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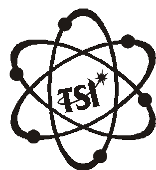

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Preparation Of Catalytic Materials By Covalent Immobilisation Of Pepsin On Cotton Fibers Activated By Tosyl Chloride

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ABSTRACT

A study was performed on the possibility of utilisation of hydrophilic cotton for the elaboration of enzymatic bio-catalytic materials. Pepsin was covalently bounded to the cotton matrix using tosyl chloride as activating agent and the stability of such pepsin modified materials was studied. The immobilized pepsin was the most stable at pH 1.5-2 as was the case of the soluble enzyme. Enzyme immobilization on the tosylated cotton followed a nucleophilic substitution mechanism as evidenced by UV and FTIR spectra and about 0.75 mg of pepsin per gram of cotton could be immobilized. However, the protein coupling efficiency was 38% (if 1 g of cotton is used). Enzyme activity was maintained to about 50% of its initial activity over one month. The continuous decrease of the activity of the immobilized pepsin was also accentuated by the successive formation followed by adsorption of the haemoglobin heme (solid particles) on the cotton fibers, which lowered the reaction between the enzyme active sites and the substrate. This study showed that proteases immobilized on cotton could be used as bioreactors. © 2006 Trade Science Inc. - INDIA

KEYWORDS

Catalytic materials;
Cotton;
Tosyl chloride;
Enzyme grafting;
Pepsin.

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INTRODUCTION

The advantages of immobilized enzymes are well known from both an academic and an industrial point of view. Enzyme immobilization provides easy recovery and reuse of the enzyme and many other advantages, including facility in product separation and continuous flow operation. For successful development and application of an immobilized biocatalyst, the enzyme support is generally considered as the most important component contributing to the performance of the reactor system. Although continuous immobilized enzyme reactors give higher productivities, minimize downtime, enzyme costs, and capital investment, large-scale applications of immobilized enzymes are limited, largely because the support materials used for enzyme immobilization are either too expensive or difficult to use in industrial scale. Therefore, development of new techniques for enzyme immobilization based on inexpensive and industrially applicable carriers is of economical significance and relevance.

Numerous synthetic and natural polymeric supports have been used in these studies, including acrylic copolymers[1], graft copolymers[2], modified polymer rubber[3], and other inorganic supports such as alumina, titanium, stainless steel, and iron oxide[4, 5]. Hydrophilic supports have been more useful owing to the increased stability of the immobilized enzymes. Ion exchange textiles have been already used for the immobilisation of enzymes such as glucose oxidase and urease which gave good results[6, 7].

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[8-11]. Lately, cotton covered by polyethyleneimine has been used as a support for the immobilization of glucose oxidase[12], urease[13, 14], and invertase[15] by ionic adsorption followed by reticulation with glutaraldehyde. Cotton fibers having a skeleton of cellulose (Figure 1) can be activated with a variety of reagents such as cyanogens bromide (CNBr), sulfonyl chlorides, and periodates to form covalent bonding with the enzyme[16, 17]. The activation of cellulose by CNBr gives a slightly reactive cyclic imidocarbonate, with a labile bond which provokes the release of the coupled catalyst[17-23]. However, CNBr is poisonous, and its use implies some serious sanitary risks during the activation by the possible presence of cyanate residues during the food ingredient production[24-26].

Cotton was chosen in our work for its highly porous fibrous structure and its high mechanical strength. Hence, it allows high flow rates and efficient mass transfer through the matrix, which are advantageous not only during treatments for chemical activation and enzyme immobilization, but also for the applications of immobilized enzymes. For this reason, in the present work we are interested in the elaboration of cotton catalytic materials on which the protease pepsin will be immobilized in an irreversible manner using the covalently bonding by tosyl chloride activation.

EXPERIMENTAL

Materials and chemical reagents

Chloride p-toluenesulfonyl (tosyl chloride), sodium hydroxide, hydrochloric acid, trichloroacetic acid (TCA), acetone and dry pyridine were commercial products of Sigma (St Louis, MO).

Pepsin (E.C. 3.4.23.1) obtained from Sigma (St Louis, MO) was the enzyme used in this study. The solutions used for the preparation of the enzyme were HCl (pH 2-5). Doubly distilled water was used.

Porcine haemoglobin from Sigma was used as the substrates of Pepsin. One unit will produce an absorbance change at 280 nm of 0.001 per minute at pH 2 and 37°C measured as TCA-soluble products using haemoglobin as substrate.

The cotton used was obtained locally and had a density of 1.27 g/cm^3. The value of specific surface for the cotton fibres was about 55×10^3 m^2 kg^{-1}[29] (Figure 1).

Tosylation of cotton and enzyme immobilization

The immobilization procedure described elsewhere[8] consisted of four main steps: mercerization of cotton with NaOH, pyridine pre-treatment, tosyl activation of cotton, and enzyme coupling to cotton fibres.

For mercerization, 0.1 g of cotton was soaked in 10 ml of 3N NaOH solution for several hours (24 hours but about 4 h can be sufficient[8]). The cotton

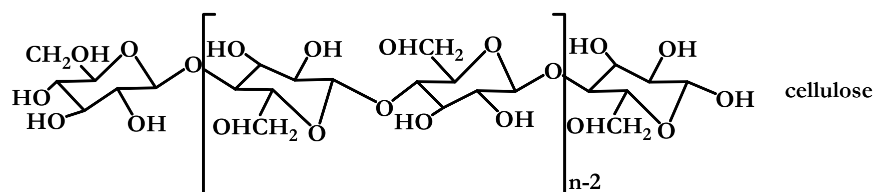


Figure 1: Basic structure of cellulose molecules

was then rinsed thoroughly with distilled water to remove the excess of NaOH. The wet cotton was blotted between paper towels to remove as much water as possible. Then the blotted cotton was further rinsed with dry acetone to remove water. A sample of 10 ml dry pyridine was then added to the cotton and allowed for a period of incubation (Pyridine pre-treatment) of 18 hours.

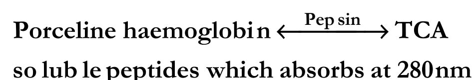
For tosylation of the cotton, 10 ml of a 5M tosyl solution (in excess) in dry acetone was added to 0.1 g of the cotton pre-treated with pyridine without removing pyridine during 48 hours. When tosylation was completed, the cotton was removed from the reaction mixture and washed first with acetone and then with excess amounts of a 10 mM HCl solution to remove tosyl and pyridine residues from the cotton. Tosyl-activated cotton was kept in 10 mM HCl solution and stored at 4°C until used for enzyme immobilization.

Enzyme immobilization was carried out by immersing 0.1 g of cotton for 2 hours in 4 ml of a 10 mM HCl solution containing 2mg of enzyme. After immobilization, the cotton was rinsed with copious amounts of the same acid or buffer solution and kept in it. Enzyme activity was determined immediately and followed during time. All reactions during tosyl activation and enzyme immobilization were carried out in 20 ml flasks at room temperature and shacked at 200rpm. The flasks were tightly closed with rubber stoppers to prevent evaporation of reactants and solvents.

The proposed mechanism for cotton tosylation followed by enzyme immobilisation is described in figure2.

Enzyme activity assay

The activity of immobilized enzyme on cotton was measured with porcine haemoglobin and BAPNA as substrates. The specific reactions are schematized as follows:



The assay procedure of pepsin activity was that described in Sigma product information (Sigma quality control test procedure)[31].

It consists in using a solution of 2% haemoglobin in 10 mM HCl as substrate. A volume of 1 ml enzyme extract in HCl was incubated at 37°C with 5 ml of substrate for 10 min. The reaction was terminated using 10 ml of 5% TCA (Trichloro Acetic Acid) and left for incubation for 5 min. The mixture was then filtered. Absorbance was recorded at 280 nm. For blank experiments, TCA was added to substrate prior to the addition of enzyme extract. Specific activity (U) was expressed as:

$$\text{Absorbance value (supernatant)} = \frac{\text{Absorbance value(blank))x1000 x dilution factor}}{\text{10 mn x mg protein in the assay}}$$

In the case of pepsin immobilized on the cotton, the substrate solution was circulated continuously into an experimental set-up containing the enzyme fixed on the cotton. After a reaction time of 10 minutes the solution was collected and analysed as before. TCA was not added to the cotton to avoid the denaturation of the enzyme attached to the cotton fibers.

Analyses

Absorbancies at 280 nm of the products solutions were measured with a UV-visible spectrophotometer (KONTRON, Uvikon 940). The amount of the enzyme coupled onto the tosylated cotton was determined from the initial protein amount present in the enzyme-coupling solution subtracting the final total protein amounts present in the remaining coupling and washing solutions. The coupling yield

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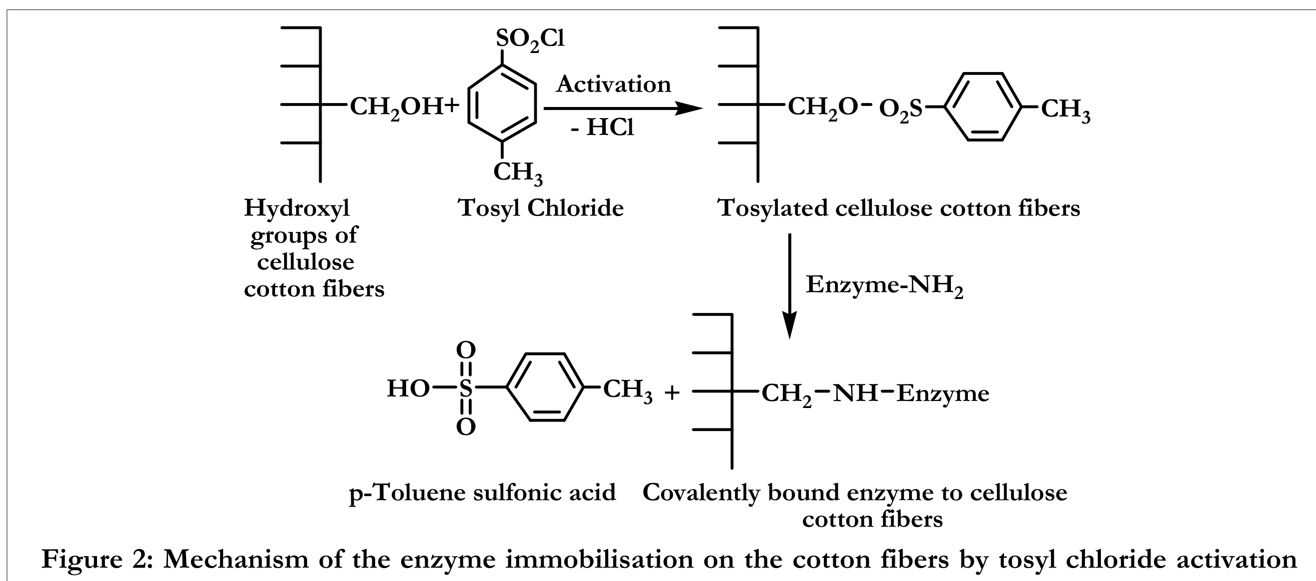


Figure 2: Mechanism of the enzyme immobilisation on the cotton fibers by tosyl chloride activation

(%) of the enzyme was then calculated from the amount of enzyme coupled to the cotton cloth divided by the initial total amount of the enzyme present in the coupling solution. After calculating the enzyme activity, we can convert it to a concentration of transformed products knowing that one unit catalyzes the hydrolysis of 1 μmol of substrate per minute at given temperature and pH.

FTIR spectra were recorded on a Nicolet 710 spectrophotometer. SEM observations were made using a JEOL JSMT-T 330A apparatus.

RESULTS AND DISCUSSIONS

FTIR and UV-visible characterization

FTIR spectra performed on the cotton before and after contact with tosyl chloride are shown in figure 3. These spectra showed that specific peaks related to -C-O-SO_2-C- were observed at 1180 and 1350 cm^{-1} on the spectrum of the tosylated cotton. It indicated that tosyl chloride reacted with the primary hydroxyl group of the cotton according to the mechanism described in figure 2.

The enzyme solution during the course of enzyme coupling to tosylated cotton was scanned in the UV-visible range from 200 to 700 nm. The spectra taken before contact with the tosylated cotton, and after 2 and 6 hours of contact are shown in figure 4. As it can be seen in this figure, the enzyme peaks (at 280 nm) was higher for the solution initially containing enzymes. However, after 2 and 6h of contact a big part of enzyme disappeared and an increase in tosyl content in the solution was observed, expressed by an increase of the absorbance of the specific peak at 261 nm. The simultaneous increase in tosyl and decrease in enzyme concentrations clearly suggested that the degree of enzyme coupling was accompanied with the displacement of tosyl, following the nucleophilic substitution mechanism for enzyme immobilization. Therefore, it could be concluded that tosyl was covalently bound to the hydroxyl groups and behaved as a leaving agent and was later on replaced by the enzyme leading to a covalent linkage according to the mechanism of figure 2.

SEM analysis

Scanning electron micrographs (SEM) were recorded to compare the morphology of the modified cotton with that of the unmodified one. All samples were dried in vacuum and gold-coated. Figures 5a and 5b show the top surfaces of both cotton samples. We could not see any difference in the morphology of both materials which demonstrated that no change occurred in the physical structure of the cotton fibers by activating the hydroxyl radicals via tosylation.

pH effect on the pepsin activity

The optimum pH for pepsin activity was determined by preparing the enzyme solutions, haemoglobin substrate in various solutions (HCl solution) of different pH values. Figure 6 shows that pepsin had

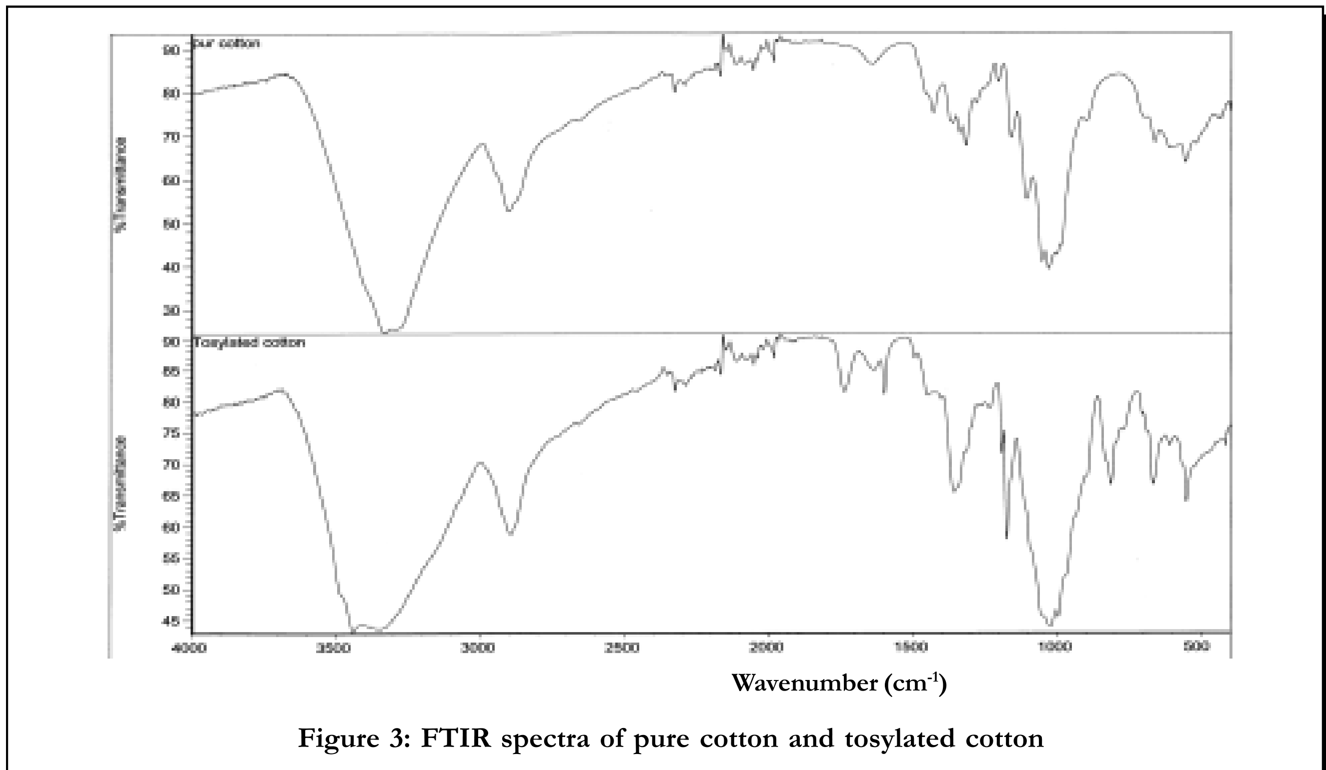


Figure 3: FTIR spectra of pure cotton and tosylated cotton

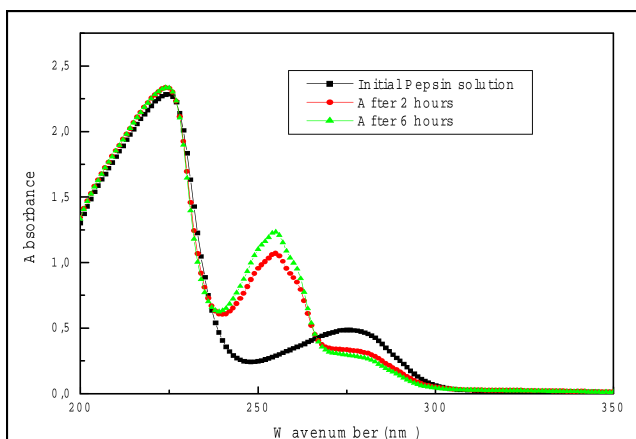


Figure 4: Scanning UV spectra of the enzyme-coupling solutions taken before contact with the cotton and at 2 and 6 hours after the coupling reaction

the highest activity at pH 1.5 and it then decreased with increasing pH. Over pH 3, almost 90% of the relative activity was lost and no activity was detected beyond pH 5. These values are in accordance with the data reported in literature[3, 5]. This process, loss of activity by changing the pH of the solution, was irreversible for the enzymes in solution as well as for the immobilized enzyme.

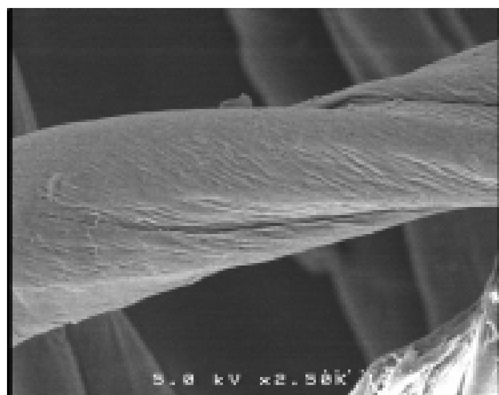
Exchange capacity of the cotton fibres

The number of exchangeable hydroxyl groups or by unit of weight (exchange capacity) of the cotton fibers was determined by shaking 0.18 g of cotton in 10 ml of a solution of NaOH 1M during 24 hours to ensure that a maximum of the cotton OH sites have been exchanged (substitution of H^+ by Na^+). Then, the cotton was rinsed thoroughly to remove the excess of NaOH. It was then shacked during 24 hours in 10 ml of a solution of HCl 1N to exchange Na^+ ions by H^+.

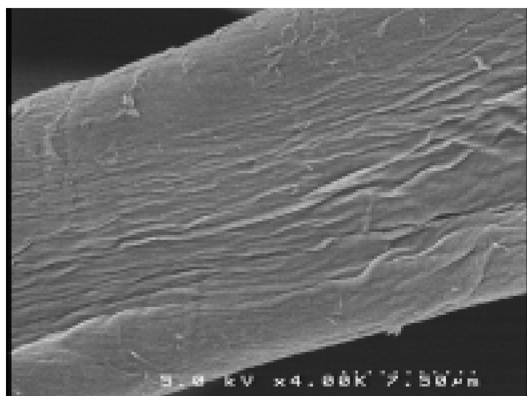
The difference in pH (or difference in Na^+ concentration) before and after exchange allowed us to calculate the number of active sites and the exchange capacity of the cotton fibers.

The value found was 6.56×10^{18} protons exchanged on 0.18 g of cotton which corresponds to 6×10^{-3} active sites/$Å^2$ [29] (i.e. about 5 meq of protons/g of cotton), and corresponding to an exchange capacity of 3.6 $\times10^{19}$ protons/g of cotton (0.18 g of cotton used corresponds to a surface of 9.9×10^{20} $Å^2$). This value was used for calculating the yield of enzyme immobilization achieved with 0.1 g cotton.

TABLE 1 gives the quantities of enzymes fixed on the cotton fibers doing the assumption that the



(a)



(b)

Figure 5: Scanning electron micrographs of the cotton fibers: (a) before and (b) after contact with the Tosyl chloride solution

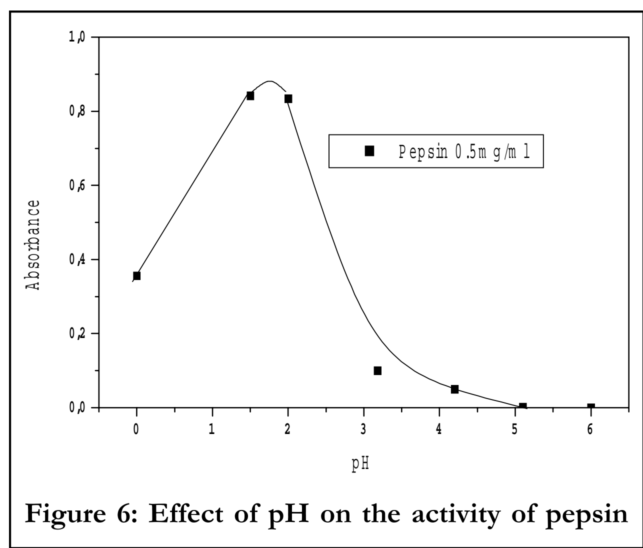


Figure 6: Effect of pH on the activity of pepsin

specific activity of the immobilized enzyme was the same as that in solution.

These values, brought back to 1 g of cotton, gave a protein coupling efficiency of 38%.

Kinetic analysis

The evolution of products formation was linearly dependent with time during the first minutes of reaction. Form the slopes at different substrate concentration the initial rates Vi can be deduced and ploted versus the concentration of the substrate This plot obeyed to a Michaelis-Menten kinetics model described by the following equation:

$V_i = V_m[S]/([S]+K_m)$, [S] being the substrate concentration

The calculation of Michaelis-Menten constants K_m of pepsin and maximal rates Vm were calculated by from the plot 1/Vi versus 1/[S]. V_m corresponds to the value of the inverse of the ordinate to origin and $-K_m$ corresponded to the inverse value of the intersection of the straight line with the abscissa (see figure 7). In the equation 1/Km was calculated when $1/V_i=0$. Km represents the complex enzyme-substrate dissociation constant and also the inverse of the affinity of enzyme for the substrate.

The different results obtained for the enzyme in solution and immobilized on the cotton fibers are plotted on figures 7 and summarized in TABLE 2.

V_m and K_m values of immobilized enzyme were lower than those of the free enzyme in solution. These results are quite classical for enzyme immobilisation on surfaces and likely due to the following factors. The immobilized enzymes have not the same accessibility to the substrates than the soluble enzymes. It could explain that a higher concentration of substrate is needed to enhance the efficiency of the collisions with the immobilized enzyme as compared to the same amount of soluble enzyme, and as a result an increased Km value. Adsorption of substrates such as haemoglobin could be also responsible for the decrease of Vm as compared to the free enzyme.

Long-term stability of the catalytic materials

Stabilities of the enzyme in solution and immobilized on the cotton fibers were estimated following the respective enzyme activities in the course of time. Figure 8 shows a decrease of the activity with time in both cases. However, contrary to the kinetic parameters the activitiy of immobilized enzymes on cotton fibers remained higher than that in solution (about 1.66 times higher). A lifetime over one month for the enzyme modified cotton bio-catalytic material,

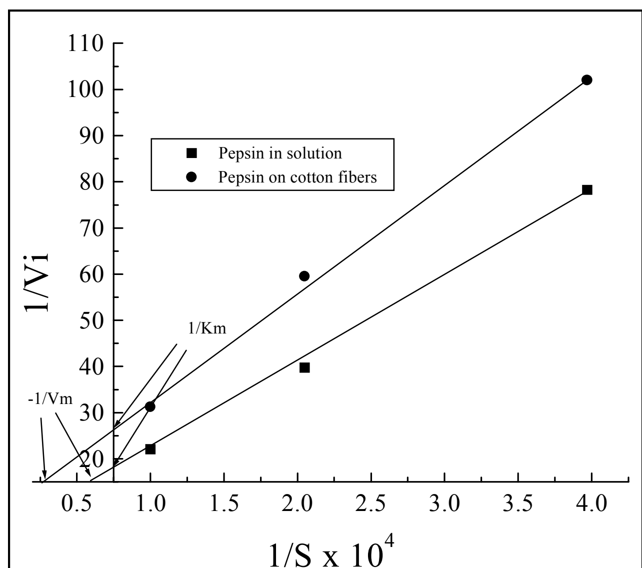


Figure 7: Determination of K_m and V_m for Pepsin in solution and immobilized on cotton fibers

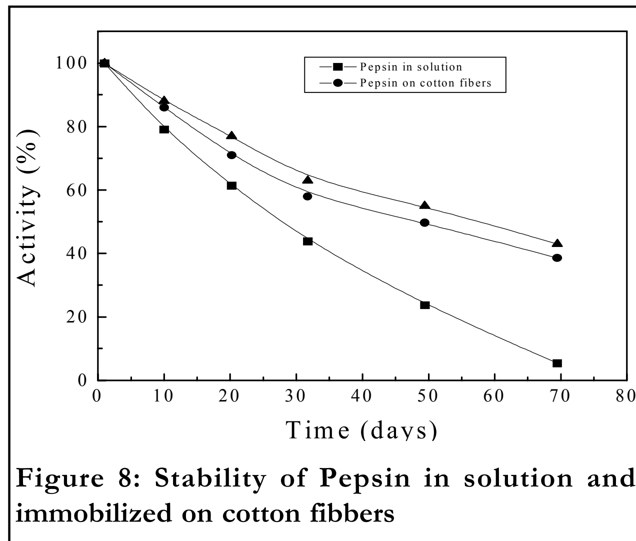


Figure 8: Stability of Pepsin in solution and immobilized on cotton fibbers

corresponding to a loss of only the half of the initial activity was observed, which represents to some extent an appreciable stabilization of the enzyme reactivity by the immobilization process.

CONCLUSION

We have demonstrated in this work, the feasibility and the efficiency of the elaboration of an enzymatic membrane made of proteinase immobilized on cotton fibbers by tosyl activation. With this procedure the immobilization of pepsin was successfully performed.

Cotton was activated with tosyl chloride and used as a novel fibrous matrix for biocatalysts immobi-lization. The described procedure was simple, inexpensive and industrially applicable and moreover provided active and stable immobilized biocatalysts supports. The optimal conditions weredetermined as 3N NaOH for mercerization of cotton fibers, 10 ml pyridine and 0.5M of tosyl/g of cotton for tosylation, pH 2 for Pepsin coupling.

Infrared spectrophotometric data suggested that enzyme immobilization was assisted by the substitution of tosyl from the cotton. The Michaelis constants of the soluble enzyme were higher than those of the immobilized enzyme due to the fact that the haemoglobin molecules have not the same accessibility to the immobilized enzyme molecules than the soluble enzymes, which include the orientation of the grafted enzyme molecules with respect to the cotton fibers surface. However, on one hand the enzyme stability has been increased by immobilizing the enzyme. On the other hand, the decrease of the immobilized enzyme activity is also accentuated by a fouling of the material by Heme aggregation. The Heme being a product of the haemoglobin hydrolysis reaction.

The very simple method for the immobilization using tosyl-activated cotton and the appreciable working life-time (over one month) of the cotton-immobilized enzyme reactor should have many applications in industrial bio-catalysis (e.g. peptides production with pepsin and synthesis of insulin with Trypsin).

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