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Functional Change of Dextran-modified α-amylase from *Bacillus acidocaldarius*

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α-AMYLASE from *Bacillus acidocaldarius* was modified by covalent coupling to activated dextran with retained activity of 77.7 %. After conjugation, the enzyme was stable within a broader pH range than the native enzyme and its optimum temperature increased by 10°C compared to the native enzyme. The conjugated α-amylase exhibited a higher K_m (Michaelis constant), lower V_{max} (maximal reaction rate) and lower E_A (activation energy) than the native enzyme. Covalent attachment of α- amylase to activated dextran protected the enzyme against heat inactivation. 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 more than that retained by the native enzyme (50.3 %) under the same conditions. The calculated $t_{1/2}$ (half-life time) values of heat inactivation energy at 50, 60 °C were 89 and 56 min, respectively for the conjugated enzyme, whereas at these temperatures the native enzyme was less stable ($t_{1/2}$ 60 and 47 min, respectively). The deactivation rate constant at 80 °C for the conjugated α-amylase is about $11.9x10^{-3}$/ min, which is lower than that of the native enzyme ($14.8x10^{-3}$/ min). Conjugated α-amylase was more stable against chemical denaturation than the native enzyme, and retained 70.6% of its activity in presence of $CuSO_4$ (10 mM) while the native form of retained only 34.1%

Keywords: α-amylase, *B. acidocaldarius*, Glycosylation, Thermal stability.

Amylases are among the most important hydrolytic enzymes for all starch based industries, and the commercialization of amylases is oldest with first use in 1984, as a pharmaceutical aid for the treatment of digestive disorders (Gupta *et al.*, 2003). In present day, amylases find application in all industrial processes such as in food, detergents, textiles, paper industries, fermentation, pharmaceutical industries and have completely replaced chemical hydrolysis in the starch

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processing industry (Alva *et al*., 2007 ; Ahmed *et al*., 2008 and De Souza & Magalhaes, 2010). α-amylase (endo-1,4-α-D- glucan glucanohydrolase EC 3.2.1.1) are extra-cellular endo-enzymes that randomly cleave the 1,4-α linkages between adjacent glucose unit in the linear amylose chain and ultimately generates glucose, maltose and maltotriose units (Gupta *et al*., 2003). The stability of enzymes *in vitro* remains a critical issue in biotechnology. Both storage and operational stabilities affect the usefulness of enzyme-based products (Fagain, 2003). Therefore, there is a continuing demand to improve the stability of enzymes and to meet the requirements set by specific applications (Prakash & Jaiswal, 2010 and David *et al.,* 2011). 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 and Ahmed *et al.,* 2007). However, critical analysis leads to the conclusion that frequently the observed stabilization was due to chemical modification or reduction of autolysis (Klibanov, 1979). Historically, carbohydrate compounds have been extensively used as modifying agents for enzymes (Gomez *et al.,* 2000). It is postulated that one of the functions of these carbohydrate residues is to stabilize the three-dimensional structure of the protein moiety of the glucoenzyme (Klibanov, 1979). Immobilized enzymes bound to a polymeric matrix are often prepared for the purpose of changing some of their properties, in particular to obtain complexes which exhibit an increased stability towards thermal denaturation, proteolytic degradation and other unfavorable conditions. An increase in thermal resistance and changes in the pH activity profile have been observed for enzymes immobilized on soluble supports such as polysaccharides (dextran, CM-cellulose and DEAE-dextran) (Lenders & Crichton, 1984 and Fernandez *et al.,* 2004). This is connected with their availability, easy modification, and hydrophily (Manaev *et al.,* 1985). In this study, we have addressed the possibilities of using activated polysaccharides to improve α-amylase stabilization via covalent attachment, focusing our attention on the enzymatic properties of the native and conjugated enzyme.

Materials and Methods

Microorganism

Bacillus acidocaldarius was isolated by Dr. Mona 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 hr and stored at 4°C.

Growth medium and cultivation

Basal medium 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 of a 24 hr -old culture and incubated at 40°C for 42 hr with shaking at 200 rpm. The cultures were then centrifuged at 10000 x g for 15 min in a refrigerated centrifuge at 4°C.

Fractional precipitation with acetone

The crude enzyme prepared as described above was added slowly to two fold cold acetone (v/v) under constant stirring. The mixture was allowed to stand for 1hr at 4°C and the enzyme fraction was dried over anhydrous calcium chloride under decreased pressure at room temperature. The fraction was tested for enzyme activity and was used for enzyme chemical modification.

Assay for α-amylase activity

α- amylase activity was determined according to Apar & Ozbek (2005) 200 µl of the enzyme were incubated with 1ml of 0.2 % soluble starch in acetate buffer (0.05 M; pH 5.9) at 40 °C for 10 min. 200 µl of the reaction mixture was added to 5 ml of an iodine solution to stop the reaction. 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.

Protein estimation

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

Carbohydrates used for the enzyme chemical modification

Pectin (MW $30x10^3$-$100x10^3$), dextran (MW 70 $x10^3$) and dextran (MW 80 $x10^3$) were from Sigma Chemical Co., USA.

Preparation of sodium periodate activated polysaccharides

α- amylase was coupled to soluble polysaccharides by the method reported by Ben Ammar *et al*. (2002) as follows: 250 mg of each polysaccharide were dissolved in 10 ml of 0.25 M sodium periodate solution and allowed to stand at 30°C for 6 hr, then 0.3 ml of ethylene glycol were added and allowed to react for 1 hr. The reaction mixture was dialyzed against water at 4°C overnight, and then lyophilized.

Enzyme coupling with activated 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.

Temperature profiles

Enzyme activities of both native and conjugated form were determined at indicated temperatures (30-80°C). The temperature data were replotted in the form of Arrhenius plots and the slope is related to the activation energy (E_A) for the molecule by the relationship:

$$\text{Slope} = E_A / 2.303\ R$$

where R is the gas constant (R = 1.976 Cal/mol).

Thermal stability

The 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. Determination of the half life another important parameter related to enzyme stability. 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:

$$t_{1/2} = 0.3 / \text{Deactivation rate constant}$$

Effect of reaction pH and pH stability

The effect of pH value on the activity of free and conjugated was investigated using buffer (0.05M) with different pHs (from 5.0 to 8.0). The pH stability of α-amylase was determined after pre-incubating the enzyme for 1hr at 30°C with buffer (0.05 M) at different pHs (from 5.0 to 9.5), followed by adjusting the pH to the value of standard assay system.

Results and Discussion

The stabilization of enzymes is very important, especially when the stability is required for the industrial use of the enzyme of interest. In this study, a method to stabilize α- amylase from *B. acidocaldarius* was achieved using simple and quick method. It depends on the oxidation state of different polysaccharides with periodate to produce a dialdehyde with two free hydroxyl groups able to link with the α-amylase molecule with any cross-linkage agent. It was reported that protein glycosylation might affect the hydrophilic/ hydrophobic balance and/ or net charge at the protein surface, leading to changes in protein-solvent and protein-protein interactions. These, in turn, could lead to changes in folding, stability, protease resistance, or biologic activity of glycosylated proteins (Pedrosa *et al.*, 2000). Among all preparations (Table 1), the enzyme conjugated to dextran (MW 70 $x10^3$) showed the highest retained activity (77.7 %) of the specific activity of the native enzyme and was selected for further investigations. The 23 % decrease in specific activity after glycosylation could be attributed to the rigidification of the enzyme protein conformation (Lenders & Crichton, 1984 and Fernandez *et al*., 2004). On the other hand, Gottschalk & Jaenicke (1991) deduced that the covalent attachment of the enzyme to the immobilization matrix would also lead to a decrease in catalytic activity owing to the decrease in the flexibility of the enzyme molecule. The drop of the specific activity after the glycosylation of other enzymes were previously reported (Abdel-Naby, 1999 and Ben Ammar *et al*., 2002).

Properties of the native and conjugated α- amylase enzyme

The optimum pH of the native and dextran conjugated α- amylase was studied using different pHs values ranging from 5.0 to 8.0. The results (Fig. 1) indicated that the two forms of α- amylase expressed an activity optimum at pH 7.25. This means that the ionization of the amino acid residues at the active site remains unaffected by the glycosylation process. Similar result was reported for other glycosylated amylase (Srivastava, 1991).

TABLE 1. Covalent coupling of *B. acidocaldarius* α-amylase to activated polysaccharides.

Activated polysaccharides	Coupled enzyme		Specific activity of conjugates (U/ mg protein)	Recovered activity (%)
	Protein (mg)	Activity (U)		
Pectin $30x10^3$-$100x10^3$	2.7	1382.4	512	31.4
Dextran $70\ x10^3$	1.9	2408.5	3965.39	77.74
Dextran $80\ x10^3$	1.45	1608.5	1109.3	68.03

Enzyme added to one gram activated polysaccharides in 1.40 mg protein containing 7141.17 U

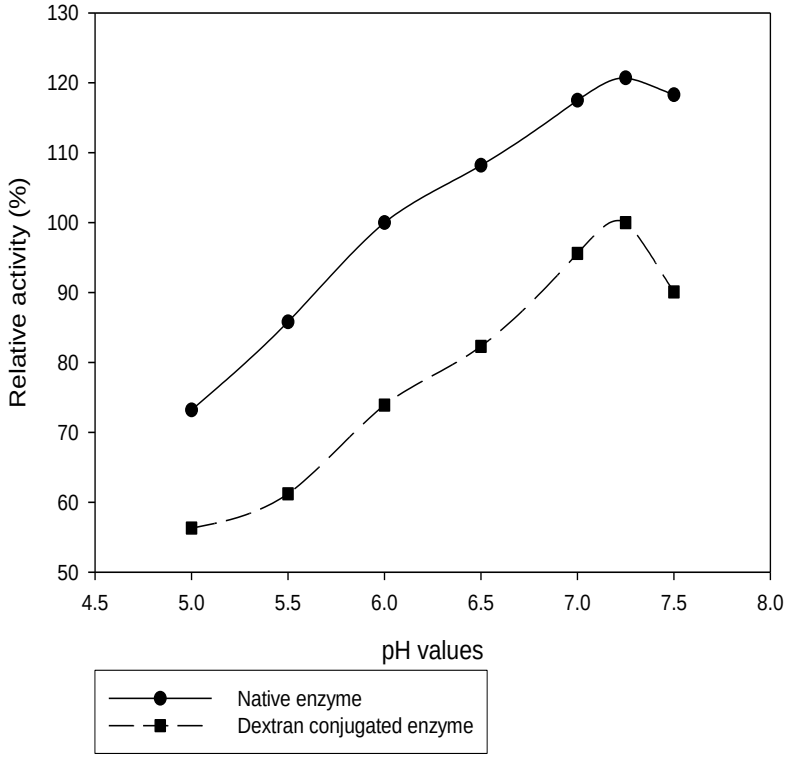

Fig.1. Effect of pH of the reaction on the activity of native and dextran conjugated α-amylase.

Activities of native and conjugated α- amylase were assayed at various temperatures (30- 70°C). The temperature optimum of the enzyme shifted from 50°C to 60°C upon conjugation (Fig. 2). The increase of the temperature is probably a consequence of enhanced thermal stability. El-Refai (2000) reported the changes in the optimal temperature of bacterial cyclodextrin glucosyltransferase from 65 to 80°C after glycosylation. Fernandez *et al*. (2004) reported changes in the optimal temperature of α-chymotrypsin after glycosylation. The plots for both the native and conjugated enzyme were linear (Fig. 3) and the values of the energy of activation were calculated as 2.37 and 2.23 Kcal/mol, respectively. This result indicated that the applied glycosylation procedure introduced changes in the structure of the enzyme molecule which enhanced its catalytic properties. A similar result was reported for other enzymes by Ahmed *et al*. (2007). Abdel-Naby (1999) pointed to the decreased in activation energy of *Aspergillus niger* cellobiase enzyme after glycosylation.

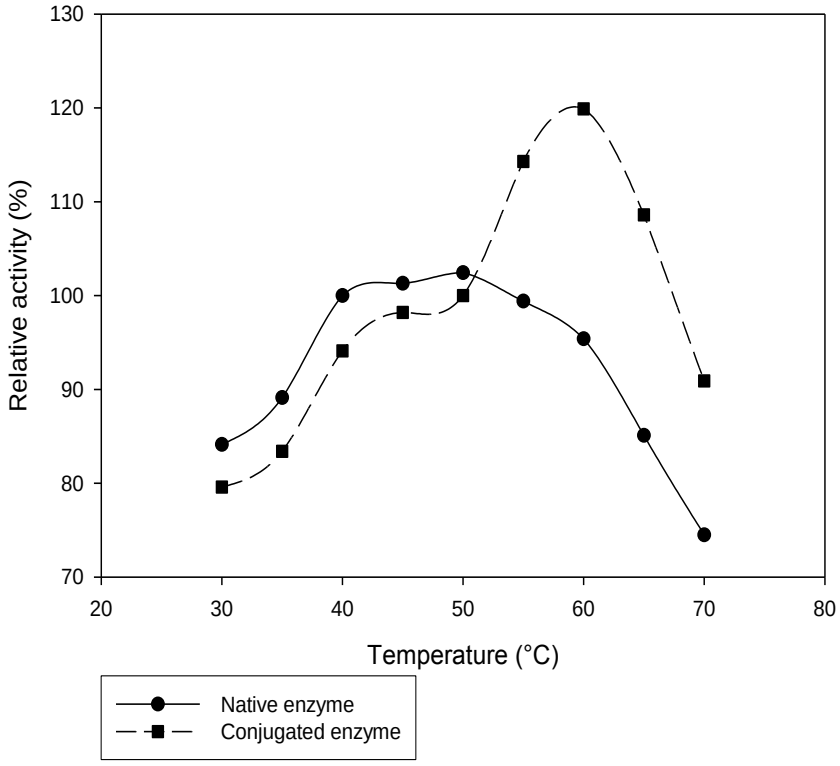

Fig.2. Effect of temperature of the reaction on the activity of native and dextran conjugated α-amylase.

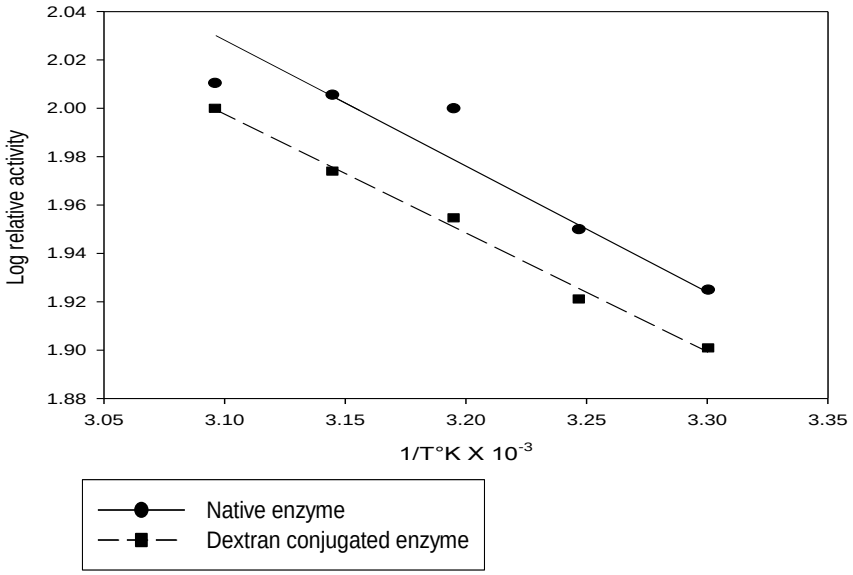

Fig.3. Arrhenius plots for the native and dextran conjugated α-amylase .

Lineweaver-Burk plots of the native and conjugated α- amylase with potato starch (Fig. 4**)** gave K_m (Michaelis constant) of 0.86 and 0.92 mg/ml, respectively. The V_{max} (maximal reaction rate) of the native and conjugated α- amylase were 144.1 and 122.7 U/mg protein, respectively. The apparent increase of K_m value after conjugation 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. Consequently, the maximum reaction rate of the enzyme-catalyzed reaction was lower than the native enzyme (Gottschalk & Jaenicke, 1991). The increase of K_m value and decrease of the V_{max} after stabilization of cellobiase by covalent coupling to soluble polysaccharide was reported by Abdel-Naby (1999).

The rates of heat inactivation of the native and conjugated α- amylase were investigated (Table 2). In general, covalent attachment of dextran to α- amylase molecule protected the enzyme against heat inactivation. For example, the calculated half-life values ($t_{1/2}$) showed that the heat inactivation of the conjugated enzyme at 50 and 60°C were 89 and 56 min, respectively whereas the native enzyme was less stable ($t_{1/2}$ of 60 and 47 min, respectively). The deactivation rate constant at 80°C for the conjugated α- amylase is about $11.9x10^{-3}$ min which is lower than that of the native α- amylase ($14.8x10^{-3}$ min). The mechanism involved in the carbohydrate-induced stability of glycosylated proteins by rigidification of the conformation has already been discussed (Klibanov, 1983). 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. Fernandez *et al*. (2004) reported that the modified α-chymotrypsin was more resistant to thermal inactivation at different temperatures.

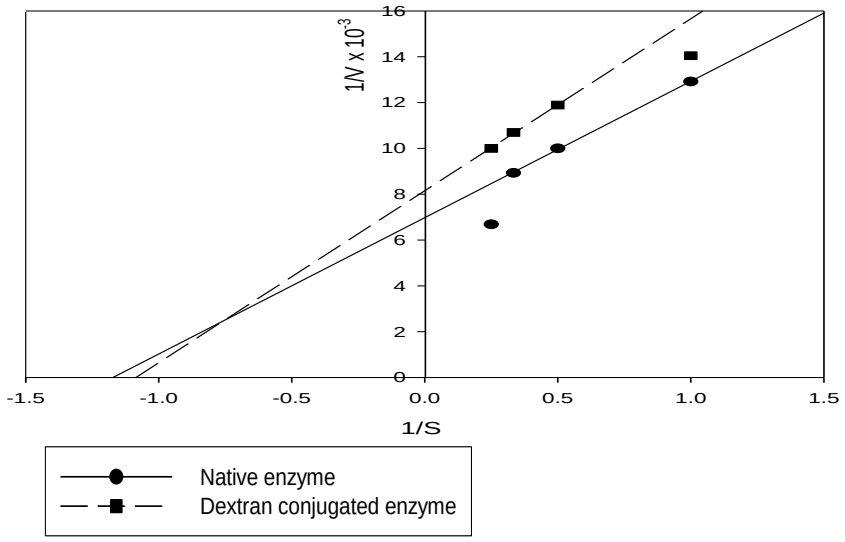

Fig.4. Lineweaver-Bulk plot of native and dextran conjugated α-amylase.

TABLE 2. Thermal stability of native and dextran conjugated α-amylase.

Temperature (°C) / Time of heating (min)	Residual activity (%)							
	Native enzyme				Conjugated enzyme			
	50	**60**	**70**	**80**	**50**	**60**	**70**	**80**
Zero	100	100	100	100	100	100	100	100
15	85.0	80.5	67.2	57.5	89.9	84.7	79.6	66.6
30	71.1	65.1	50.3	30.6	78.5	71.6	68.2	57.6
45	65.0	50.5	34.9	2.1	70.2	66.5	54.7	33.7
60	58.5	37.5	33.5	0	63.1	54.6	48.6	20.4
75	25.4	13.6	4.3	0	48.3	37.8	21.6	11.9
90	4.5	0	0	0	23.6	17.9	11.6	7.0

The results shown in Fig. 5 demonstrate that α- amylase conjugated with dextran was significantly more stable against incubation at different pH values (6.0-9.5) than the native enzyme. Fernandez *et al.* (2004) found that, α-chymotrypsin stabilized by chemical modification was more stable at pH 9.0 than the native enzyme.

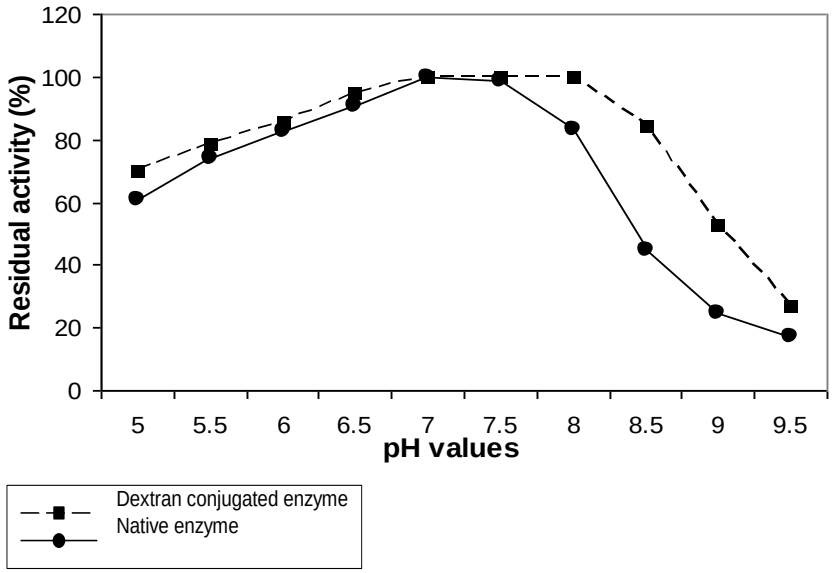

Fig. 5. pH stability of native and dextran conjugated α-amylase .

The enzyme activity was assayed after incubating with various metal ions (10 mM) at 30°C for 30 min (Fig. 6). In general, it was observed that the inhibitory effect of the investigated metal ions was less pronounced with the conjugated enzyme as compared with the free α-amylase. The results pointed to the inhibitory effect of most metal ions tested on the activity of native and conjugated enzyme. Treatment of proteins with high water-binding salts (like $MgSO_4$) reduces the water shield surrounding them and consequently, the stability was negatively affected. In case of the conjugated α-amylase water shield was probably preserved due to the hydrophilic nature of the polysaccharide attached to it, the water shield was probably preserved. A similar explanation was given by Srivastava (1991). On the other hand, Cu^{2+} ion decreased the enzyme activity, this inhibitory effect was higher in the native enzyme (65.9 %) than the modified enzyme (29.4 %). The results revealed that the glycosylation of α-amylase formed stable covalent bonds that led to achievement of resistance against chemical and thermal denaturation.

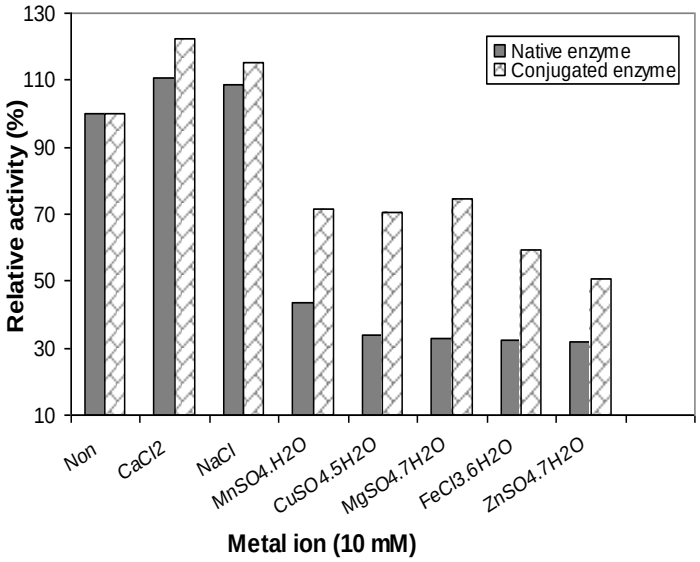

Fig.6. Effect of some metal ions on the activity of native and dextran conjugated α-amylase.

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التغير الوظيفى لانزيم ألفا أميليز المعدل بالدكستران والمنتج بواسطة *Bacillus acidocaldarius*

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قسم كيمياء المنتجات الطبيعية والميكروبية - المركز القومي للبحوث– الدقي و*قسم الكيمياء- كلية العلوم- جامعة حلوان و** مركر البحث والتطور-جامعة مصر للعلوم والتكنولوجيا- القاهرة - مصر.

تهدف هذه الدراسة إلى محاوله تعديل انزيم ألفا أميليز المنتج بواسطة سلالة *Bacillus acidocaldarius* ثم دراسة التغير الوظيفى الناتج عن هذا التعديل. وقد تم ذلك عن طريق الأرتباط التساهمى للأنزيم مع بعض السكريات العديدة المنشطة. أظهر البحث أن الأنزيم المرتبط مع الدكستران له أعلى قدرة على الاحتفاظ بالنشاط الأنزيمى (٧, ٧٧٪) مقارنه بالسكريات الأخرى، وقد أظهر الأنزيم المعدل درجة ثبات أعلى لدرجات أس هيدروجينى مختلفة كما ارتفعت درجة الحرارة المثلى للتفاعل بمقدار ١٠ درجات مئوية مقارنة بالأنزيم الأصلى. وأدت عملية الأرتباط مع الدكستران إلى زيادة قيمة ثابت ميخائيل (K_m) وانخفاض قيمة معدل السرعة القصوى للتفاعل (V_{max}) بالاضافة إلى انخفاض طاقة التنشيط (E_A). وقد أدت عملية التعديل إلى حماية الانزيم من التثبيط الحرارى. كما أظهر الإنزيم المعدل درجة ثبات حراري عالية عند التعرض لدرجات حرارة مختلفة وفترات زمنية مختلفة مقارنة بالأنزيم الأصلى حيث أعطى ٦٨,٢٪ نشاط متبقي بعد التحضين لمدة ٣٠ دقيقة على ٧٠°م فى حين اظهر الأنزيم الأصلى ٥٠,٣ ٪ فقط. أشارت الدراسة إلى أن الإنزيم المعدل له درجة ثبات حرارى أعلى حيث أعطى قيم أعلى عند حساب فترة نصف الحياة $t_{1/2}$ (٨٩، ٥٦ دقيقة) عند درجات حرارة ٥٠، ٦٠°م على التوالى، على الجانب الأخر أظهر الأنزيم الأصلى درجة ثبات أقل (٦٠، ٤٧ دقيقة)، على التوالى عند نفس درجات الحرارة وقد تم تقدير قيمة ثابت معدل التثبيط عند ٨٠°م للأنزيم المعدل وكانت أقل من الأنزيم الأصلى (١١,٩×١٠$^{-٣}$، ١٤,٨×١٠$^{-٣}$ / دقيقة).أدت عملية التعديل إلى زيادة ثبات الانزيم المعدل للتثبيط الكيميائى (فى وجود أيونات بعض المعادن) مقارنة بالأنزيم الأصلى ففى وجود أيونات Cu^{2+} احتفظ الأنزيم المعدل بحوالى ٧٠,٦ ٪ من النشاط فى حين احتفظ الأنزيم الأصلى بحوالى ٣٤,١ ٪.