Brazilian Journal of Microbiology 46, 1, 23-28 (2015)
ISSN 1678-4405
DOI: http://dx.doi.org/10.1590/S1517-838246120120462

Research Paper

Chemical modification of *Aspergillus niger* β-glucosidase and its catalytic properties

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Submitted: December 24, 2012; Approved: June 6, 2014.

Abstract

Aspergillus niger β-glucosidase was modified by covalent coupling to periodate activated polysaccharides (glycosylation). The conjugated enzyme to activated starch showed the highest specific activity (128.5 U/mg protein). Compared to the native enzyme, the conjugated form exhibited: a higher optimal reaction temperature, a lower Ea (activation energy), a higher K_m (Michaelis constant) and Vmax (maximal reaction rate), and improved thermal stability. The calculated $t_{1/2}$ (half-life) values of heat in-activation at 60 °C and 70 °C were 245.7 and 54.5 min respectively, whereas at these temperatures the native enzyme was less stable ($t_{1/2}$ of 200.0 and 49.5 min respectively). The conjugated enzyme retained 32.3 and 29.7%, respectively from its initial activity in presence of 5 mM Sodium Dodecyl Sulphate (SDS) and *p*-Chloro Mercuri Benzoate (*p*-CMB), while the native enzyme showed a remarkable loss of activity (retained activity 1.61 and 13.7%, respectively). The present work has established the potential of glycosylation to enhance the catalytic properties of β-glucosidase enzyme, making this enzyme potentially feasible for biotechnological applications.

Key words: β-glucosidases, glycosylation, soluble polysaccharides, enzyme stability.

Introduction

β-glucosidases are enzymes that hydrolyze cellulose (β-1,4-glucan or β-D- glucosidic linkages) resulting in the formation of glucose, cellobiose, cellooligosaccharides, and the like (Su *et al.*, 2010; Cai *et al.*, 2012). Efficient hydrolysis of cellulose requires the synergistic activities of three types of enzymes: cellobiohydrolase, endo- β-1,4-glucanase, and β-glucosidase (Chauve *et al.*, 2010; Singh *et al.*, 2011; Cai *et al.*, 2012). β-glucosidase (EC 3.2.1.21), catalyses the final step of cellulose hydrolysis (*i.e.*, the breakdown of cellobiose to glucose). The supplementation of this enzyme to cellulose preparations, in order to obtain higher rates and extent of saccharification of cellulose, has been recommended (Wood and Wiseman, 1982). Cellulases have a great application prospect in many fields, such as medicine, textile, chemical engineering, paper engineering, food and fermentation industries, commercial detergents, tobacco, waste water treatment, feed additives etc. (Howard *et al.*, 2003; Su *et al.*, 2010). The application of cellulase has very important significance in solving problems related with raw materials in industry and agriculture, the energy crisis, and environmental pollution and so on (Cai *et al.*, 2012). An efficient cellulose hydrolysis requires a stable β-glucosidase for the utilization of abundant cellulosic waste material on an industrial scale (Rashid and Siddiqui, 1996). There are several major and principally different routes to obtain cellulases with improved stability properties to date: (a) by selecting organisms living in appropriate extreme environments (Huang *et al.*, 2005; Kubartova *et al.*, 2007), (b) by gene recombination and genetic engineering (Liu *et al.*, 2006; Kim *et al.*, 2007), (c) by immobilization (Sinegania *et al.*, 2005; Singh *et al.*, 2011), and (d) by chemical modification (Yang *et al.*, 2009; Cai *et al.*, 2012). Chemical modification is the process of covalent attachment of special groups of modifiers to the side-chain group of certain amino-acid residues in the enzyme. There are few reports about stabilization of β-glucosidase by co-

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valent coupling to soluble polysaccharides (Rashid and Siddiqui, 1998; Abdel-Naby, 1999).

In the present study, *Aspergillus niger* β-glucosidase was covalently immobilized on to water soluble carrier (polysaccharides). The catalytic properties and the stability of modified enzyme have been compared to those of native enzyme.

Materials and Methods

Microorganism

Aspergillus niger strain was obtained from the Center of Culture Collection of National Research Center, Cairo, Egypt, and sub-cultured every 3 weeks and stored at 4 °C in PDA slants.

Enzyme production

For inoculum preparation, spores from *A.niger* were produced on PDA slants for 5 days at 30 °C. One mL of spore suspension (6 x 10^{10} spores) was then inoculated in 250 mL-Erlenmeyer flasks containing 50 mL production medium. The production medium was prepared from a mixture of 3 g NH_4Cl, 17 g citric acid, 3 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g $CaCl_2$, 0.2 mL triton and 10 mL milk whey, in 1000 mL wheat bran extract (1% w/v) with pH adjusted to 7.0. The flasks were shaken at 150 rpm and 30 °C for 7 days. The culture was filtered through cloth to remove the mycelia. The crude supernatant was used for enzyme modification.

Oxidation of carbohydrate used for the modification

Agar, dextran A (200 x 10^3-275 x 10^3), dextran 70 x 10^3, glucomannan, galactomannan, pectin and soluble starch were used in the present study, all were obtained from Sigma. Activation of polysaccharide was done according to Ben Ammar *et al.* (2002) with some modification as follows: 1 g of each polysaccharide was dissolved into 40 mL of 0.25 M periodate solution and gently shaked at 50 rpm at 37 °C for 6 h. After that, 1.2 mL of ethylene glycol were added and allowed to react for 1 h. The reaction mixture was dialyzed against water at 4 °C for 2 h then lyophilized.

Covalent coupling of β-glucosidase to activated polysaccharide

Forty units of enzyme and 150 mg oxidized polysaccharide were combined in citrate buffer pH 4.8. The mixture was allowed to stand at 4 °C overnight. Modified β-glucosidase was precipitated with 90% acetone, centrifuged at 4 °C and 3000 rpm for 15 min, the precipate was dried and used as modified enzyme.

Enzymatic assay

β-glucosidase activity was determined according to Abdel-Naby (1999) using 0.4% (w/v) cellobiose solution in citrate buffer (0.05 M, pH 4.8). The glucose released was determined by glucose oxidase / peroxidase reagent. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of glucose per minute under assay conditions.

Protein determination

Protein contents of enzyme were estimated by method of Lowry *et al.* (1951).

Thermal stability

Both native and conjugated β-glucosidase were held at different temperatures (40-70 °C) for 2 h before activity assay, with samples being drawn for the assay at 15-min intervals.

pH stability

The pH stability was examined by measuring the residual activity of the enzyme after incubation at 0.05 M buffer with different pHs using citrate buffer (pH 2.5-6.0), citrate phosphate buffer (pH 6.5-7.5), and phosphate buffer (pH 7.5-8.0).

Results and Discussion

The variation of activity obtained for glycosylated β-glucosidase was affected by the types of modifiers and the molecular weight of carbohydrate residue linked to the enzyme (Figure 1). Among all preparations tested, the enzyme coupled to activated soluble starch showed higher specific activity (128.5 U/ mg protein) than the native enzyme (29.6 U mg/ protein). This result was due to the conjugation to the active protein in crude enzyme preparation. It is clear from the results that the crude enzyme contains a variety of proteins in addition to the β-glucosidase. Similar result was obtained by Singh *et al.* (2011) on immobilized β-1,4-glucosidase onto functionalized silicon oxide nanoparticles.

Properties of native and starch conjugated cellobiase

Optimum temperature

The activities of native and conjugated enzymes were assayed at various temperatures (30-75 °C). The native enzyme had an optimum temperature of 40 °C, whereas that of the conjugated enzyme was shifted to 50 °C. The increase of the optimum temperature is probably a consequence of enhanced thermal stability. Similar result was reported for other glycosylated cellobiases (Abdel-Naby, 1999). The temperature data were replotted in the form of Arrhenius plot (Figure 2) which is related to the activation energy (Ea) by the relationship: Slope= Ea / 2.303 R, where R is the gas constant. Activation energy for both native and conjugated enzymes was 4.55 and 3.87 cal/ mol, respectively. Rashid and Siddiqui (1996) reported that poly-

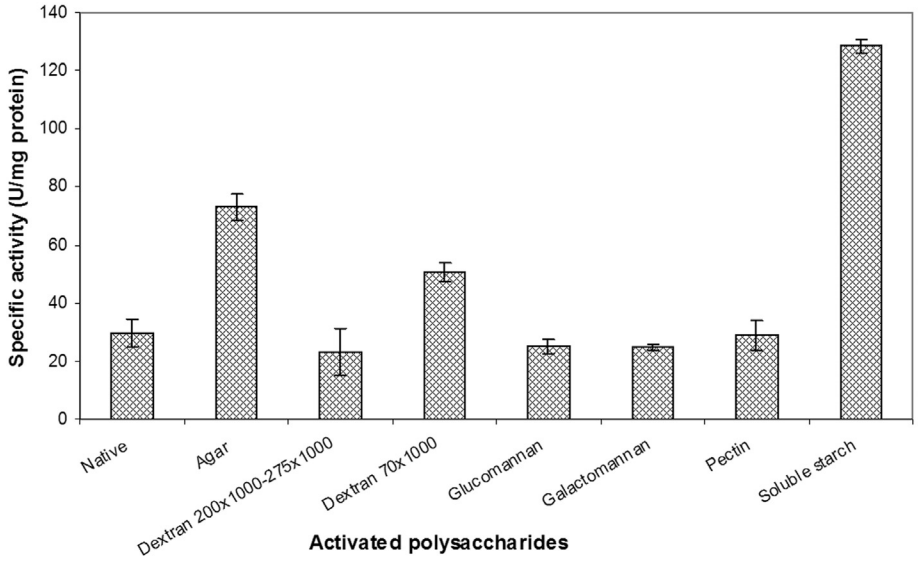

Figure 1 - Covalent coupling of *Aspergillus niger* β-glucosidase to periodate activated polysaccharides.

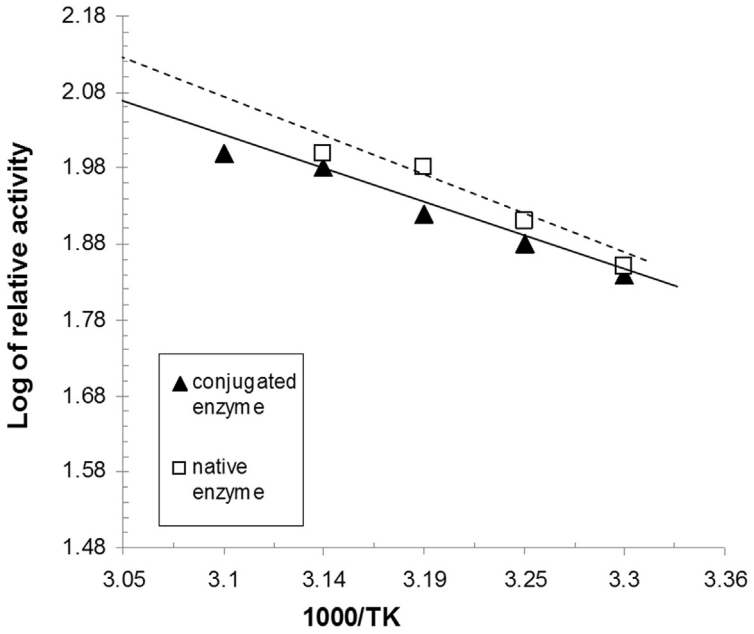

Figure 2 - Arrhenius plots for the native and starch conjugated *Aspergillus niger* β- glucosidase.

saccharide-β-glucosidase had lower Ea (55 kJ/ mol) as compared with the native form (61 kJ/ mol).

Optimum pH

Optimum pH of the native and modified β-glucosidase was studied using 0.05 M of different buffers with different pH values. Both native and modified enzymes were optimally active at pH 4.0 (Figure 3). This means that the ionization of the amino acid residues at the active site remained unaffected by the glycosylation process. Abdel-Naby (1999) found that there was no change of the pH optimum for the activity of the native and glycosylated cellobiase.

Thermal stability

The rates of heat inactivation of the native and conjugated β-glucosidase were presented in Figure 4A. Covalent coupling of β-glucosidase to activated starch appeared to enhance its thermal stability as shown total activity of the conjugated enzyme which was retained after 2 h of heating at 55 °C, while the native enzyme lost about 11% of its initial activity. This is probably because the formation of mul-

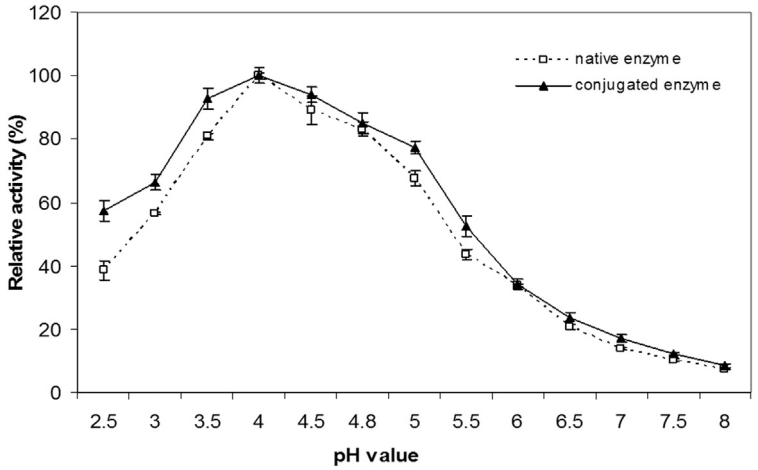

Figure 3 - Optimum pH of native and starch conjugated *Aspergillus niger* β-glucosidase.

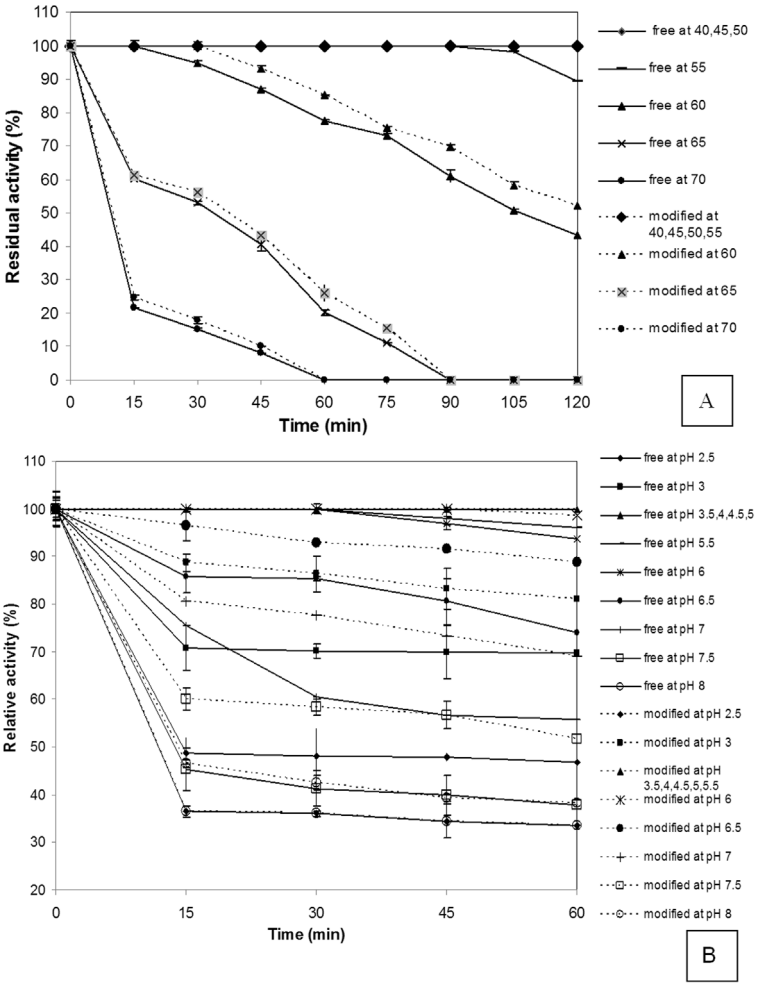

Figure 4 - Thermal stability (A) and pH stability (B) of native and starch conjugated *Aspergillus niger* β-glucosidase.

tiple covalent bonds between the β-glucosidase and polysaccharide reduces conformational flexibility and thermal vibrations, thus preventing the conjugated protein from unfolding and denaturing (Wong *et al.*, 2009; Singh *et al.*, 2011). Wongkhalaung *et al.* (1985) reported that cellulase conjugated to soluble polymer retained all initial activity after heating at 60 °C for 20 min, while the native cellulase lost about 18% of its activity at the same condition. For ex-

ample, the calculated half-life ($t_{1/2}$) value shows that the heat inactivation of conjugated enzyme at 65 °C was 67.2 min whereas at the same temperature the native was less stable ($t_{1/2}$ 55.3 min). Glycosylation of β-glucosidase protected the enzyme against heat in-activation as its half-life for thermal deactivation was increased. Conjugation to polysaccharide induced stability of modified proteins by rigidification of the conformation, which could retain its denaturation (Bund and Singhal, 2002). On the other hand, Srivastava (1991) argued that the hydration effect of the attached carbohydrate may be responsible for improving the stability of conjugated enzymes. Hydrogen binding between the polysaccharide and protein surface as well as intra molecular cross links between protein and polysaccharide has been suggested as causes of thermal stabilization (Zhang *et al.*, 2009).

pH stability

The stability of the conjugated β-glucosidase at various pH values was higher than that of the native enzyme (Figure 4B). At pH 3.0 and 8.0 after 30 min the residual activity of the conjugated enzyme was 86.4 and 44.3% which was higher than that of the native enzyme (70.1 and 36.0%, respectively). In general, the glycosylated enzyme was more resistant to acid or alkaline pH values. In contrast, Abdel-Naby (1999) reported that no change of pH stability of cellobiase after glycosylation process.

Kinetics of native and conjugated cellobiase

According to the Michaelis-Menton kinetics, the v_{max} (maximum reaction rate) and k_m (Michaelis constant) of enzymes are determined at optimum assay conditions. Lineweaver-Burk plots of the native and conjugated β-glucosidase (Figure not shown) gave k_m of 40 mM and 46.5 mM, respectively. With cellobiose, the v_{max} of the native and conjugated enzyme were 416.7 and 1111.0 U / mg protein, respectively. Increasing the k_m value after glycosylation is most likely a consequence of conformational changes in the enzyme introduced by the glycosylation procedure which renders its active site less accessible to the substrate. In contrast Zhang *et al.* (2009) reported that k_m of modified cellulase is smaller than that of natural cellulase. It illustrates that the affinity of the modified form for the substrate is larger than that of the natural form. The specific activity of conjugated β-glucosidase is higher than that of the native enzyme, consequently, v_{max} of modified enzyme is higher than that of native enzyme. Rashid and Siddiqui (1998) found that no significant difference could be detected in the specificity constant (k_m and v_{max}) between free and glycosylated β-glucosidase.

Effect of various metal ions

In this experiment, both the native and conjugated β-glucosidase were incubated with the tested metal (5 mM) at 30 °C for 60 min before mixing with the substrate. The results listed in (Figure 5) indicated that the inhibitory effect of the investigated metal ions was less pronounced with the conjugated enzyme as compared to the native enzyme. This may be due to the protection of the enzyme by immobilization. In presence of Sodium Dodecyl Sulphate (SDS) the conjugated β-glucosidase retained 32.3% from its initial activity, while the native enzyme showed a remarkable loss of activity (retained activity 1.61%). The native enzyme lost 13.8 and 34.4% of its initial activity in presence of $ZnCl_2$ and Iodo Aceto Amide (IAA). However, under the same conditions, the conjugated β-glucosidase was more stable with retained activity of 113.3 and 80.3%, respectively. The higher recovered activity of the conjugated form after treatment with chemicals compared to the native enzyme could be attributed to the hydrophilic nature of the polysaccharide attached to it (Srivastava, 1991). Perutz (1978) reported that protein hydration may have a positive effect on stability.

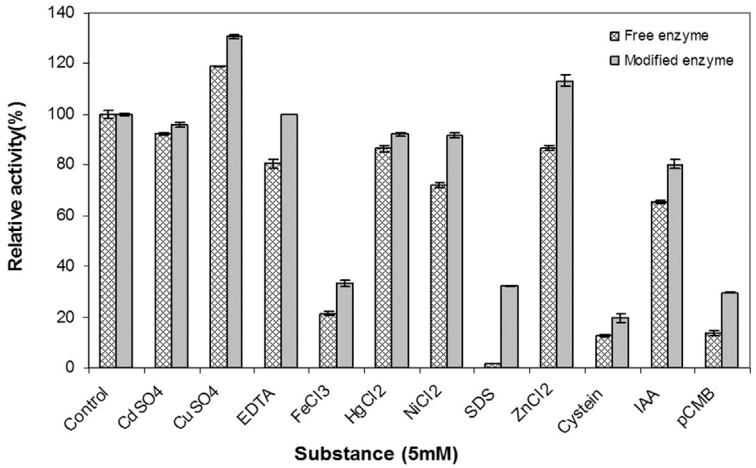

Figure 5 - Effect of metal salts and inhibitors on the activity of native and starch conjugated *Aspergillus niger* β-glucosidase. EDTA (Ethylene Diamine Tetra Acetic Acid), SDS (Sodium Dodecyl Sulphate), IAA (Indo Aceto Amide), *p*-CMB (*p*-Chloro Mercuri Benzoate).

Conclusion

There are few reports about modification of the β-glucosidase enzyme by conjugation to soluble polysaccharides (glycosylation). To our knowledge, this study produced stabilized (modified) β-glucosidase by covalent coupling to activated polysaccharides. Starch is safe, cheap, readily available on the markets and suitable for industrial application. The glycosylation process improved *Aspergillus niger* β-glucosidase stabilities toward temperature, pH, inhibitors which make it more suitable for industrial applications.

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Associate Editor: Rodrigo da Silva Costa