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Immobilization of endo-inulinase on poly-D-lysine coated $CaCO_3$ micro-particles

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ABSTRACT

Inulinase is widely applied to produce high fructose syrup (HFS) which is used in the food and pharmaceutical industries. Numbers of different techniques such as physical adsorption, entrapment, ion exchange and covalent bonding have been used to immobilize enzymes on solid supports. In this study, an efficient technique for inulinase immobilization was developed which consists of adsorbing a cationic polyelectrolyte poly-D-lysine (PDL) on negatively charged calcium carbonate micro-particles ($CaCO_3$-MPLs) and immobilizing inulinase through electrostatic interactions between negatively charged amino acids of inulinase and positively charged [$CaCO_3$/PDL-MPLs] complex. The most effective $CaCO_3$-MPLs which had the smallest diameter were manufactured using ultrasonic spray pyrolysis; a new application of this method to manufacture the enzyme support. This method enabled us to reduce the particle size to 2.9 μm which showed the highest average protein loading capacity of 21.3 ± 0.1 mg or 32.0 ± 1.3 U enzyme per gram of $CaCO_3$-MPLs. Two sets of binding sites are available on the [$CaCO_3$/PDL-MPLs] for inulinase, both resulted in positive cooperation with Hill coefficients of 2.3 and 6.8 for the first and second sets of binding sites, respectively. For the immobilized enzyme, the optimum temperature of activity increased from 50 to 55 °C and immobilization resulted in higher thermal stability. Storage stability of immobilized inulinase increased more than two-fold as demonstrated by half-lives ($t_{1/2}$) which increased from 65.2 to 148 days for the free and immobilized inulinases, respectively.

1. Introduction

Although a variety of supports and methods exist for the immobilization of enzymes, the application of composite nano- and micro-structures for the enzyme immobilization is still in its infancy. Nowadays, these structures have attained more attention due to their multifunctionality and versatility. Here the application of a composite consisting a cationic polyelectrolyte poly-D-lysine (PDL) on negatively charged calcium carbonate micro-particles ($CaCO_3$-MPLs) to immobilize enzyme is reported. $CaCO_3$-MPLs could be easily recovered from the medium which had the practical advantages by reducing downstream processing costs. They are inexpensive, non-toxic, biocompatible and resistant to microbial contamination. It has been reported that binding of the enzyme to this support is also reversible under high salt concentration and the support can be reloaded with the fresh enzyme to be used in further rounds of applications (Sawangpanya, Muangchim, & Phisalaphong, 2010).

High fructose syrup (HFS) is used in food and pharmaceutical industries as a sweetener because of its abundance, ease of dissolution in water and inexpensive nature to manufacture (Lima et al., 2011). Inulinase is used to convert inulin into fructose and fructo-oligosaccharides in industry (Neagu & Bahrim, 2011). Inulin is composed of linear chains of β 2 → 1-D-fructofuranose molecules with a glucose residue at the reducing end. In nature, inulin is found in plant species such as Jerusalem artichoke, dahlia and chicory as a reserve carbohydrate (Danial, Elnashar, & Awad, 2010).

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Inulinases belong to the glycoside hydrolase family 32 (GH32). They are classified as exo-inulinases (β-D-fructan fructohydrolase, EC 3.2.1.80) and endo-inulinases (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7) based on their mode of actions (Basso et al., 2010). Their isoelectric point is 3.85 and they contain two key domains; N-terminal 5-fold β-propeller catalytic domain with four β-sheets and C-terminal β-sandwich domain organized in two β-sheets with five β-strands whose function is not well known (Nagem et al., 2004). N-terminal group is more common among the different inulinases. Several loops are close to the catalytic site and may take part in the substrate binding (Pouyez et al., 2012). Enzymatic hydrolysis of inulin with inulinase can be an efficient and a single step process to produce HFS with 95% purity (Chi, Chi, Zhang, Liu, & Yue, 2009).

Adsorption, encapsulation, entrapment, cross-linking and covalent binding have all been used to immobilize enzymes on solid supports. Each has its own advantages and disadvantages. Adsorption is usually simple and inexpensive and results in the least chemical changes to the enzyme which may affect its activity. It is also possible to remove the deactivated enzyme and reload the support with the fresh enzyme. There are however some limitations, importantly, enzyme leakage from the support. Covalent binding and cross-linking have the advantage of strong irreversible binding of the enzyme to the supporting agent which could prevent the enzyme leaching. This strong binding to the support matrix comes with the risk of reducing enzyme activity, although in many applications this reduction is negligible. However, irreversible cross-linked enzymes could only be used until the enzyme inactivates which may involve using one or more cycles. Then both the support and inactivated enzyme should be discarded (Sassolas, Blum, & Leca-Bouvier, 2012).

Small size supports enhance enzyme activity by increasing enzyme binding capacity due to the increased surface area for binding per support mass and reducing substrate mass transfer resistance to the active site of the enzyme, which is proportional to $1/L^2$, where L is the diffusion path to the active site (Tsang, Yu, Gao, & Tam, 2006). Microparticles have been widely used to immobilize proteins and enzymes (Goradia, Cooney, Hodnett, & Magner, 2005). We report here an inulinase immobilization through electrostatic interactions between negatively charged amino acids of inulinase and positively charged PDL on the surface of [$CaCO_3$/PDL-MPLs] complex. $CaCO_3$-MPLs were synthesized with three different methods which resulted in $CaCO_3$-MPLs with different sizes then, comparisons were made to find the best method of support synthesis method and support size to immobilize inulinase. The optimum temperature, thermal stability, and storage stability of the immobilized inulinase were estimated and compared with the free inulinase.

2. Material and methods

2.1. Chemicals and reagents

Sodium potassium tartrate, endo-inulinase (EC 3.2.1.7) from *Aspergillus niger*, 3,5-dinitrosalicylic acid (DNS) and poly-D-lysine were obtained from Sigma Chemical Company (St. Louis, MO, USA). Inulin (Chicory inulin) was from MP Biomedicals Company (Santa Ana, USA). $CaCl_2$ and K_2CO_3 were obtained from Fisher Chemicals. All other reagents were of analytical grade. Distilled deionized water was used in all the experiments. All the results are the average of at least three independent measurements.

2.2. Preparation of $CaCO_3$-MPLs coated with PDL

$CaCO_3$-MPLs were synthesized using three different methods in order to develop the best support for the enzyme immobilization; these include precipitation of $CaCO_3$, precipitation accompanied by ultrasound treatment and ultrasonic spray pyrolysis, then their properties were investigated.

For this reason, 0.3 M $CaCl_2$ and 0.5 M K_2CO_3 were mixed at ambient temperature while stirring to form an amorphous precipitate. They were then dried in an oven (50 °C) immediately after the formation of precipitate. To produce $CaCO_3$-MPLs with the smaller particle size, sonication was applied in ultrasonic bath for 10 min after forming the precipitate (Gustafsson, Johansson, Barrabinoa, Oden, & Holmberg, 2012; Volodkin, Petrov, Prevot, & Sukhorukov, 2004). Ultrasonic spray pyrolysis was used to develop the smallest size particles, and a solution of calcium nitrate tetra hydrate [$Ca(NO_3)_2 \cdot 4H_2O$] in ethanol (0.25 M) was ultrasonically nebulized (the custom nebulizer base was constructed at the University of Illinois at Urbana-Champaign and contains a 1.65 MHz fixed frequency piezoceramic). In general, spray pyrolysis involves the thermal reactions of aerosols (i.e., solid or liquid droplets suspended in a gas) generated by a nebulizer (e.g. pneumatic, ultrasonic, or electrostatic) carried in a gas flow through a furnace. USP technique is a solution based method wherein high frequency ultrasound is passed through a liquid precursor solution, impinging on a liquid–gas interface, which forms an aerosol of micron-sized liquid droplets. The ultrasonically generated droplets of reaction precursors thus become individual chemical micro reactors as they are carried by gas flow into a heated furnace where reactions occur (Bang, Didenko, Helmich, & Suslick, 2012). A flow of argon carries the droplets through a heated tubular reactor (the tubular furnace tube is made of quartz and it is located inside a pre-heated furnace set at 600 °C during pyrolysis). Solvent evaporation and precursor decomposition occur within the furnace. The final $CaCO_3$ product was collected in four bubblers in a series filled with ethanol solvent (Bang & Suslick, 2010; Messing, Zhang, & Jayanthi, 1993).

The effect of ultrasound treatment was evaluated to understand its likely effects on the particle size of $CaCO_3$-MPLs. Negatively charged $CaCO_3$-MPLs were coated with a layer of positively charged PDL by adding a PDL solution to $CaCO_3$-MPLs. The layer of PDL molecules on the surface of $CaCO_3$-MPLs is responsible for the positive charge that attracts the negatively charged amino acids of inulinase molecules. Loading of inulinases on this support is carried out via electrostatic interactions between these opposite charges (Bolivar & Nidetzky, 2012). The best particle size to support inulinase was selected for additional optimum temperature, storage, thermal and functional stability studies.

2.3. Characterization of the $CaCO_3$-MPLs

$CaCO_3$-MPLs were degassed at 300 °C under vacuum for approximately 3 h to remove any moisture or volatiles within the pores of the material, then exposed to liquid N_2 to measure Brunauer–Emmett–Teller (BET) isotherms. The BET surface area was calculated from the adsorption isotherm of N_2 using a surface analyzer (Quanta Chrome Instruments Nova 2200e, Odelzhausen, Germany). The specific surface area was determined using the multipoint BET method (Coghetto et al., 2012). The particle size distribution was determined by laser diffraction particle size distribution analyzer (LDPSDA) (Partica LA-950, Horiba Ltd, Kyoto, Japan). Isopropyl alcohol was used in order to disperse the samples.

Scanning electron microscopy (SEM) was used to characterize the morphology of the particles. The samples were prepared by putting the micro-particles on the slides then coating them with a thin layer of Au–Pd by magnetron sputtering. Measurements were conducted using a Hitachi instrument (S-4700, Japan, Tokyo) at an operation voltage of 2.0 kV and a working distance of 8 mm (Gustafsson et al., 2012).

Zeta potential of the enzyme and the polyelectrolyte in 50 mM sodium acetate buffer solution at pH 5.4 was determined using the phase analysis light scattering (PALS) mode of a Zeta PALS system (Brookhaven Instruments Co., USA). Electrophoretic mobility (EPM)/Zeta potential of $CaCO_3$-MPLs and [$CaCO_3$/PDL–MPLs] samples were conducted using ZS-90 Zetasizer (Malvern, UK). 1 mM KCl was used to dissolve the $CaCO_3$-MPLs samples. Five