Improving the Properties of Chitosan as Support for the Covalent Multipoint Immobilization of Chymotrypsin

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Received March 14, 2008; Revised Manuscript Received May 26, 2008

Changing gel structure and immobilization conditions led to a significant improvement in the covalent multipoint attachment of chymotrypsin on chitosan. The use of sodium alginate, gelatin, or κ-carrageenan, activation with glutaraldehyde, glycidol, or epichlorohydrin, and addition of microorganisms followed by cellular lysis allowed the modification of the gel structure. Immobilization yields, recovered activities, and stabilization factors at 55 and 65 °C were evaluated. Enzyme immobilization for 72 h at pH 10.05, 25 °C and reduction with $NaBH_4$ in chitosan 2.5%−carrageenan 2.5%, with addition of *S. cerevisiae* 5% and activation with epichlorohydrin led to the best derivative, which was 9900-fold more stable than the soluble enzyme. This support allowed an enzyme load up to 40 mg chymotrypsin × g_{gel}^{-1}. The number of covalent bonds, formed by active groups in the support and lysine residues of the enzyme, can explain the obtained results. SEM images of the gel structures corroborate these conclusions.

Introduction

The specificity of enzymes and their ability to catalyze reactions at low temperatures and neutral pH make them attractive for applications in the biochemical industry. Nevertheless, enzymes are usually expensive and have low operational stability, and the recovery of the free enzyme from the medium is not economically feasible in general. These drawbacks have somehow hindered their application in large-scale processes.

The immobilization of the enzyme on insoluble supports allows not only the reuse of the protein, but also the modulation of the catalyst properties. Besides, the use of an insoluble enzyme derivative enables the operation of continuous processes in industry and allows the fast quenching of the reaction by the removal of the catalyst in batch processes, thus helping to control the formation of products.[1]

In particular, multipoint immobilization, through the covalent link of each enzyme molecule to several activated groups of the support, can lead to a high degree of stabilization. Therefore, this technique may contribute to solve or, at least, to decrease negative aspects of the enzymatic processes, making feasible their more widespread use in industry.

Multipoint covalent immobilization requires the interaction of several residues of the same enzyme molecule with active groups of the support. The formed bonds will increase the rigidity of a small part of the molecule surface (e.g., 10−20%), which will make more rigid the overall three-dimensional structure.[2–5] Then, enzyme stabilization is achieved. Aldehyde and oxirane groups in the support and amine groups in the enzyme are a good choice to promote the multipoint attachment of the enzyme molecule and, therefore, to obtain highly stable enzyme derivatives. Increasing the number of enzyme−support covalent bonds, a higher stabilization degree of the enzyme is expected, although an upper limiting number of links may exist, because the accessibility of the active site by the substrate, and even its catalytic action may be affected. The number of linkages depends on the degree of activation of the support (concentration of aldehyde or oxirane groups on the support surface) and on the concentration of available amine groups of the enzyme that have no role in the catalytic mechanism. Furthermore, an adequate geometrical congruency between enzyme and support is also important: the greater the enzyme−support congruence, the higher the possibility to achieve an intense multipoint attachment. Therefore, the internal area of the support is one important variable to obtain active and stable enzyme derivatives.

Chymotrypsin (EC 3.4.21.1), an endoprotease with 25 kDa, cleaves protein chains providing peptides mainly with aromatic amino acids (tryptophan, tyrosine, or phenylalanine) at the C-terminal position. This protease may be used to produce tailor-made protein hydrolysates, for instance, with interesting nutritional and pharmaceutical properties. The hydrolysis may enhance nutritional and textural characteristics of the product, change its solubility, remove allergenic properties, and reduce bitterness and undesirable flavors.[6,7] The literature reports several works dealing with chymotrypsin immobilization.[8–11] However, if this enzyme is intended to be used in large-scale processes, as it is the case of the production of protein hydrolysates, it is very important to reduce biocatalyst costs.

The production of an active and stable enzyme derivative, using a simple immobilization protocol and a nonexpensive support, is the main goal when studying enzyme immobilization, mainly if large amounts of biocatalyst are required. In this context, chitosan, a cheap and abundant linear polysaccharide composed of randomly distributed β-(1−4)-linked D-glucosamine (deacetylated) and N-acetyl-D-glucosamine (acetylated) units, is a very interesting support for enzyme immobilization.[12–14] This cationic biomaterial is produced by deacetylation of chitin, a polymer present in the cell wall of fungi and in the shells of shellfish (mainly crab, shrimp, lobster, krill), wastes

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10.1021/bm8002754 CCC: $40.75 © 2008 American Chemical Society
Published on Web 07/17/2008

of the seafood industry. The water-insoluble chitin, after removal of more than 60% of the acetyl groups, is called chitosan and becomes soluble at acidic pH, due to the presence of the charged amine groups. At basic pH, the amine groups of chitosan become uncharged (pK_a 6.5) and the polymer is insoluble in aqueous medium, forming a gel.

The internal structure of the chitosan gel can be modified by interaction with other biopolymers such as gelatin, alginate and carrageenan, with which chitosan may form hybrid gels. Different size distributions of pore beads may be obtained through the covalent or physical interactions of the two polymers.[15] The biopolymers gelatin, alginate, and κ-carrageenan have groups that are negatively charged at neutral pH and can interact with the positively charged amine groups of chitosan, forming different internal nets.[16] Gelatin is a protein, alginate is a linear copolymer with homopolymeric blocks of (1−4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, respectively, covalently linked in different sequences or blocks. Carrageenans are a family of linear sulfated polysaccharides extracted from red seaweeds. They are large, highly flexible molecules that curl, forming helical structures that give them the ability to form a variety of different gels at room temperature, in the presence of some cations like potassium. κ-Carrageenan can form strong, rigid gels. The hydrogen bonds formed between chitosan, alginate, or κ-carrageenan modify the porosity of the gel. They also change the chitosan molecule conformation, which becomes more resistant to drastic conditions of pH and temperature.[17] Alginate induces the formation of stable polyelectrolytes with chitosan that are broken at strict pH and temperature conditions.[18]

Another way to modify the gel internal structure is its formation in the presence of a material that can be further removed. The preparation of macroporous beads and membranes with the presence of silica during gel formation, followed by the removal of the silica after dissolution at alkaline pH, has been already reported in the literature.[19] When a similar approach is used, the gel could be formed in the presence of cells of microorganisms, followed by cell lysis. After removing the cellular material, the resulting polymer may have a higher internal surface area, favoring enzyme immobilization.

Chitosan, pure or forming hybrids with other polymers, contains a high density of primary amine and hydroxyl groups that can be chemically modified, generating aldehyde groups to form Schiff bases with the amine groups of the enzyme. The activation of the hybrid support with oxirane reactants followed by oxidation with sodium periodate generates aldehyde (glyoxyl) groups, using glycidol, or both aldehyde (glyoxyl) and oxirane groups, if activated with epichlorohydrin. Amine groups of chitosan may also be directly activated with glutaraldehyde. Aldehyde groups produced after activation with glutaraldehyde are very reactive and can immobilize the enzyme after linking with only one amine group. Although less reactive than aldehydes from glutaraldehyde, oxirane groups can react with different residues, while glyoxyl will only be able to keep the enzyme linked if at least two bonds with amine groups are formed.[13,20] Therefore, glutaraldehyde can react with the terminal amine group of the protein, while with glyoxyl-aldehyde groups, the ε-amino of the lysine residues have to be available to make feasible the immobilization. However, uncharged amine groups are required to react, which makes the multipoint attachment dependent on pH.

The pK of lysine amine groups is 10.5, thus, a pH above 10 is needed to achieve a significant concentration of these groups to form the Schiff bases.[5,8] Supports containing epoxy or oxirane groups are also able to react with different nucleophilic groups on the protein surface (e.g., amino, hydroxy, or thiol moieties), forming extremely strong linkages (secondary amino bonds, ether bonds, and thio-ether bonds) with minimal chemical modification of the protein: pK values of the new secondary amino groups are very similar to those of the preexisting primary amino groups. The high reactivity of glutaraldehyde is useful to immobilize enzymes that are not stable at alkaline pH. However, its reactivity makes the multi-interaction process difficult to control, generally leading to lower stability factors of the enzyme that the ones obtained with glyoxyl and oxirane.[21,22]

The aim of this work was to improve the properties of chitosan as a support for enzyme immobilization by modifying the polymer structure and testing different activation and immobilization protocols. Different gel structures were obtained by using chitosan, either pure or mixed with another biopolymer: alginate, gelatin, or carrageenan. The influence on the final gel structure of the presence of *Saccharomyces cerevisiae* or *Bacillus megaterium* during gel formation, followed by the removal of the cells, was also tested. The gels were activated with glutaraldehyde, glycidol, or epichlorohydrin and, after enzyme immobilization, different chymotrypsin derivatives were obtained and their properties were evaluated and compared with the soluble enzyme.

Materials and Methods

Materials. Powdered chitosan, 85.2% deacetylation degree, was purchased from Polymar (Fortaleza, Brazil); sodium alginate, glutaraldehyde 25% (m/v) from Vetec (São Paulo, Brazil); κ- carrageenan, glycidol, epichlorohydrin, chymotrypsin (EC 3.4.21.1) type II from bovine pancreas 120 $U_{BTEE} \cdot mg^{-1}$ of lyophilized enzyme (0.92 mg of protein mg^{-1} of lyophilized enzyme) and benzoyl-L-tirosine ethyl ester (BTEE) were purchased from Sigma Chemical Company (St Louis, MO); alcalase 2,4L type FG (liquid form) 28 $U_{BTEE} \cdot mg^{-1}$ of lyophilized enzyme was a gift from Novo Nordisk A/S (Bagsvaerd, Denmark). Powdered commercial gelatine from Oetker was purchased (Brazil), *Bacillus megaterium* ATCC 14945 was donated by Tropical Foundation (Campinas, Brazil), and commercial *Saccharomices cerevisiae* (baker's yeast) was purchased from Fleischmann (Brazil). All other reagents were of analytical grade.

Preparation of Chitosan Beads. Chitosan beads were prepared by dissolving powder chitosan in an acetic acid 5% v/v solution. The obtained solution of 2.5−5.0% (m/v) was sprayed into a gently stirred NaOH 0.1 M solution for 24 h at room temperature and washed with distilled water and, after, with Milli-Q water. Higher concentrations of polymer were not used due to the high viscosity, which makes the formation of beads difficult.

Preparation of Hybrid-Chitosan Beads. Hybrid-chitosan beads were prepared by dissolving powder chitosan in acetic acid 5% (v/v) solution. Afterward, commercial gelatin, sodium alginate or carrageenan was added to the solution, which was stirred for 10−30 min. The obtained solutions were sprayed into a gently stirred NaOH 0.1 M solution for 24 h at room temperature and washed with distilled water and, after, with Milli-Q water. The obtained supports were chitosan 2.5% and 5.0%, as well as, chitosan 2.5%−alginate 2.5%, chitosan 2.5%−carrageenan 2.5%, and chitosan 4.0%−gelatin 3.0%, being all concentrations expressed as % (m/v).

Preparation of Hybrid-Chitosan Beads Using Cells. Chitosan beads were prepared by dissolving powder chitosan in an acetic acid 5% (v/v) solution. Afterward, commercial gelatin, sodium alginate or carrageenan was added to this solution, followed by addition of microorganisms, *Saccharomyces cerevisiae* (Sc) or *Bacillus megaterium* (Bm). The resultant solution was stirred during 30 min, and sprayed into a

gently stirred NaOH 0.1 M solution for 24 h at room temperature, followed by washing with distilled water and, after, with Milli-Q water. To remove proteins and membrane cell debris, two treatments were used. In the first one, the beads were treated with an alkaline solution containing 20% of 1 M NaOH, 10% of sodium dodecyl sulfate (SDS), and 70% of distilled water (v/v) for 24 h at room temperature, using 0.30 mL of solution per milligram of added microorganism. The last step was proteolysis with Alcalase 0.05% (v/v) in sodium phosphate buffer 0.1 M, pH 8.0, at room temperature for 24 h, followed by washing with distilled water. To reduce treatment time and costs, a second methodology was tested using commercial powder soap that usually contains proteases and lipases secreted by *Bacillus subtilis* and *Bacillus licheniformis* immobilized by encapsulation and SDS,[23] instead of Alcalase. After treatment with the NaOH solution, as described above, and setting the pH at 9.0 and temperature at 40 °C, commercial soap powder was added (1% (m/v)). The suspension was kept under mild stirring for 24 h. The beads were washed with excess of distilled water and, after, with Milli-Q water. The supports prepared with cells were chitosan 5.0%−Sc 5.0%, chitosan 2.5%−alginate 2.5%−Sc 1.75%, chitosan 2.5%−alginate 2.5%−Bm 5.0%, chitosan 2.5%−carrageenan 2.5%−Sc 1.25%, chitosan 2.5%−carrageenan 2.5%−Sc 5.0%, chitosan 2.5%−carrageenan 2.5%−Bm 5.0%, chitosan 4.0%−gelatin 3.0%−Sc 1.75%, chitosan 4.0%−gelatin 3.0%−Bm 5.0%, with all concentrations expressed as % (m/v).

Activation of Hybrid-Chitosan Beads Using Glutaraldehyde. Activation was made by contacting hybrid-chitosan beads with sodium phosphate buffer 0.1 M, pH 7.0, containing glutaraldehyde 5% (m/v) using a ratio V_{beads}/V_{total} of 1/10 during 1.0 h at 25 °C and washing first with distilled water to remove the excess of the activating agent and after with Milli-Q water.

Activation of Hybrid-Chitosan Beads Using Glycidol and Epichlorohydrin. Glyceryl-supports were prepared by mixing beads under stirring with an aqueous solution containing NaOH 1.7 M and $NaBH_4$ 0.75 M (glycidol)[5] or NaOH 2 M and $NaBH_4$ 0.12 M (epichlorohydrin)[19] in ice bath. Then, 0.48 mL of glycidol or 2.00 mL of epichlorohydrin per gram of bead were added, kept under mechanical stirring for 18 h, and washed until neutrality. Glyoxyl/oxirane-supports were obtained by contacting beads with 2.00 mL of 0.1 M $NaIO_4$ solution per gram of gel for 2.0 h at room temperature.[21] Afterward, they were washed with excess of distilled water until neutrality and, in sequence, with Milli-Q water.

Determination of the Aldehyde Content on Supports. A total of 10 mg of support were added to 1 mL of Schiff reagent containing basic fucsin 0.67% (m/v), sodium bisulphate 0.67% (m/v), and concentrated hydrochloric acid until pH 4.5. Then, supports were kept at rest for 24 h. Afterward, 2 mL of water were added to the suspension. After decanting for 10 min, the supernatant absorbance was measured at 450 nm. Aldehyde group concentration in glyoxyl-agarose can be calculated after measuring the consumption of sodium periodate. However, for glyoxyl-chitosan, this methodology is not accurate. Therefore, chitosan aldehyde concentrations were measured comparing the absorbance changes during the Schiff reaction of chitosan supports with the ones of glyoxyl-agarose supports,[6] prepared with different known concentrations of aldehyde$\cdot g^{-1}$ of gel.

Determination of the Oxirane Content on Supports. Analysis of oxirane groups were performed according to Sundberg and Porath[24] with slight modifications. The release of hydroxyl groups was followed by titration with 0.1 M hydrochloric acid. Oxirane in solution was determined as follows: the oxirane-containing gel (100 mg) was added to 15 mL of 1.3 M sodium thiosulphate solution at pH 7.0 (by addition of hydrochloric acid) until the reaction was over. The amount of oxirane present in the solution was then calculated from the amount of hydrochloric acid needed to maintain neutrality.

Scanning Electron Microscopy. To observe the surface morphology of hybrid chitosan supports, scanning electron microscopy (SEM) was used. The samples were freeze-dried to extract water from the pores without collapsing their structures. The particles were instantaneously frozen in liquid nitrogen using Heto FD 2.5 equipment under 5×10^{-3} mBar vacuum and −45 °C. After drying, the samples were covered with a thin layer of gold (10 nm) using a sputter coater (Veeco VE-300) and were observed using a JEOL JSM-35C scanning electron microscope (20 kV).

Multipoint Covalent Attachment of Chymotrypsin to Supports Activated with Glutaraldehyde. Chymotrypsin (5 mg of enzyme per gram of bead) was added to the activated support (ratio m/v of 1/10) in sodium phosphate buffer 0.1 M, pH 7.0, at 4 °C or bicarbonate buffer 0.1 M, pH 10.05, at 25 °C for 2 h. Derivatives incubated in pH 10.05 were reduced by addition of $NaBH_4$ (0.1% m/v), under gentle stirring, for 30 min at room temperature and washed with abundant distilled water to eliminate residual sodium borohydride and with Milli-Q water in sequence.

Multipoint Covalent Attachment of Chymotrypsin on Supports Activated with Glycidol or Epichlorohydrin. Chymotrypsin (5 or 10 mg of enzyme per gram of bead) in bicarbonate buffer 0.1 M, pH 10.05, was added to the activated support (ratio m/v of 1/10). The preparation was kept under mild stirring at 25 °C and different incubation times (24, 48, 72, and 96 h). After that, the best derivative was reduced by addition of $NaBH_4$ (0.1% m/v) during 30 min at room temperature, followed by washing with abundant distilled water to eliminate residual sodium borohydride[25–27] and, after, with Milli-Q water. All buffer solutions and suspensions were prepared using Milli-Q water.

The number of enzyme units/mg of lyophilized enzyme and the protein mass/mg of lyophilized enzyme specified by Sigma were checked using the standard assays (enzyme activity and Bradford method[28]). The mass of enzyme and gel were weighted and the offered enzyme load could be calculated ($U\cdot g_{gel}^{-1}$ and mg of lyophilized enzyme per gram of gel).

The immobilization yield was calculated by measuring protein concentrations and enzyme activities in the blank solution and in the supernatant.

Blank assays were run with the soluble enzyme in the same conditions used in the immobilization assays. It was observed that chymotrypsin always preserved 100% activity. Both protein concentration and enzyme activity in the supernatant were used to calculate the immobilization yield.

The percentage of enzyme not immobilized was calculated dividing the remaining activity in the supernatant by the enzyme activity measured in the blank, taking into account the dilution due to the addition of gel (9 mL of enzyme solution + 1 g of gel). Because the offered enzyme load was known, the number of enzyme units theoretically immobilized per gram of gel ($At_{theoretically\ immobilized}$) could be calculated. After finishing the immobilization, the apparent gel activity At_{app} (enzyme units$\cdot g_{gel}^{-1}$) was measured and compared to the theoretically immobilized. The recovered activity was then calculated as $At_{app}(U\cdot g_{gel}^{-1})/At_{theoretically\ immobilized}(U\cdot g_{gel}^{-1})$. These values were always in agreement with the measured disappeared protein.

Chymotrypsin Activity Assays. Enzyme activity was assessed via spectrophotometer using BTEE 8 mM. The product released by the ester hydrolysis, in 0.1 M sodium phosphate buffer, pH 7.0 at 25 °C, was measured at 258 nm. The activity was calculated by using a molar extinction coefficient of 870 L/(mol × cm). One unit (U_{BTEE}) corresponds to the amount of enzyme that hydrolyzes 1 μmol of ester per minute at pH 7.0 and 25 °C.

Thermal Stability Assays. Soluble enzyme was incubated in a sodium phosphate buffer 0.1 M and pH 8.0 at 55 °C, while immobilized derivatives were incubated at 55 and 65 °C. Periodically, samples were withdrawn and their residual activities were assessed as described above. The single-step nonfirst-order model, proposed by Sadana and Henley[29] was fitted to the experimental data. This model considers that a single-step inactivation leads to a final state that exhibits a residual activity, which is very stable. The activity-time expression is

$$a_R = (1 - \alpha) \times \exp(-kt) + \alpha \tag{1}$$

where a_R is the activity (dimensionless); α is the ratio between the specific activity of the final state, E_1, and one of the initial state, E; and k is the first-order deactivation rate constant (time^{-1}). The parameter

Table 1. Influence of the Polymer Composition on the Immobilization of Chymotrypsin at pH 7.0, 4 °C, for 2 h[a]

biocatalyst	I_Y (%)	R_A (%)	A_{app} U·g_{gel}^{-1}	$t_{1/2}$ (h)	S_F
soluble enzyme				0.02	1
chitosan 2.5%	24	51	73	0.57	29
chitosan 5.0%	78	17	80	2.63	132
chitosan 4.0%−gelatine 3.0%	65	29	113	3.34	167
chitosan 2.5%−alginate 2.5%	62	59	219	4.32	216
chitosan 2.5%−carrageenan 2.5%	82	58	285	2.84	142

[a] Supports activated with glutaraldehyde. Offered enzyme load: 5 mg of lyophilized enzyme·g_{gel}^{-1} (600 U·g_{gel}^{-1}). Immobilization parameters: immobilization yield (I_Y), recovered activity (R_A), apparent activity (A_{app}), half-life ($t_{1/2}$), and stabilization factor (S_F) at 55 °C.

k should describe the unfolding or the inactivation process and the parameter α describes the long-term level of activity.[8] Stabilization factor (S_F) was given as the ratio between the half-life of the immobilized derivative and the half-life of the soluble enzyme at the same conditions. However, due to the quick deactivation of the free enzyme at 65 °C, the stabilization factor of the most active derivative was calculated as ($t_{1/2}$ of derivative/$t_{1/2}$ of reference derivative) × stabilization factor of reference derivative at 55 °C. The reference derivative was chitosan 2.5%−alginate 2.5% glutaraldehyde at pH 10.05.

Chymotrypsin Maximum Theoretical Immobilized Loading. Chymotrypsin maximum theoretical immobilized loading was assessed for the best derivatives, activated with glutaraldehyde, glycidol, and epichlorohydrin, respectively. Offered chymotrypsin loads ranged from 5 to 200 mg of enzyme·g^{-1} of support.

Amino Acid Analysis of Soluble and Immobilized Chymotrypsin. Soluble and stabilized chymotrypsin derivatives (both containing approximately 2 mg of protein) were hydrolyzed in HCl (6.8 M) at 105 °C for 24 h. The mixture was filtrated and dried. The amino acid extract was dissolved in 0.2 M sodium citrate buffer, pH 2.2. HPLC amino acid analysis protocol followed the Waters Pico-Tag method. Before separation in the column, the amino acids present in the sample were reacted with phenylisothiocyanate (PITC) to form phenylthiocarbamyl (PTC) derivatives. The HPLC system consisted of a Model 717 plus multisystem with a 486 UV−vis detector (Waters, Milford, MA) with a 300 mm × 3.9 mm reversed-phase column (Pico Tag; Waters) at 50 °C. A binary gradient using solvents (A) 0.14 M sodium acetate containing 0.5 μM EDTA and 6% acetonitrile (pH 5.7) and (B) 60% acetonitrile was carried out at a flow rate of 1.0−1.5 mL·min^{-1} as follows: 0.0−12.0 min, linear gradient from A/B (99:1) to A/B (0:100); 12.0−12.5 min, elution with A/B (99:1).

Results and Discussion

Activation with Glutaraldehyde: Influence of the Polymer Composition on the Multipoint Covalent Attachment of Chymotrypsin. Pure and hybrid chitosan gels were prepared in the presence and absence of microorganism cells. The supports were activated with glutaraldehyde and used for chymotrypsin immobilization at pH 7.0 and 4 °C for 2 h. Table 1 presents the immobilization parameters for these chymotrypsin derivatives. It can be seen that the most significant variable was the polymer concentration. The increase of the chitosan concentration from 2.5 to 5.0% led to an increase in the immobilization yield from 24 to 78% and in the stabilization factor of the derivatives from 29 to 132. These results may be explained by the increase of aldehyde groups available to link to the amine groups of the enzyme. The higher the polymer concentration, the higher the number of available amine groups in the support to react with glutaraldehyde. However, the high reactivity of this activating reactant also might have led to excessive cross-linking in the matrix and the formation of small pores. In consequence, the apparent activity of the immobilized enzyme did not increase. The results of Table 1 also show that the formation of hybrid gels by mixing chitosan with κ-carrageenan, alginate, or gelatine for the same total polymer concentration (5.0%) may have improved the internal structure of the matrix, as the obtained hybrid derivatives presented higher activity and thermal stability when compared to pure chitosan. The most active derivative was obtained using κ-carrageenan, 285 U_{BTEE}·g_{gel}^{-1} and S_F = 142, while the most stable was obtained using alginate, 216-fold more stable than the soluble enzyme and activity of 219 U_{BTEE}·g_{gel}^{-1}.

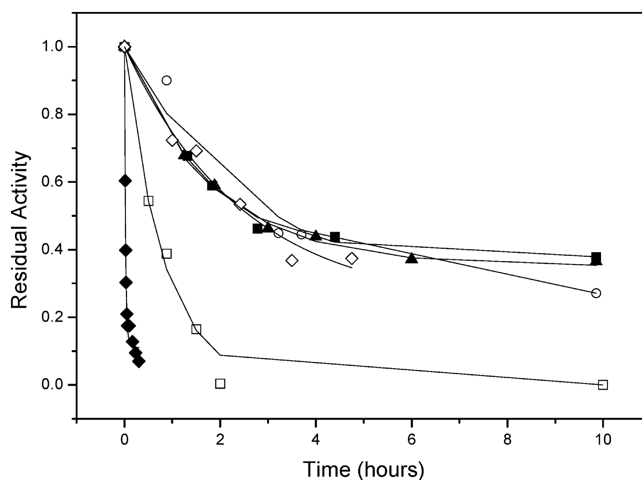

Figure 1. Thermal inactivation of pure and hybrid chitosan-glutaraldehyde derivatives. Chymotrypsin immobilization at pH 7.0, 4 °C, for 2 h, offered enzyme load of 5 mg of lyophilized enzyme·g_{gel}^{-1} (600 U·g_{gel}^{-1}). Soluble enzyme and derivatives were incubated at 55 °C and pH 8.0: (◆) soluble chymotrypsin; (□) chitosan 2.5%; (◇) chitosan 5.0%; (○) chitosan 4.0%−gelatine 3.0%; (■) chitosan 2.5%−alginate 2.5%; (▲) chitosan 2.5%−carrageenan 2.5%. The lines correspond to the fitting of the Sadana−Henley model.

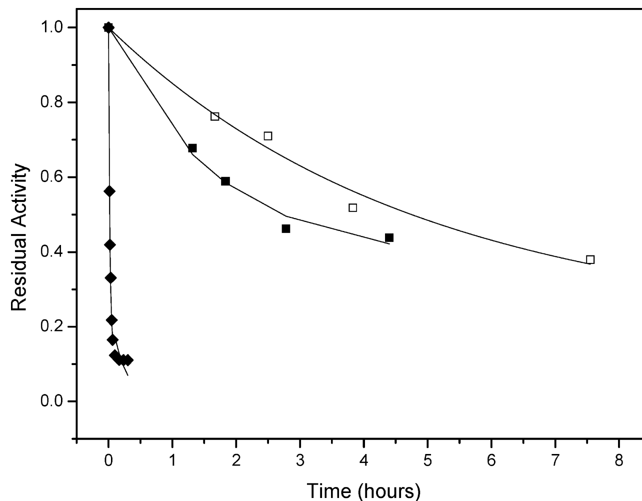

Figure 2. Influence of the temperature and pH of immobilization on the thermal stability of chitosan 2.5%−alginate 2.5%−glutaraldehyde−chymotrypsin derivative, after 2 h of immobilization reaction, with offered enzyme load of 5 mg lyophilized enzyme·g_{gel}^{-1} (600 U·g_{gel}^{-1}), incubated at 55 °C and pH 8.0: (◆) soluble chymotrypsin; (■) immobilization at pH 7.0, 4 °C; (□) immobilization at pH 10.05, 25 °C. The lines correspond to the fitting of the Sadana−Henley model.

The thermal stability of the produced derivatives was studied at 55 °C, pH 8.0. The Sadana−Henley two-parameter deactivation model was fitted to the data (enzyme residual activities for different incubation times at 55 °C). Figure 1 shows the results

Table 2. Influence of the Polymer Composition on the Immobilization of Chymotrypsin on Supports Activated with Glycidol[a]

biocatalyst	I_Y (%)	R_A (%)	A_{app} $U \cdot g_{gel}^{-1}$	$t_{1/2}$ (h)	S_F
soluble enzyme				0.02	1
chitosan 5.0%	50	13	78	1.89	95
chitosan 2.5%−alginate 2.5%	17	43	88	8.50	425
chitosan 2.5%−carrageenan 2.5%	34	76	310	4.90	243

[a] Immobilization conditions: pH 10.05, 25 °C, 24 h, 10 mg of lyophilized enzyme·g_{gel}^{-1} (1200 U·g_{gel}^{-1}). Immobilization parameters: immobilization yield (I_Y), recovered activity (R_A), apparent activity (A_{app}), half-life ($t_{1/2}$), and stabilization factor (S_F) at 55°C.

Table 3. Influence of the Polymer Composition on the Immobilization of Chymotrypsin on Supports Activated with Epichlorohydrin[a]

biocatalyst	I_Y (%)	R_A (%)	A_{app} $U \cdot g_{gel}^{-1}$	$t_{1/2}$ (h)	S_F
soluble enzyme				0.02	1
chitosan 5.0%	77	19	176	9.00	455
chitosan 2.5%−alginate 2.5%	65	24	187	6.70	335
chitosan 2.5%−carrageenan 2.5%	71	22	187	10.34	517

[a] Immobilization conditions: pH 10.05, 25 °C, 24 h, 10 mg of lyophilized enzyme·g_{gel}^{-1} (1200 U·g_{gel}^{-1}). Immobilization parameters: immobilization yield (I_Y), recovered activity (R_A), apparent activity (A_{app}), half-life ($t_{1/2}$), and stabilization factor (S_F) at 55 °C.

of the model fitting to all data. It can be observed that the model fits quite well to the experimental data for all derivatives, as well as for the soluble enzyme. The calculated half-lives for the obtained biocatalysts are shown in Table 1.

The calculated half-lives for the chitosan derivatives that are presented in Table 1 are much lower than the one reported for chymotrypsin immobilization on agarose, 3 h at 25 °C.[30] These values may be explained by the immobilization conditions used in these experiments, pH 7.0, 4 °C, and 2 h for immobilization time. At this pH, the concentration of uncharged ϵ-amino lysine groups is expected to be quite low and, therefore, the possibility of multipoint covalent attachments is not very high. Besides, the correct alignment of the enzyme for the multipoint attachment requires immobilization times longer than 2 h. Finally, a low temperature was used for the immobilization reaction, 4 °C, and higher temperatures would help to promote more intense interactions. Then, to improve the multi-interaction between enzyme and support, an immobilization assay was run at pH 10.05, for 24 h, 25 °C, even though under this pH the stability of the support was not so good due to the glutaraldehyde chemical properties.[31] The support chosen to test the influence of pH was chitosan 2.5%−alginate 2.5%. According to Figure 2, it can be noticed that at pH 10.05 the derivative stabilization factor was 240, while at pH 7.0 it was 216. Therefore, a very significant improvement was not obtained. Although at pH 10.05 there might be a higher number of amine groups (uncharged lysine residues) of the enzyme able to react with the aldehyde groups of the support, it seems that there was no formation of a correspondently higher number of Schiff bases. However, the immobilization yield increased from 65% at pH 7.0 to 100% at pH 10.05, indicating that more enzyme molecules could be linked to the support through the lysine residues. The lower recovered activity obtained at the more basic pH (12.2%) was already expected, because the higher reactivity of glutaraldehyde in this immobilization condition may lead to an increased grade of cross-linking, with consequent higher mass transfer effects. Although the immobilization had been followed up to 24 h, the activity of the immobilized enzyme after 2 h of immobilization was very low, and only the thermal stability for the 2 h derivative was determined.

Activation with Glycidol: Influence of the Polymer Composition. Table 2 shows the immobilization parameters for chymotrypsin in pure and hybrid chitosan, activated with glycidol. Activation with glycidol led to an increase of almost 100% in the stability factor, when compared to the activation with glutaraldehyde. The enzyme immobilization through reaction between glyoxyl aldehyde groups of the support and amine groups of the enzyme requires the formation of at least two simultaneous bonds, which act in a synergistic way. This behavior may explain the observed increase in the stability factor of the chitosan glyoxyl derivatives. Probably, immobilization with glutaraldehyde was unipoint and, with glyoxyl, at least through two points. The chitosan−carrageenan derivative was the most active, presenting 34% immobilization yield, 76% of recovered activity with apparent activity of 310 $U_{BTEE} \cdot g_{gel}^{-1}$, and stabilization factor of 243 at 55 °C. Although being 425-fold more stable than the soluble enzyme, the chitosan−alginate gel showed a very low immobilization yield (17%).

Activation with Epichlorohydrin: Influence of the Polymer Composition. Results in Table 3 show that activation of pure and hybrid chitosan with epichlorohydrin led to a significant improvement in the thermal stability of the derivatives, when compared to the other two tested activating agents. With glycidol, the hybrid gel chitosan 2.5%−alginate 2.5% had a much higher stability factor than pure chitosan 5% (see Table 2). Table 3 shows that using epichlorohydrin pure chitosan derivatives were more stable than the hybrid chitosan-alginate. On the other hand, the most stable derivative, using epichlohydrin, was the hybrid chitosan−carrageenan. Hence, the presence of different reactive groups in each polymer and the difference in reactivity of the activating agents have caused this nonmonotonic behavior. Besides the hydroxyl groups, the polymers have other different reactive groups: amine groups in chitosan, acidic groups in alginate, and sulfate groups in κ-carrageenan. The reaction of the amine groups of chitosan with epichlorohydrin generates epoxide groups, which are able to link to the enzyme, as well are the glyoxyl groups. Glycidol has epoxy and hydroxyl groups while epichlorohydrin has epoxy and chloride groups, being the latter one more reactive. On the other hand, chitosan has also two reactive groups, amine and hydroxyl, being the former ones more reactive than hydroxyl. Therefore, after the reaction with the epoxide reactants, probably many amine groups also reacted with the activating agents. Although it has been already reported that only few chitosan amine groups react with epichlorohydrin, the reaction conditions used in this work were stronger and it was expected that more amine groups were transformed into amino-diol.[13] As epichlorohydrin is more reactive than glycidol, more aldehyde groups in the support might be formed using this activating agent, which allowed the formation of more bonds between enzyme and support, thus explaining the increase in the stability factor. The best results were obtained for chitosan 2.5%−carrageenan 2.5%, which presented a stabilization factor of 517 (at 55 °C). The different reactivity of the involved groups and the helicoidal conformation of κ-carrageenan, which may lead to the formation of a better internal gel structure, may be responsible for this result.[18]

Influence of the Addition of Microorganism Cells During Gel Formation. The influence of the presence of cells during the formation of the internal gel structure was studied for the pure and the chitosan-hybrid gels that led to the best chymotrypsin derivatives, after activation with glutaraldehyde,

Table 4. Influence of the Addition of Microorganisms Cells to the Polymers During the Immobilization of Chymotrypsin on Pure and Hybrid Chitosan[a]

biocatalyst	activating agent	I_Y (%)	R_A (%)	A_{app} (U. g_{gel}^{-1})	$t_{1/2}$ (h)	S_F
soluble enzyme					0.02	1
chitosan 5.0%	GLU	78	17	80	2.63	132
chitosan 2.5%−alginate 2.5%	GLU	62	59	219	4.32	216
chitosan 5.0%−Sc 5.0%	GLU	88	17	90	2.25	114
chitosan 2.5%−alginate 2.5%−Sc 5.0%	GLU	54	37	120	3.38	169
chitosan 2.5%−alginate 2.5%−Bm 5.0%	GLU	69	38	157	5.22	261
chitosan 4.0%−gelatine 3.0%−Bm 1.75%	GLU	69	41	170	4.02	201
chitosan 5.0%	GLY	50	13	78	1.89	95
chitosan 2.5%−carrageenan 2.5%	GLY	34	76	310	4.90	243
chitosan 5.0%−Sc 5.0%	GLY	54	10	65	3.50	177
chitosan 2.5%−carrageenan 2.5%−Sc 1.25%	GLY	38	47	214	8.38	419
chitosan 2.5%−carrageenan 2.5%−Sc 5%	GLY	72	31	268	8.84	442
chitosan 2.5%−carrageenan 2.5%−Bm 5%	GLY	20	46	110	6.90	345
chitosan 5.0%	EPI	77	19	176	9.00	455
chitosan 2.5%−carrageenan 2.5%	EPI	71	22	187	10.34	517
chitosan 5.0%−Sc 5.0%	EPI	76	17	155	12.00	610
chitosan 2.5%−carrageenan 2.5%−Sc 1.25%	EPI	82	49	482	21.04	1052
chitosan 2.5%−carrageenan 2.5%−Sc 5.0%	EPI	91 (±2)	57 (±10)	622 (±62)	26.8 (±1.4)	1379 (±44)
chitosan 2.5%−carrageenan 2.5%−Bm 5.0%	EPI	69	33	273	8.30	415

[a] Activated with glutaraldehyde, glycidol and epichlorohydrin, at the same immobilization conditions used in Tables 1, 2, and 3, respectively. Immobilization parameters: immobilization yield (I_Y), recovered activity (R_A), apparent activity (A_{app}), half-life ($t_{1/2}$), and stabilization factor (S_F) at 55 °C.

Table 5. Maximum Theoretical Immobilized Chymotrypsin ($^*C_{MAX}$: mg of Lyophilized Enzyme·g_{gel}^{-1}) for Different Support Activation Processes[a]

support	activating agent	$^*C_{MAX}$	R_A(%)	A_{theor}(U·g_{gel}^{-1})	A_{app} (U·g_{gel}^{-1})
chitosan 2.5%−alginate 2.5%	glutaraldehyde	42	n.d.	n.d.	n.d.
chitosan 2.5%−alginate 2.5%−Bm 5%	glutaraldehyde	52	14.2	6240	886
chitosan 2.5%−carrageenan 2.5%	glycidol	22	6.0	2640	158
chitosan 2.5%−carrageenan 2.5%−Sc 5.0%	glycidol	21	18.0	2520	454
chitosan 2.5%−carrageenan 2.5%	epichlorohydrin	30	17.0	3600	612
chitosan 2.5%−carrageenan 2.5%−Sc 5.0%	epichlorohydrin	40	35.0	4800	1680

[a] R_A, recovered activity; A_{theor}, theoretical activity; A_{app}, apparent activity. A total of 1 mg of lyophilized enzyme·g_{gel}^{-1} corresponds to 120 U·g_{gel}^{-1}.

Table 6. Oxirane and Aldehyde Groups Concentrations for Different Derivatives

activated support	μmol of aldehyde·g_{gel}^{-1}	μmol of oxirane·g_{gel}^{-1}
chitosan 2.5%−alginate 2.5% glutaraldehyde	83 ± 1.0	
chitosan 2.5%−carrageenan 2.5%−Sc 5% glycidol	49 ± 5.0	
chitosan 2.5%−carrageenan 2.5%−Sc 5% epichlorohydrin	66 ± 0.5	68 ± 2.5

glycidol, or epichlorohydrin. The addition of *Saccharomyces cerevisiae* or *Bacillus megaterium* in two concentrations was tested. Table 4 shows the obtained results. It was expected that the presence of cells could promote a significant improvement in the intraparticle conditions, increasing the support porosity and allowing the attachment of a higher number of enzyme molecules to the activated support. However, for the activation with glutaraldehyde, the immobilization parameters were not affected as expected. The high reactivity of this activating agent may be responsible for these poor results. Side reactions may form hydrophobic side products, which make it more difficult to achieve the correct alignment of the reactive groups and, consequently, decrease enzyme stabilization.[32] Results from Table 4 also indicate that, using glycidol for support activation, the immobilization parameters were affected using cells, as expected. The best results were obtained for chitosan−carrageenan−Sc 5.0%, which presented 72% immobilization yield, 31% recovered activity, and stability factor of 442 compared to free enzyme at 55 °C. *Saccharomyces cerevisiae* led to better results than *Bacillus megaterium*. Although being yeast and bacterium, respectively, the tested microorganisms have similar sizes: *S. cerevisiae* with around 40 μm^3 of volume[33] and *B. megaterium* around 60 μm^3 of volume.[34] Therefore, differences between the cell wall compositions of these two microorganisms may explain these results. *B. megaterium* is a large gram-positive bacterium and its cellular wall is mainly composed by a thick teichoic acid−peptidoglycan complex without lipids,[35] with the thickness between 24 and 37 nm.[36] In *S. cerevisiae*, the cell wall contains β(1→3)-D-glucan, β(1→6)-D-glucan, chitin, and mannoprotein(s). Kolla′r et al.[37] studied the cell wall of *S. cerevisiae* and reported that the polysaccharides appear to have a structural function, whereas the mannoprotein(s) may act as a "filler" and are important for the permeability of the cell wall. It seems that the cell lyses of *S. cerevisiae* were more efficient due to its different structure, leading to a better internal gel structure. Yet, although the gel was treated and washed, part of the large biomolecules may be retained inside of the gel, thus contributing with hydroxyl groups able to be activated with glycidol.

The best enzyme stabilization results with the addition of cells were obtained for activation with epichlorohydrin. It is observed that the best biocatalyst was chitosan 2.5%−carrageenan 2.5%−Sc 5.0%, which presented 91 and 57% of immobilization and recovered activity, respectively, being 1335-fold more stable than soluble enzyme at 55 °C.

Influence of the Activation Degree when Using Epoxides as Activating Agents. Cell debris may have increased the concentration of polymers susceptible to be activated with epoxides. The good results obtained for derivatives prepared with epoxide activation led us to study the activation conditions with glycidol and epichlorohydrin. When the support chitosan 2.5%−carrageenan 2.5%−Sc 5.0% was used, different glycidol and sodium periodate concentrations were tested, offering a high enzyme load (50 mg of enzyme·g_{gel}^{-1}) for immobilization.

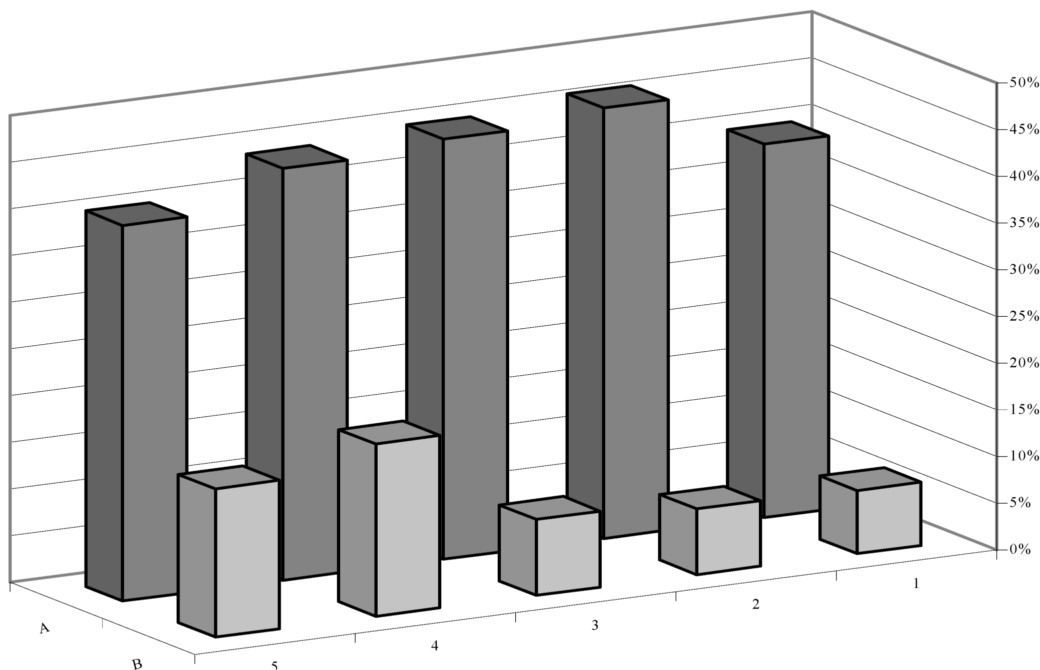

Figure 3. Influence of cell lysis treatment on immobilization yield (A) and recovered activity (B) for chymotrypsin immobilization on chitosan 2.5%−carrageenan 2.5% and activation with glycidol, in the following cases: (1) without cells; (2) support with cells without treatment; (3) SDS treatment before activation of support; (4) SDS and alcalase treatment before activation of support; (5) cell lysis and treatment after support activation. Offered enzyme load (50 mg lyophilized enzyme$\cdot g_{gel}^{-1}$, 6000 U$\cdot g_{gel}^{-1}$).

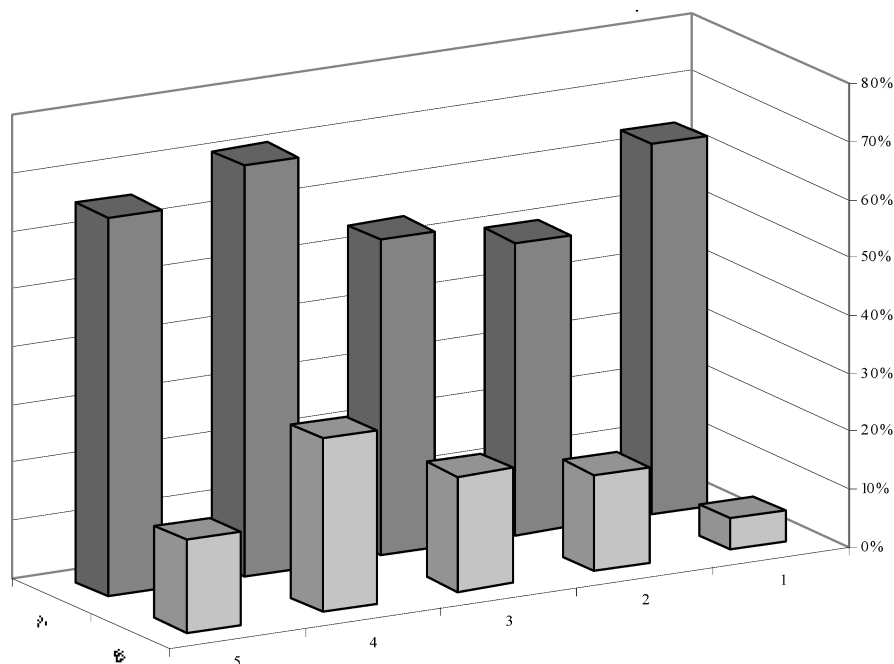

Figure 4. Influence of cell lysis treatment on immobilization yield (A) and recovered activity (B) for chymotrypsin immobilization on chitosan 2.5%−carrageenan 2.5% and activation with epichlorohydrin, in the following cases: (1) without cells; (2) support with cells without treatment; (3) SDS treatment before activation of support; (4) SDS and alcalase treatment before activation of support; (5) cell lysis and treatment after support activation. Offered enzyme load (50 mg lyophilized enzyme$\cdot g_{gel}^{-1}$, 6000 U$\cdot g_{gel}^{-1}$).

Glycidol ranged from 0.48 to 0.96 mL$\cdot g^{-1}$ of gel and periodate from 2 to 4 mL$\cdot g^{-1}$ of gel. Immobilization yields varied from 44 to 46% and recovered activities from 19 to 22%, showing that the conditions that were being used in this work were already adequate. Next, different concentrations of epichlorohydrin and $NaIO_4$ 0.1 M, during the steps of activation and oxidation, respectively (1−3 mL$\cdot g_{gel}^{-1}$), were tested. For epichlorohydrin, concentrations above 2 mL$\cdot g_{gel}^{-1}$, which had been used, did not improve significantly the immobilization parameters. Periodate concentrations above 2 mL$\cdot g_{gel}^{-1}$ led to mechanically fragile gels and should not be used.

Maximum Theoretical Amount of Immobilized Enzyme. The maximum enzyme load is an important property of the support. Assays of maximum theoretical amount of immobilized chymotrypsin were performed for the best derivatives obtained by activation with glutaraldehyde, glycidol, and epichlorohydrin. In Table 5, it can be observed that the derivative with the highest amount of immobilized protein is chitosan 2.5%−alginate

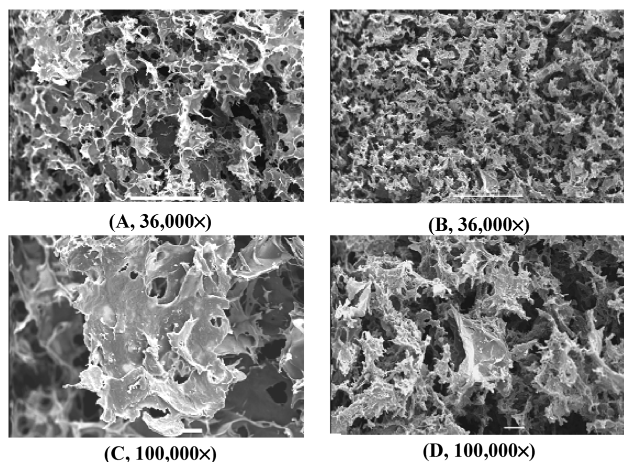

Figure 5. Scanning electron microscopy (SEM) of fractured surface of chitosan 2.5%−carrageenan 2.5% (A, C) and chitosan 2.5%−carrageenan 2.5%−Sc 5% (B, D) with magnification of 36000× (A, B) and 100000× (C, D).

2.5%−Bm 5% (glutaraldehyde), with 52 mg of enzyme·g^{-1} of bead. The derivative that presented the highest stability factor, chitosan 2.5%−carrageenan 2.5%−Sc 5.0%−epichlorohydrin, reached an enzyme load of 40 mg of enzyme·g^{-1} of bead. The derivative obtained by activation with glycidol reached the lowest load, around 20 mg of enzyme·g^{-1} of bead. The number of reactive groups generated in the support using the three different protocols was determined and the obtained results are presented in Table 6. As it can be observed comparing Tables 5 and 6, the obtained maximum enzyme loads, as well the highest stability factors (Tables 1–3), agree with the number of reactive groups (aldehyde and oxirane) available to link to the enzyme in each support. As it can be seen, glutaraldehyde generates the highest concentration of aldehyde groups when compared to the other activating agents. However, the high reactivity of glutaraldehyde makes the immobilization process hard to control and causes the formation of subproducts. In consequence, lower stabilization factors are obtained with this aldehyde. On the other hand, activation with epichlorohydrin generates not only high aldehyde concentrations but also

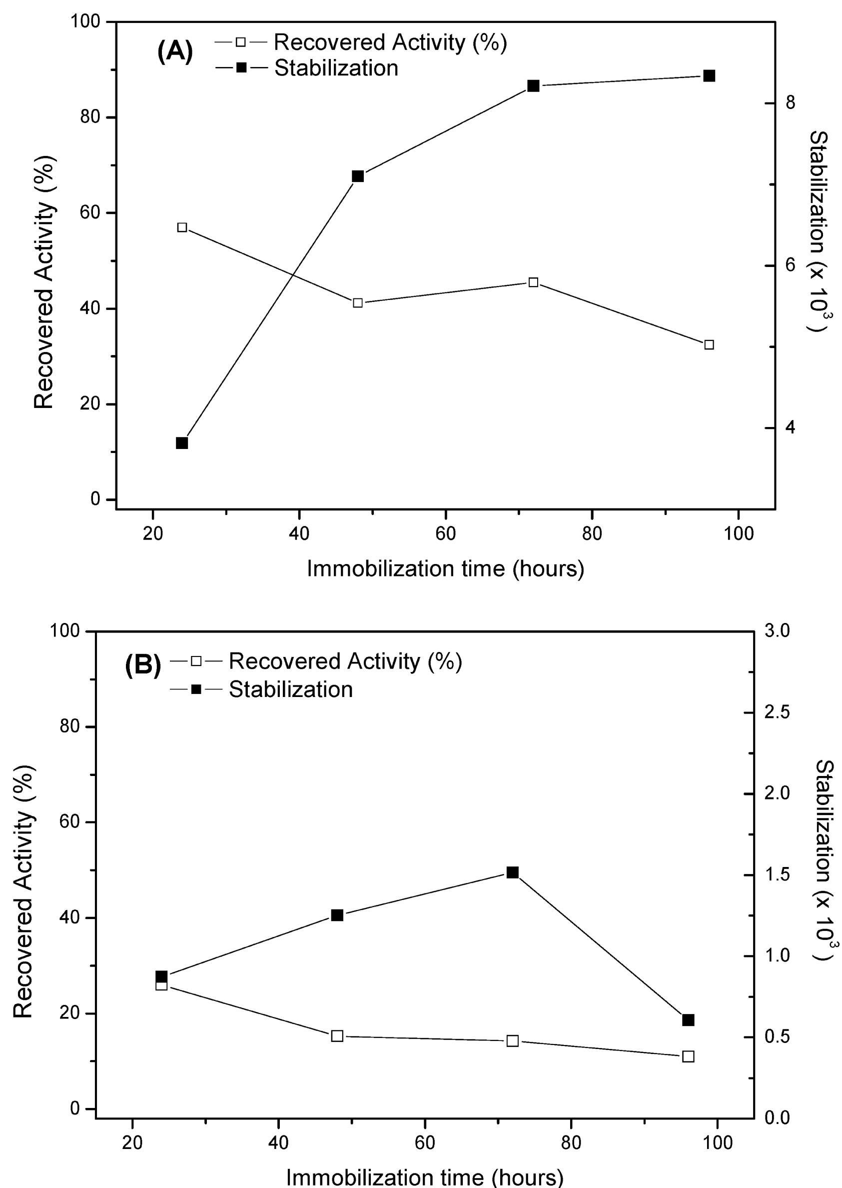

Figure 6. Influence of the immobilization time on the thermal stability and recovered activity of chymotrypsin derivatives. Half-lives ($t_{1/2}$) determined at 65 °C, pH 8.0. Offered enzyme load: 5 mg of lyophilized enzyme·g_{gel}^{-1}. Chitosan 2.5%−carrageenan 2.5%−Sc 5% epichlorohydrin (A) and chitosan 2.5%−carrageenan 2.5%−Sc 5% glycidol (B).

Table 7. Influence of the Reduction with $NaBH_4$ on the Immobilization Parameters[a]

biocatalyst	I_Y (%)	R_A (%)	$A_{app}U \cdot g_{gel}^{-1}$	$t_{1/2}$ (h) at 65 °C	S_F
reference derivative[b]	100	12	72	0.19	240[c]
not reduced	100	45	270	6.50	8211[d]
reduced	100	34	204	7.80 ± 0.5	9853 ± 631[d]

[a] Chitosan 2.5%−carrageenan 2.5%−Sc 5.0%, epichlorohydrin activated. Enzyme load: 5 mg of lyophilized enzyme$\cdot g_{gel}^{-1}$ (600 U$\cdot g_{gel}^{-1}$). [b] Chitosan 2.5%−alginate 2.5% glutaraldehyde at pH 10.05. [c] Stabilization factor of reference derivative at 55 °C. [d] ($t_{1/2}$ of derivative at 65 °C/$t_{1/2}$ of reference at 65 °C) × stabilization factor of reference derivative at 55 °C.

generates oxirane reactive groups, which explain the good results obtained with this epoxide activating reactant.

Influence of Cell Lysis Treatments. The addition of cells during gel formation, followed by cell lysis, had the purpose of obtaining a more open gel structure. The support was submitted to a sequence of different enzymatic and chemical treatments to eliminate as much as possible of the cell contents. The obtained results are presented in Figures 3 and 4 for the supports activated with glycidol and epichlorohydrin, respectively. The best chymotrypsin derivative obtained in this work (chitosan 2.5%−carrageenan 2.5%−Sc 5.0%) was for an offered enzyme load of 50 mg of chymotrypsin$\cdot g^{-1}$ of bead. Figure 3 shows the influence of different protocols on the immobilization parameters of derivatives obtained after chymotrypsin immobilization on supports activated with glycidol. It can be seen that the immobilization yield changed only about 7% for all the conditions tested, not a very expressive effect. On the other hand, the recovered activity improved after the use of SDS and Alcalase, which indicates that the presence of cells during gel formation, with subsequent removal of debris, can improve characteristics of the gel pore surfaces.

Results of Figure 4 show that the addition of cells and removal of debris in supports activated with epichlorohydrin improved not only the recovered activity but also the immobilization yield. This may be a consequence of the higher number of reactive groups in the support when compared to activation with glycidol. However, it can be observed that the treatment is important mostly to improve the recovery activity of the immobilized enzyme. It was already shown that the thermal stability increased with the addition/removal of baker's yeast for activation with glycidol and epichlorohydrin. When the latter one was used, the effect is more pronounced due to the higher reactivity of this activating agent, forming more bonds between enzyme and support.

To simplify the procedure for removal of cellular contents, another methodology was tested. The gel with cells was submitted to a cheaper, faster, and easier treatment using commercial powder soap at pH 9.0 and 40 °C for 24 h, followed by abundant washing with distilled water and, after, with Milli-Q water, before the activation reaction. The resulting immobilization parameters were very similar to the ones showed in Figures 3 and 4, and the new procedure was used in the rest of this work.

To observe the modification of the gel structure after addition of cells and removal of debris, chitosan 2.5%−carrageenan 2.5% hybrid gels, prepared in the absence and presence of cells (followed by cell debris removal) were submitted to SEM (scanning electron microscopy). Figure 5 shows pictures without (A, C) and with addition of cells and removal of debris (B, D). From these pictures, it can be observed that the pores of the material are quite irregular in form and size. It would be very difficult to estimate an average pore size with statistical significance. On the other hand, it can be observed that the presence of cells during gel formation, followed by the removal of debris, led to a clear change in the gel structure. The gels prepared in the absence of cells (A, C) presented a lower available internal surface than the gels prepared with cells (B, D), which agrees with the better immobilization parameters obtained with the latter ones.

Influence of Immobilization Time. The multi-interaction between enzyme and support is a slow process. The longer the immobilization time, the higher the number of covalent bonds formed and, consequently, a higher thermal stability of the derivative is expected. The derivatives obtained by immobilization on supports activated with epoxide reactants, at pH 10.05, are able to make multi-interactions, because at this pH there is a significant number of lysine amine groups available to react with the aldehyde groups of the support. All epoxide derivatives previously presented were prepared using 24 h of contact time between enzyme and activated support. Results of new assays to study the influence of immobilization time are in Figure 6. As expected, for the same derivative higher thermal stabilities and lower recovered activities were observed when the immobilization time was extended. Derivatives obtained using the two tested activation methodologies presented this same behavior. Nevertheless, the influence of the immobilization time was much more significant for activation with epichlorohydrin. When this activation was used, a derivative 8000-fold more stable than the soluble enzyme was obtained for an immobilization time of 72 h.

The influence of the immobilization time on the derivative stability its correlation with the number of bonds formed between enzyme and support was already studied for trypsin[38] and lipase[13] and the obtained results agreed with the ones presented in Figure 6 for chymotrypsin. Our results also show that the derivative chitosan 2.5%−carrageenan 2.5%−Sc 5.0%, activated with epichlorohydrin, has higher stability (Figure 6) and recovered activity than the derivative obtained using activation with glycidol. These are indications that the former derivative has more bonds with the enzyme than the latter one. This fact may also explain the observed decrease of the recovered activity; because no substrate or enzyme inhibitor was present during the immobilization process to protect the active site of the enzyme, the formation of several bonds with the support may have distorted the active site of the enzyme.

The results presented in Figure 6 also indicate that 72 h is the most adequate reaction time because incubation for longer

Table 8. Amino Acid Analysis of the Hydrolysates of Soluble and Immobilized Chymotrypsin

derivatives	amino acid content (±σ), relative to the soluble enzyme				
	Asp	Glu	Pro	Phe	Lys
soluble chymotrypsin	1.00 ± 0.10	1.00 ± 0.06	1.00 ± 0.02	1.00 ± 0.10	1.00 ± 0.10
chitosan 2.5%−alginate 2.5% glutaraldehyde at pH 7.0	0.84 ± 0.10	0.89 ± 0.06	1.00 ± 0.02	1.00 ± 0.10	0.90 ± 0.10
chitosan 2.5%−alginate 2.5% glutaraldehyde at pH 10.05	0.88 ± 0.10	0.75 ± 0.06	1.00 ± 0.02	1.20 ± 0.10	0.65 ± 0.10
chitosan 2.5%−carrageenan 2.5%−Sc 5% glycidol	0.93 ± 0.10	0.93 ± 0.06	1.00 ± 0.02	0.94 ± 0.10	0.64 ± 0.10
chitosan 2.5%−carrageenan 2.5%−Sc 5% epichlorohydrin	1.10 ± 0.10	0.95 ± 0.06	1.00 ± 0.02	1.00 ± 0.10	0.45 ± 0.10

times neither improves the thermal stability nor increases the recovered activity.

Finally, the best derivative was reduced with $NaBH_4$ to verify its influence on the immobilization parameters (Table 7).

$NaBH_4$ reduction decreased the residual activity of the immobilized enzyme. On the other hand, the reduction reaction increased the stabilization factor. Other authors have also reported loss of derivative activity after reduction with $NaBH_4$, which improved the derivatives stability, nonetheless.[8,21]

Amino Acid Analysis of Soluble and Immobilized Chymotrypsin. Table 8 shows the results of the amino acid analysis of the hydrolysates of soluble chymotrypsin and of some chymotrypsin derivatives. Lysine contents changes according to the activation and immobilization condition employed. The chitosan 2.5%−carrageenan 2.5%−Sc 5.0% epichlorohydrin, the most stable derivative, presented the higher percentage of lysine residues involved in the covalent enzyme-support attachments, approximately 55% of the total.

This is a clear indication that the increase in enzyme stability was correlated to the formation of multipoint covalent linkages between enzyme and support. Because chymotrypsin has 14 lysine residues per molecule and the enzyme used in this work was pure, it is believed that 7−8 lysine residues per molecule of enzyme were linked to the support. The derivatives obtained at pH 7.0 and 10.05 with glutaraldehyde had one and five lysine residues per molecule of enzyme linked to the support, respectively, while chitosan 2.5%−carrageenan 2.5%−Sc 5.0% glycidol presented five lysine residues per molecule. The results obtained for the glutaraldehyde-activated derivatives confirm the importance of the pH during immobilization: at pH 10.05, an intense multipoint attachment is favored when compared to pH 7.0. The results for glycidol- and epichlorohydrin-activated derivatives shown in Table 8 agree with the determined number of reactive groups generated in these supports (Table 6). When epichlorohydrin is used, a higher number of reactive groups was generated in the support. In consequence, more residues of the enzyme were involved in the multipoint attachment, which led to a higher stability.

Conclusions

In this work, a very significant improvement in thermal stability of chymotrypsin was achieved after the covalent attachment of the enzyme on chitosan. The half-life of immobilized chymotrypsin could be increased from 0.57 h, at 55 °C, to 7.8 h, at 65 °C. The less stable derivative was obtained using pure chitosan 2.5% activated with glutaraldehyde at pH 7.0, 4 °C, for 2 h, without reduction of the final derivative with sodium borohydride. The more stable chymotrypsin was immobilized on a hybrid gel, chitosan 2.5%−carrageenan 2.5%, with addition of 5% of *S. cerevisiae*, followed by the removal of cell debris after the formation of the gel, activation of the support with epichlorohydrin, immobilization for 72 h, at pH 10.05 and room temperature, and with reduction of the final derivative. This best derivative was 9900-fold more stable than the soluble enzyme, with a maximum load of 40 mg chymotrypsin$\cdot g^{-1}$ beads. The determination of the number of aldehyde and oxirane groups generated in the support and of the lysine groups of the enzyme involved in the multipoint attachment, as well as analysis of SEM images of the gel structures, agrees with the obtained results.

Acknowledgment. The authors would like thanking the financial support of the Brazilian Research Agencies CNPq, CAPES, FINEP, and FAPESP.

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BM8002754