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Invertase stability in alginate beads
Effect of trehalose and chitosan inclusion and of drying methods

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ABSTRACT

The purpose of present work was to analyze the effect of trehalose and chitosan on invertase activity preservation in wet and dried alginate beads, by taking advantage of the well known trehalose protective effect of labile compounds during drying and chitosan improvement on mechanical properties of alginate beads. The beads were subjected to different drying methods (freeze-drying, vacuum and forced air circulation drying) and to a mild thermal treatment (50 °C). The size and morphology of the beads were analyzed by optical microscopy and by scanning electron microscopy. The size of the beads was affected by drying at an extent which depended more on the drying method than on the beads composition. Freeze-drying allowed better maintenance of the diameter and circularity of the beads, which showed smoother surface and smaller pores than those generated by vacuum or air drying. The inclusion of chitosan in the gelation media improved enzymatic activity recovery after beads generation; this fact was attributed to a higher mechanical resistance provided by the interaction between alginate and chitosan. There was no effect of trehalose inclusion during this step. Interactions between alginate and chitosan, as well as the effect of trehalose presence were manifested in the FT-IR spectra, and on the increase of alginate decomposition temperatures as detected by DSC. In the alginate beads the enzymatic activity was not enough protected from the damage caused by drying or by heat treatment. The thermal stability of invertase in the dried beads was independent on the type of drying employed, being mainly affected by beads composition. Although trehalose was not effective to prevent enzyme functionality damage during beads generation, it was essential for achieving adequate invertase protection during freezing, drying (freeze-, vacuum or air drying) and thermal treatment.

1. Introduction

In search of suitable matrices for biomolecule stabilization, delivery and controlled release, ionically crosslinked hydrogels have been thoroughly investigated (Deladino, Anbinder, Navarro, & Martino, 2008; Drush & Mannino, 2009; Han, Guenier, Salmieri, & Monique Lacroix, 2008; Vidhyalakshmi, Bhakyaraj, & Subhasreey, 2009). Alginate is one of the most popular anionic polyelectrolyte used for bead preparation since it is non toxic, biodegradable and biocompatible (Endress, Mattes, & Norz, 2006; Ward & Hanway, 2006). Alginate is widely used in encapsulation applications due to its ability to form gels in the presence of certain divalent cations such as calcium, barium, and strontium by ionotropic gelation (Braccini & Perez, 2001). The immobilization procedure on alginate beads is not only inexpensive but also very easy to carry out and provides extremely mild conditions, so that the potential for industrial application is considerable (Zhou, Li, & Li, 2010). However, some disadvantages are often associated with this carrier, including high biomolecule leakage, low mechanical strength and large pore size (Elnashar, Danial, & Awad, 2009). In order to optimize the encapsulation efficiency and control release of enzymes from the gel matrix, new formulations incorporating chitosan, modified polymers or proteins have been studied (Han et al., 2008; Matto & Husain, 2009; Ortega, Perez-Mateos, Pilar, & Busto, 2009).

Chitosan is a cationic polysaccharide obtained from partial deacetylation of chitin. It forms polyelectrolyte complexes with polyanionic polymers such as alginate (Zhou et al., 2010) and has been used increasingly as a biomimetic material (Ravi Kumar, 2000) as well as excipient in cosmetics (Lang & Clausen, 1989) and in food processing (Roller & Covill, 1999). Alginate beads coated with chitosan were used for encapsulation and release of different proteins (Aral & Akbuga, 1998; Coppi & Iannuccelli, 2009; Kumar, Dwevedi, Arvind, Kayastha, & 138–145, 2009; Mansfeld, Forster, Hoffman, & Schellenberger, 1995; Zhou et al., 2010). The direct reaction between these two biopolymers forms beads with improved mechanical properties (Rha & Rodríguez-Sanchez, 1988). In general, the prolongation of drug release from the beads coated with chitosan was observed, but Polk, Amsden, Yao, Peng, and Goosen (1994) showed that the release rate of bovine serum albumin was accelerated on increasing the chitosan concentration from 0.1% to 0.2% (w/v). These contrary results may be related to the variation in the preparation procedures (Polk et al., 1994), as well as other factors, like

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the properties of the encapsulated biomolecule and/or its interactions with the wall material.

In areas of biotechnology, food and pharmacology, there is an important increase in the use of peptides and proteins based drugs (Zhou & Li Wan Po, 1991). Invertase (β-fructofuranosidase) is an enzyme of high technological impact in the production of invert sugar, and is widely used in the pharmaceutical and food industries (Mansfeld et al., 1995; Tomotani & Vitolo, 2007), and also for the development of analytical devices (Rodríguez, Aguilar, & Pérez Padilla, 2000). For these important applications, alginate beads may help in the production of stable and reusable invertase form, in order to reduce costs and increase the productivity of the overall process.

The conservation of labile proteins in dehydrated or frozen systems has been generally performed in presence of saccharides, being trehalose as one of the most used excipient.

The action of sugars can be ascribed to both kinetic and specific effects. At the kinetic level, they promote the formation of amorphous systems. At the specific-interaction level, sugars and particularly trehalose interact by hydrogen bonding with biological structures, stabilizing them during drying (Crowe, Reid, & Crowe, 1996; Longinotti, Mazzobre, Buera, & Corti, 2002). Several enzymes have been stabilized through immobilization in glassy sugar matrices (Aguilera & Karel, 1997; Crowe et al., 1996; Suzuki, Imamura, Yamamoto, Satoh, & Okazaki, 1997), however, they are not effective to control the release or the delivery of the encapsulated compound. Therefore, the incorporation of sugars to polyelectrolyte beads emerges as an interesting option.

The purpose of present work was to improve the stability of invertase via encapsulation in alginate beads containing trehalose and chitosan. The inclusion of trehalose and chitosan to the calcium gelation media was evaluated on enzyme stability and on the physical characteristics of the beads subjected to different drying methods and to thermal treatment.

2. Materials and methods

2.1. Materials

Sodium alginate (from *Laminaria hyperborean*, molecular mass of 1.97×10^5 Da, mannuronate/guluronate ratio = 0.6) was from BDH, Poole, UK. Chitosan with a degree of amidation 86% and molecular mass of 1.8×10^5 Da was obtained from Cicarelli S.A., Argentine. The enzyme invertase from *Saccharomyces cerevisiae* (β-fructofuranosidase, E.C. 3.2.1.26, 1840 U/mg), molecular mass 270 kDa was from Fluka, Buchs, Switzerland. One enzymatic unit was defined as the amount of enzyme needed to hydrolyze 1.0 μmol of sucrose per minute at pH 4.6 at 37 °C. D-trehalose dihydrate (α-α-trehalose) (T), was from Hayashibara Co, Ltd., Japan.

All other reagents were commercially available and used as received.

2.2. Gel beads preparation

Beads were prepared by ionotropic gelation accordingly to the drop method described elsewhere (Austin, Bower, & Muldoon, 1996). 1% w/v sodium alginate was prepared in 50 mM acetate buffer pH 3.8 and invertase solution was added to a final concentration of 0.0636 mg/mL. Acetate buffer pH 3.8 was selected in order to guarantee an electrostatic interaction between the polymer and the enzyme. Since the isoelectric point of invertase is between 4.0 and 4.5 (Kizilyar, Akbulut, Toppare, Özden, & Yagci, 1999; Schülke & Schmid, 1988), and the pKa values of alginate are 3.38 and 3.65 (Smidsrød, Larsen, Painter, & Haug, 1969), alginate is negatively charged at pH 3.8 and the enzyme is positively charged. A peristaltic pump was used to drop 10 mL of the alginate–enzyme mixture to 100 mL of $CaCl_2$ solution. 2.5% w/v $CaCl_2$ was prepared in acetate buffer (with or without trehalose 20% or chitosan 0.5% w/v). Beads generation was performed in a cold bath under constant stirring, with a needle size 0.25 (diameter) × 6 (length) mm (Novofine 31 G, Novo Nordisk A/S, Bagsvaerd, Denmark); the distance of the needle above the solution was 6 cm and the speed of the pump was 9.0 ± 0.1 rpm. After generation, the beads were hardened 15 min in the $CaCl_2$ solution (Deladino et al., 2008), washed 5 times with cold water and stored in microcentrifuge tubes at 4 °C till the correspondent treatment.

2.3. Loading efficiency of the enzyme in the beads

The amount of enzyme loaded in the beads after generation was determined through the loading efficiency parameter (L.E.) defined in Eq. (1).

$$\text{L.E.} = \frac{L}{L_0}\times 100 \quad (1)$$

where L is the amount of enzyme in the beads and L_0 is the initial amount of enzyme dissolved in the alginate solution. Bradford method (Bradford, 1976) was used to determine the amount of protein in each fraction. For L determination, 15 beads were dissolved in 0.25 mL NaCit 10% w/v during 1 h in constant sink. Protein concentration was normalized for a volume factor and for bead.

2.4. Beads characterization

2.4.1. Digital image analysis

The size and shape evaluation of the beads was carried out by analyzing digital images (Deladino et al., 2008) captured by a digital camera (Canon PowerShot A70 3.2 Mpix, Canon Inc., Malaysia; with zoom fixed in 3.0X) installed on a binocular microscope (magnification 7×, Unitron MS, Unitron Inc., New York, USA). The pictures were analyzed with the free software Image J (http://rsb.info.nih.gov/ij/).

Wet beads were dyed with methylene blue 9% v/v during 15 min in order to increase the contrast between the bead and background, which is critical for the analysis. Dried beads were measured as obtained. Pictures were acquired in RGB, and resolution of 1600 × 1200 pix and without flash. Area, perimeter, Feret's diameter and circularity were analyzed for at least 50 beads (wet systems) or 40 beads (dried systems). The Feret's diameter corresponds to the longest distance between any two points along the bead boundary. Circularity is defined as a value between 0 and 1 indicating how closely the shape of the particle resembles a circle. The effect of treatment (wet or drying) and the effect of composition on Feret's diameter and circularity were analyzed by ANOVA with Bonferroni post test using GraphPad Prism v 5.

The Image J software also allows performing a calibration in length units for obtaining the values of the studied parameters in adequate units (i.e. mm). A picture of a caliper section was used to perform the calibration.

2.4.2. Water content and water activity

The total water content of the beads was determined gravimetrically by difference in weight before and after drying in vacuum oven for 48 h at 96 °C ± 2 °C. These drying conditions were selected in previous studies (Santagapita et al., 2008) and they were adequate to determine water content in the studied systems with a confidence interval of 6% for a 95% certainty. For wet beads, the water content was expressed in wet basis (w.b., amount of water related to the total amount of sample). Instead, for dried beads, the water content was expressed in dry basis (d.b., amount of water related to the dried matter).

Water activity (a_w) was determined by means of an Aqualab instrument (Decagon Devices, Inc, USA). A special sampler holder was used to reduce the number of beads to be placed, and the corresponding calibration curve was performed with salts of known a_w (Greenspan, 1977).