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Inulin hydrolysis by inulinase immobilized covalently on magnetic nanoparticles prepared with wheat gluten hydrolysates

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ABSTRACT

Inulinase can produce a high amount of fructose syrup from inulin in a one-step enzymatic process. Inulinase from *Aspergillus niger* was immobilized covalently on Fe_3O_4 magnetic nanoparticles functionalized with wheat gluten hydrolysates (WGHs). Wheat gluten was enzymatically hydrolyzed by two endopeptidases Alcalase and Neutrase and related nanoparticles were prepared by desolvation method. Magnetite nanoparticles were coated with WGHs nanoparticles and then inulinase was immobilized onto it using glutaraldehyde as crosslinking agent. Parallel studies employing differential scanning calorimetry and field emmision scanning electron microscopy were carried out to observe functional and structural variations in free inulinase during immobilization. Optimum temperature of immobilized inulinase was increased, while, pH and K_m values were decreased compared to free enzyme. Overall, a 12.3 folds rise was detected in enzyme half-life value after Immobilization at 75 °C and enzyme preserved 70% of its initial activity after 12 cycles of hydrolysis with 75% of enzyme loading.

1. Introduction

Microbial inulinases belong to an important class of industrial enzymes that have gained increasing attention in the recent years. A series of microorganisms such as fungi, yeasts, and bacteria can produce inulinases [1]. Inulin represents a source for the production of high fructose syrup through enzymatic hydrolysis by either single exo-inulinase (EC 3.2.1.80; β-D-fructofuranosidase) or cooperative action with endo-inulinase (EC 3.2.1.7; β-fructan fructanohydrolase) [2]. This enzyme is stored in the underground parts of chicory, dahlia, and Jerusalem artichoke [3]. Inulin as a polysaccharide composed of fructose units appears to be an attractive source of fructose syrup. The use of inulinase has made it possible to obtain pure fructose syrup by one-step enzymatic process instead of using acid hydrolysis of the inulin and prevention of difructose anhydrides formation at low pH of process as well as the costly, uneconomical, and multi-stage enzymatic process for starch hydrolysis performed by α-amylase, glucoamylase, pullulanase, and glucose isomerase [4,5]. Inulin has low solubility and high capability to microbial contamination in water at room temperature. Therefore, industrial hydrolysis of inulin needs to be carried out at higher temperatures since it permits the use of higher inulin substrate concentration due to the increased solubility [6]. Thus, inulin would be hydrolyzed more efficiency by thermostable inulinases as inulinolytic enzymes for food and chemical industry usages. There are many reasons for the immobilization of the produced enzymes such as easy separation of the enzyme from the product, reuse of the enzyme from the reaction medium, enhancement of enzyme stability against pH, temperature, solvents, contaminants, and impurities, ideality for multi-enzyme reaction systems, and ease of controlling enzymatic process [7]. Immobilization of enzymes commonly is accomplished by four methods including physical adsorption of enzyme molecules on a support material, entrapment or encapsulation of the enzyme in polymers, covalent binding to a support (that provides more strong, stable and irreversible linkages compared to other methods), and carrier- free immobilization by cross-linked enzyme aggregates (CLEAs or CLECs) procedure [8,9].

Recently, considerable attention has been paid to immobilization of enzymes on nanomaterials such as nanopolymers, nanofibers, and nanoparticles. In fact, reducing the size of the enzyme

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bonded to the carrier increases mass transfer and improve the efficiency of the enzyme. Smaller particles provide a larger surface to enzyme loading per unit mass of the particles [10].

Using magnetic nanoparticles as a carrier provides the possibility of easy and low-cost collecting of enzyme from the reaction medium by the magnetic field. Moreover, surface modification of magnetic nanoparticles with functional groups is a useful strategy for suitable attachment of the enzyme to the carrier, increasing the stability of nanoparticles against oxidation and acidic environments and reducing the accumulation of nanoparticles to each other [11]. Above that, functional groups such as protein amino acid residues (for instance lysine ε-amino groups) provide magnetic nanoparticles with a more reactive surface for enzyme immobilization.

In the present study, magnetic wheat gluten hydrolysates nanoparticles were fabricated using the co-precipitation and desolvation methods and immobilized inulinase on it through covalent binding via surface lysine ε-amino groups by glutaraldehyde as a crosslinking agent. Then, the properties of the immobilized enzyme compared with free form was studied [12].

The hypothesis is that, wheat gluten hydrolysates (WGHs) nanoparticles can produce an effective coating on the surface of the magnetic iron oxide nanoparticles which, on the one hand, prevents aggregation of magnetic nanoparticles and, on the other hand, has been created an ideal environment for enzyme binding to the carrier and immobilization of the inulinase to provide a new method for inulin hydrolysis.

2. Materials and methods

2.1. Materials

Inulinase from *Aspergillus niger* (EC 3.2.1.7 and EC 3.2.1.80) was obtained from Sigma-Aldrich. Glutaraldehyde (25% v/v in water) while sodium-potassium tartrate and 3,5-dinitrosalicylic acid (DNS) were purchased from Merck. Inulin (from Chicory roots) was received from Fluka Company (Fluka, Switzerland). Wheat gluten hydrolysates were produced via enzymatic hydrolysis of wheat gluten by Endopeptidases (Alcalase 2.4 L and Neutrase 0.8 L, provided from Novozymes Company) by Food Science and Technology Lab of Department of Chemical Technologies, Iranian Research Organization for Science and Technology (IROST, Tehran, Iran). All other reagents and solvents used were of analytical grade and obtained from Merck and Sigma-Aldrich.

2.2. Methods

2.2.1. Magnetite nanoparticles (MNPs) preparation

Magnetic nanoparticles were prepared by co-precipitation of Fe^{2+} and Fe^{3+} salts in 2:1 molar ratio under the presence of nitrogen gas. Ferric and ferrous chloride ($FeCl_3{\cdot}6H_2O$ and $FeCl_2{\cdot}4H_2O$) in 2:1 molar ratio were dissolved in deionized water at a pH of 9.5 adjusted by adding 1N sodium hydroxide (NaOH) solution. After stirring (150 rpm) for 30 min, the black precipitation of iron oxide was formed in the solution at room temperature under the presence of N_2 gas. The particles were separated by high-speed centrifuge at 13,000 × *g* for 20 min, washed with deionized water for three times at pH, 8, and dried in hot air oven at 70 °C for 10 h, respectively. FE-SEM analysis was performed for evaluation of the prepared MNPs [13].

2.2.2. Gluten hydrolysates nanoparticles preparation

The wheat gluten hydrolysates (WGHs) nanoparticles were prepared by the desolvation method. Wheat gluten hydrolysates were previously produced by the Department of Food Science and Technology of IROST through the enzymatic hydrolysis of wheat gluten by endopeptidases (Alcalase and Neutrase) in phosphate buffer pH 6.5 at 60 °C for 7 h. The molecular weight of gluten hydrolysates specified by the SDS-PAGE method and mostly was between 35 and 45 KDa. For this reason, WGHs were solubilized in 100 ml of deionized water (0.1-1% w/v) and its pH was adjusted to 10 with 1 M NaOH solution under stirring at 500 rpm. Then, ethanol was added dropwise to WGHs aqueous solution with ethanol/water ratio 7:3 and 0.5% (w/v) of Tween-80 was added to the mixture as a surfactant to stabilize the preparation. Afterward, most of the ethanol was eliminated by evaporation with a rotavapor and its final volume was adjusted to 100 ml. Desolvation method was accomplished by sonication technique (400 Hz, 5 min, 25 ± 5 °C) and then large aggregates were separated by centrifuge at 11337 × g for 15 min. Then, the supernatant containing WGHs particles was dispersed by three sessions of 5 min ultrasonic waves. Next, the resulting WGHs nanoparticles were crosslinked with 197 μl of glutaraldehyde (25% v/v in water) and the stirring was kept for 2 h at a room temperature. The particle size, size distribution, and zeta potential of particles were determined by DLS method. Also, the morphology of nanoparticles was specified by FE-SEM technique [14].

2.2.3. Magnetic nanoparticles functionalization by gluten hydrolysates nanoparticles

Magnetic nanoparticles were coated with gluten hydrolysates nanoparticles by adding 0.06 g of prepared Fe_3O_4 nanoparticles, to 0.05 g of gluten hydrolysates NPs (1.2:1 ratio) with 197 μl of glutaraldehyde (25% (v/v)) into 25 ml sodium phosphate buffer (pH 7.4). The solution was subjected to ultrasonic waves for 5 min and then stirred at room temperature for 24 h [15].

2.2.4. Immobilization of inulinase on Fe_3O_4-Gluten hydrolysates nanoparticles

In this step, 4 ml inulinase enzyme (4.47 mg protein/ml) was added to magnetic gluten hydrolysates nanoparticles solution containing glutaraldehyde and was mixed under a gentle stirring speed at 4 °C for 24 h. The covalently immobilized enzyme was centrifuged and washed three times with phosphate buffer solution [3].

2.2.5. Protein content and inulinase activity assay

Protein content was determined according to Bradford's method using bovine serum albumin (BSA) as the protein standard [16]. The activities of free and immobilized inulinase were estimated according to the dinitrosalicylic acid (DNS) method. Soluble inulin (1% w/v) in sodium acetate buffer (pH 5.5) was used as a substrate. The assay mixture contained 100 μl inulinase, 1000 μl of soluble inulin (1%w/v), and 9000 μl sodium acetate buffer (pH 5.5). The cocktail was hydrolyzed for 0–60 min in a water bath shaker at 38–40 °C. The reaction was terminated by adding 500 μl of DNS reagent to 500 μl of hydrolyzed mixture followed by incubating it at 97–98 °C for 10 min. The activity was determined (μmol min^{-1}) by reading the absorbance intensity at 575 nm using Perkin Elmer, Lambda 25 UV/VIS spectrophotometer against the blank [17].

2.2.6. Effect of pH and temperature on enzyme activity

The optimum temperatures regarding enzymatic activity of free and immobilized inulinases were determined in the range from 35 to 75 °C. The optimum pH for inulinase activity was tested by incubation of enzyme at 40 °C in sodium acetate buffer (pH 5–6) and phosphate buffer (pH 7–8) using inulin suspension (1% w/v) as the substrate. Samples were withdrawn at a determined time and the enzyme activity was determined by DNS method as described previously [18].