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Polyelectrolyte complex formation mediated immobilization of chitosan-invertase neoglycoconjugate on pectin-coated chitin

Abstract *Saccharomyces cerevisiae* invertase, chemically modified with chitosan, was immobilized on pectin-coated chitin support *via* polyelectrolyte complex formation. The yield of immobilized enzyme protein was determined as 85% and the immobilized biocatalyst retained 97% of the initial chitosan-invertase activity. The optimum temperature for invertase was increased by 10 °C and its thermostability was enhanced by about 10 °C after immobilization. The immobilized enzyme was stable against incubation in high ionic strength solutions and was 4-fold more resistant to thermal treatment at 65 °C than the native counterpart. The biocatalyst prepared retained 96 and 95% of the original catalytic activity after ten cycles of reuse and 74 h of continuous operational regime in a packed bed reactor, respectively.

Keywords Invertase · Chitin · Enzyme immobilization · Enzyme stability · Polyelectrolyte complex

Introduction

Nowadays, the development of novel procedures for preparing stable enzyme forms received considerable attention in order to design more economic and efficient production processes catalyzed for these biomolecules. In this context, the attachment of carbohydrate moieties to the protein surface constitutes a successful approach for stabilizing enzymes [1–3]. These neoglycoenzymes are able to work in homogeneous aqueous solutions under extreme physicochemical conditions, [3–5] but show several technological disadvantages when comparing with those immobilized on solid supports. In fact, insolubility of immobilized enzymes allows for their (1) multiple reuse, (2) easy separation from the reaction media, (3) controlled product formation, (4) continuous operation of enzymatic processes, (5) rapid termination of reactions, and (6) greater variety of engineering designs [6].

Immobilization of enzymes on charged supports via electrostatic interactions constitutes a common and not expensive approach for preparing industrial biocatalyst [7–9]. This immobilization method also allows the reuse of the matrix [10]. However, the electrostatic immobilization of enzymes on commercial supports is mediated by weak forces and the proteins trend to be desorbed under operational conditions [11]. For this reason, it is necessary to design novel approaches for immobilizing enzymes on charged solid supports via electrostatic interactions. In order to provide a major electrostatic attraction for improving the technological disadvantages previously mentioned, in this paper we propose a combined stabilization–immobilization strategy for enzymes. It is based on the previous modification of invertase (EC 3.2.1.26) with chitosan [12], a positive-charged polymer, and its further immobilization on a support coated with the anionic polysaccharide pectin. Covalent coupling with chitosan has been previously reported as a successful strategy for preparing stable enzyme derivatives [12, 13].

As target enzyme for the present work we selected invertase, enzyme widely employed for producing edulcorant syrups at industrial levels [14].

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Experimental section

Materials

Invertase from *S. cerevisiae* (1840 U/mg) and pectin from citrus fruits were purchased from Fluka (Buchs,

Switzerland). Analytical data for pectin were: molecular weight = 1.03×10^5 [15], degree of de-esterification = 79% [16]. The purity of the enzyme used was checked by SDS-PAGE. Chitin from lobster shells (degree of de-acetylation = 10% [17], average particle size = 30 μm) was obtained from Empresa Mario Muñoz (Havana, Cuba). Chitosan was prepared by alkali deacetylation of chitin [18]. Analytical data were: molecular weight = 2.1×10^4 [19], degree of deacetylation = 90% [18]. All other chemicals were of analytical grade.

Preparation of chitosan-invertase conjugate [12]

A reaction mixture containing 10 mg of invertase, dissolved in 25 ml of 50 mM sodium acetate buffer, pH 5.0, and 213 mg of sodium metaperiodate was stirred for 30 min in the dark. The reaction was stopped by adding 800 μl of ethylene glycol, and the mixture was left for 2 h. The solution was dialyzed against 2.5 l of 200 mM sodium acetate buffer, pH 5.0 in the dark. The activated enzyme solution was mixed with 40 mg of chitosan, dissolved in 2.0 ml of 3% (v/v) acetic acid, and stirred in the dark for 4 h. A solution of 80 mg of $NaBH_4$, dissolved in 1.0 ml of distilled water, was dropped with continuous stirring and the reaction was left for 4 h.

The conjugated enzyme was obtained in solution after dialysis against 2.5 l of 200 mM sodium acetate buffer, pH 5.0. All procedures described were carried out at 4 °C.

Enzyme immobilization

For preparing the pectin-coated support for invertase immobilization, 600 mg of the anionic polysaccharide was dissolved in 60 ml of potassium phosphate buffer, pH 6.0, and then 150 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) was added. The solution was stirred for 1 h at room temperature and further mixed with a suspension of chitin (3 g) in 30 ml of distilled water. The reaction was maintained at 25 °C for 16 h under continuous stirring. The solid was collected by centrifugation, washed several times with distilled water until the carbohydrates were not detected in the wastes, and finally suspended in 90 ml of 50 mM sodium acetate buffer, pH 4.5. The amount of attached pectin was determined by quantification of soluble carbohydrate before and after coupling reaction [20].

For preparing the immobilized biocatalysts, 2 mg of chitosan-modified invertase were added to the solution containing 1 g of pectin-coated support and the mixture (total volume 5 ml) was shaken during 3 h at 4 °C. After the immobilization, the support with the immobilized enzyme was repeatedly washed with 20 mM of sodium acetate buffer, pH 4.5, and the amount of adsorbed enzyme was estimated by difference obtained after measuring the non-immobilized enzyme [21].

SEM spectroscopy

SEM measurements were done with a TESLA BS 343A scanning electron microscope instrument at 15 kV. The gold-coated substrate with a 20-nm-thick layer was mounted on an Al support by using EMS-550, automated sputter coater. All samples were carefully spread on this support and observed by SEM.

Assays

The enzymatic activity of the native, modified, and immobilized invertase forms was determined by adding 100 μl of enzyme solution (or suspension) to 400 μl of 200 mM sucrose in 50 mM sodium acetate buffer, pH 4.6. After 10 min at 37 °C the reaction was stopped by adding 3,5-dinitrosalicylic acid and the reducing sugars were determined as previously described [22]. One unit of activity was defined as the amount of enzyme required to hydrolyze 1.0 μmol of sucrose per minute under the described conditions. Total carbohydrates were determined by the phenol–sulfuric acid method using glucose as standard [20]. Invertase concentration was estimated from $A_{280\ nm}$ (1 mg/ml) = 2.25 [21].

pH optimum

The hydrolytic activity of the enzyme preparations (0.5 U/ml final concentration, corresponding to 100% in the graphic) toward sucrose were measured at 37 °C in 50 mM citric acid/Na_2HPO_4 buffer solution with pH ranging from 2.2 to 6.2.

Temperature optimum

The hydrolytic activity of the enzyme preparations (0.5 U/ml final concentration, corresponding to 100% in the graphic) toward sucrose was measured at different temperatures ranging from 20 to 80 °C. The corresponding values of optimum temperature were calculated from Arrhenius plots.

Thermal stability properties

For determining the thermal stability profile, all invertase forms were incubated at selected temperatures from 30 to 85 °C in 50 mM sodium acetate buffer, pH 4.6 (0.5 U/ml final concentration, corresponding to 100% in the graphic). Aliquots were removed after 10 min of incubation, chilled quickly, and assayed for enzymatic activity. On the other hand, the kinetics of thermal inactivation at 65 °C was determined by incubating the enzyme at this temperature under the same conditions described above. Aliquots were removed at scheduled times, chilled quickly, and assayed for enzymatic activity.

Operational stability and reuse of the immobilized invertase in a packed bed reactor

For determining the operational stability, a solution of sucrose (200 mM) in 50 mM sodium acetate buffer, pH 4.6, was introduced into a column reactor packed with chitin immobilized invertase (length 10 cm, diameter 1.2 cm) at a flow rate of 20 ml/h through the upper inlet part. The reactor was operated under continuous regime during 74 h at 30 °C. The solution leaving the reactor was collected at scheduled times and assayed for invertase activity.

For evaluating the reusability properties of the immobilized biocatalyst, a similar reactor was used and operated during 1 h at 30 °C under the same conditions described above. At the end, the solution leaving the reactor was collected and assayed for invertase activity and protein concentration. The reactor was washed with running buffer solution and kept at 4 °C until next reuse after 1-day storage. The activity of the immobilized enzyme was expressed as a percentage of its residual activity compared to the initial activity in the first cycle.

Results

Pectin was covalently attached to chitin particles through a carbodiimide-catalyzed reaction in order to prepare a matrix suitable for invertase immobilization. According to this reaction, the carboxylate groups from pectin were linked to the amino group located at the surface of the chitin particles through the formation of stable amide bonds. Since only 10% of the monosaccharide units in chitin are deacetylated and are able to react with pectin, it is expected that only few anionic polysaccharide chains can be covalently attached to the solid support. In this sense, the amount of pectin coating the support was estimated to be 30 mg per gram of chitin, as determined by colorimetric quantification of total water-soluble polysaccharide before and after reaction. Pectin-coated chitin was further employed as support for immobilizing invertase, previously modified with chitosan moieties [12]. The overall strategy used for preparing the immobilized biocatalyst is illustrated in Scheme 1.

Optimum parameters for enzyme immobilization were determined by measuring the effects of different experimental conditions on the immobilized enzyme activity. Figure 1a shows the influence of pH on the immobilized enzyme activity in the incubation solutions. Higher catalytic activity was observed for invertase immobilized at pH above 4.5, and further experiments were performed at pH 4.5. The time course of invertase immobilization is shown in Fig. 1b. The degree of immobilized enzyme increased when the time of incubation was increased, reaching maximal value after 2 h. The effect of initial enzyme concentration on the amount of immobilized invertase in the bulk solution was determined by incubating the enzyme with the support during 2 h at pH 4.5 and 4 °C. As is illustrated in Fig. 1c, the immobilized activity increases progressively when the concentration of the invertase-chitosan conjugate in the solution increases, reaching maximal activity at values higher than 0.2 mg/ml of enzyme protein concentration. Consequently, this value of initial invertase-chitosan conjugate concentration was selected as optimum for further experiments.

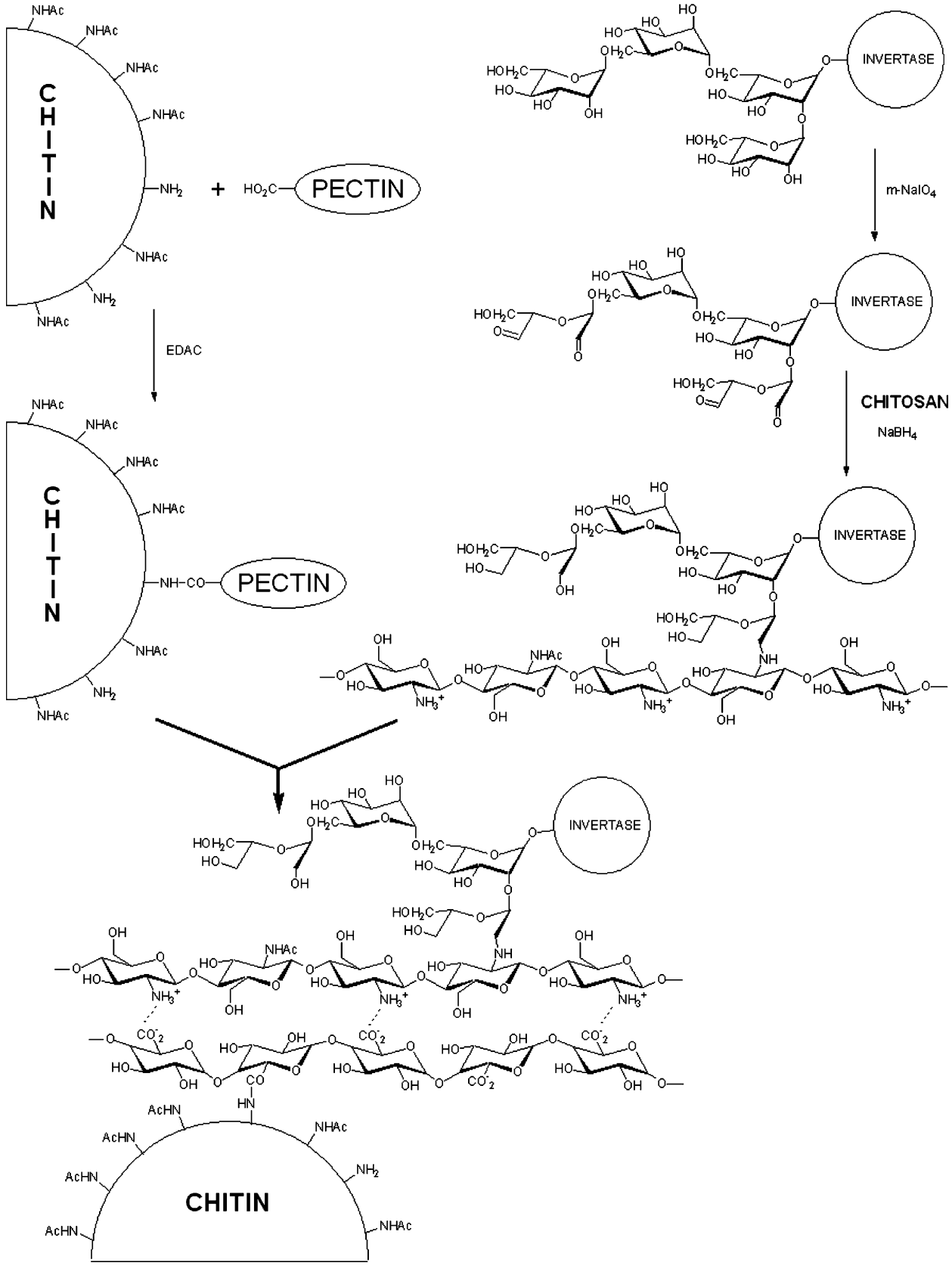
All chitin-based materials were analyzed by SEM. The thin metallic coating, usually applied by sputter coating, is typically 20–30 nm in thickness. The samples were analyzed without further purification. It should be noted that the drying and metal coating processes used in the preparation of some polymeric materials might alter surface morphology, particularly those surfaces that may undergo changes in a hydrated environment; however, it seems this is not the case. The SEM analysis of chitin, chitin-pectin, and chitin-pectin-chitosan-invertase particles allows us to characterize the surface morphology of those systems. As is illustrated in Fig. 2a, chitin shows an irregular appearance in size and form, its particle sizes varied between 10 and 100 μm and shows a compact rough surfaces. Nevertheless, chitin-pectin and chitin-pectin-chitosan-invertase samples show a fibrillar and wrinkle surfaces with the same appearance in size and form, respectively (Fig. 2b, c).

Under optimal conditions (0.2 mg/ml enzyme protein concentration, pH 4.5 and 2 h of reaction time), an average of 1.07 mg of invertase per gram of support was immobilized, representing 54% of the initial amount of incubated enzyme. On the other hand, the enzyme retained 79% of the initial non-modified specific activity, representing 98% of the chitosan-invertase complex activity. It should be noted that the amount of non-modified invertase loaded on the pectin-modified chitin was significantly lower, and this preparation was not further considered in the present study.

The affinity for sucrose was slightly increased in invertase after covalent glycosidation with chitosan: native and modified forms gave apparent K_m values of 15.6 and 12.4 μM, respectively. On the contrary, the affinity for substrate was 2-fold reduced for the immobilized invertase preparation with an apparent K_m value of 30.0 μM.

Figure 3 shows the influence of pH on the activity of native, modified, and immobilized enzyme forms. The optimum range of pH for invertase was slightly increased and shifted to acidic values of pH after modification with chitosan: optimum pH of native enzyme lies in the range of 4.2–4.6, whereas optimum pH of the modified form lies in the range of 3.8–4.6. On the other hand, optimum range of pH for invertase activity was reduced and shifted to lower values of pH after immobilization of the polymer–enzyme complex on chitin-based support, ranging from 3.4 to 4.2.

The temperature-activity profile for all invertase preparations is shown in Fig. 4. The temperature for



Sch. 1 Preparation of the immobilized biocatalyst

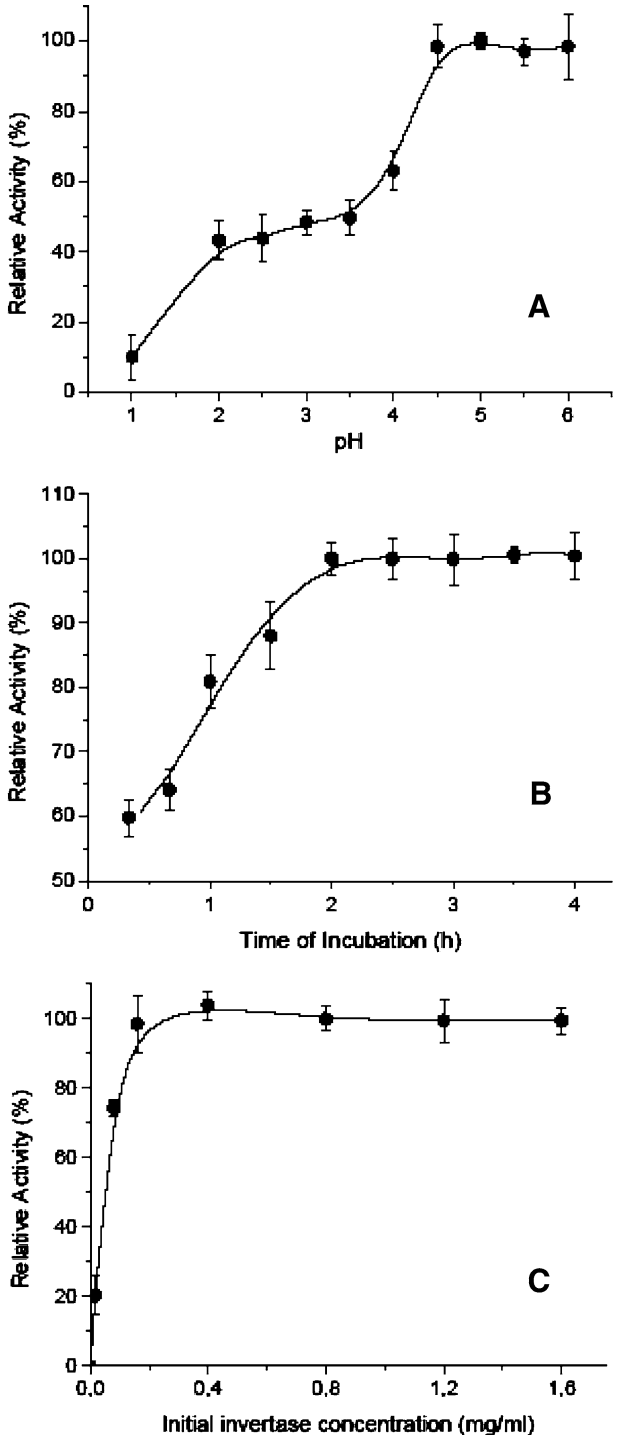


Fig. 1 Influence of pH (**a**), incubation time (**b**), and initial protein concentration (**c**) on the immobilization yield of chitosan-invertase conjugate on pectin-coated chitin support. Data represent the means and standard deviations of three different experiments

maximal rate of sucrose hydrolysis was increased from 55 to 65 °C for the enzyme after covalent modification with chitosan, but optimum temperature was only increased to 5 °C for the immobilized form.

Figure 5 shows the thermal stability profile of all invertase preparations, determined for the activity retained after heating the enzymes at different temperatures during 10 min. The immobilized invertase was significantly more resistant to heat treatment at temperatures higher than 50 °C, in comparison with native counterpart. Consequently, the value of T_{50}, defined as the temperature at which 50% of the initial activity was retained, was increased from 57 to 67 °C for the enzyme after immobilization on the negative-charged support. It should be noted that thermal stabilization showed by the immobilized invertase preparation was lower when compared with the corresponding chitosan-modified enzyme form ($\Delta T_{50} = 17$ °C for the chitosan-invertase conjugate in comparison with native enzyme).

The kinetics of thermal inactivation of all enzyme forms exposed at 65 °C is shown in Fig. 6. All invertase preparations lost activity progressively with time according to a biphasic inactivation kinetics. However, the half-life time of invertase at this temperature was increased from 5 to 87 min after immobilization on the solid support. Interestingly, this thermal stabilization was lower when compared with the corresponding chitosan-invertase complex [12].

The operational stability of immobilized invertase was studied in a packed bed reactor for 74 h under continuous regime at 30 °C. It should be noted in Fig. 7 that the activity of the immobilized enzyme was slightly decreased according to a first-order process, with an operational inactivation rate constant of $k_{opi} = 1.2 \times 10^{-3}$ h^{-1}. Consequently, the half-life time for continuous operational regime of this reactor was estimated about 24 days.

Figure 8 shows the reusability properties of the immobilized biocatalyst in a packed bed reactor, operated at 30 °C and stored at 4 °C between each reuse cycle. As can be observed, immobilized enzyme showed high stability when it is repeatedly used for sucrose hydrolysis, retaining about 96% of its initial activity after ten cycles of reuse.

It should be noted that invertase was not released from the support during both experiment previously described, according to the results achieved by measuring protein concentration in the solutions leaving the reactor. These results support the formation of strong polyelectrolyte interactions between the modified enzyme and the pectin-coated support.

The immobilized biocatalyst was resistant to incubation at 1 M NaCl ionic strength in 50 mM sodium acetate buffer, pH 4.6, retaining full enzymatic activity after 4 h incubation at 30 °C under these conditions. On the other hand, the storage stability of invertase at 37 °C was significantly improved after immobilization on pectin-coated chitin, retaining about 85% of its activity after 50 days of storage in 50 mM sodium acetate buffer, pH 4.6 (Fig. 9).

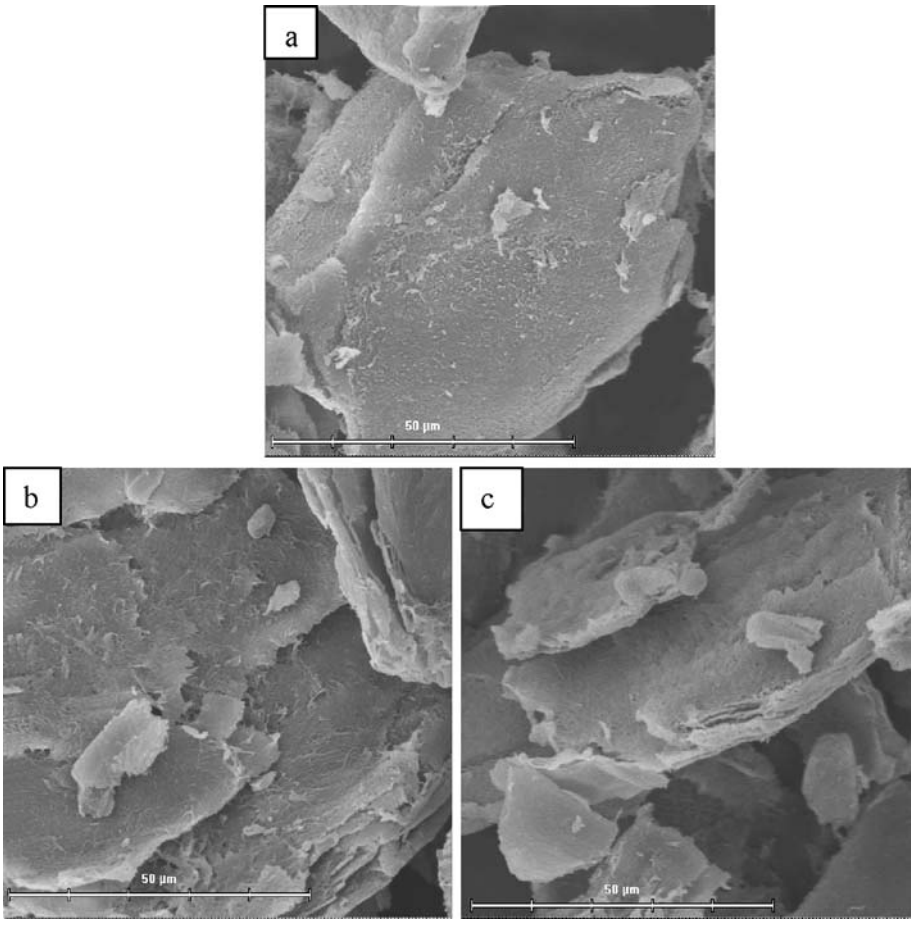


Fig. 2 SEM images of chitin (**a**), chitin-pectin (**b**), and chitin-pectin-chitosan-invertase (**c**)

Discussion

In the present study, chitin was coated with citrus pectin through the formation of amide linkages. This material was further used as support for immobilizing a chitosan-invertase conjugate (Scheme 1). Pectin forms highly stable polyelectrolyte complexes with chitosan, which are only dissociated under very extreme conditions [23]. Through such kind of polyelectrostatic interactions, we prepared an immobilized biocatalyst resistant to high

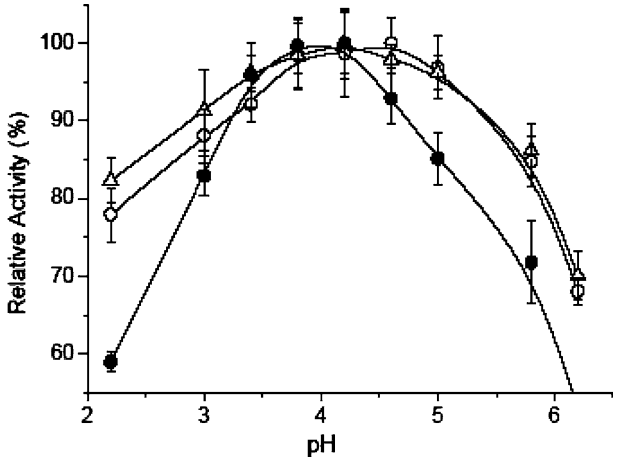


Fig. 3 Optimum pH for the native (*open circle*), modified (*open diamond*), and immobilized (*filled circle*) invertase preparations. Data represent the means and standard deviations of three different experiments

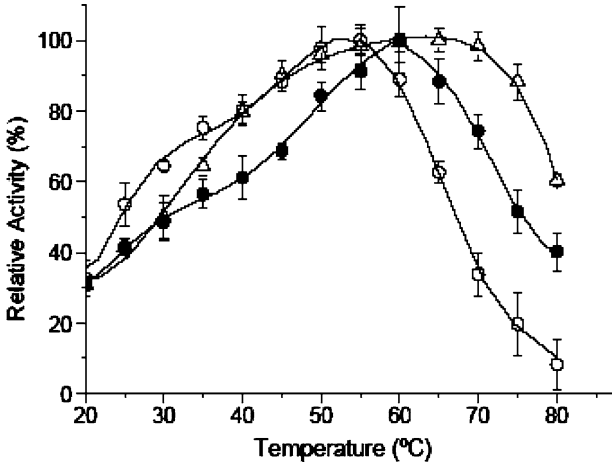


Fig. 4 Optimum temperature for the native (*open circle*), modified (*open diamond*), and immobilized (*filled circle*) invertase preparations. Data represent the means and standard deviations of three different experiments

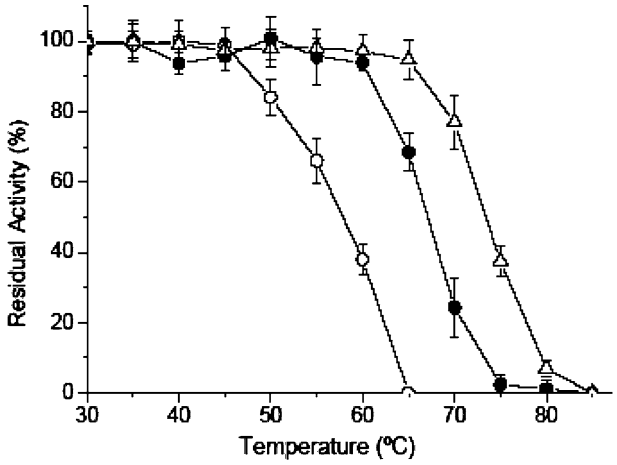


Fig. 5 Thermal stability profile of the native (*open circle*), modified (*open diamond*), and immobilized (*filled circle*) invertase preparations. Data represent the means and standard deviations of three different experiments

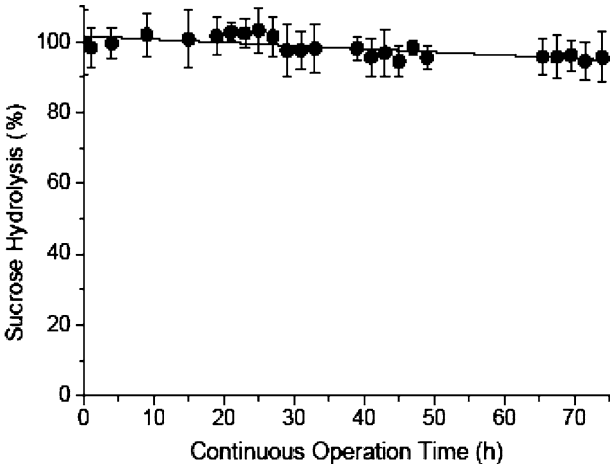


Fig. 7 Operational stability of the immobilized invertase in a packed bed reactor at 30 °C. Data represent the means and standard deviations of three different experiments

ionic concentrations and having excellent storage stability (Fig. 9).

The affinity of the enzyme for sucrose was reduced after immobilization. Diffusional effects, caused by the three-dimensional structure of the support and the pectin chains, may be mainly responsible for the increase in K_m value. This factor should significantly contribute to the lower catalytic activity showed by the immobilized invertase form.

The optimum pH of both the chitosan-modified and the immobilized enzyme forms was shifted to lower values, in comparison with native invertase. This phenomenon could be justified by the attachment of the cationic polyelectrolyte to invertase, in agreement with the general observation that the positive-charged supports displace pH-activity curves of the enzymes attached to them towards acidic pH values [24, 25].

The immobilized enzyme was more resistant to thermal treatment than the native and the chitosan-modified counterparts. The later could be explained considering that the heat resistance showed by invertase-chitosan conjugate was mediated by the contribution of several structural factors; including the formation of intramolecular electrostatic interactions between the positive-charged polysaccharide and the anionic residues at the protein surface [12]. In fact, it was also demonstrated that invertase form stable electrostatic complexes with cationic polymers [26]. In the present study, part of the stabilizing positive charges from the polymer chains are involved in the formation of polyelectrolyte complexes with pectin, then decreasing the thermal stability properties of the immobilized enzyme.

The release of high amount of enzyme under continuous or cyclic operation regime, and consequently the

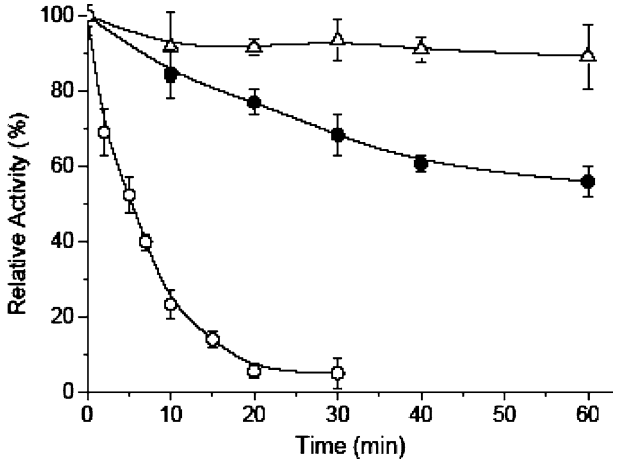


Fig. 6 Kinetics of thermal inactivation of the native (*open circle*), modified (*open diamond*), and immobilized (*filled circle*) invertase preparations at 65 °C. Data represent the means and standard deviations of three different experiments

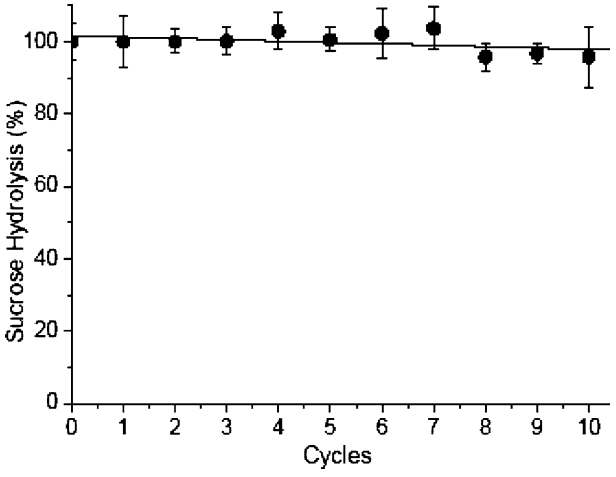


Fig. 8 Cycles of reuse of the immobilized invertase in a packed bed reactor at 30 °C. Data represent the means and standard deviations of three different experiments

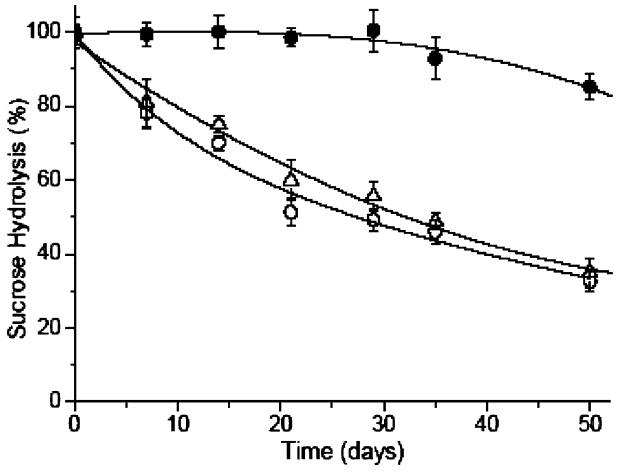


Fig. 9 Storage stability of the native (*open circle*), modified (*open diamond*), and immobilized (*filled circle*) invertase preparations at 37 °C. Data represent the means and standard deviations of three different experiments

reduction of catalytic activity, constitutes one of the most important limitations of the non-covalent immobilization methods for enzymes [6]. In our case, the formation of high stable polyelectrolyte complexes between the positive-charged neoglycoenzyme and the pectin-coated support led to a biocatalyst that was able to retain high catalytic activity under continuous or discontinuous operation regime in a packed bed reactor (Figs. 6, 7).

Conclusions

In the present paper we described a new immobilization method for enzymes chemically modified with ionic polysaccharides, based on the formation of polyelectrolyte complexes with supports coated with opposite-charged polymers. The biocatalyst prepared by loading chitosan-modified invertase on pectin-coated chitin showed excellent thermal, storage and operational stability properties. These results suggest that the electrostatic immobilization approach described might be a useful tool for improving the functional and operational properties of further enzymes. In addition, our strategy constitutes an alternative to other methods used for immobilizing enzyme on polysaccharide-based support, such as covalent attachment to polymeric beads, encapsulation, and physical adsorption.

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