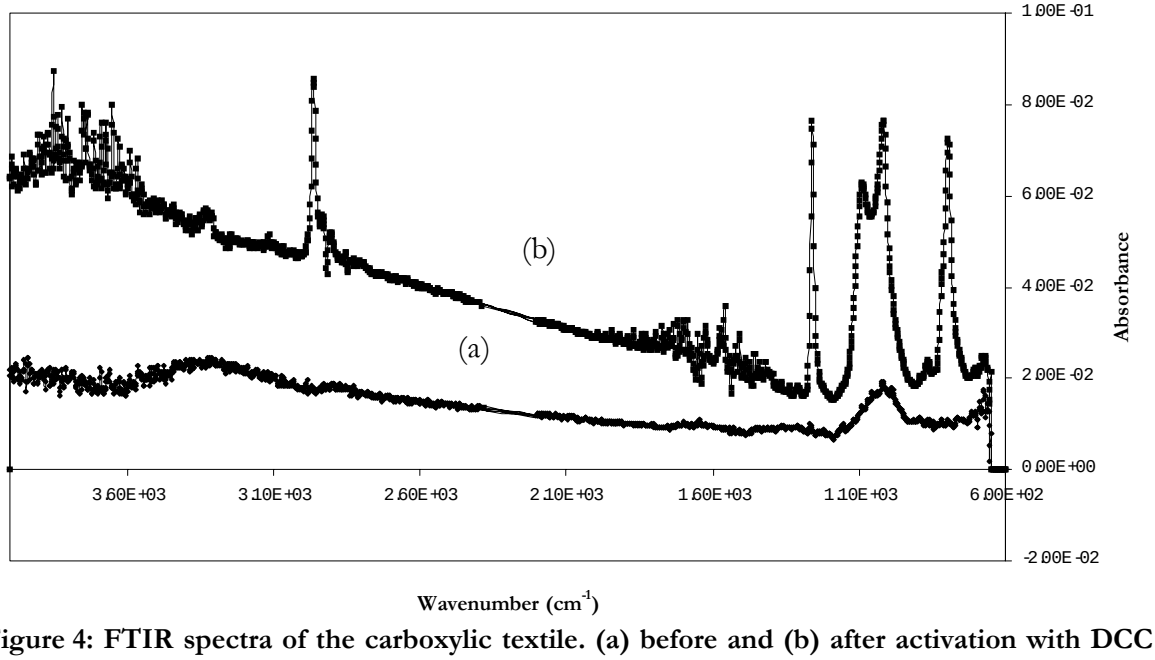


Figure 4: FTIR spectra of the carboxylic textile. (a) before and (b) after activation with DCC

several tris-HCl buffers of different pH values. Figure 5 shows that the optimal pH is about 8. For a pH lower than 4 and higher than 12 no activity of trypsin was measured. These values are in accordance with the data signalled in literature[17]. On the other hand, this process (loss of activity by change of pH) proved to be irreversible. We fixed the pH therefore to 7.5 for the following parts of the work.

The evolution of the products concentrations in time obtained with the different materials is represented on the figure 6. The formation of products is linearly dependent on time during the first minutes of reaction. The evolution of these graphs obeys to a Michaelis-Menten kinetics and their slopes represent the values of Vi.

The Michaelis-Menten Km and Vm have been calculated by plotting 1/Vi versus 1/[S], S reefers to the substrate (BAPNA). Km represents the enzyme-substrate complex dissociation constant and also the inverse of the affinity of the enzyme for the substrate.

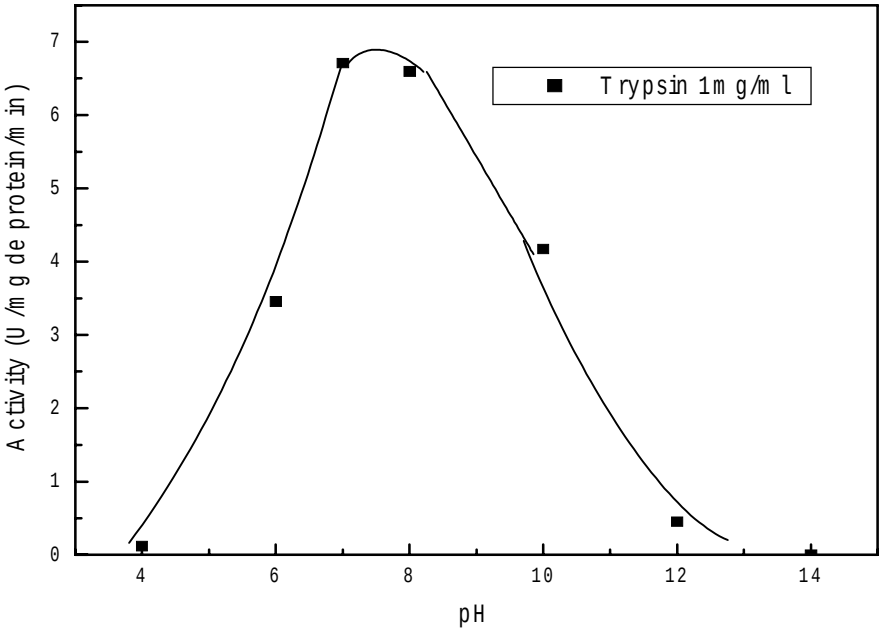


Figure 5: Effet du pH sur l'activité de la Trypsine en solution

TABLE 1: Quantities of trypsin in the different phases. Initial quantity 1mg in 4ml of tris-HCl buffer

Support	Solution after immobilisation (mg)	Washing solutions (mg)	Immobilized quantities (mg)
Textile, m = 0.0625 g	0.020	0.977	0.0035
Carbone tube m = 1,408 g	0.363	0.633	0.003

TABLE 1, collects the trypsin quantities immobilized on the different supports used.

Quantities of materials used not being the same and in order to compare their efficiency to immobilize the trypsin we brought back immobilized enzyme quantities to 1g of material. Results regrouped in the TABLE 2. The carboxylic textile seems to be the one which gives the biggest rates of fixing. It could be due to the importance of the specific surface of this material compared to the carbon matrix. It is necessary to also underline that quantities of immobilized enzyme have been calculated supposing that the specific activity of the immobilized enzyme was the same than that of the enzyme in solution.

TABLE 2: Quantities of trypsin immobilized for 1g of matrix.

Support	Immobilized quantity (mg of trypsin/g of support)
Textile carboxylique	0.056
Tube de Carbone	0.0021

FULL PAPER

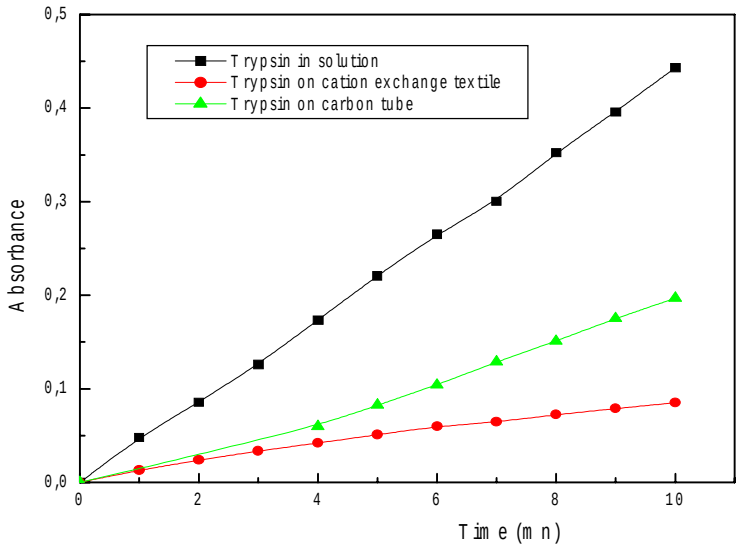


Figure 6: Kinetic of reaction in solution and on the different supports. [BAPNA] = 0.98 x 10^{-3} M, pH = 7.5

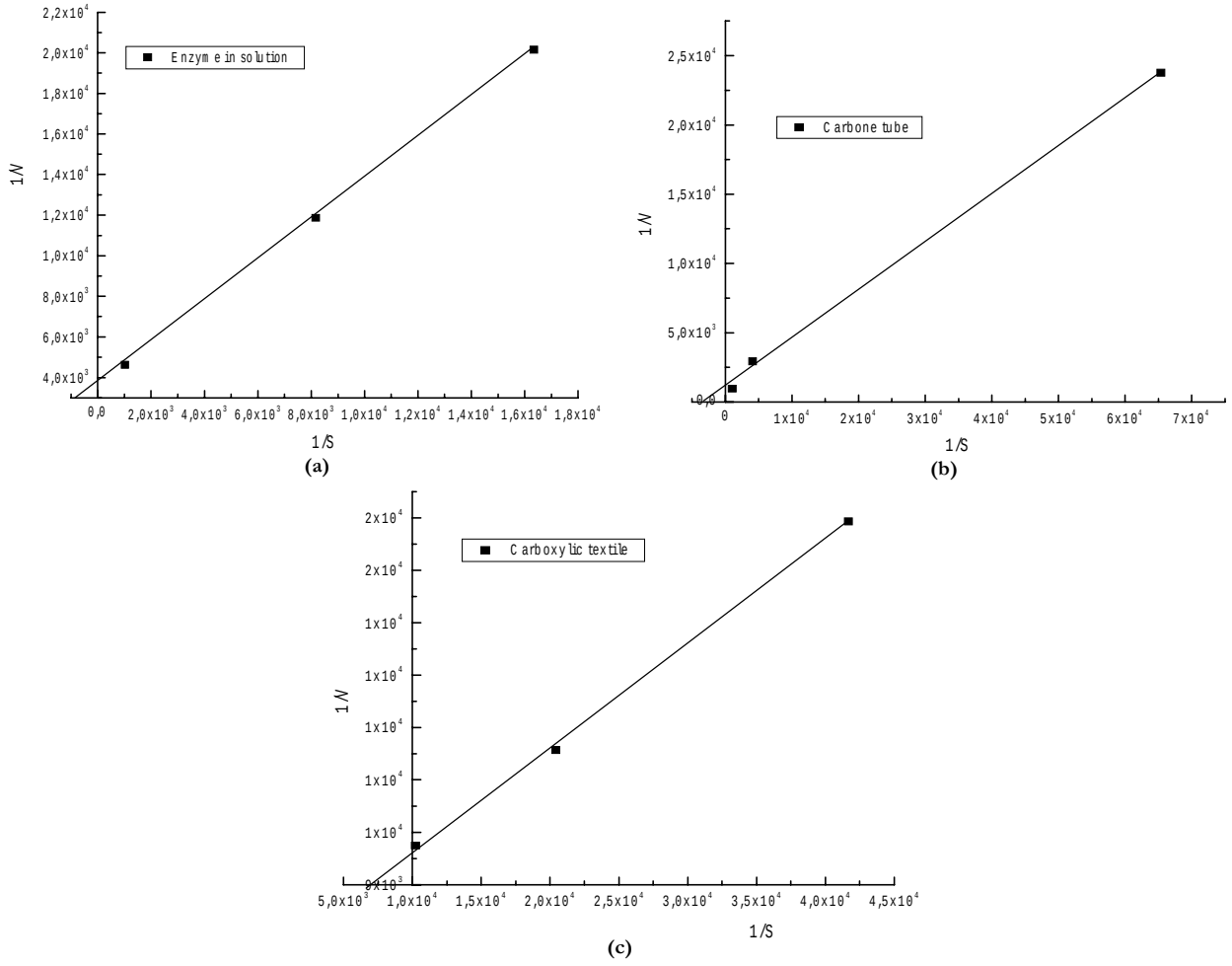


Figure 7: Determination of michaelis-menten constants, K_m et V_m. pH=7.5

FULL PAPER

TABLE 3: Values of K_m and V_m

Support	V_m (mol/l/mn) x 10^{-3}	K_m (mol/l) x 10^{-3}
Carbone tube	0.10	1.166
Textile	0.08	0.144
Trypsin in solution	0.26	1.220

The results obtained are represented on figures 7a-c and TABLE 3.

The values of Vm and Km determined for the enzyme in solution are higher than that of the immobilized enzyme. This is probably due to the fact that the immobilized enzyme does not have the same accessibility to substrate than the soluble enzyme. It can be due also to the orientation of the enzyme active site after immobilization. According to the disposition of the enzyme molecule on the support the active site can be free or more cluttered.

CONCLUSION

We demonstrated during this work, the feasibility and the efficiency of the development of a catalytic membrane by covalent immobilisation of trypsin on different types of supports. The activation by DCC proved to be efficient. The stability of the enzyme was considerably improved when it was grafted to a carboxylic or carbon matrixes. It keeps about 40% of its activity for a long time more than 80 days. However this immobilisation method opens a large perspective for the utilisation of these biomaterials at a large scale.

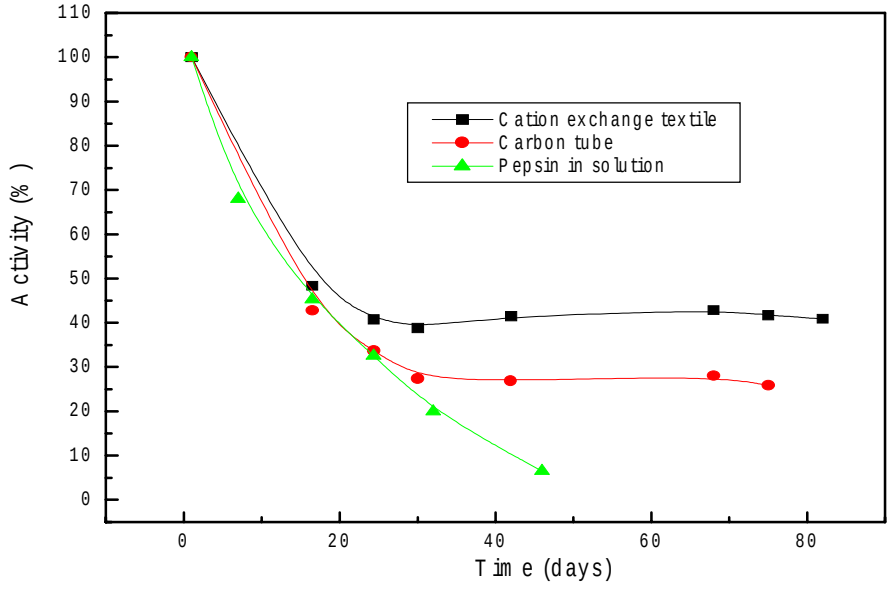


Figure 8: Evolution of trypsin activity in time.

The stability of the trypsine in solution and immobilized on the different supports is evaluated by following its activity during time. Figure 8 shows that in any case, the activity of the trypsine decreases during time. However in the case of the immobilized trypsine on the solid matrix the activity takes a value nearly constant beyond 20 or 30 days and the enzyme preserves until 40% of its activity.

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FULL PAPER

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