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Functional change of *Bacillus acidocaldarius* α-amylase chemically modified with periodate oxidized polysaccharides

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ABSTRACT

α-amylase from isolated bacterial strain (*Bacillus acidocaldarius*) was chemically modified by covalent coupling to several periodate oxidized polysaccharides. Conjugated enzyme with oxidized dextran (MW 84 kDa) retained the highest activity (77.4%) and the highest specific activity (3948.4 U/mg protein). Compared to the native enzyme, the dextran conjugated α-amylase exhibited higher optimum temperature, higher K_m (Michaelis constant), lower V_{max} (maximal reaction rate), lower V_{max}/K_m (catalytic efficiency), lower E_a (activation energy), lower deactivation constant rate (k_d), higher half-life time ($t_{1/2}$), and higher decimal reduction time values (D). Covalent attachment of α- amylase to oxidized dextran brought about significant enhancement of thermal stability, stability at extreme pHs, and resistance against the inhibitors. In the presence of the substrate, the conjugated enzyme retained 68.2% of its original activity after incubation at 70 °C for 30 min which was 1.4-fold higher than that retained by the native enzyme (50.3%) under the same conditions. The K_d value at 80 °C for the dextran conjugated α-amylase was 2.5-fold lower than that of the native enzyme. Dextran conjugated α-amylase was more resistance against the inhibitors than the native enzyme, and retained 70.6% of its activity in presence of 10 mM $CuSO_4$, while the native form retained only 34.1%.

1. Introduction

α-amylase is an extracellular enzyme that randomly cleaves the 1,4 α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain (Zhang et al., 2017). It possesses approximately 25–33% share of the world's marketable enzymes. A large number of industrial amylases are derived from bacteria belonging to the very wide and diversified genus *Bacillus*. The demand for amylases is constantly increasing owing to the variety of industrial applications such as food, detergent, paper, removing environmental pollutant, bakery, alcohol, pharmaceutical and desizing of textile (Homaei, 2015; Ahmed et al., 2017; Rodrigues et al., 2017). The stability of enzymes *in vitro* remains a critical issue in biotechnology. Operational stabilities affect the usefulness of enzyme-based products. Therefore, there is a continuing demand to improve the stability of enzymes to meet the requirements set by specific applications (Prakash and Jaiswal, 2010; David et al., 2011). Three methods are used to increase the enzyme stability (1) immobilization (2) chemical modification (3) engineering of the protein. 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 (Abdel-Naby et al., 2017). Polysaccharide-protein interaction is a simple and an efficient technique to enhance the enzyme stability. Moreover, by chemical modification, the interaction of enzyme with substrate is not hindered by the presence of matrix consequently, the decrease of enzyme activity can be minimized. Modified enzyme using water soluble polyols as thermoprotectant additives appears as the most economic approach, taking into account the simplicity, availability and the lower cost of this method. Carbohydrates (pectin, amylopectin, dextran – etc.) are the most polyols used successfully as additives for stabilizing enzymes. It was demonstrated in a number of studies that the stability of some enzymes is enhanced using covalent attachment to water soluble polymers (Ben Ammar et al., 2002; Jadhavand Singhal, 2013; Abdel-Naby et al., 2017). Chemical modification by glycosylation of the enzymes has been reported as a method to improve their catalytic and thermal stability. Carbohydrates in glycoproteins have been shown to stabilize the core protein even under denaturing conditions and have profound effect on their biological and physicochemical properties. In addition, covalent binding of the enzymes with polysaccharides causes a shielding of the protein surface from water thus restricting conformational motions

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(Flores-Fernández et al., 2010). Also, glycosylation helps in forming long range contacts between amino acids, which are separated in sequence and thus provides a folding nucleus (Kaushik et al., 2011).

In previous work, an active α-amylase was produced by isolated bacterial strain *Bacillus acidocaldarius* (Ahmed et al., 2008). In this study, we have addressed the possibilities of using oxidized polysaccharides to improve α-amylase enzyme stability via covalent attachment, focusing our attention on the enzymatic properties of the chemically modified and native enzyme.

2. Materials and methods

2.1. Microorganism and carbohydrates

Bacillus acidocaldarius was isolated by Prof. Mona A. Esawy (Department of Chemistry of Natural and Microbial Products, NRC, Egypt). It was identified according to Bergey's Manual in Micro Analytical Center, Cairo University, Egypt. The culture was maintained on nutrient agar medium at 30 °C for 48 h and stored at 4 °C. Citrus pectin (MW 35KDa) was obtained from El-Nasr Co., Egypt. Dextran (MW 84 kDa) and dextran (MW 185 kDa) were from Sigma Chemical Co., USA

2.2. Growth medium and cultivation

Basal medium as mentioned in the previous search (Ahmed et al., 2008) for liquid culture consists of (g/l): Starch, 10; nutrient broth, 2.5; $CaCl_2$, 0.5 and the pH was adjusted to 7.0 before autoclaving. The same medium was also used for inoculum preparation. Cultivation was in 250-ml Erlenmeyer flasks containing 50 ml of sterile medium. The flasks were inoculated with 1 ml (24 h old culture) and incubated at 40 °C and 200 rpm for 42 h. The cultures were then centrifuged at 10,000 × *g* for 15 min in a refrigerated centrifuge at 4 °C.

2.3. Fractional precipitation with acetone

The crude enzyme was added slowly to 2-fold cold acetone (v/v) under constant stirring. The mixture was allowed to stand at 4 °C for 1 h and the enzyme fraction was dried over anhydrous $CaCl_2$ under decreased pressure at room temperature. The fraction was tested for enzyme activity and was used for preparation of the conjugated enzyme.

2.4. Assay for α-amylase activity

α-amylase activity was determined according to Apar and Ozbek (2005). 200 µl of the enzyme were incubated with 1 ml of soluble starch 0.2% in 0.05 M acetate buffer pH 5.9 at 40 °C for 10 min. To stop the reaction, 5 ml of an iodine solution was added to 200 µl of the reaction mixture. The degradation of starch by the enzyme was measured at 620 nm. One unit of α-amylase activity was defined as the quantity of enzyme required to hydrolysis of 0.1 mg starch under assay conditions. All the results reported are the mean of at least three separate experiments.

2.5. Protein estimation

Protein was determined according to the Lowry et al. (1951) method using BSA (bovine serum albumin) as standard.

2.6. Chemical modification (glycosylation) of α-amylase enzyme

α-amylase was coupled to soluble polysaccharides by the method reported by Ben Ammar et al. (2002).

2.6.1. Preparation of oxidized polysaccharides by sodium periodate ($NaIO_4$)

250 mg of polysaccharide (citrus pectin and dextran) were dissolved in 10 ml of 0.25 M $NaIO_4$ solution and incubate for 6 h at 30 °C, and to equalize the excess of $NaIO_4$, 0.3 ml of ethylene glycol was added and allowed for 1 h to react. The reaction mixture was dialyzed against distilled water at 4 °C overnight, and then dried.

2.6.2. Enzyme coupling with oxidized polysaccharides

Partially purified α-amylase (0.4 mg) and oxidized polysaccharides (100 mg) were combined in acetate buffer (0.05 M; pH 5.9). The reaction mixture was allowed to stand at 4 °C overnight. The conjugates were precipitated at 50% ethanol and lyophilized.

2.7. Enzyme properties

2.7.1. pH profiles

The effect of pH value on the activity of native and dextran conjugated enzyme was investigated using 0.05 M buffer with different pHs (5.0–8.0).

2.7.2. Temperature profiles

The optimal reaction temperature of the native and dextran conjugated enzyme was investigated in temperature range 30–80 °C. The activation energy (E_a) was calculated from the slope of the Arrhenius plot according to the following equation:

$$Slope = E_a/2.303\ RT \quad (1)$$

where: R is the gas constant (R = 8.314 KJ/mol) and T is the absolute temperature (Kelvin).

2.7.3. Determination of kinetic constants

Various substrate concentrations from 0.1% to 0.6% in 0.05 M acetate buffer (pH 8.0) were used for the kinetic analysis of native and dextran conjugated form. The enzyme kinetic parameters, Michaelis–Menten constant (K_m), maximum reaction velocity (V_{max}) and the catalytic efficiency (V_{max}/K_m) were determined using Lineweaver–Burk plot method (Lineweaver and Burk, 1934) at optimum assay conditions.

2.7.4. Effect of conjugation on enzyme stability

2.7.4.1. Thermal stability. In the absence of substrate, thermal stability was determined by measuring the residual activity of the enzyme exposed at a temperature range from 50 to 80 °C for different periods (15–90 min). Plotting activity data, Log of residual activity (%) as a function of the time, and the slope is related to the deactivation rate constant (k_d).

$$k_d = slope \quad (2)$$

The enzyme half life ($t_{1/2}$) corresponds to the time period necessary for the residual enzyme activity to decrease to 50% of its initial value and it was calculated from the following equation:

$$t_{1/2} = ln\ 2/K_d \quad (3)$$

where: K_d is deactivation rate constant

Decimal reduction time (*D*-value) was defined as the time needed to reduce the initial activity by 90% at a specific temperature is given by the expression:

$$D = ln\ 10/K_d \quad (4)$$

2.7.4.2. pH stability. The pH stability of α- amylase was determined after pre-incubating the enzyme for 1 h at 30 °C with 0.05 M buffer at different pHs (5.0–9.5), followed by adjusting the pH to the value of standard assay system.