Immobilization of inulinase from *Aspergillus niger* on octadecyl substituted nanoporous silica; Inulin hydrolysis in a continuous mode operation

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Abstract

Inulinase hydrolyzes inulin and produce high fructose syrup in a single-step reaction. However, finding the best working conditions and the highest inulinase stability, especially in continuous mode operations are of critical value. Native (endo-inulinase (EC 3.2.1.7)) and chemically modified inulinases (endo-inulinase stabilized with pyridoxal 5'-phosphate /ascorbic acid in a semi-rational chemical modification approach) were immobilized on octadecyl substituted nanoporous silica (with the average pore size of 66 nm) by using dioxane to extend the hydrophobic interactions between inulinase refolding intermediates and the hydrophobic alkyl moieties of the support. Native and modified inulinases revealed their maximum surface hydrophobicity at 40 and 50 % dioxane in water, respectively. Immobilized inulinases (native and modified) showed 7.89 and 3.73 fold increase in their half lives compared to the free native sample., higher reusability compared to free inulinases (native and modified) and also could retain their activities after 10 hydrolysis cycles in a continuous mode of experimental conditions.

Key words: inulinase, immobilization, nano-porous silica

1. Introduction

Inulin is a storage polyfructan found in the underground parts of *Compositae* and *Gramineae* families containing linear (2→1) linkages between fructose residues and a sucrose moiety at the end of its chain (Ronkarta et al., 2007). Due to its low solubility in water at room temperature and its susceptibility to microbial contamination, industrial hydrolysis of inulin should be performed at higher temperatures. (Gill et al., 2006). There are two different subclasses of inulinase: exo-inulinase (EC 3.2.1.80) which hydrolyses the terminal bonds of inulin chain to produce fructose and endo-inulinase (EC 3.2.1.7) which cleaves the internal bonds of inulin to produce fructose and fructooligosaccharide (Cruz et al., 1998; Barthomeuf et al., 1991; Nakamura et al., 1995; Pandey et al., 1995; Krieger & Fontana, 1999). Tertiary structure of inulinase consists of N-terminal catalytic domain with a 5-bladed β -propeller fold and C-terminal domain which folds into a sandwich-like structure. A short polypeptide linker connects these two parts (Nagem et al., 2004; Kim et al., 2005).

Free enzymes have restricted applications in industry because they have restricted operational and storage stability, their recovery or reusability is not possible, they could be irreversibly inactivated through aggregation (Risso et al., 2010; Knubovets et al., 2009) and since there is always a risk of allergic reactions due to final product contamination with proteins, (Vasileva & Godjevargova, 2005). Inulinase hydrolyzes inulin in a single-step reaction and produces high fructose syrup (HFS) which has a wide range of applications in food and pharmaceutical industries. Fructose is a GRAS sweetener, which is sweeter and than sucrose, and has functional properties which could enhance product flavor, color and stability, thus could be easily used instead of sucrose (Rocha et al., 2006). There is a growing interest in producing inulinases with improved thermal stability to ensure appropriate hydrolysis rate and also to

inhibit microbial growth in inulin, so in this research, inulinase modified with pyridoxal 5'-phosphate/ascorbic acid in a semi-rational chemical approach was used which has 8.1°C higher melting temperature and improved thermal stability compared to native inulinase (Torabizadeh et al., 2010). Since high yield processes are preferred in industry, inulinase immobilization seems helpful in multi-usage of the enzyme, and in overcoming the protein-protein interactions and aggregations (Risso et al., 2010; Knubovets et al., 2009). Enzyme immobilization offers higher stability, reusability and makes the continuous application of the enzyme, possible. Enzymes has been immobilized through a wide variety of the strategies (Ettalibi & Baratti, 2001; Rocha et al., 2006; Barranco-Flrido et al. 2001; Uzunova et al., 2002) like adsorptive binding which mainly suffers from leakage, but at the same time is the easiest and the least denturing method (Sassolas et al., 2012). Covalent binding, cross linking using glutaraldehyde (Karimi et al, 2014 a) and electrostatic attachment (Karimi et al, 2014 b) of the inulinase to the carrier have been reported. Using silica nanoparticles as support to immobilize enzymes offers some advantages owing to its high surface area, large pore volume, uniform and tunable pore sizes, outstanding physicochemical constancy, well-regulated shape, adaptable surfaces, nontoxic nature and well biocompatibility (Tourné-Péteilh et al., 2003). Presence of dioxane makes octadecyl groups of silica support, exposed and at the same time inulinase refolding intermediates with extended hydrophobic surfaces forms that could easily adhere to each other through adsorptive forces. However, in this work we reported a novel adsorptive immobilization strategy that was used for the first time to immobilize both native and PLP/Ascorbate modified inulinases, then applying them in a continuous bioconversion of inulin in a fluidized bed mini-bioreactor.

2. Materials and Methods

2.1. Chemicals

Nano porous silica, Trichloro (octadecyl) silane (OTS) and dioxane were purchased from Merck (Darmstadt, FRG). 8-Anilino-1-naphthalen sulfonic acid (ANS), sodium potassium tartrate and 3, 5-Dinitrosalicylic acid (DNS) were prepared from Sigma Chemical Company (St. Louis, MO, USA). Inulin (Chicory), pyridoxal 5'-phosphate (PLP) and inulinase (EC 3.2.1.7) from *Aspergillus niger* were obtained from Fluka (Switzerland). Ascorbic acid was from Acros Organics (New Jersey, USA). All other chemicals were of analytical grade. Acetate sodium buffer (50 mM), pH 5.5 was prepared in 0–60% (v/v) ratios of dioxane to water. All the experiments were repeated three times and the results are the mean value of three experiments.

2.2. Protein assay

Protein content was estimated using the Bradford method (Bradford, 19761) and the bound protein was calculated by subtracting the total loaded protein from the protein recovered in the supernatant and washing solutions (Nabati et al., 2011).

2.3. Inulinase activity assay

Concentration of the liberated sugar after the hydrolysis action by free and immobilized enzymes, was estimated with DNS (3, 5-Dinitrosalicylic acid) method. To prepare solution of 1% DNS; 10 g DNS, 1 g phenol, 0.5 g Na₂SO₃, 10 g NaOH and 1 L of distilled water were mixed and gently stirred until a clear solution. was developed. Reaction mixture contained 400 µL of inulin 0.2% and 100 µL of inulinase (native or modified) in sodium acetate buffer (pH 5.5, 50 mM). The reaction mixture was incubated at 45°C for 30 minutes (Nakamura et al., 1997) then 500 µL of DNS reagent was added and the mixture was heated for 10 minutes in a boiling water bath to stop the reaction. To stabilize the color, 166 µL of sodium potassium tartrate 40%

was added, then the mixture was cooled to the ambient temperature and the color intensities were measured by Shimadzu UV-3100 spectrophotometer (Shimadzu Corp., Kyoto, Japan) in cells with 1-cm path length at 575 nm against the control (control was 400 μ L of inulin 0.2%, 100 μ L of sodium acetate buffer pH 5.5, 50 mM) (Miller, 1959).

Immobilization efficiency was estimated through assaying the protein concentration and/or inulinase activity in the supernatants after 5 minutes of centrifugation at 5000 rpm at room temperature (Heraeus Labofuge 400 R, Germany). The Pellets were washed three times with sodium acetate buffer (pH 5.5, 50 mM) to exclude the trapped enzyme molecules. Specific activities were determined based on the milligram of the protein present in the reaction mixture or adsorbed on the support for the free and immobilized enzyme, respectively (Norouzy et al., 2010). The amount of enzyme which liberates 1 mmol of fructose per minute under the standard assay condition (reaction mixture containing 400 μ L of inulin 0.2% and 100 μ L of native or modified inulinases in sodium acetate buffer pH 5.5, 50 mM and incubation at 45°C) was defined as one unit of inulinase (IU).

2.4. Alkylation of the nanoporous silica

Surface area and the porosity of the nanoporous silica were characterized using Brunauer-Emmett-Teller (BET) method. About 0.5 g of the sample was degassed under vacuum for approximately 2.5 h prior to the adsorption/desorption measurements to remove any moisture or volatiles within the pores of the alkylated nanoporous silica and then exposed to liquid N₂ using a surface analyzer (Quantachrome instruments Nova 2200 e, Odelzhausen, Germany) (Coghetto et al., 2012). For alkylation, 250 mL toluene was dried by agitating with 10 g of anhydrous sodium for 24 hours, followed by distillation. Then, OTS dissolved in the dried toluene, was added to the anhydrous nanoporous silica, and left overnight under refluxing to complete the

alkylation procedure. The alkylation of the support was confirmed using FTIR spectroscopy (Equinox 55, Bruker Company) and IR spectra were recorded using KBr pellets in the range of 4000-400 cm^{-1} . The result was calculated from a total of 18 scans. Thermo gravimetric analysis (TGA)(TA instrument Q50) was carried out to verify the grafted organic groups on the surface of silica nanoparticles in which, temperature increases by time at 10 $^{\circ}\text{C}/\text{min}$. At about 100 $^{\circ}\text{C}$, the initial weight loss occurs which is due to the desorption of physisorbed water, the weight loss at the temperature range of 200-600 $^{\circ}\text{C}$ is due to the burning of organic moieties of the sample. The breakage that occurs at 350 $^{\circ}\text{C}$ indicates the functional groups grafted on the surface of silica nanoparticles (Norouzy et al., 2009; Bahrami et al., 2015).

2.5. PLP/ascorbate modification of inulinase

Endo-inulinase (50 μL ; 0.11 $\text{mg}\cdot\text{mL}^{-1}$) was incubated with 450 μL of PLP (1 mM in 50 mM sodium phosphate buffer, pH 7.5) for 30 min at 25 $^{\circ}\text{C}$ in the dark. Then the enzyme-PLP complex was reduced by ascorbic acid (1 mM in 50 mM phosphate buffer, pH 7.0). The solution was kept at 4 $^{\circ}\text{C}$ for 15 min in the dark. Overnight dialysis was performed (MWCO 12 kDa) against a 50 mM sodium acetate buffer (pH 6.0) at 4 $^{\circ}\text{C}$ (Torabizadeh et al., 2010). Modified inulinase was compared with the native counterpart using intrinsic fluorescence and thermal stability analysis. The intrinsic fluorescence was recorded in the emission wavelength ranging from 280 to 530 nm at a concentration of 1 $\text{mg}\cdot\text{mL}^{-1}$, by excitation at 280 nm using Hitachi spectrofluorimeter (Model MPF-4, Tokyo, Japan). The slit width for both excitation and emission was set at 5 nm. For thermal stability analysis of the modification products, the aliquots of the native and modified inulinases were incubated in 50 mM sodium acetate buffer solution, pH 5.5 in the temperature range of 25-65 $^{\circ}\text{C}$ for 130 min, then their activities were assayed at 37 $^{\circ}\text{C}$ in the

same buffer. Assaying pH was not reproducible for the multiple repeats, since all the assays were done in dioxane containing buffers and adjusting pH was not reliable.

2. 6. Inulinase immobilization using dioxane-induced unfolding/refolding method

Extrinsic fluorescence analysis was performed using spectrofluorimeter in a quartz cuvette with 1 cm path length at 25°C using ANS as an indicative of the hydrophobic pockets at the enzyme concentration of 0.1 mg*ml⁻¹ in sodium acetate buffers containing 0-60% dioxane and the final concentration of ANS at 88.8 µM. Excitation and emission wavelengths were 350 and 400-600 nm respectively and the maxima of the differential spectra (ΔFI) were plotted versus dioxane concentration.

Samples of the native and modified inulinases (200 µL) at 0.3 mg*ml⁻¹ in sodium acetate buffer (50 mM, pH 5.5) containing 0-60 % (v/v) dioxane were added to 100 mg of hydrated octadecyl nanoporous silica and incubated for 2 hours at 4°C in the rotary mixer. They were then centrifuged at 5000 rpm for 5 min in 4°C. The supernatants were collected for further analysis and the pellets were washed three times with sodium acetate buffer solution to remove unbound enzymes. Subsequently the immobilized inulinase was stored at 4°C until use (Nabati et al., 2011; Norouzy et al., 2010).

2.7. Characterization of the immobilized inulinases

Storage stability, reusability and the performance of free and immobilized inulinases in a continuous catalytic operation were studied. Storage stability of the enzymatic processes, principally depends on their ability to retain their bio-catalytic activities over time, thus 6 sets of aliquots (in triplicate) were prepared and stored at 4°C. The assay was done in sodium acetate buffer (pH 5.5) containing 0.2% inulin for 30 minutes at 37°C with 1 day intervals up to 6 days. The half-life of an enzyme which is the time required to lose 50 percent of the enzyme initial

activity was calculated according to the equation $t_{1/2} = \ln 2 / k_{in}$ (Torabizadeh et al., 2011). Also, the reusability analysis of the immobilized inulinases (native and modified) was investigated for 10 hydrolysis cycles, while each cycle lasted 8 hours in a continuous operation mode. Immobilized inulinases were stored in 4°C during 10 day intervals. To perform continuous operation, solid particles of the support must be retained by the filter designed at the top of the hydrolysis chamber of the bioreactor. For the continuous catalytic operation, sodium acetate buffer, pH 5.5 containing 2% inulin at 45°C (temperature was maintained by circulating water through the jacket) was pumped at a flow rate of 250 $\mu\text{L} \cdot \text{min}^{-1}$ through the fluidized bed mini-bioreactor containing 100 mg of inulinase loaded nanoporous silica, then the fractions of the continuous hydrolysis were collected after achieving the steady state up to 8 hours. The presented values are the mean value of five independent continuous operations assessments. The relative activities were expressed as the ratio of the residual activity to the initial activity in percent (Gill et al., 2006; Norouzy et al., 2010; Norouzy et al., 2009; Catana et al., 2007; Arzeni et al., 2012).

2.8. HPLC analysis of the bioconversion products of the free and immobilized inulinases

Products of the inulin bioconversion by free and immobilized inulinases (native and modified) were characterized using Reversed-Phase HPLC and the detection was performed through refractive index (Knauer K-2301, Germany) at 30°C, flow rate of 0.5 $\mu\text{L} \cdot \text{min}^{-1}$, with Eurotech columns and H_2SO_4 (0.01 N) as the mobile phase (Rawat et al., 2015; Trytec et al., 2015)

3. Results and Discussion

Table 1 shows the products of inulinases (native and modified) bioconversion in the free and immobilized forms using RP-HPLC. Although, the catalytic efficiency decreased upon immobilization, inulin hydrolysis improved in modified inulinases: the yield of fructose production increased 1.11 and 1.48 folds for the modified (free and immobilized) inulinases, compared to native (free and immobilized) respectively.

Results of BET for average grain size, pore size, pore volume and surface area were 22.01 μm , 66 nm, 0.488 cm^3g^{-1} and 33.62 m^2g^{-1} , respectively. Schematic figure representing the dioxane induced interactions between inulinase and nanoporous silica which results in immobilization of inulinase on the alkyl groups of the support (Fig. 1). Alkylation of the matrix was confirmed by FTIR spectroscopy; at 2880 cm^{-1} region C-H stretching vibration of methylene groups are seen that approves the presence of the functional groups. A broad band at around 1100 cm^{-1} is related to the Si-O-Si asymmetric stretching vibration. The adsorption band at 3400 cm^{-1} is due to the stretching vibrations of the silanol groups on the surface of the silica support (Norouzy et al., 2009; Bahrami et al., 2014) (Fig. 2). The observed peak at 1720 cm^{-1} could represent the capture of CO_2 on the sample surface which is mostly seen in functionalized silica particles (Kim et al., 2015)

Specific activities of the inulinases, loaded on the nanoporous silica support were 1.42 ± 0.21 , 1.61 ± 0.24 , 3.52 ± 0.15 and 3.78 ± 0.28 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for free native, free modified, immobilized native and immobilized modified inulinases, respectively. Improvement in the specific activity following the adsorption and immobilization has also been reported by Nabati et al., 2011 and Norouzi et al., 2010.

Preferential chemical modification of the non-catalytic domain of endo-inulinase to enhance the thermostability was done through semi-rational chemical modification of the accessible lysine residues by adding PLP to form Schiff bases, then reducing them by ascorbate (Torabizadeh et al., 2010; Torabizadeh et al., 2011). We have previously reported supporting results on the inulinase modification, including the increase in α -helix content and the melting temperature (T_m) using spectropolarimetric (CD) and calorimetric (DSC) methods in details (Torabizadeh et al., 2011; Shapiro et al., 1968). However, to ensure the modification procedure, intrinsic fluorescence after excitation at 280 nm and also endo-inulinase thermal stability analysis were done, the results of which are presented in Fig. 3, confirming the altered emission and more efficient activity retaining capacity of inulinase after modification. This could be due to the development of the new intra-molecular electrostatic interactions between covalently attached PLP-Lys₃₈₁ with Arg₅₂₆ and Ser₃₇₆ which advances the structural, functional and thermal stability of the PLP-modified endo-inulinase. Applying molecular dynamics stimulation (by using the GROMACS program version 4.0.3). make the simulation of the PLP-modified inulinase, possible which proves the formation of the new contacts between covalently attached PLP-Lys₃₈₁ with guanidinium group of Arg₅₂₆ and the hydroxyl group of Ser₃₇₆ as a result of modification which are considered to be the reason for improved thermostability of the plp-modified enzyme. In addition, the results of intramolecular interactions analysis of inulinase gained by docking approach after PLP-modification at the Lys₃₈₁ using the LIGPLOT program proves the formation of new hydrogen bonds between the phosphate group of PLP and Arg₅₂₆ and Ser₃₇₆ as well(Torabizadeh et al., 2011). A significant improvement in the thermal stability of the enzyme after immobilization has already been reported (Catana et al., 2007; Bajpal & Margaritis, 1985; Kim et al., 1982; Wenling et al., 1999) which was so remarkable in the

chemically modified inulinase compared to the native one. Structural analysis of the native and modified inulinases were done at each ratio of dioxane to find the best solvent polarity that forms inulinase intermediate state molecules and exposed substituted alkyl chains. It was found that 40% and ~50% (v/v) of dioxane in sodium acetate buffer for the native and modified inulinases deeply affects the emission of ANS which is a reporter of the surface hydrophobicity of the proteins (Hu et al., 2013; Arzeni et al., 2012; Kato & Nakai, 1980). Differential analysis of ANS fluorescence emission confirmed the surface hydrophobicity increase in native and modified inulinases at 40% and ~50% (v/v) of dioxane, respectively (Fig. 4a). The observed minimum intensity in ANS fluorescence emission could be due to the decrease in the protein surface hydrophobicity caused by the strengthening of the hydrogen bonds at 20% and 35% dioxane for the native and modified inulinases, respectively. As Fig. 4b shows, maximum activities pertaining to the native and modified inulinases on the exposed octadecyl moieties of the alkylated nano porous silica were observed in 40 and 50% dioxane (v/v) as well. Thus, it could be concluded that the refolding intermediates express the optimum hydrophobic interaction with exposed alkyl groups of the support at the above mentioned polarities (Nabati et al., 2011). However, at dioxane concentrations beyond what is required to form intermediate state, a protein molecule unfolds, denatures and deactivates, which explains the observed decrease in the extent of enzyme immobilization at dioxane concentrations higher than 60% (Risso et al., 2010).

The main preference of the modification and immobilization of endo-inulinase was to improve the storage stability and reusability of the modified and immobilized enzymes compared to the native and free inulinases (Torabizadeh et al., 2010). Immobilization imposes no significant activity loss (based on specific activity analysis) in spite of the mass transfer restrictions. Activity improvement of inulinase after immobilization has been recently stated by

Kovaleva et al. (2009), Artiukhov et al. (2010) and Karimi et al., (2014) a, b. The hydrolytic activity of free and immobilized inulinases were assayed in three repeats at one day intervals up to 6 days and the corresponding half-lives are presented in inset to Fig. 5. The results showed that the activity loss of the immobilized enzyme was less compared to the free enzyme (both native and modified). Activity loss could be due to enzyme leakage or intrinsic loss of activity. It has been reported that if adsorption is the only method applied to attach the enzyme to the support in inulin hydrolysis, inulinase activity would be lost in repeated batches due to leaching most of the protein to the medium (Yewale et al., 2013). Similarly, activity loss of the modified enzyme was far less than the native enzyme which confirms the stabilizing effect of the modification and immobilization of the inulinase. Considering the results presented in the inset to the Fig. 5, immobilized-modified, free modified and immobilized native samples had 7.89, 3.73 and 1.24 fold increase in their half lives in comparison with the free native sample.

Continuous catalytic operations of the inulin hydrolysis was achieved using a simple stirred fluidized bed mini-bioreactor (FBR) as depicted in Fig. 6. After 8 hours of the continuous operation at 45°C, modified and native inulinases could retain 90.32% and 80.46% of their original activities, respectively. Moreover, 40 and 30 % activity retaining was reported for immobilized inulinases (native and modified) respectively, when reusing for 10 day intervals up to 100 days. Inset to Fig. 6 shows a homemade stirred glass fluidized bed bioreactor used for the continuous hydrolysis of inulin.

4. Conclusions

Using inulinase to hydrolyze inulin is an economical and widely applied strategy to produce high fructose syrup in industry, mainly because it is a single-step reaction. In this research, the efficiency of the dioxane induced unfolding/refolding absorptive immobilization method for the immobilization of the modified/thermo-stabilized inulinase along with the native enzyme was verified. Our results support the performance of this modification/immobilization strategy for the applied approaches.

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Table 1. Results of HPLC for the products of inulinases (native and modified) bioconversion.

	Fructose	Glucose	Sucrose	Inulin	Fructose/Inulin
Native	48.04	37.80	1.68	6.19	7.7
Modified	53.30	12.40	-	4.19	12.7

Figure captions

Fig. 1. Schematic figure of inulinase immobilization on the alkyl groups of the nano porous silica support.

Fig. 2. Infrared spectra of the nano porous silica before (dashed line) and after substitution of octadecyl moieties (continuous line). IR spectra were recorded using KBr pellets and calculated from a total of 18 scans. For details please see materials and methods section.

Fig. 3. Fluorescence emission spectra of the native and modified inulinases after excitation at 280 nm. Inset to Fig. represents the remaining activities of the native (dashed line) and modified inulinases in 50 mM sodium acetate buffer solution, pH 5.5 after 130 min incubation at each temperature, then assaying their activities by incubating with 0.2% inulin in the same buffer for 30 minutes at 37 °C.

Fig. 4. ANS fluorescence emission intensities of the native and modified inulinases as function of dioxane concentrations at the final concentration of 88.8 μ M ANS (a). Data correction was performed by subtracting the ANS emission in the absence of the inulinase from the one in the presence of inulinase in sodium acetate buffers containing 0 to 60% dioxane. The ANS excitation wavelength was 350 nm and the emission spectra were between 400 and 600 nm.

Immobilization of the native and modified inulinases on octadecyl substituted nano porous silica based on the immobilized activities (b). They were also presented in the buffers containing 0 to 60% dioxane.

Fig. 5. Storage stability of the free and immobilized inulinases (native and modified). Free and immobilized aliquots were assayed after storing at 4°C up to 6 days (a). Reusability of the immobilized inulinases (native and modified) for 10 hydrolysis cycles. Immobilized inulinases were stored in 4°C between the intervals (b).

Fig. 6. Results of the continuous operations. 100 mg of inulinase loaded octadecyl substituted nano porous silica was injected into the bioreactor. The reaction mixture (sodium acetate buffer 50 mM, pH 5.5 containing 2% inulin) was pumped through the reactor at a constant flow rate of 250 $\mu\text{L}\cdot\text{min}^{-1}$ and the samples were collected and used to determine the liberated fructose content by DNS method. Inset to figure represents the homemade stirred glass fluidized bed bioreactor used for the continuous hydrolysis of inulin at 45°C.