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Modified alginate matrice for the immobilization of bioactive agents

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Bioactive agents (catalase – an enzyme, and nisin – a bacteriocin) were covalently immobilized on alginate activated by sodium periodate (oxidatively converting 2,3-dihydroxyl groups into dialdehyde residues), followed or preceded by ionotropic gelation. For the same protein coupling yield, the retained enzyme activity of the immobilized enzyme (ImE) can be markedly increased by diminishing the beads diameter, a phenomenon that illustrates the role of substrate/products diffusion through the bead gel layer. When the amount of enzyme introduced for coupling was about 15 mg/100 mg of support and the bead diameter was about 100 μm, a high retained specific activity (95-98%) was obtained. Diffusion phenomena can be markedly decreased by enzyme immobilization on the surface of microbeads (obtained by gelation of activated alginate prior to immobilization). In this case, the retained activity was approx. 75% of that of free enzyme. A slightly higher Km value of ImE suggested that the enzyme-substrate affinity was almost maintained. The profiles of ImE activities at various pH values, temperatures and when undergoing proteolysis showed an overall higher stability for the immobilized than that for the free enzyme. Nisin immobilized on the microbeads surface, when submitted to proteolysis, conserved its bacteriocin activity, strongly inhibiting the growth of *Lactobacillus sake* when subjected to an agar spot-test, whereas free nisin totally lost its activity.

Introduction

Immobilized enzymes (ImE) obtained by covalent linkage to an insoluble matrix possess several advantages over the free enzyme, for example an increase of enzyme stability and the facility of their separation from the reaction medium by filtration or centrifugation. Consequently, the enzyme reaction can be controlled without the addition of blockers (inhibitors) and thus the products are not contaminated. Furthermore, the recovered ImE can be re-used many times and stored for long periods without loss of activity [1,2].

The resistance of the catalytically active protein structure to high temperatures, extreme values of pH and other denaturing factors is an important objective for commercialisation and industrial applications of enzymes [3,4] (for example the production of various sugars, amino acids and pharmaceuticals, etc.). The limited stability of free enzymes during long-term storage has been attributed to the deleterious effects of environmental moisture and microbial contamination [5,6]. In general, the stabilisation of the enzymes can be enhanced in several ways: chemical modifications, the use of stabilizing additives [such as poly(ethylene) glycol, glycerol and BSA], protein engineering and coupling to polymers [7,8].

Immobilization represents an approach to improve enzyme stability by covalent linkage to an insoluble matrix or by entrapment into gel support under mild conditions (i.e. by ionotropic gelation or by polymerisation process). For instance, Munjal and Sawhney [9] described the use of alginate, polyacrylamide and gelatine to entrap enzymes (i.e. a tyrosinase) and showed that, under drastic conditions, the entrapped enzyme presented larger activity profiles than the free enzyme. However, the enzymes entrapped in such gels tend to leach out in time making conservation for long periods impossible. To overcome this problem, covalent immobilization of the enzyme on the support material seems more attractive, if long-term storage or continuous function of enzyme is required. There are many reports describing covalent immobilization of different enzymes on various supports [1]. The bonds are normally formed between functional groups present on the surface of matrix and functional groups of amino acid residues of protein. Such technique can lead to a considerable loss of enzyme activity [10]. The immobilization can be realised using coupling agents {i.e. carbodi-imide, Woodward's (*N*-ethyl-5-phenylisoxa-zolium-3'-sulphonate)

Keywords: bacteriocin, covalent immobilization, enzyme and peptide stabilisation, ionotropic gelation, periodate-activated microbeads.

Abbreviations used: FTIR, Fourier Transform Infrared; ImE, Immobilized enzyme; DO, Degree of oxidation

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reagent or glutaraldehyde [1,2,11]} or using already activated supports such as CNBr-Sepharose [12], *p*-benzoquinone-agarose [13]. Periodate oxidation can also be used for activation of polyhydroxylic materials. It is a highly specific reaction, detecting neighbouring hydroxylic groups, that is also used in structural analysis of carbohydrates [14]. When applied to polysaccharides, the periodate reaction cleaves the C-C links of diol groups converting them to paired aldehyde groups able to couple primary amino groups (Lysine) of proteins.

The choice of matrix type is important and depends of the application domains (biomedical, pharmaceutic or food processing). In these cases, the matrix should be generally non-toxic and biocompatible. Alginate, a natural polysaccharide extracted from seaweed, is a co-polymer of alternating sequences of β-D-mannuronic and α-L-guluronic acid residues linked by $1\rightarrow 4$ glycosidic bonds [15,16]. Several studies suggested alginate as an interesting matrix, on account of its low toxicity, biocompatibility [17,18] and its easily to form bead by ionotropic gelation [19] in the presence of bivalent or tervalent cations, such as Ca^{2+} or Al^{3+}, which ionically cross-link carboxylate groups in the uronate blocks of alginate, giving it a gel-like character. However, the immobilization by entrapment or covalent linkage could diminish enzyme activity as a result of an alteration in the catalytic site or of the steric hindrance due to the matrix, which can hinder the accessibility of the substrate to the immobilized enzyme active site. In addition, the limited diffusion of substrate and of products formed through the matrix gel layer can also decrease the catalytic activity of enzyme. This phenomenon appears more intense if the enzyme possesses a high catalytic-centre activity («turnover number»; e.g. for catalase: 5.10^8 moles of H_2O_2 decomposed/min per mole of enzyme, under optimal conditions [8]). For this reason and for its therapeutic and technological potential, catalase was selected as model enzyme for this study, which was aimed at developing methods to reduce the effects of the diffusion phenomena and thus to improve the retained activity. Catalase (*EC 1.11.1.6*) catalyses the decomposition of H_2O_2 to water and molecular oxygen. Being used in food technology [20], this enzyme was suggested as a therapeutic agent to be administered intraperitoneally [21]. In these cases, catalase is largely preferred as immobilized enzyme, being more stable to proteolysis. The use of the 3.3 kDa peptide nisin, a bacteriocin (a proteinaceous toxin secreted by certain bacteria to inhibit the grouwth of similar bacterial strains) accepted for use by the FDA (the U.S. Food and Drug Administration) and often used in food preservation, was also approached in the present study. However, the nisin activity can be severely depressed by several factors (proteases, basic pH, salts) [22,23]. It was therefore of interest to evaluate an immobilization procedure to protect these bioactive agents against destabilizing factors.

The present study describes the covalent immobilization of enzyme onto alginate activated by periodate, followed by ionotropic gelation, forming beads or microbeads, a system appropriate for utilisation in drastic environment (extreme values of pH, high temperature, proteolysis). As an alternative to internal immobilization, activated alginate was first beaded by ionotropic gelation and then the protein immobilized on to the beads surface. The novelty of the present work lies in the association of covalent coupling and ionotropic inclusion for internal or external immobilization of proteins (catalase and nisin).

Materials and methods

Materials

Sodium alginate (with a high content of guluronic acid) was from Fluka Biochemika (Buchs, Switzerland). Trypsin (porcine pancreas, *EC 3.4.21.4*), bovine liver catalase (hydrogen peroxide:hydrogen peroxide oxidoreductase, *EC 1.11.1.6*) and sodium periodate were from Sigma Chemical Co (St Louis, MO, U.S.A.). The other current chemicals (Sigma-Aldrich Chem. Co, Sheboygan, WI, U.S.A.) were reagent grade and used without further purification.

Activation of alginate by oxidation

Sodium alginate (10 g) was added to 1 liter of a solution of sodium periodate at various concentrations (20-300 mM) at 4°C in the dark for activation. The reaction was stopped after 24 h by addition of an equimolar amount of ethylene glycol [24]. NaCl (20 g) was then added to the mixture, in order to facilitate the subsequent precipitation with an excess of ethanol. The precipitate was collected, redissolved in distilled water (200 mL) and dialysed for 3 days to remove completely the sodium periodate and others chemicals or by-products. Then, the polymer was precipitated and washed three times in 100 % (v/v) ethanol, filtered, then dried with pure acetone. The activated alginate product obtained was conserved in amber bottles at room temperature.

Determination of the degree of oxidation (DO)

The DO was evaluated by determining the aldehyde groups as described by Noelting and Bernfeld [25]. Briefly, 100 mg of activated matrix were added to 3 mL of a reagent containing 2 % 3,5-dinitrosalicylic acid and sodium potassium tartrate (50 mM). The reaction mixture was incubated for 5 min at 100 °C and rapidly cooled in an ice bath before taking spectrophotometric readings at 535 nm at room temperature. Maltose was used as standard.

Enzyme immobilization and determination of coupling yield (saturation of matrix)

Different quantities of catalase (0-40 mg) were added at room temperature to 10 mL of a solution containing 0.1 M $NaHCO_3$, pH 7.5, and 1 % activated alginate (25 % OD). After 2 h, 10 mL of ethanol were added to the solution and centrifuged. The precipitate obtained was washed at

least three times with distilled water and filtered to eliminate the excess, non coupled, enzyme. The ImE was collected by centrifugation, dried at 37 °C and submitted to Fourier transform infrared (FTIR) analysis. The quantity of protein as ImE gel suspension was determined by the method of Lowry *et al.* [26] using BSA as standard. The coupling yield was expressed as:

Coupling yield (%) = Immobilized protein (mg)/ Introduced protein (mg) x 100

FTIR analysis

FTIR spectra were recorded with a Spectrum One (Perkin Elmer) equipped with an Universal Attenuated Total Reflectance (UATR) device for powders (10 mg) analysis on the spectral region (4000-650 cm^{-1}) with 64 scans at 4 cm^{-1} resolution.

Activated alginate beading

Enzyme immobilization and beads-forming solution A 150 mg portion of activated alginate was dissolved in 6 mL of 0.2 M $NaHCO_3$, pH 8.0, with mild stirring. When the solution was homogenised, a 4.55 mg portion of catalase (2200 units/mg) was slowly added and enzyme immobilization was continued at room temperature for 2 h. Finally, up to 2 mL of 50 mM Tris buffer, pH 7.0, were introduced drop-bydrop in to the solution to quench the remaining non-coupled (if any) carbonyl groups. The beads-forming solution was obtained by addition of 150 mg of native alginate to the alginate-catalase conjugate solution. Additional non-modified alginate (optimum ratio 1:1) is essential to improve the mechanical properties of beads, because the structure of activated alginate was altered during periodate oxidative activation process [27].

Matrix beading Alginate matrix beading was performed as described by Palmieri et al. [28]. A syringe provided with a needle was filled with the mixture and the beads were obtained by dropping the bead-forming solution into a solution of $CaCl_2$ (4 %) and mildly stirring for 30 min to allow the beads harden. The beads were then washed with distilled water and stored in acetate buffer (50 mM, pH 6.8-7.0) at 4 °C. To make different sized beads, needles of 26G½ (0.5 mm diameter), 20G1½ (0.9 mm) and 16G1½ (1.6 mm) were used to obtain beads of respectively 1, 2 and 3 mm (±0.3-0.4) average diameter. The beads diameter was measured with an ID-110E Digimatic Indicator (Mitutoyo Mfg, Ltd., Tokyo, Japan) and, for each sample, at least ten beads were used to obtain a statistical evaluation. The ImE concentration in the bead was determined by Lowry method after dissolving the beads overnight in 0.5 M phosphate buffer, pH 8.0. Furthermore, the stability of beads formed by gelation with $CaCl_2$ was also examined at 25 °C by keeping them immersed in 0.1 M phosphate buffer, pH 7.2, as described by Munjal and Sawhney [9].

Microbeads preparation

Internal enzyme immobilization A portion (10 mL) of microbeads-forming solution (prepared as above with catalase-alginate conjugate and native alginate in a 1:1 ratio) was slowly incorporated in 40 mL Canola (Canada oil low acid) oil [29] for 30 min with moderate stirring at 4 °C. A portion (10 mL) of 4 % $CaCl_2$ was added drop-by-drop in the mixture and stirred during 30 min for ionotropic gelation to obtain microbeads. The mixture was centrifuged and the oil was eliminated by decantation. To remove the oil completely, 5.0 mL of hexane were added for 5 min under mild stirring. The microbeads were collected by centrifugation, washed three times in distilled water to completely remove the solvent and conserved in 50 mM acetate buffer, pH 6.8-7.0, at 4 °C. The microbeads diameter was microscopically determined with a scale micrometer and the ImE concentration was evaluated by Lowry method, as above.

Enzyme immobilization on the surface of microbeads The microbeads of activated alginate were prepared as described above (but without enzyme), washed twice, dried with acetone and stored in amber bottles. For enzyme immobilization on the surface, microbeads were rehydrated in carbonate buffer (0.1 M, pH 7.5) for 20 min prior to the addition of the appropriated quantity of enzyme to the solution for the coupling (2 h at room temperature) and microbeads collected by centrifugation.

Optical microscopy images

The ImE were stained with the Coomassie Blue solution, washed and photographed with an optical microscope (*Dialux 20*; Leitz, Wetzlar, Germany) equipped a *Wild MPS 45 Photoautomat Camera Controller* (Vermont Optechs Inc., Charlotte, VT, U.S.A.).

Enzyme activity assay

The catalase activities of free and ImE were spectrophotometrically determined at 240 nm by monitoring the decrease of absorbance caused by the decomposition of H_2O_2 during catalysis [30]. One enzyme unit was defined as the amount of enzyme decomposing 1.0 μmole of H_2O_2/min at pH 7.0 and 22 °C. Before mixing in the reactional medium, the ImE beads or microbeads were equilibrated in 50 mM phosphate buffer, pH 7.0, for about 5 min at room temperature. These preparations (collected by decantation) were mixed in a final volume of 3 mL of reactional medium containing 50 mM potassium phosphate, pH 7.0, and 0.035 % (w/w) H_2O_2. The V_{max} and the Michaelis constant (K_m) of free or ImE were determined from the Lineweaver-Burk plots.

Retained activity

Retained activity represents the specific activity of ImE as a proportion (%) of the specific activity of soluble (free) enzyme used for immobilization, calculated as follows:

Retained activity (%)
= Specific activity of ImE (units/mg)/
Specific activity of free enzyme (units/mg)

Stability studies

PH-dependency of free and immobilized enzyme Free catalase and the ImE-microbeads obtained were incubated at room temperature (22 °C) for 30 min at different pH values in appropriate 50 mM buffers: glycine-HCl, pH 2.0-4.0; acetate, pH 4.0-6.0; sodium phosphate, pH 6.0-8.0; Tris-HCl, pH 9.0; and carbonate, pH 9.0-11.0. The specific activity was then measured under standard conditions (pH 7.0) as described for the enzyme assay.

Thermal stability evaluation The thermal stability of free and immobilized catalase incubated for 30 min at different temperatures (22, 30, 40, 50, 60 and 70 °C) after 30 min incubation was studied. The specific activity was then determined under standard conditions (22 °C) as described above.

Stability at proteolysis of free and immobilized enzyme Proteolysis studies were carried out with free and ImE, the enzyme activities in the absence and in the presence of trypsin being determined at various intervals. Aliquots of 100 µL were taken at various intervals (15, 30, 60, 120, 240 and 360 min) and retained activities were determined as described above. The trypsin/catalase ratio was of 1:10 in 20 mL Tris buffer, pH 7.2. The specific activity of trypsin was 1230 units/mg, one enzyme unit being considered the amount of enzyme which increases the absorbance at 253 nm by 0.001/min at pH 7.6 and 25°C (after hydrolysis of N-benzoyl-L-arginine ethyl ester as substrate).

Microbiological assay: resistance of immobilized nisin to proteolysis

Strain and culture preparations *Lactobacillus sake* ATCC (American Type Culture Collection, Manassas, VA, U.S.A.) 15521 was stored frozen at -80 °C in Lactobacilli MRS (De Man, Rogosa and Sharpe) broth (Difco, Detroit, MI., U.S.A.) supplied with 10 % glycerol. Prior to use for experiments, one cryovial was thawed and cultured twice during 18 h at 35 °C in 9 ml of MRS broth. The protease preparation (actinase E and pronase E activities, *EC 3.4.24.31*) was from *Streptomyces griseus* (Type XIV, 4.9 units/mg solid, Sigma-Aldrich, Oakville, ON, Canada). One unit hydrolyses casein to produce a colour (given by Folin-Ciocalteu reagent) equivalent to that of 1.0 µmol (181 µg) of tyrosine/min, at pH 7.5 and 37 °C. Nisin (650 units/mg solid material) was from ICN Biomedicals Inc. (Cleveland, OH, U.S.A.).

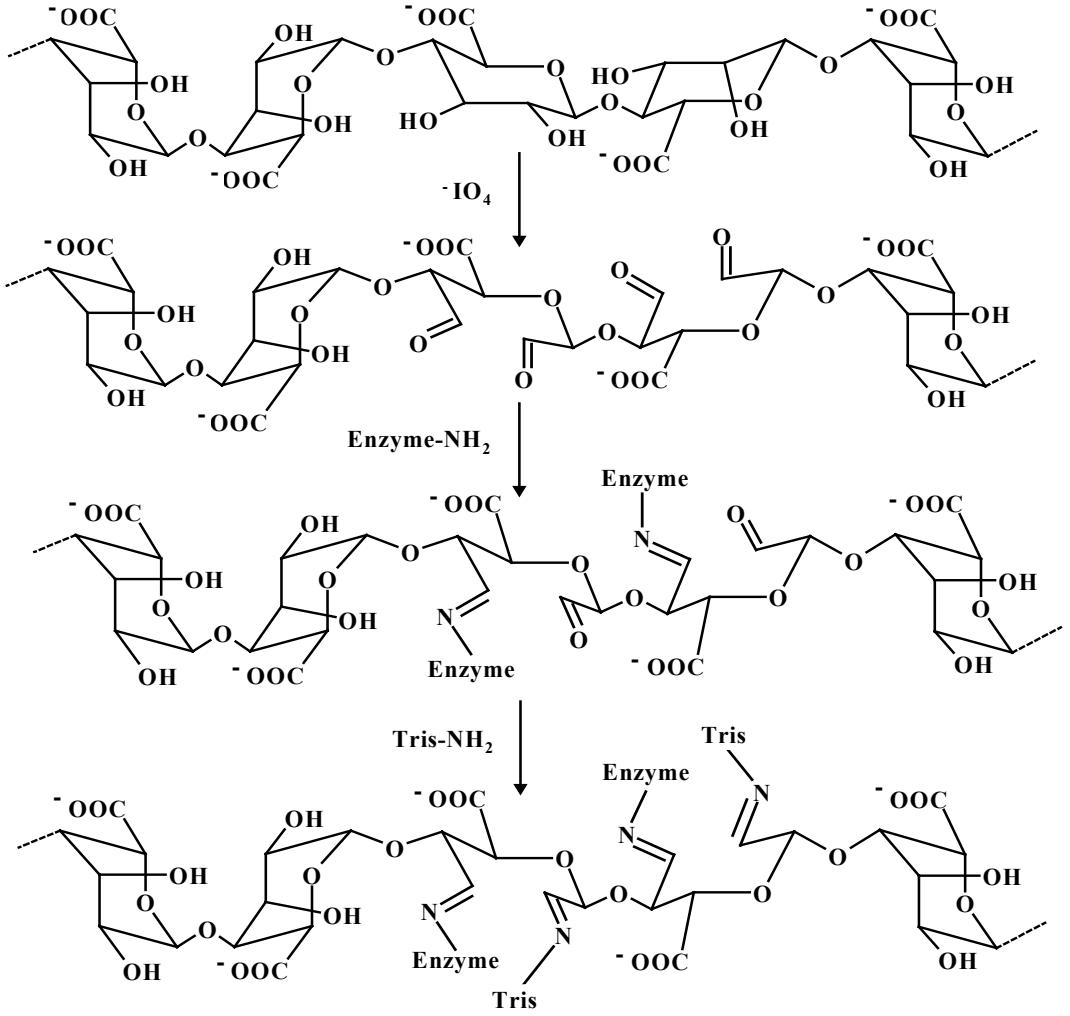
Immobilization of nisin and agar spot test Nisin was immobilized on the surface of microbeads as described above for enzyme immobilization. Portions of free or immobilized nisin (1500 units) were incubated with 10 mL of *Streptomyces* proteases (1 mg/mL) solution (50 mM Tris-HCl, pH 7.2) at 37 °C. A sample (1 mL) was taken at various times (0, 0.5, 1, 2, 4 and 6 h) and boiled for 10 min (on water bath) to inactivate the residual *streptomyces* protease used for proteolytic treatment. The aliquot was then assayed with the agar spot-test [31], with slight modifications [32]. Briefly, 100 µL of an 18 h culture of *L. sake* was mixed with 5 mL of Lactobacilli MRS soft agar (0.75 %) and poured into Petri dishes. Approx. 150 units of free or immobilized nisin previously treated with *Streptomyces* proteases were separately deposited on the surface of the bacterial lawn. Similar processing was done with the control microbeads without nisin (but treated 2 h in the Tris buffer) to ensure that alginate was not toxic to bacteria. The Petri dishes were incubated during 36 h at 35 °C, and a positive inhibition was considered when a clear zone of 0.5 mm or more was observed around the spot inoculation.

Results and Discussion

Alginate activation with $NaIO_4$, generates neighbouring dialdehyde residues (Scheme 1) able to react with amine groups of enzyme forming a *Schiff's* base linkage.

Characterisation of activated alginate matrix

The DO of alginate activated with various $NaIO_4$ concentrations (Figure 1A) shows that, at periodate concentrations up to 50 mM, the percentage oxidation was linearly increased, with a profile slightly stabilised at concentrations between 50 and 100 mM and increasing again at concentrations higher than 100 mM. The initial increase in percentage oxidation could be due to the great availability of diol groups, which were then diminished at higher $NaIO_4$ concentrations, mainly due to the diol (C-C) cleavage and probably to the hemiacetal [33] formation from reaction between the dialdehyde formed and some remaining hydroxyl groups of uronate units or water. Further increase of $NaIO_4$ concentrations generate highly oxidized alginate, but the mechanical properties were poor and beads rapidly disintegrated in phosphate buffer. The decrease in mechanical stability could be due to the oxidative degradation (breaking of C-C bonds) and eventually hydrolysis of the polymer. Several studies [27,34] reported alteration in polysaccharide oxidation giving hemiacetal structures susceptible to hydrolysis with a decrease in molecular mass [27], thus explaining the alginate beads disintegration when activated at periodate concentrations higher than 100 mM. Therefore, for this study, the selected $NaIO_4$ concentration was of 100 mM of alginate, which generated 25 % DO and satisfactory mechanical properties of beads. The stability of alginate beads was good enough, no deterioration of spheres being noticeable after 12 h of incubation in 0.1 M phosphate buffer (pH 7.2) at 25 °C. The changes in chemical structure of alginate induced by periodate oxidation were also examined by FTIR (Figure 1B). The characteristic band of carbonyl group appeared in the 1740-1720 cm^{-1}



Scheme I **Enzyme immobilization to alginate activated by periodate, generating dialdehyde residues able to react with amine groups of enzyme forming *Schiff's*-base linkages and quenching thenon-reacted aldehyde groups with Tris**

region, with a band increasing as a distinct peak related to increasing oxidation level.

Evaluation of the amount of immobilized enzyme

The effect of the initial amount of free enzyme introduced for immobilization on to the matrix was investigated in terms of the protein coupling yield. Figure 2A shows that the amount of catalase bound to activated alginate increases with the concentration of introduced enzyme. The maximum amount of coupled protein was achieved at an initial free enzyme concentration of about 18 mg/100 mg of activated alginate (giving a 85 % coupling yield) and a retained activity of approx. 94 %. A further increase in enzyme/support ratio gives a constant coupling yield (plateau), probably due to saturation of binding sites of the matrix.

FTIR spectra of activated alginate and of immobilized catalase on alginate at various initial amounts of introduced enzyme (Figure 2B) show a slight increase of absorption at 1680 cm^{-1} corresponding to the C=N group. A minor symmetric vibrational band for the aldehyde group of activated alginate at 1740-1720 cm^{-1} disappears after covalent linking with enzyme amino group to form *Schiff's* base, which appears as a shoulder at 1680 cm^{-1}. In addition, a new band at 1650 cm^{-1} can be assigned to amide I band of the enzyme. On a further increase in the ImE concentration, bands at 1680 and 1650 cm^{-1} become more evident.

Effect of bead size on immobilized enzyme activity

Important loss (80-90 %) of the enzyme specific activity has been noticed for the ImE beads of 1 mm or greater diameter (Figure 3). This low ImE retained activity (10-20

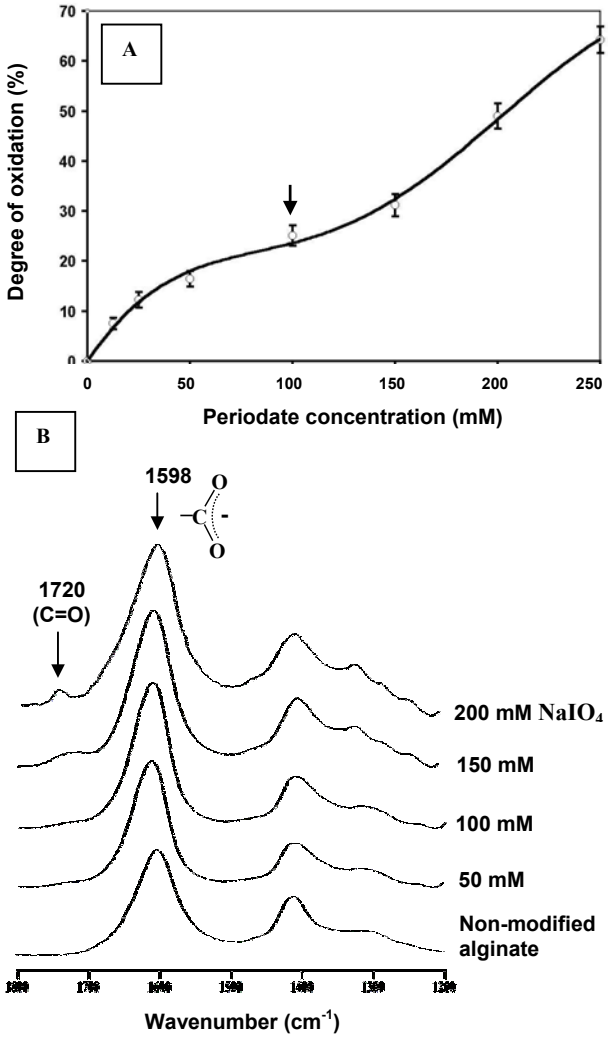


Fig. 1 **Degree of oxidation (A) and FTIR spectra (B) of native and oxidized alginate at various concentrations of periodate**

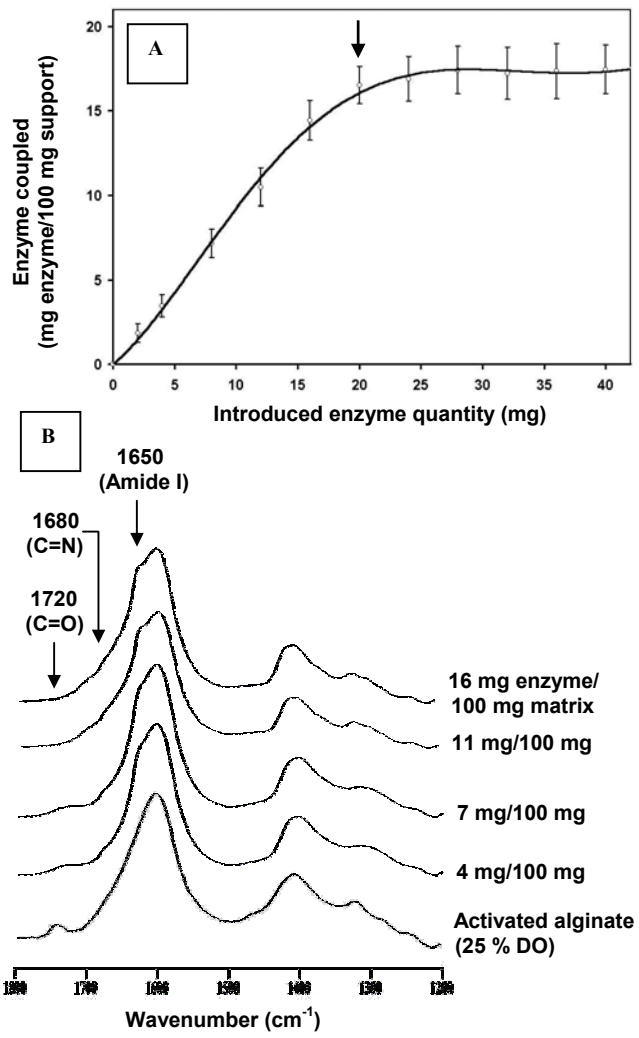


Figure 2: **Enzyme coupling (A) and FTIR Spectra (B) of alginate activated only and coupled with various amounts of catalase**

%) was not due to enzyme de-activation, since an enzyme assay after dissolving the beads (in 0.5 M phosphate buffer, pH 7.5) showed a specific activity of at least 60 % from that of the free enzyme. Consequently, this loss of activity in ImE beads can be related to several factors such as the diffusion through the matrix of substrate (H_2O_2) from the external environment into gel layer and after enzyme catalysis, of products (O_2 and H_2O) from beads to the medium. Other factors are probably related to the steric hindrance of the matrix, which can limit the accessibility of the substrate to the enzyme active site, thus lowering the reaction rate. Structural deformations or denaturation of the enzyme during immobilization and, eventually, an alteration of catalytic site, can also occur [8-10].

It was suggested [2,10,35] that the diffusion phenomenon can be diminished by increasing the amount of enzyme and by decreasing the thickness of the gel layer. When the amount of ImE was increased approx. 6 fold (Table 1), retained activities for beads with diameters greater than 1 mm were just doubled with respect to their initial activity (Figure 3). The slight specific activity increase could be related to an excess of ImE on the beads surface (more accessible to substrate). These results suggest that an increase in the enzyme/matrix ratio does not appear to be a determining factor in the improvement of ImE specific activity. However, the retained specific activity of catalase (10-20 %) on beads of 1-3 mm was markedly increased (98 %) when the enzyme was immobilized on beads of low diameter (<100 µm). Consequently, the increase of enzyme/matrix ratio and, at

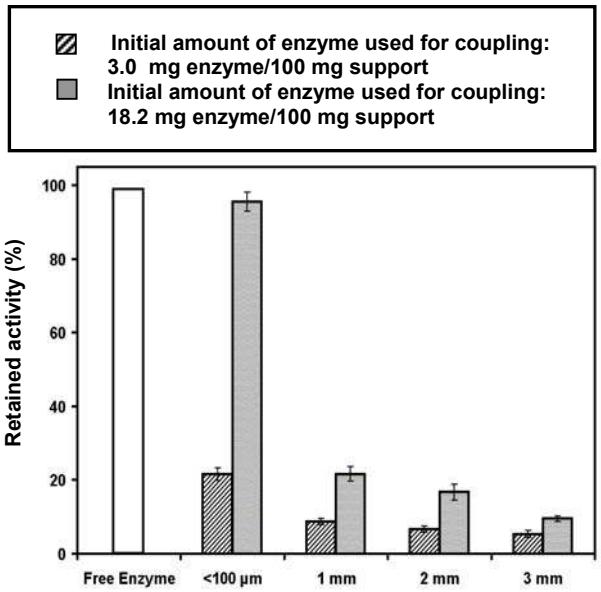


Figure 3: Activities of free and immobilized catalase with various diameters of alginate beads

The same letter and the same number respectively at the top of white and dark columns indicate no significant difference at $p \leq 0.05$

the same time, a decrease in the bead size, permits an improvement in ImE specific activity.

Kinetic parameters

Free and immobilized catalase show very similar Km values (Table 2; 25 and 27 mM); only a slight decrease in substrate affinity and no major structural alterations, occurred as result of covalent immobilization. The Vmax for free enzyme was determined as 24000 units/mg, whereas for ImE (internal immobilization), the Vmax was of 2040 units/mg (Table 2). This difference may be related to diffusion phenomena and to steric hindrance Enzyme immobilization on the matrix surface (Figure 4) can eliminate, at least in part, diffusion constraints.This surface ImE had a retained activity of 75 % with a Vmax of about 19800 units/mg, close (about 80 %) to that of the free enzyme. These values reflect well marked decrease in diffusion effects.

Table 2 Kinetic parameters of free enzyme and ImE

Enzyme	Coupling yield (%)	Retained specific enzymatic activity (%)	Kinetic parameters	
			K_m (mM)	V_{max} (units/mg)
Free enzyme (native)	-	100	25.0	24390
ImE (Internal) *	85.5	98	27.0	2040
ImE (surface)	89.8	75	28.5	19800

*** The amount of enzyme introduced for coupling was six times higher than that for surface coupling**

PH-stability

The free and ImE activities at various pH (Figure 5A) indicated a maximal activity for both at pH 7.0. However, pH-dependency profiles indicated that the activity of ImE was maintained over a wider pH range than that of free enzyme. For instance, at pH values higher than 10.0, ImE retained more 70 % of its activity, whereas the free enzyme retained only 50 %. Consequently, the higher stability of ImE at both acidic and alkaline treatments suggests that the ImE is less sensitive to the alterations induced by pH than the free enzyme. This behaviour can be related to the covalent linkage and physical interactions (ionic and polar stabilization, hydrogen bonding, etc.) between the enzyme and the polymeric matrix, which enhance the stability of ImE structure at various of pH values.

Table I Characteristics of immobilized catalase on activated supports as a function of coupling conditions

Type of enzyme	Amount of enzyme (mg) for 100 mg of support		Specific activity (units/mg)	Retained specific enzymatic activity (%)
	Introduced	Coupled		
Free enzyme	3.0	-	2200	100.0
Enzyme coupled on:				
Activated gel (prior to beading)	3.0	2.78 ± 0.15	2068	94.0
Beads (> 1 mm diameter) internally	3.0	2.64 ± 0.12	110-190	5.0 - 8.5
Microbeads (< 100 µm diameter) internally	3.0	2.57 ± 0.10	475	21.6
Beads (> 1mm diamater) internally	18.2	16.75 ± 0.10	220-470	10.0 - 21.5
Microbeads (< 100 µm diameter) internally	18.2	15.56 ± 0.91	2156	98.0
Microbeads (< 100 µm diameter) surface	18.2	2.72 ± 0.11	1650	75.0

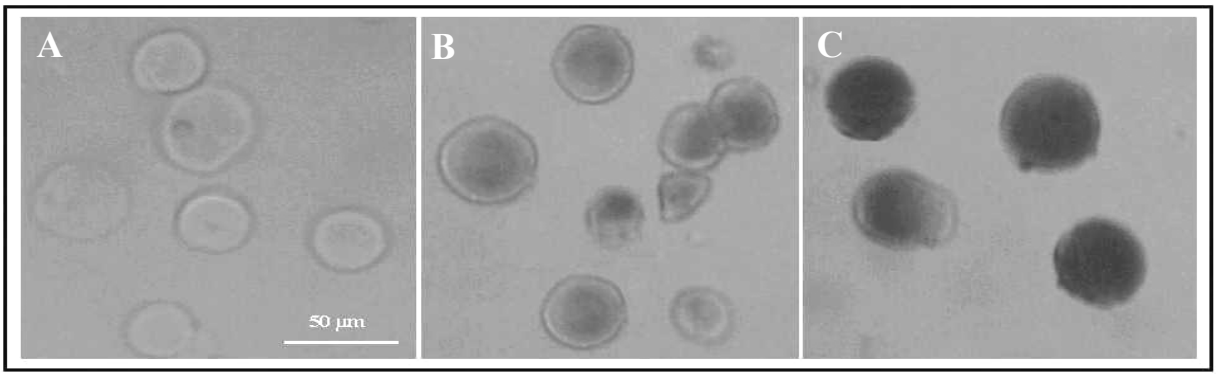


Figure 4: Optical microscopy images of alginate based-microbeads and the immobilized enzymes were detected by Coomassie blue

(A) Alginate without enzyme; (B) internal immobilization; and (C) surface immobilization

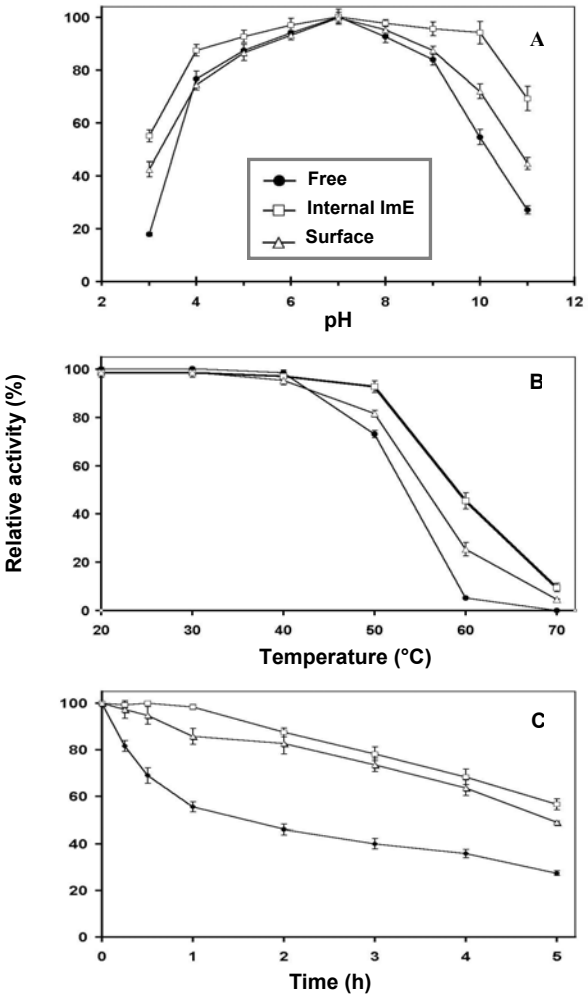


Figure 5 Stabilities of free and immobilized catalase at various pH values, temperatures and proteolysis times

The relative activity of 100 % was considered the enzyme activity determined at pH 7.0 and 22 °C, without incubation at various pH values, temperature or proteolytic attack

Thermal stability

The activities of free and immobilized catalase were stable up to 40 °C (Figure 5B). When heated at 60 °C, the immobilized catalase (internally) retained approximately 40 % of its control activity (at 22 °C) and the catalase immobilized on the surface 22 %, whereas the free enzyme activity was only 5 % (completely de-activated at 70 °C). The higher stability of the ImE on heating may be ascribed to the stabilising effects of the covalent and physical interactions between the enzyme and the matrix, ensuring multipoints articulation of the enzyme on the support frame.

Stability to proteolysis

The behaviour of free and ImE on proteolysis with trypsin clearly indicated a higher resistance of ImE (Figure 5C). The retained activity of free enzyme progressively declined at about 45 % after 2 h of trypsinolysis, whereas both internally and surface immobilized catalase retained approximately 85 % of their initial activities in the same time period. Further increase of the incubation time induced a pronounced degradation of free enzyme. For instance, after 5 h of proteolysis, the free enzyme retained only 22 % of its control activity, while ImE retained more than 50 % of its activity. The higher stability of ImE could be due to several factors: a steric hindrance generated by the matrix can prevent the access of protease to the substrate proteolysis sites on the ImE. Furthermore, alginate as an anionic polymer is susceptible to interact with trypsin by ionic interactions, possibly leading to a certain decrease in protease activity.

Proteolysis resistance of immobilized nisin

The free or immobilized nisin was submitted to a proteolytic attack by preincubating it for various times (0-6 h) in presence of a bacterial protease (*S. griseus*). At the established times (2, 4, 6 h), the nisin preparations were spotted onto the surface of agar plates containing *L. sake* (indicator cells) and incubated for 36 h until a bacterial

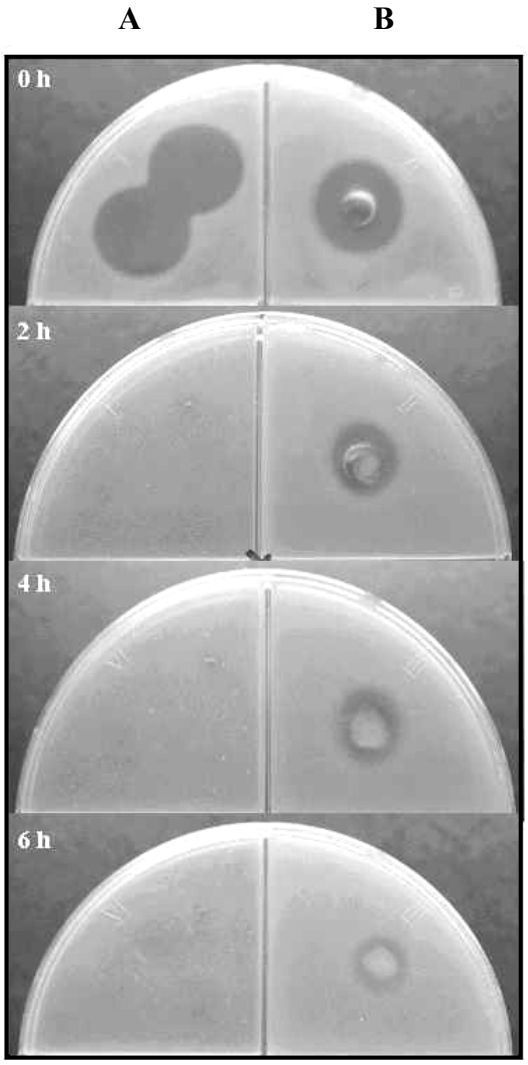


Figure 6 Stability of free (A) and immobilized (B) nisin incubated (0-6 h) to proteolysis with *S. griseus* protease

After proteolysis, the material was added to *L. sake* as indicator cells cultures on MRS Petri dishes. The remaining antibacterial activity of nisin was evaluated by the presence at inoculation point of an inhibitory zone after 36 h of incubation at 35 °C

lawn appeared. In absence of protease (0 h), both free and immobilized nisin showed large inhibition zones indicating high antibacterial activities (Figure 6). For the samples incubated for 1 h in a solution of the bacterial protease, no activity was detected for free nisin (not shown), whereas the immobilized nisin still remained active as a bacteriocin even after 6 h of incubation in protease solution. These results show the efficiency of the immobilization procedure in enhancing the stability of the bioactive agents against degradation or denaturing factors.

In conclusion, the immobilization of bioactive agents by simple entrapment via gelation is not-recommended for long-term conservation, because of the tendency for the entrapped proteins to leach out of gel over a period of time as mentioned by several authors [9,10]. Immobilization by covalent linkage and entrapment into alginate appears to be a good method to overcome this problem. Results obtained with enzyme immobilized into microbeads (<100 µm) showed a higher specific activity than other preparations. Higher enzyme/support ratios when immobilizing were found to overcome diffusion phenomena. The enzyme immobilization on the microbeads surface could be a promising method to ensure the protection of bioactive agents (not as high as in case of internal immobilization, but satisfactory for certain applications). Microbeads activated with periodate could be of commercial interest as ready-to-use supports. The beaded material, after activation, can be dried and stored for months. The immobilization can be simply done by swelling the activated support in mild aqueous conditions (pH 7.0-7.5), followed by the addition of the protein or peptide based-bioactive agent for a few hours at room temperature. The immobilization of bioactive agents on activated alginate microbeads appears, therefore, as a good candidate for biomedical, biotechnological or food applications.

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