Immobilization of Enzymes into a Polyionic Hydrogel: ChitoXan

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Immobilization of Enzymes into a Polyionic Hydrogel: ChitoXan

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ABSTRACT: Three enzymes were immobilized onto polyionic hydrogel, ChitoXan, obtained by complexation between chitosan and xanthan. The biocatalysts used were two proteases (protease type XIX from Fungal d'*Aspergillus sojae* and the trypsin type II.S from Porcine Pancreas) and a lipase (lipase Type VII from *Candida rugosa*). The immobilization efficiencies and the relative activities were investigated for these enzymes. The immobilization efficiencies changed with each enzyme and varied between 53 and 80%. Good relative activities were found for the lipase Type VII from *Candida rugosa* and the protease type XIX from Fungal d'*Aspergillus sojae.* For the latter, the influence of several factors were studied: molarity of the storage buffer, storage temperature and time of hydrogel, and the enzyme concentration. For the immobilized lipase, hydrolysis of olive oil in aqueous and organic media has been compared. This study confirmed that the lipase modified the external and internal structure of the hydrogel from fibrillar to the formation of globular structures in the presence of lipases.

KEY WORDS: protease immobilization, lipase immobilization, chitosan, xanthan, hydrogel polyionic.

INTRODUCTION

Enzymes immobilization into solid and porous supports is a widely used technique that allows to use them as valuable biocatalysts. These biologically active proteins and their bioinert carriers form a confined bioreactor system. Biopolymers such as chitosan and chitin are often

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used as bioinert carriers, especially to immobilize enzymes [1]. Enzymes immobilization into solid supports provides several advantages: a repeated use of the bio-catalyst, an easy separation of enzyme from products, the possibility of continuous processes, the rapid stop of reactions, and the improvement of enzymes stability [2]. These advantages may be very interesting for many applications in biotechnology [3], in biomedicine [3], for analytical systems [4], and for enzyme therapy [5].

ChitoXan is a polyionic hydrogel with a solid and porous matrix that is used to immobilize enzymes in the laboratory [2]. ChitoXan is formed by ionic bondings and van der Waal interactions between two biopolymers: chitosan and xanthan [2]. Chitosan is a linear binary heteropolysaccharide composed of β-(1→4)-2-amino-2-deoxy-D-glucopyranose and of β-(1→4)-2-acetamino-2-deoxy-D-glucopyranose, obtained by alkaline deacetylation of chitin, poly[β-(1→4)-2-acetamino-2-deoxy-D-glucopyranose]. Chitin is a natural polysaccharide, that is the major material found in the shell of crustaceans (such as, crabs and shrimps), the cuticule of insects and the cell walls of fungi. Chitin is the second most abundant biopolymer after cellulose. Consequently, chitin and chitosan may be interesting products for biomedical, biotechnological, and industrial applications.

Xanthan is an extracellular heteropolysaccharide produced by *Xanthomonas sp*. It consists of D-glucose, D-mannose, D-glucuronic acid, and pyruvate. Xanthan contains cellulosic backbone and a trisaccharide side chain on every second glucose residue. An α-mannose, a glucuronic acid, and a β-mannose compose the trisaccharide side chain. Pyruvic acid diketal groups are located at the 4,6-positions of the terminal mannose. Xanthan is one of the most commonly used polysaccharide with applications in food, pharmaceutical, and biomedical fields.

Proteases are widely used in industrial applications and trypsin is a hepatic enzyme of interest in medical applications. The immobilization of α-chymotrypsin, subtilisin BNP, and subtilisin Carlsberg has been studied in porous chitosan bead: the immobilized enzymes have shown higher catalytic activities than free enzymes for amino acid esterification [6]. The thermolysin like-protease has been immobilized and its stabilization was found to be strongly dependent on the site of attachment of the enzyme to the support [7]. Four other enzymes: polyphenol oxidase, trypsin, acid phosphatase, and peroxidase have been adsorbed onto chitosan and chitin; these immobilized proteases generally show an enhanced activity in organic cosolvent mixtures particularly at low concentrations of the organic solvent [8].

Immobilized proteases have been used in veterinary medicinal products; such as, Lysosubtilin, a lytic enzyme [9].

Lipases have received much attention in a large range of applications [10–13]. Lipases have been immobilized by different methods, such as, covalent attachment, adsorption, entrapment, and crosslinking on various supports [11]. The immobilization of lipases has been studied with a porous polypropylene support: the activity yields were 100 and 0.4% for the hydrolysis of *p*-nitrophenyl acetate and *p*-nitrophenyl palmitate, respectively [13]. Porous chitosan beads have been used to covalently immobilize lipoprotein lipase: its activity was higher than that of the free lipase [1]. Porcine pancreatic lipase has been immobilized on Celite for the synthesis of butyl butyrate in a nonaqueous system; the activity of the immobilized lipase was less than the activity of the free lipase [14]. Lipase immobilization on microemulsion-based polymer gel has been studied by Xenakis and Stamatis [15]. The potential applications of lipases for the production of several high-value chemical products and the advantages associated with the use of these biocatalysts in nonaqueous media, has motivated us to study the lipase activity of a ChitoXan/lipase complex in organic media [16].

In the present work, the immobilization of protease type XIX fungal from *Aspergillus sojae* (PRO XIX), trypsin II.S from porcine pancreas (TRY) and Lipase VII from Candida rugosa (LIP) in ChitoXan is described. The immobilization efficiency, the enzymatic activities, and the stability of the immobilized enzymes are presented. For the determination of enzymatic activity of the proteases, the hydrolysis of hemoglobin is used; for trypsin, the substrate is BAEE and for lipase, it is an olive oil emulsion. Finally, a microscopic study has allowed to determine the structure of ChitoXan spheres with and without lipase.

MATERIALS AND METHODS

Materials

Chitosan samples were provided by Kemestry Inc. (Sherbrooke, Canada). The xanthan was obtained from Monsanto Pharmaceutical Ingredients. Chitosan and xanthan were used without further purification. The enzymes used in this study were; endopeptidase: PRO XIX = protease type XIX fungal from *Aspergillus sojae* (Sigma), serine protease, TRY = trypsin II.S from porcine pancreas (EC 3.4.21.11) (Sigma) and lipase, LIP = Lipase VII from Candida rugosa (EC 3.1.1.3) (Sigma). All other chemicals and solvents were analytical grade and were used without further purification.

Preparation of Chitosan and Xanthan Solutions

Chitosan preparation and solutions of chitosan and xanthan were prepared as described in a previous study [2]. The 0.65 wt.% solutions of chitosan and xanthan were prepared in double-distilled water.

Hydrogel Preparation

The hydrogels were prepared as spheres in the following way: A mixture of enzyme (1 g) and 100 g of 0.65 wt.% xanthan solution (previously degassed) were added dropwise, from a syringe, into 200 mL of 0.65 wt.% chitosan solution. Spheres formed while under mild stirring for 2 h at room temperature: The hydrogel spheres were washed to eliminate excess free chitosan: two washings for 20 min in an acetate buffer (pH 6.5) and one washing for 20 min in an enzyme buffer were carried out. The hydrogels were kept at 4°C in the enzyme buffer [2].

Enzymes Assays

Protease Type XIX Fungal from Aspergillus sojae Assay

Substrate preparation: a 2% hemoglobin solution was prepared by dissolving 5 g of hemoglobin substrate powder (Sigma) in a solution containing 80 g of urea in 80 mL of water. After incubation at 37°C for 60 min, 50 mL of 0.25 M phosphate buffer were added. The pH was adjusted to pH 7.2 and the solution was then diluted to 250 mL with water. The solution was stored at 4°C.

Protease activity: a temperature-equilibrated substrate solution (5 mL) was added to 0.02 g of immobilized protease and mixed thoroughly but gently to prevent frothing. The mixture was incubated at 37°C for <10 min. Then 10 mL of 5% Trichloroacetic acid were added to the assay mixture to terminate the reaction. Over a 30 min period, the solution was decanted and filtered through WHATMAN N° 4 paper. One unit of protease activity was defined as the amount of enzyme required to cause a unit increase in absorbance at 280 nm across a 1 cm path length [17].

Tests of stability of the immobilization of the protease type XIX fungus from *Aspergillus sojae* were performed. Immobilized protease (2 g) were mixed in 100 mL of phosphate buffer (0.02 M, pH 7.2 at 37 C). At specific times, 1 mL of the buffer was analyzed by Bradford's

method in order to determine the quantity of protease released by the ChitoXan.

Trypsin Assay

Substrate preparation: BAEE (*N*-benzoyl-L-arginine ethyl ester, a synthetic substrate) was dissolved in 67 mM sodium phosphate buffer at pH = 7.6 to form a 0.025 mM solution.

Trypsin activity: Temperature-equilibrated substrate solution (3 mL) and 0.2 mL of buffer were added to 0.02 g of immobilized trypsin and mixed thoroughly but gently to prevent frothing to obtain a homogeneous mixture. Then, the mixture was incubated at 25°C for 5 min. One unit of enzyme activity was defined as the amount of enzyme required to cause a unit increase in absorbance at 253 nm across a 1 cm path length [18].

Lipase Assay

Substrate preparation: The substrate for the lipase activity (Sigma, cat No. 800-1) was stabilized olive oil emulsion 50% v/v with 0.1% sodium azide as preservative.

Lipase activity: Temperature-equilibrated substrate solution (2.5 mL) and 2 mL of 0.55 M phosphate buffer were added to approximately 0.02 g of immobilized lipase and mixed thoroughly but gently to prevent frothing. The mixture was incubated at 37°C for 30 min. Then 2 mL of 6 M hydrochloric acid were added to the assay mixture and then heated at 100°C for 5 min to stop the reaction. The solution was cooled and 15 mL of isooctane were added; the mixture was agitated for 2 min and centrifuged at 4000 rpm for 10 min. Then 5 mL of the organic phase were added to 1 mL of copper-pyridine solution. The mixture was vortexed for 90 min. The supernatant was analyzed by spectrophotometry at 715 nm [16].

The relative activity of all the enzymes were defined as the activity of the immobilized enzymes in the hydrogel divided by the activity of the free enzymes.

Microscopy Characterization

The optical microscope used to observe the sphere was a Leica (Germany). Preparation has been necessary to observe the surface of the sphere. Scanning electron microscopy samples were prepared by cryopreparation: The ChitoXan spheres were put into liquid nitrogen and then freeze-dried. The freeze-dried samples were fixed on an aluminum sample holder with carbon-based adhesive tape and coated

Table 1. Immobilization efficiency of three enzymes. The weight percentage of PRO XIX in xanthan solution is 0.8% and for LIP and TRY this percentage is 1%.

Enzymes	Immobilization Efficiency (%)					Average (%)	Isoelectric Point
PRO XIX	78	80	75	79	74	77.2	>7
LIP	51	44	40	81	50	53.2	4.9
TRY	92	62	85	89	73	80.2	10.2

with Au–Pd. Samples were observed with a JEOL JSM-840A scanning electron microscope and with a Hitachi S-3000N scanning electron microscope.

RESULTS AND DISCUSSION

Immobilization Efficiency

The capacity of ChitoXan to immobilize the three enzymes is defined as the immobilization efficiency shown in Table 1. The immobilization efficiency was estimated as the ratio between the activity of the complex xanthan/enzyme and the sum of the activities following complex xanthan/enzyme, free enzymes in excess in the chitosan solution and free enzymes in the washing solutions.

$$E(\%) = \frac{\text{xant.act} \times 100}{\sum (\text{xant.act}; \text{chit.act}; \text{wash.act})} \quad (1)$$

where E is the immobilization efficiency, xant.act is the activity of the complex xanthan/enzyme, chit.act is the activity of free enzymes in excess in the chitosan solution, and wash.act is the activity of free enzymes in the washing solutions.

The immobilization efficiencies for PRO XIX and TRY which have a basic isoelectric point were higher than for LIP: which has an acidic isoelectric point. An assay with another enzyme with an acidic isoelectric point was made and the immobilization efficiency was also small ($< 25\%$).

Nonimmobilized enzymes were found in the chitosan preparation solution and those adhering to the outside layer of bead were removed in the washing solutions. Shown in Figure 1, is the enzyme found in the free chitosan solution and in the washing solutions. For PRO XIX, the enzymes in the chitosan solution and in washing solutions represent 8 and 20% respectively (Figure 1(a)). For TRY (Figure 1(b)), these

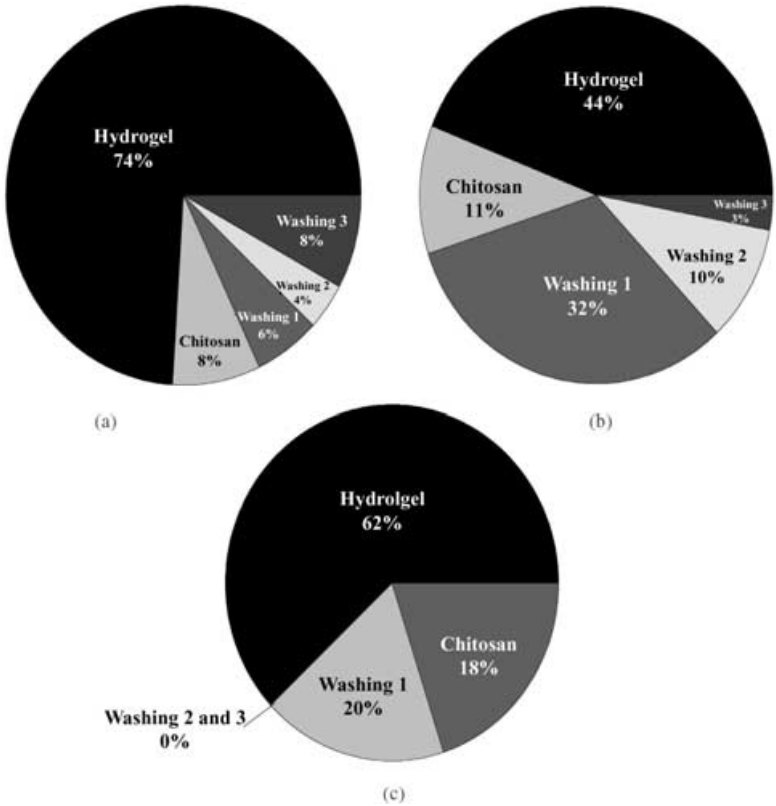

Figure 1. An example of enzyme distribution in the hydrogel and the amount of enzyme in the chitosan preparation solution and in the washings. Figure 1(a) is PRO XIX hydrogel prepared with 0.8% Pro XIX in xanthan solution. Figure 1(b) represents the LIP distribution for a hydrogel prepared with 1% of lipase in the xanthan solution. Figure 1(c) is for a hydrogel prepared with 1% of trypsin.

values are 18 and 20%, respectively (20% of enzymes were lost in the first washing whereas no enzyme was lost in the two last washings). For LIP, the excess chitosan solution contained 11% of the free lipase and the washings contained 45% of free lipase with a large amount in the first washing. For the three enzymes, the amount in the chitosan solution varied between 8 and 18% of the enzymes used in the initial xanthan solution.

The immobilization efficiency were determinated by measuring the protein concentration. Biuret's method [19], measuring the absorbance at 280 nm [20], were not accurate enough to obtain good values. Bradford's determination [21] could not be used since there is an interaction between Bradford's reagent and chitosan.

Determination of the Stability of Immobilization

A kinetic test has been performed in order to determine the stability of the Protease type XIX fungal from *Aspergillus sojae* immobilized into ChitoXan. In phosphate buffer 0.02 M, pH 7.2 at 37°C, no protein has been detected by the Bradford method after 8 h. The test was conducted for 24 h. After 9 h of mixing, protease was observed in the buffer. It is possible, that after 9 h of mixing, that the ChitoXan spheres had begun to get degraded which would explain the presence of enzyme in the buffer. The immobilization of protease type XIX fungal from *Aspergillus sojae* into ChitoXan provided a continuous process for 8 h.

Immobilization of Protease Type XIX Fungal from *Aspergillus sojae*

Relative activity is defined as the ratio between the hydrogel activity and the free protease activity. To calculate the ChitoXan activity, the percentage of enzyme immobilized was taken into account. In the first experiment, three factors were tested: the enzyme percent (0.2–1 wt.%) in xanthan solution, the storage temperature (4 and 25°C) and the molarity of the storage buffer (0.05 and 0.2 M). Shown in Figure 2 are the results of these tests. The better storage buffer was 0.2 M; for free protease the storage buffer is normally of 0.05 M. This phenomenon is due to PO_4^{3-} diffusion into the ChitoXan [22]. The ChitoXan complex is charged, and therefore, there are electrostatic, hydrophobic, and hydrophilic interactions. Moreover, the protease activities were higher when the ChitoXan with enzymes were stored at 4°C. This observation is consistent with the fact that the proteases in the ChitoXan complexes undergo degradation more readily than the free enzymes. To analyze the effect of enzyme concentration, two cases were considered. For a storage buffer of 0.2 M, the activities increased until 0.8 wt.% of protease and decreased thereafter. On the other hand, with a storage buffer of 0.05 M, the activities increase until 1%. It is possible that an interaction with enzyme and PO_4^{3-} exists.

Shown in Figure 3 is the influence of chitosan and the reaction time between the solution of xanthan and PRO XIX in the chitosan solution. The influence of chitosan was tested in solutions of chitosan that were kept for upto two weeks at room temperature. The immobilization of PRO XIX have been made with chitosan A (a solution kept for 24 h at room temperature), chitosan B (a solution kept for 7 days at room temperature) and chitosan C (a solution kept for 14 days at room

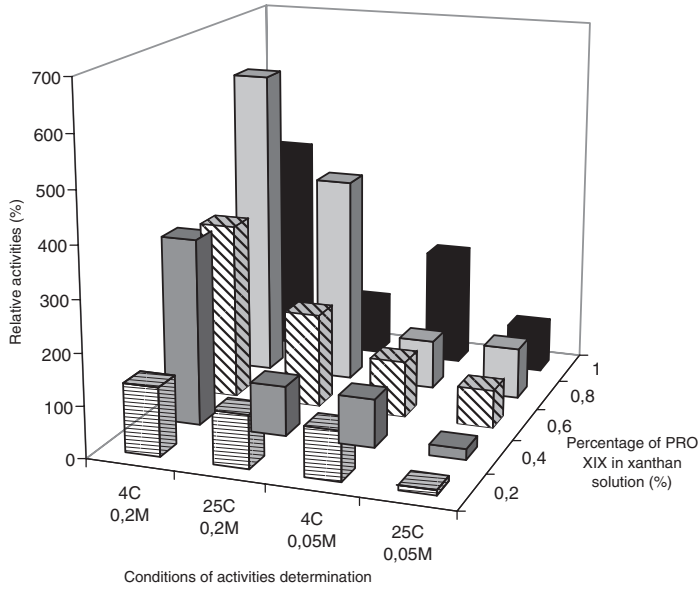

Figure 2. The influence of the PRO XIX concentration (between 0.2 and 1% of PRO XIX in xanthan solution), the storage temperature (4 and 25°C) and the molarity of storage phosphate buffer (0.05 or 0.2 M).

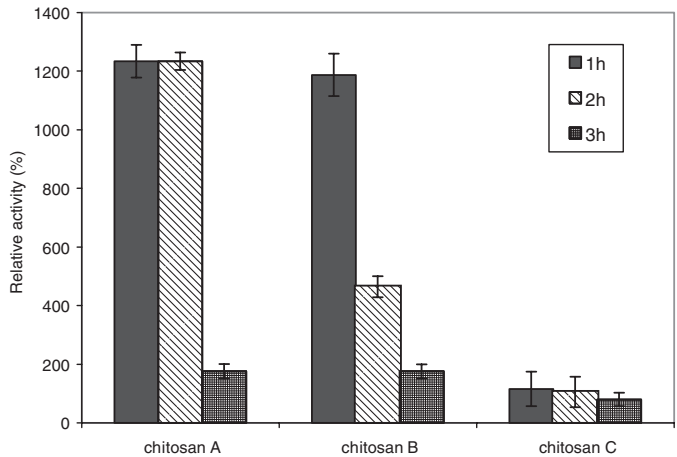

Figure 3. Influence of the storage of the chitosan solution and reaction time. The concentration of PRO XIX in xanthan solution is 0.8%. The storage buffer of hydrogel and enzyme immobilized were phosphate buffer 0.2 M. Chitosan A: the solution was kept for 24 h at room temperature, Chitosan B: for 7 days at room temperature and Chitosan C: for 14 days at room temperature.

temperature). The preservation of chitosan with time greatly decreased the relative activities of PRO XIX. The activity of the immobilized enzymes decreased with reaction time after a specific time. This phenomenon is due to the relation of the physical structure of the matrix and the time of reaction. In fact, greater the reaction time, greater is the complexation, increasing the matrix compactness and decreasing the size of the pores. Consequently, the substrate cannot diffuse as well into the matrix and interact with the enzymes. The best activities were found for a reaction time of 1 h but at this time the spheres are still fragile. The optimum time was 2 h because with this time the activities were still high (>100% in the three cases) and the physical characteristics of the matrix was better. The molecular weight of chitosan and the time of reaction may play a role on the porosity of the hydrogel: this porosity is important since the substrates and products may diffuse through the membrane of the bead.

The degradation with time was studied; presented in Figure 4 are the results of ChitoXan (with protease) in a phosphate buffer (0.2 M at 4°C). All values were greater than 100% so the immobilization of the protease in ChitoXan prevented temporal degradation compared at a solution of enzyme, that is rapidly degraded. The decrease in activity was greater between the first day and the third day (Figure 4) whereas the decrease

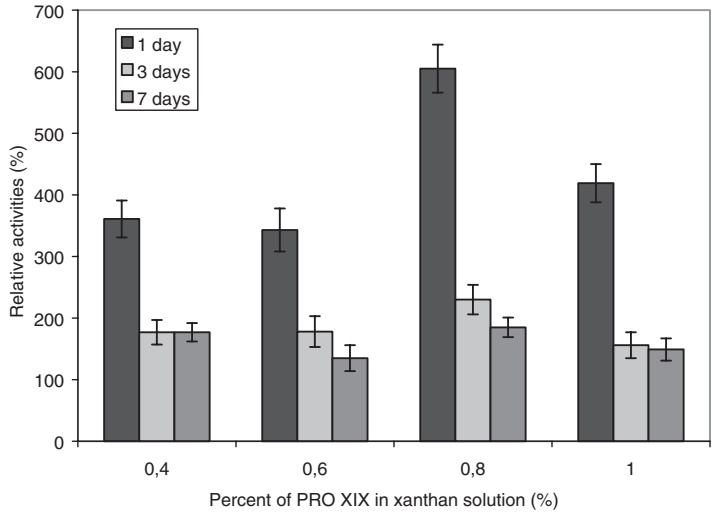

Figure 4. Temporal protection of the enzymes by the hydrogel. Concentration of PRO XIX in xanthan solution was 0.8%. The hydrogel was stored at 4°C in 0.2 M phosphate buffer.

between day 3 and 7 was very small. This temporal protection of the enzyme by the hydrogel may be useful for repeated processes.

Immobilization of Lipase

The lipase immobilization in the ChitoXan complex has been previously studied [16]. The immobilization of lipase in ChitoXan doubled the enzymatic activity compared to the free enzyme (Figure 5).

Shown in Figure 6 is the thermal denaturation of the enzymes in aqueous medium. Free lipase rapidly denatures at temperatures higher than 37°C. The optimum temperature for the free lipase is 37°C. For the immobilized lipase in ChitoXan, the thermal denaturation is very slight. In addition, between 30 and 55°C the lipasic activity of the immobilized enzyme was always greater than the activity of free enzymes at 37°C.

The hydrolysis of olive oil (substrate for lipase) in organic solvents by immobilized lipases is illustrated in Figure 7. Three solvents were used: iso-octane, toluene, and cyclohexane. In these tests, olive oil in the emulsions was 20%. The highest activity was found in cyclohexane, followed by iso-octane, and toluene; the lipase hydrolyses the triacylglycerol into glycerol and fatty acids. The lipase immobilized into ChitoXan can make this hydrolysis due to the presence of water in the matrix. The activity in organic media is very interesting since degradation of olive oil occurs in the immobilization matrix even if no water is present in the system.

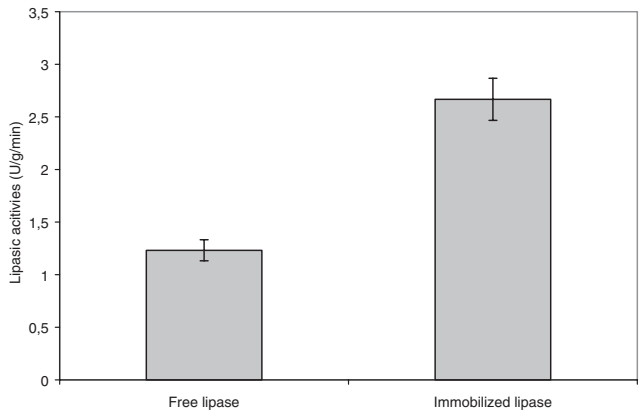

Figure 5. Comparison of lipasic activities of free lipase and immobilized lipase in aqueous medium. Concentration of LIP in xanthan solution was 1%. The storage buffer was 0.55 M phosphate buffer at 4°C.

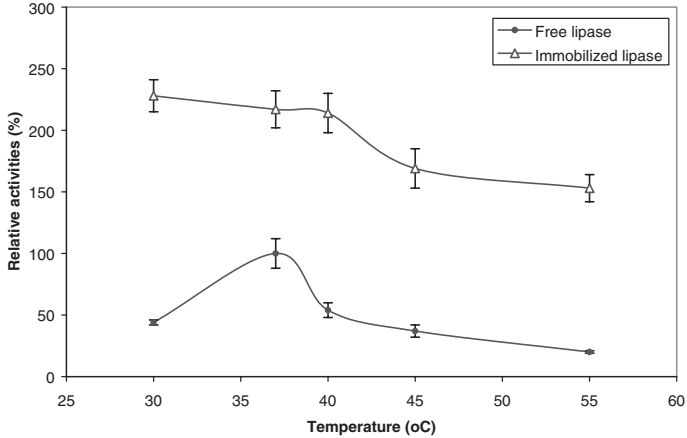

Figure 6. Thermal denaturation of free and immobilized lipase in aqueous medium. Concentration of LIP in xanthan solution was 1%. The storage buffer was 0.55 M phosphate buffer at 4°C.

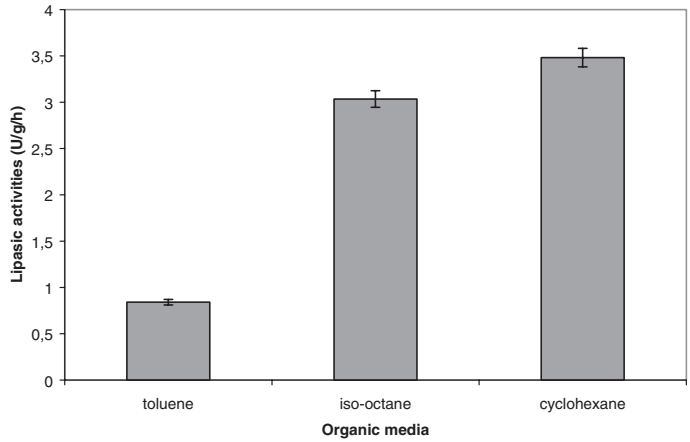

Figure 7. Lipase activities in organic media. Concentration of LIP in xanthan solution was 1%. The storage buffer was 0.55 M phosphate buffer at 4°C.

Shown in Figure 8 is the influence of the water added to the iso-octane emulsion and to the toluene emulsion. The experiment could not be done with cyclohexane because the emulsion with water was not stable. The profiles for iso-octane and toluene are different. With the iso-octane and water emulsion, when the water concentration was 8% and above, the activity of immobilized lipase in iso-octane was similar as the activity in 100% of iso-octane. With toluene, the best activity was determined with

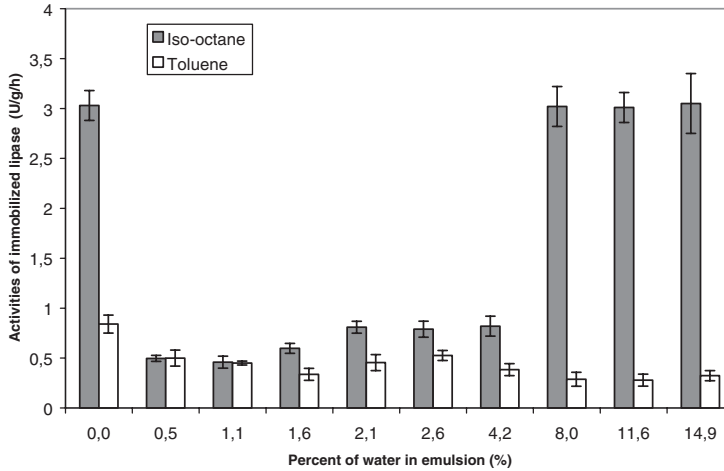

Figure 8. Influence of the quantity of water added in the iso-octane emulsion and toluene emulsion on the activity of immobilized lipase. Concentration of LIP in xanthan solution was 1%. The storage buffer was 0.55 M phosphate buffer at 4°C.

2.6% water in the emulsion, however adding more water did not improve the activity in the toluene.

Immobilization of Trypsin

For trypsin, immobilization efficiency was the highest obtained for the ChitoXan matrix with a value of 80% (Table 1). However, the activity of the immobilized trypsin was low, due to an interaction between ChitoXan and the active site of trysin.

Microscopy Characterization

Shown in Figures 9 and 10 respectively, are the external structures of the spheres obtained without and with lipase by optical microscopy. The advantage of optical microscopy is that no treatment is required to observe the sphere and the native structure is observable. The spheres without lipase were more compact than the spheres with lipase. In Figure 9 a sphere is shown with a smooth external structure while a fibrillar network is visible. In Figure 10, the external structure of the sphere with lipase is formed in a regular pattern. The presence of lipase in the sphere modified the structure of the hydrogel. This modification was observed with an electronic microscope (SEM). The SEM of the sphere surfacess in Figures 11 (hydrogel without lipase)

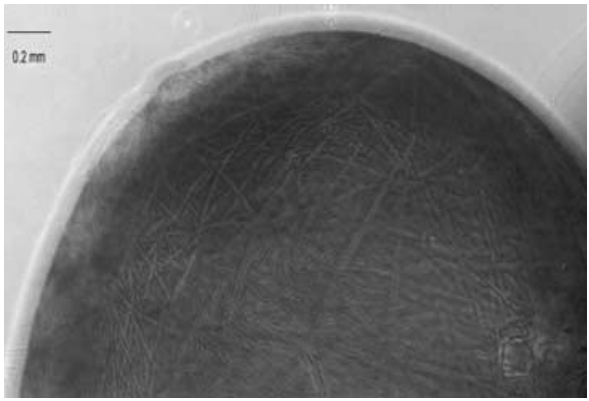

Figure 9. Observation of external structure of a sphere without lipase by optical microscopy.

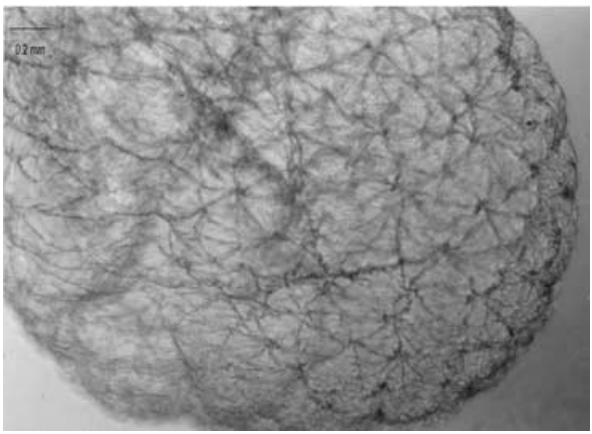

Figure 10. Observation of external structure of a sphere with lipase by optical microscopy.

and 12 (hydrogel with lipase) are similar. In both cases, there are regular arrangements of the external structure. However, it is possible to see in Figure 12, that the number of foldings is different; the presence of lipase modified the structure of the sphere surface. This folding arrangement is very interesting because when the specific surface of the sphere was increased and the enzymatic reaction increased too.

The internal structure of a sphere without lipase in scanning electron microscopy is shown in Figure 13. The internal structure of the latter is

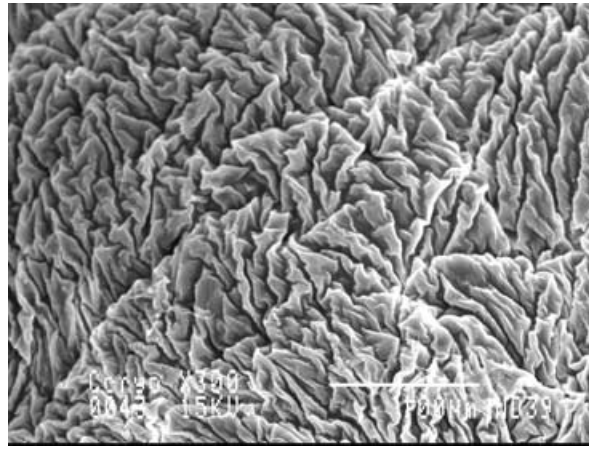

Figure 11. Observation of external structure of a sphere without lipase by scanning electron microscopy.

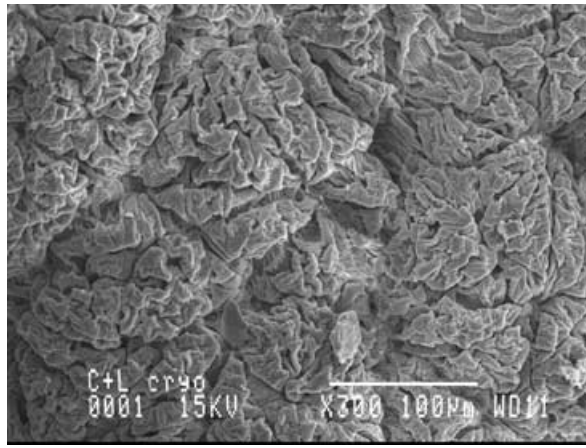

Figure 12. Observation of external structure of a sphere with lipase by scanning electron microscopy.

a porous network composed of fibers. A similar observation is made in Figures 14 and 15 for the internal structure of a sphere with lipase. In this case, it is possible to see the presence of fibers as well as new globular like structures. These globular structures have been previously observed [23]. The presence of these globular structures indicates that a triple complexation exists between the chitosan, the xanthan, and the enzymes.

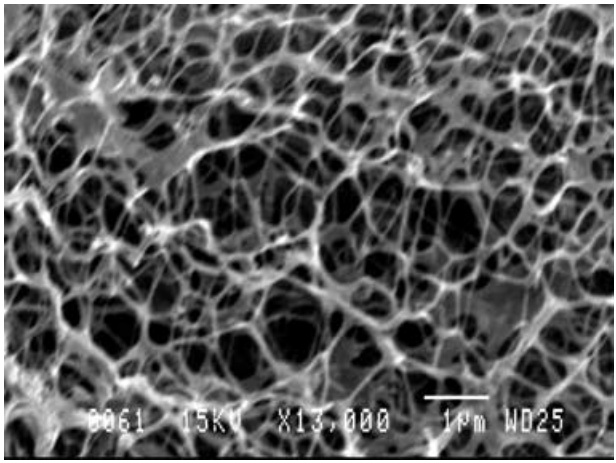

Figure 13. Observation of internal structure of a sphere without lipase by scanning electron microscopy.

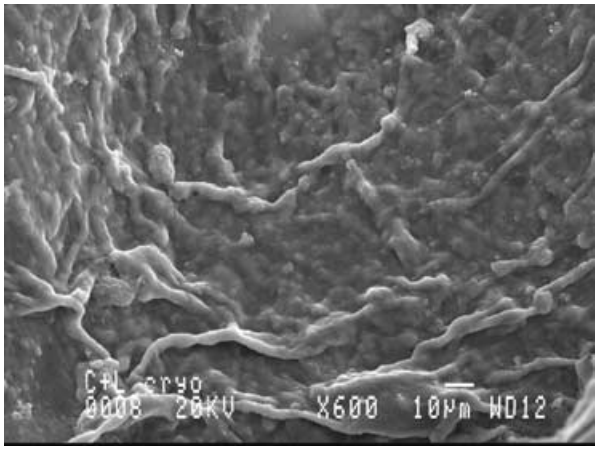

Figure 14. Observation of internal structure of a sphere with lipase by scanning electron microscopy.

CONCLUSION

Hydrogels based on the complexation between chitosan and xanthan are very interesting matrices for the immobilization of enzymes. The efficiency of immobilization into this matrix is specific for each enzyme. Several factors influence the relative activity of immobilized enzymes, such as, the conditions of storage (molarity of buffer and temperature), the percent enzyme in the matrix and the molecular weight of chitosan.

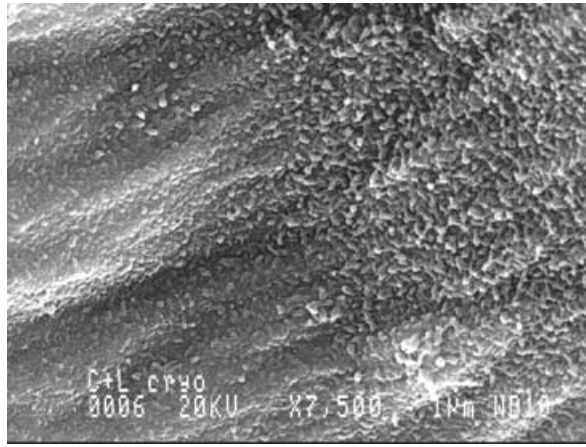

Figure 15. Observation of internal structure of a sphere with lipase by scanning electron microscopy.

The ChitoXan plays an important role on the physical structure of the matrix which is influenced by the molecular weight of ChitoXan. The immobilization of enzymes into this matrix has many advantages: the relative activity of immobilized PRO XIX with respect to the free enzyme is greater that 100% after 7 days; for lipase, the relative activity between 30 and 55°C varies between 230 and 150%, so this matrix thermally protects the lipase. Moreover, it has been shown possible for immobilized lipases to be active in organic media. Finally, the major interest of this immobilization on to ChitoXan is the high increase of catalytic activity of enzymes. This is the first publication that has presented data showing an increase in activity for LIP and PRO XIX compared to the free enzymes. During the immobilization of enzymes on to ChitoXan, there is no covalent bond between the biopolymer system and the enzymes but only polyionic interactions allow the formation of the matrix and the immobilization. Consequently, the triple complexation between chitosan, xanthan, and enzymes provides an increase in enzyme activity, the thermal protection of the enzyme, the activity in organic media, and the protection with time.

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