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High operational stability of invertase from *Saccharomyces cerevisiae* immobilized on chitosan nanoparticles

Sheila G. Valerio[a], Joana S. Alves[a], Manuela P. Klein[a], Rafael C. Rodrigues[b], Plinho F. Hertz[a,*]

[a] Enzymology Lab, Institute of Food Science and Technology, Federal University of Rio Grande do Sul, Av. Bento Gonçalves 9500, P.O. Box 15090, ZC 91501-970 Porto Alegre, RS, Brazil
[b] Biocatalysis and Enzyme Technology Lab, Institute of Food Science and Technology, Federal University of Rio Grande do Sul, Av. Bento Gonçalves 9500, P.O. Box 15090, ZC 91501-970 Porto Alegre, RS, Brazil

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ABSTRACT

Invertase (E.C.3.2.1.26) from *Saccharomyces cerevisiae* was covalently immobilized on chitosan nanoparticles prepared by the ionotropic gelation method and activated with glutaraldehyde. The support was characterized and it was studied its load capacity, the influence of the presence of substrate during immobilization, and determined the biocatalyst kinetic parameters and stabilities. The light scattering analysis (LSA) and transmission electron microscopy (TEM) techniques indicated a mixture of chitosan nano and aggregated nanoparticles, providing high superficial area for enzyme immobilization. The thermal and storage stabilities, the optimal pH and temperature of the enzyme were not altered. K_m increased 3-fold, while V_{max} remained unaltered. The immobilized biocatalyst was reused for 59 batches with maximal invertase activity, the highest operational stability so far described in the literature. These results fulfill some important aspects for the enzyme immobilization: the simplicity of the protocols, the conservation of the enzyme activity, and the high operational stability.

1. Introduction

Invertase or β-D-fructofuranosidase (E.C. 3.2.1.26) from *Saccharomyces cerevisiae* is an enzyme that has major specificity for sucrose hydrolysis. This reaction results in an equimolar mixture of α-D-glucose and β-D-fructose, known as invert syrup. As a consequence, the crystallization phenomenon is avoided and, as fructose is sweeter than sucrose, the sweetness of the product is higher. The prevention of crystallization justifies the main food industrial application of the enzyme in the manufacture of fillings of sweets, keeping the softness. Also, invertase can be applied in fermentation process, when sucrose is the substrate, in the manufacture of artificial honey, humectant agent for candies production, besides other applications as in cosmetic, paper and drug industry (Kotwal & Shankar, 2009).

The solubility of free enzymes makes their uses for large-scale relatively costly since in a mixture containing the substrate, products and other components, their recoveries are difficult, being economically unattractive (Kotwal & Shankar, 2009). Nevertheless, biocatalysts are increasingly being employed because of their high selectivity and potential as a greener alternative to chemical catalysts (Polizzi, Bommarius, Broering, & Chaparro-Riggers, 2007), which could result in the formation of undesirable color and flavoring agents.

The interest for enzymatic processes is over growing, which also reflects the great interest for biocatalysts immobilization. Taking into account distinct applications, there are diverse immobilization techniques with different methods of linkages among enzymes and supports. It is noteworthy that each protein and support has typical characteristics, so there is no universal ideal method of immobilization. For example, when the support for the immobilization is solid and non-porous, the size of the particles needs to be as small as possible, considering that nanoparticles provide a reasonable enzyme load capacity (Garcia-Galan, Berenguer-Murcia, Fernandez-Lafuente, & Rodrigues, 2011). In general, the enzyme immobilization aims at keeping or increasing storage, temperature, pH, and operational stabilities.

Chitosan has been used as a support for enzyme immobilization (Klein et al., 2012; Kuo et al., 2012; Li, Cai, Zhong, & Du, 2012; Muzzarelli, 1980; Orrego et al., 2010). Chitosan, isolated from chitin, is the linear and partly acetylated (1–4)-2-amino-2-deoxy-β-D-glucan (Muzzarelli, 1977, 2012; Muzzarelli et al., 2012). Certain chitosan salts are soluble in water, thus they form gels and polyelectrolyte complexes, in particular with proteins (Krajewska, 2004). Invertase was encapsulated in chitosan microbeads (Siso et al., 1997), and immobilized covalently via carbohydrate moiety (Hsieh, Liu, & Liao, 2000). The enzyme was also modified with chitosan and immobilized in sodium alginate-coated chitin support, and in hyaluronic-acid-coated chitin support (Gomez, Ramirez,

* Corresponding author. Tel.: +55 51 3308 7094; fax: +55 51 3308 7048.
E-mail address: plinho@ufrgs.br (P.F. Hertz).

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Cabrera, Simpson, & Villalonga, 2008; Gómez, Ramírez, Villalonga, Hernández, & Villalonga, 2006). Chitosan was also prepared as films and utilized for the biocatalyst immobilization (Teodor, Radu, Dan, & Stanciu, 2006). Biró, Németh, Sisak, Feczkó, and Gyenis (2008) described protocols for the preparation of chitosan support suitable for biocatalysts immobilization, applying to β-galactosidase. The ionotropic gelation method proposed for nanoparticles production, modified from the one described by Berthold, Cremer, and Kreuter (1996), showed the highest enzyme activity, serving as model for the chitosan preparation described in the present study. Recently, it was studied the effect of support size for enzyme immobilization on chitosan (Klein et al., 2012). Macro and nanoparticles of chitosan were prepared and characterized for β-galactosidase immobilization. The authors found a high operational stability for lactose hydrolysis for both preparations.

Thus, the objective of this work was to immobilize the invertase from *S. cerevisiae* on chitosan nanoparticles. The evaluation of the enzyme properties and thermal and operational stability was also studied.

2. Materials and methods

2.1. Materials

Invertase from *S. cerevisiae* (Maxinvert L 10000, batch 409200451) was kindly donated by DSM Food Specialties (The Netherlands). Chitosan from shrimp shells (>75% deacetylated) was purchased from Sigma–Aldrich (St. Louis, USA). Sucrose and all other chemicals (acetic acid, dinitrosalicylic acid, ethylene glycol, glutaraldehyde, magnesium chloride, polysorbate 80, potassium phosphate, sodium and potassium tartrate, sodium chloride, sodium hydroxide and sodium sulfate) were of analytical grade and purchased either from Merck or Fluka (São Paulo, Brazil). All the following tests were performed in duplicate.

2.2. Preparation of chitosan particles

Chitosan support was prepared by ionotropic gelation according to a procedure already described (Berthold et al., 1996) with some modifications: 0.5 mL of sodium sulfate aqueous solution (1.4 M) were added dropwise into 9.5 mL of chitosan (0.25%, w/v) dissolved in 0.35 M acetic acid containing Tween 80 (1%, v/v) under sonication (30 min, 40 kHz, 25 °C). This suspension was magnetically stirred for 2 h (500 rpm) and the particles formed were collected by centrifugation (3500 × *g*, 15 min, 4 °C). The particles obtained were washed with distilled water and activated by suspending them in 10 mL of glutaraldehyde (1.25%, v/v) in 0.1 M phosphate–potassium buffer (pH 7.0) under agitation during 30 min. The glutaraldehyde excess was removed with successive washings using the same buffer.

2.3. Dry weight and support size determination

Support dry weight was performed by lyophilization, after activation with glutaraldehyde. The further immobilization results are expressed as units of enzyme activity by dry mass of support.

Two different techniques were used for support size determination, considering the procedures involved in the preparation of the samples for analysis and the sensitivity of each equipment. Results of the mean particle size before activation were performed using filtered sample (membrane of 45 μm of diameter) in light scattering analysis (LSA) on a Brookhaven Instruments standard setup (BI-200M goniometer, BI-9000AT digital correlator) with a He–Ne laser (k = 632.8 nm) as light source. Using a JEOL JEM 1200ExII transmission electron microscope (JEOL, Tokyo) operating at 120 kV, the transmission electron microscopy (TEM) was done for attainment morphological images of chitosan particles before and after activation. The support was suspended in distilled water and after homogenized with ultrasonic bath for 5 min, the samples were negatively stained with uranyl acetate solution (0.047 M). At room temperature the samples were air-dried.

2.4. Invertase covalent immobilization

Covalent immobilization of invertase on chitosan beads was carried out incubating 0.05 g (dry weight) of the chitosan activated support with 10 mL of each enzyme solution in acetate buffer (0.1 M; pH 4.5) overnight under gentle stirring, at room temperature. Then, the immobilized enzymes were centrifuged (15 min, 4 °C, 3000 × *g*) and washed with solutions of acetate buffer (0.1 M; pH 4.5), sodium chloride (1 M), ethylene glycol (5.38 M), and again with acetate buffer, until activity was no longer detected in the washing solutions. The immobilization yields were calculated using Eq. (1):

$$\mathrm{IY} = \frac{U_i - (U_3 + U_w)}{U_i} 100 \quad (1)$$

where IY is the immobilization yield, U_i is the initial enzyme activity in the solution, U_s the activity in the supernatant after immobilization, and U_w the activity in the washing solutions.

The efficiency of immobilization was defined as:

$$\mathrm{Ef} = \frac{U_i}{\mathrm{IY}} 100 \quad (2)$$

where Ef is the immobilization efficiency, U_i the activity in the immobilized enzyme, and IY the immobilization yield.

Except for free enzyme, all aliquots were submitted to the washing solutions.

2.5. Enzymatic activity assay

Based on a described method (Bryjak, Liesiene, & Stefuca, 2008), activities of free and immobilized enzyme were assayed by the addition of invertase suspension (1 mL of final volume) in acetate buffer (0.1 M; pH 4.5) to 2 mL of sucrose (8% in the same buffer). The samples were incubated during 5 min at 55 °C in a water bath (with agitation for the immobilized derivatives), and ice bath (5 min) was used to stop the reaction. An aliquot of 100 μL was withdrawn and analyzed with 1 mL of DNS reagent by DNS method (Miller, 1959), in order to quantify released reducing sugars by the sucrose hydrolysis. The absorbance was measured at 540 nm and the results were calculated using glucose as standard. The enzyme activity unit (U) was defined as the amount of enzyme liberating 1 μmol of reducing sugars per minute under the assay conditions. All samples were analyzed in duplicate.

2.6. Determination of optimal pH, temperature and kinetic parameters

For the determination of optimal pH and temperature of free and immobilized invertase, each parameter was individually changed (pH from 3.0 to 7.0; temperature from 40 °C to 70 °C). The buffers concentrations were 0.1 M, and except for pH 3 (citrate buffer) and for pH 7 (phosphate buffer), the others measurements were performed with acetate buffer.

The Michaelis–Menten constant (K_m) and V_{max} were calculated under optimal conditions, using Lineweaver–Burk plot.

2.7. Thermal stability

The thermal stability of free and immobilized invertase was measured at 55 °C and 65 °C. Samples were collected periodically