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Immobilization of endo-inulinase on non-porous amino functionalized silica nanoparticles

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ABSTRACT

Endo-inulinase (EC 3.2.1.7) from *Aspergillus niger* as a key enzyme in catalytic hydrolysis of inulin and production of high fructose syrup (HFS) was immobilized on aminated non-porous silica nanoparticles (NPs) with particle sizes of 50, 100 and 200 nm. These particles were synthesized and Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and dynamic light scattering (DLS) were used to characterize them. The immobilization was carried out by three methods including non-covalent adsorption (nC), covalent attachment (C) and cross linking (CL) using glutaraldehyde (GA). The immobilized enzymes were functionally characterized in terms of their optimum temperature, thermal stability and reusability. Results revealed that 50 nm silica NPs had the highest efficiency in immobilizing inulinase. As a result, thermal stability was improved and the activity was counter correlated with the particle size in immobilization products. Results of thermodynamic analysis showed that E_{in}, ΔH° and ΔG° for all the immobilized forms were higher than that of the free enzyme suggesting that a higher level of energy is required to denature the immobilized enzyme, making the immobilized enzymes substantially more stable than the free enzyme. Results of the operational stability analysis showed that the enzyme immobilized via non-covalent adsorption, covalent attachment and cross linking preserved 56.45, 65.45 and 80.75% of their initial activities after 7 cycles of hydrolysis, supporting the fact that the cross linking method is the superior method. The optimum temperature was shifted from 50 °C for the free and non-covalent products to far higher temperatures for the covalent and the cross linking products. As a result, immobilization and brightly cross linking strategy brings about thermal stability and elevates the optimum temperature which is necessary for endoinulinase application in industry.

1. Introduction

Inulinase belongs to glycoside hydrolase family GH32 (1-β-D-fructan hydrolase, EC 3.2.1.7). They have been widely applied as biocatalysts to hydrolyze inulin to produce primarily fructose with a small amount of glucose [1]. Inulin is a reserve carbohydrate in the roots and tubers of Jerusalem artichoke, chicory, dahlia and other sources and is industrially important to produce high fructose syrup (HFS) [2]. It consists of linear chains of D-fructose with a glucose terminal group at the reducing end [3].

Immobilization of enzymes facilitates recovery of products, enables the reutilization of the high value enzyme and makes industrial processing economical [4–6]. Various immobilization strategies have been reported which could be classified as non-covalent (adsorption) and covalent immobilization methods. Covalent immobilization is usually preferred if it retains the enzyme activity by avoiding enzyme leakage from the support. Nanoparticles (NPs) have been widely applied to host enzymes because they offer high surface area to volume ratio which results in higher enzyme loading with low mass transfer resistance [7].

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Glutaraldehyde (GA) activation of amino-functionalized supporting agents is one of the most efficient enzyme immobilization strategies [8] which acts as a cross linker to attach the enzyme to the support by forming Schiff bases [8–10]. In this study we applied noncovalent, covalent and cross linking strategies to prepare, then compare the immobilization products. Non-covalent adsorption of inulinase occurs through interactions like hydrogen bonds, electrostatic forces and hydrophobic interactions [11]. Reducing the size of the supporting material, provides greater surface to volume ratio of particles, and results in much greater loading of the enzyme per unit mass of the support [12]. Among them, nano-sized silica particles have received much attention as efficient host materials for enzyme immobilization [13,14] owing to their uniform shape, chemical and mechanical stabilities, small diffusion limitation and their small size, high surface to volume ratio which increases the enzyme loading per unit mass of the support.

In cross linking immobilization, first layer of enzyme is covalently attached to the supporting agent that could be aggregated in the presence of excess free enzyme using GA treatment. These cross linked immobilized-enzyme aggregates could overcome the limitation in maximum enzyme loading capacity in covalent attachment which is due to the mono layer coverage of the enzyme molecules on the supporting agent and at the same time, could improve the enzyme activity and stability. Kim et al. developed a unique approach for the fabrication of enzyme aggregate coatings on the surfaces of electrospun polymer nanofibers. They coated α-chymotrypsin on nanofiber electrospun and showed that the initial activity of α-chymotrypsin-aggregate-coated nanofibers was nine times higher than nanofibers with just a layer of covalently attached α-chymotrypsin molecules [15].

Kim et al. developed a biocatalytic system using polymer nanofibers coated with trypsin aggregates. First, an initial layer of trypsin covalently attached to the polymer nanofibers, then more trypsin molecules cross linked to the first layer using GA treatment. This process resulted in an increase in trypsin activity compared to covalently attached trypsin which retained a high level of activity after a year of repeated recycling [16].

In this paper, silica NPs with particle sizes of 50, 100 and 200 nm were synthesized and used for immobilization of inulinase by three methods then, comparisons were made to find the best immobilization method to produce HFS.

2. Materials and methods

2.1. Materials

Sodium potassium tartrate, 3,5-dinitrosalicylic acid (DNS), endo-inulinase (EC 3.2.1.7) from *Aspergillus niger*, glutaraldehyde, tetraethylorthosilicate (TEOS), 3-aminopropyltriethoxysilane (APTS) and potassium bromide were from Sigma Chemical Company (St. Louis, MO, USA). Ammonium hydroxide solution and methanol were obtained from Fisher Scientific. Inulin (from chicory) was received from MP Bio-Medicals, LLC Company (Santa Ana, USA). All other chemicals were of analytical grade. Milli-Q water was used in all the experiments.

2.2. Methods

2.2.1. Preparation and characterization of the nonporous silica materials

Uniform silica NPs with controlled sizes of 50, 100 and 200 nm were synthesized in a reaction mixture of Milli-Q water, methanol, ammonium hydroxide (28–30%) to which, 62.5 μl TEOS, was quickly added while stirring (~400 rpm) then left gently under stirring (100 rpm) overnight at room temperature. Size and morphology of the particles were examined using scanning electron microscopy (SEM) (Hitachi S-4700, Japan, Tokyo) at a voltage of 20.0 kV after coating the samples with a thin layer of Au–Pd by magnetron sputtering. Particle size and distribution were measured using dynamic light scattering (DLS) (Brookhaven Instruments Co., USA).

Amino functionalizing of the surface of silica NPs was achieved through introducing the amino groups on the silanols of the silica NPs followed by adding 4 μl APTS and 1 μl TEOS to the reaction mixture while stirring overnight. NPs were then centrifuged (20,000 × *g*, 10 min) and washed twice with 1 ml of anhydrous ethanol and centrifuged (20,000 × *g*, 10 min), then washed twice with 1 ml of Milli-Q water and centrifuged (20,000 × *g*, 10 min) and finally dispersed in 1 ml of Milli-Q water. To prepare 50 nm NPs, 1 ml methanol, 360 μl Milli-Q water and 90 μl ammonium hydroxide was used while for 100 nm NPs: 1 ml methanol, 360 μl Milli-Q water and 120 μl ammonium hydroxide was used and for 200 nm NPs: 1 ml methanol, 270 μl Milli-Q water and 240 μl ammonium hydroxide was used. The whole procedure was scaled up to 5× [17,18]. To modify the primary amino groups of the supporting agents with GA molecules, 10 mg of amino-functionalized silica NPs was incubated with 100 μl of 1% (v:v) GA solution in 50 mM sodium acetate buffer at room temperature under constant stirring for 2 h. Samples were then washed with 50 mM sodium acetate buffer at pH 5.4 and centrifuged (20,000 × *g*, 10 min) to remove excess of GA [11,19,20]. Fourier transform infrared spectroscopy (FTIR)confirmed the amine and the amine-aldehyde functionalization and also inulinase immobilization on the silica NPs using Perkin Elmer LS 55 (Perkin Elmer, Inc., USA) at 4 cm^{-1} resolution in the range of 500–4000 cm^{-1} [11].

2.2.2. Immobilization of enzyme on silica NPs

Three kinds of enzyme immobilization procedures were used to bind inulinase to 50, 100 and 200 nm silica NPs. These are noncovalent adsorption of the enzyme on the surface of non-porous silica NPs, covalent attachment using GA as a cross linking agent and cross linking strategy in which GA is applied in two steps which could result in a higher concentration of enzyme aggregates. First, inulinase was exposed to the aldehyde groups of GA activated amino functionalized silica NPs to form covalent linkages which could act as an anchor for the enzyme aggregates that are cross linked by exposing inulinase to the cross linker that added at the second step.

For non-covalent adsorption, an enzyme solution (100 μl) at 2.5 mg ml^{-1} in 50 mM sodium acetate buffer at pH 5.4 was added to 10 mg of the amino-functionalized silica NPs and stirred for 24 h at 4 °C [10]. The covalent immobilization was performed by mixing 10 mg of GA-activated amino-functionalized silica NPs and 100 μl of enzyme solution at the same concentration and stirring for 24 h at 4 °C. In enzyme cross linking strategy, covalently immobilized preparation was prepared as previously described then, 100 μl of 0.2% (v:v) GA in 50 mM sodium acetate buffer at pH 5.4 was introduced into the mixture and stirred continuously for 10 h at 4 °C [21]. Inulinase-loaded amino-functionalized particles were then recovered by centrifugation (20,000 × *g*, 10 min).

In order to remove trapped non-bound enzymes, the products obtained by centrifugation were rinsed three times with 50 mM sodium acetate buffer solution in each of the applied strategies. The buffer solution for the rinse was collected to determine the loading of inulinase molecules on the supporting agents [22,23]. To verify the covalent bonds as the main attaching forces between the enzyme and the supporting agents, the enzyme loaded particles with different methods of immobilization were incubated with 1 M NaCl and the leakage of an enzyme in the presence of NaCl was evaluated by measuring the activity of the supernatants pertained to each of the inulinase loaded particles [20].