Effect of Metal Ions on Kinetics and Thermo Stability of α-Amylase Isolated from Aspergillus oryzae

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Abstract

Alpha amylase produced from Aspergillus oryzae after thermal treatment has been rarely investigated. The purpose of current research was to study the effect of metal ions on the activity and stability of α-amylase. Supplementation of Ca2+ and Co2+ improved the enzyme activity up to 2 folds, while rest of the metals did not affect the enzyme activity. The Michaelis-Menten constants (Vmax, Km and Vmax/Km) for soluble starch hydrolysis by α-apo-amylose were 65 Umin⁻¹ mg⁻¹, 2.01% (w/v) and 32.26 Umg⁻¹, respectively. The thermodynamics constants (ΔH*, ΔG* and ΔS*) of irreversible thermal inactivation for Ca2+ bonded amylose were 154.49 kJ mol⁻¹, 106.09 kJ mol⁻¹ and -147 J mol⁻¹ K⁻¹, respectively. The Ca2+ ions made the α-amylase about 53 folds more stable as compared to the control. The thermostability of α-amylase was also increased after thermal treatment and become more heat tolerant which is necessary requirement for α-amylase to perform ideal result in different industries. © 2018 Friends Science Publishers

Keywords: Enzyme production; Aspergillus oryzae; Kinetic parameters; Submerged culture; Metal ions; Thermostability

Introduction

Aspergillus oryzae is a probiotic filamentous fungus, widely used to produce a variety of proteins known for beneficial effects in pharmaceutical, beverages and conventional food industries (Lene et al., 2000; Bozic et al., 2011). Aspergillus sp. has gained much attention in modern biotechnology due to easy availability, proteins production, higher productivity and suitability for genetic manipulations. Therefore, different species of the genus Aspergillus such as A. niger, A. oryzae, A. flavus, A. tamarii, A. fumigatus and A. kawachii are being frequently used for the production of α-amylase, protease and glucoamylase etc. (Nagamine et al., 2003; Ramachandran et al., 2004; Rasooli et al., 2008; Bakri et al., 2009).

Amylases (EC 3.2.1.1) correspond to about 25–33% of the total enzymes used for starch hydrolysis in various industries to produce maltose, glucose and a variety of alpha-limit dextrin containing α-1,6 bond and a combination of malto oligosaccharides (Elayaraja et al., 2011; Haq et al., 2012; Shah et al., 2014). Amylases are classified into exo amylases (β-amylase, glucoamylase), endo amylases (α-amylases) and debranching enzymes (isoamyolase, pullulanase). Most of α-amylases need a metallic ion as cofactor, thus named as metalloenzyme. Mostly they need calcium ions for optimal function, prolonged stability and structural integrity. The α-amylase family is roughly categorized in two groups, one of which hydrolyses the starch while other modifies it. The enzymatic hydrolysis is favoured in starch processing industry used for acid hydrolysis due to many different benefits including stability of the generated products, the reaction specificity, removal of neutralization steps and minimum requirements for energy (Sathyanarayana et al., 2005).

At industrial scale enzymes may be obtained from plant, animals and microbes, but the concentration of enzyme obtained from first two sources is limited while, starch processing industries require higher amounts of α-amylase. Hence, microbial sources have gained much attention for enzyme production in abundant quantities to meet the necessary industrial requirements. Furthermore, microbial enzymes are also quite useful due to enhanced features (Hussain et al., 2013). Different species of the genus Aspergillus, such as A. niger and A. oryzae are being used for the production of alpha amylose. Owing to rising demand for these enzymes transgenic strains have become focus of a number of industries. The enzyme produced by these mutant strains has higher stability to survive under
extreme pH and temperature fluctuations at industrial level (Umbreen et al., 2013). Furthermore, it has been demonstrated that such strains require more dispositive ion as Ca\(^{2+}\) for thermostability (Niaz et al., 2010). Therefore, present study deals with the determination of metalo nature and kinetic mechanism of activation/inhibition of α-amylase from transgenic strain of Aspergillus oryzae (Oryzae mutant) induced by addition of calcium and cobalt ions.

Materials and Methods

All the chemicals used were of analytical grade and obtained from Sigma Chemical Company, USA. Soluble starch was purchased from Rafhan Maize Products (Pvt) Ltd, Faisalabad. The transgenic strain of Aspergillus oryzae (oryzae mutant) was obtained from IBD, NIBGE Faisalabad, culture was maintained on potato-dextrose agar medium (PDA) and preserved at 4°C (Rashid and Saddique, 1998).

Enzyme Production and Harvesting

For production of enzyme soluble starch (1% w/v) was added in 45 mL of Vogel’s medium in 250 mL elnermeyer flask and was set at pH 5.0. About 7 glass beads were added in each flask after washing with water to rupture the mycelium. All flasks were capped with cotton, wrapped with aluminium foil and autoclaved at 121°C for 20 min and 1.05 kg/cm\(^2\) pressure. The flasks were inoculated with the spores of Aspergillus oryzae and incubated on orbital shaker (orbital shaker incubator TI-OSI-HR) at 120±5 rpm, 30°C for 72 h. After 72 h of incubation, α-amylase was harvested, filtered and centrifuged at 10,000 rpm at 4°C for 20 min. Finally obtained culture was concentrated by freeze drying (Lypholizer Alpha1-5).

Enzyme Assay

The amylase activity was determined as described by (Huma et al., 2012) using 1% soluble starch as a substrate and amount of released product was estimated by using di-nitro-saliclyc acid (DNS) method. The reaction mixture (2100 μL) contained 1 mL of sodium acetate buffer (50 mM, pH 5), 1 mL of soluble starch (1% W/V) and 100 μL of enzyme extract. The reaction tubes were incubated at 45°C for 30 min, after incubation the test tubes were dipped in boiling water for 5 min. The reaction was quenched rapidly by adding 2 mL of DNS solution and volume was made up to 4.1 mL and then boiled in water bath for 10 min. The tubes were cooled in ice bath and the absorbance was measured accurately by spectrophotometer at 550 nm. The amount of maltose released was determined by maltose standard curve.

One unit of alpha amylase activity was defined as the amount of enzyme required to release reducing sugars 1 μmole of maltose per minute at pH 5.0 at 45°C.

Bradford Method was used to determine the total protein content in the solution (Bradford, 1976) using bovine serum albumin (BSA) as standard.

Optimization and Characterization of α-amylase from A. oryzae

Crude α-amylase obtained from the mutant strain of Aspergillus oryzae was partially purified by ammonium sulphate precipitation. Total proteins and alpha amylase activity were determined before and after dialysis of ammonium sulfate precipitation (Riaz et al., 2012). Enzyme activity of α-amylase produced by Aspergillus oryzae and purified was further analyzed for effect of different metals (CaCl\(_2\), MgCl\(_2\), FeCl\(_2\), MnCl\(_2\), CoCl\(_2\), AlCl\(_3\)) in concentration range of 1-8 mM (Niaz et al., 2010). Moreover, apo-α-amylase was assayed in the presence of varied concentrations of CaCl\(_2\)(6.0-7.0 mM) and CoCl\(_2\)(6.0-7.0 mM) with temperature range 40-60°C and 34-55°C, respectively (Rashid and Saddique, 1998). The optimum pH of the alpha amylase isolated from Aspergillus oryzae was determined by measuring activity at different temperatures ranging from (35-50°C) against various pH ranging buffers from 3.8 -10.4. The enzyme activity was determined in the presence of CaCl\(_2\) (6.0 mM) and CoCl\(_2\) (6.0 mM) (Dixon and Webb, 1979).

Kinetic Characterization of Enzyme

The activation energy for starch hydrolysis was determined by plotting the data of temperature optimum according to Arrhenius as described (Rashid and Saddique, 1998; Siddiqui et al., 2000). Effect of calcium and cobalt ions on Michaelis-Menten kinetic constants (\(K_\text{m}\), \(V_{\text{max}}\), \(K_{\text{cat}}\)) were determined by using different concentrations of soluble starch as a substrate (0.025-0.6% w/v), while keeping the metal and enzyme concentrations constant. Line weaver Burk plot was used to determine the Michaelis-Menten constant (Rashid and Saddique, 1998; Siddiqui et al., 2000). The enzyme was incubated at 45°C with 6 mM CaCl\(_2\) and CoCl\(_2\) in a separate test tube for 30 min. Irreversible thermal inactivation of metal treated α-amylase enzyme was estimated by incubating the enzyme at different temperatures (45, 48, 51, 57, 62, 65°C). Time course aliquots were withdrawn, cooled on ice for at least 30 min and then assayed for α-amylase activity at 45°C. The data was fitted to first order plots and analyzed (Umbreen et al., 2013).

Thermodynamics of Thermal Inactivation of α-amylase

The first order rate constant for irreversible thermal denaturation (\(K_d\)) of alpha amylase and the activation energy for denaturation (\(E_d\)) were determined using the Arrhenius plot. The thermodynamic parameters for thermostability
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were calculated by rearranging the Eyring’s absolute rate equation derived from the transition state theory (Eyring and Stearn, 1939).

**Statistical Analysis**

All the analysis was made in triplicate and data was analyzed statistically. The mean and standard deviation of the values were calculated using SPSS (Version 17, Chicago, SPSS, Inc) and Slide Write Plus (Version 7.0.1, Advance Graphic Software, Inc) was used to draw graphs.

**Results**

**Effect of Metals on α-amylase Activity**

Six different metals (CaCl₂, CoCl₂, MnCl₂, FeCl₂, AlCl₃, and MgCl₂) were used in different concentrations (1, 2 and 3 mM) to evaluate their effect on α-amylase activity. Results showed (Fig. 1) that CaCl₂ and CoCl₂ significantly (p≤0.05) activated the enzyme at all concentrations, while MnCl₂, FeCl₂, and AlCl₃ showed the inhibiting effect on the enzyme activity at all concentrations. Maximum activity of 155% and 150% was observed with CaCl₂ (2 mM concentration) and CoCl₂ (3 mM concentration) respectively, while FeCl₂ and AlCl₃ inhibited the amylase activity at all concentrations (Fig. 1). Therefore, further characterization was carried out using different concentrations of CaCl₂ and CoCl₂ only.

Various concentrations of calcium and cobalt (1–8 mM) were used to study their effect on pH and temperature optimum, kinetics of soluble starch hydrolysis and irreversible thermal stability of enzyme. Ca²⁺ showed the maximum activity (180%) at 6 mM concentration and Co²⁺ gave maximum activity (170%) at 7 mM concentration (Fig. 2).

**Effect of Metals on Optimum Temperature and Activation Energy**

The apo-α-amylase was assayed in the presence of different CaCl₂ (6-7 mM) concentrations with temperature range of 40-60°C. Effect of metals on temperature and activation energy (Eₐ) for soluble starch hydrolysis by α-amylase was determined by Arrhenius plot (Fig. 3). The activation energy of apo-α-amylase determined by Arrhenius plot was 4.58 KJ mol⁻¹ at optimum temperature for the formation of enzyme-substrate complex. Furthermore, activation energy (Eₐ) of enzyme coupled with metals at optimum temperature (55°C) for calcium using 6.0 mM concentration was calculated to be 27.2 KJ mol⁻¹ and for Co²⁺, at optimum temperature (50°C) Eₐ was 15.68 KJ mol⁻¹.

**Effect of Metals on pH Optimum**

The α-amylase activity was determined in the presence of different pH values ranging from 3.8-10.4 at various temperatures. Apo-α-amylase from *Aspergillus oryzae* showed optimum pH 5.0 and enzyme coupled with CaCl₂ and CoCl₂ showed optimum pH of 7.5 and 5.6, respectively (Fig. 4 and 5). The pKₐ₁ and pKₐ₂ of active site ionizable groups were determined by applying Dixon plot.
The pKa₁ and pKa₂ of ionizable groups of apo α-amylase were 4.7 and 5.6, respectively, while calcium and cobalt ions bound α-amylase showed pKa₁ and pKa₂ of 5.4 & 6.9 and 4.95 & 6.75, respectively (Fig. 6 and 7).

Effect of Metals on Substrate Hydrolysis

The α-amylase was assayed at various substrate concentrations at 50°C and pH 5.0. Double reciprocal plot (Line-Weaver Burk plot) was applied to determine Vₘₐₓ and Kₘ. The Vₘₐₓ and Kₘ of Apo α-amylase was 65 Umin⁻¹ mg⁻¹ protein and 2.0% (w/v), respectively. Calcium ions activated the α-amylase of A. oryzae by increasing Vₘₐₓ at all CaCl₂ concentrations (Fig. 8). Maximum activation was observed at 6.0 mM CaCl₂ and Vₘₐₓ at this concentration was 662 U min⁻¹ mg⁻¹. The value of Kₘ decreased at this concentration 10.0% (w/v). While in case of Cobalt ions, Vₘₐₓ and Kₘ decreased at 6.0 mM concentration (Fig. 9). Vₘₐₓ and Kₘ values for CoCl₂ were 246 Umin⁻¹ mg⁻¹ and 6.67% (w/v). For CaCl₂ at 6.0 mM concentration, the specificity constant (Vₘₐₓ/Kₘ) for soluble starch hydrolysis was 66.23 while in the case of CoCl₂ and apo-enzyme, the (Vₘₐₓ/Kₘ) values was 39.61 and 32.26, respectively.

Effect of Metals on Irreversible Thermal Stability

The apo-α-amylase from Aspergillus oryzae and metal treated enzyme was heated at different temperatures ranging from 45-57°C for 75 min. Aliquots were taken at regular time intervals 0 (control), 5, 10, 15, 20, 30, 40, 50, 60 and 75 min. Pseudo first order plots were applied to determine the rate of irreversible thermal inactivation (Fig. 10, 11, 12 and 13). The half life (t₁/₂) and thermodynamic parameters of irreversible thermal stability (ΔH*, ΔG* and ΔS*) of α-amylase were
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Table 1: Kinetics and thermodynamics of irreversible thermostability of apo-α-amylase from *A. oryzae*

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Temp (K)</th>
<th>(K_d) (min(^{-1}))</th>
<th>(t_{1/2}) (min)</th>
<th>(\Delta H^*) (kJ mol(^{-1}))</th>
<th>(\Delta G^*) (kJ mol(^{-1}))</th>
<th>(\Delta S^*) (J mol(^{-1}) K(^{-1}))</th>
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</thead>
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<td>15</td>
<td>95.12</td>
<td>100.73</td>
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</tr>
</tbody>
</table>

\(K_d\) \(= (\text{first order rate constant of denaturation})\) was determined from Fig. 10.
\(t_{1/2}\) \(= (\text{half-life})\) \(= 0.693/K_d\)
\(\Delta H^* = E_a - RT\) \(E_a\) of apo-amylase was 97.86 kJ mol\(^{-1}\) and was calculated from Fig. 13.
\(\Delta G^* = -RT \ln (K_d/k_bT)\)
\(\Delta S^* = (\Delta H^* - \Delta G^*)/T\)

Table 2: Effect of CoCl\(_2\) on kinetics and thermodynamics of irreversible thermostability of α-amylase from *A. oryzae*

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Temp (K)</th>
<th>(K_d) (min(^{-1}))</th>
<th>(t_{1/2}) (min)</th>
<th>(\Delta H^*) (kJ mol(^{-1}))</th>
<th>(\Delta G^*) (kJ mol(^{-1}))</th>
<th>(\Delta S^*) (J mol(^{-1}) K(^{-1}))</th>
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<td>103.96</td>
<td>-135</td>
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</tbody>
</table>

\(K_d\) \(= (\text{first order rate constant of denaturation})\) was determined from Fig. 12.
\(t_{1/2}\) \(= (\text{half-life})\) \(= 0.693/K_d\)
\(\Delta H^* = E_a - RT\) \(E_a\) of Co\(^{2+}\) bound amylase was 62.11 kJ mol\(^{-1}\) and was calculated from Fig. 4.13.
\(\Delta G^* = -RT \ln (K_d/k_bT)\)
\(\Delta S^* = (\Delta H^* - \Delta G^*)/T\)

Table 3: Effect of CaCl\(_2\) on kinetics and thermodynamics of irreversible thermostability of α-amylase from *A. oryzae*

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Temp (K)</th>
<th>(K_d) (min(^{-1}))</th>
<th>(t_{1/2}) (min)</th>
<th>(\Delta H^*) (kJ mol(^{-1}))</th>
<th>(\Delta G^*) (kJ mol(^{-1}))</th>
<th>(\Delta S^*) (J mol(^{-1}) K(^{-1}))</th>
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<td>106.09</td>
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</tbody>
</table>

\(K_d\) \(= (\text{first order rate constant of denaturation})\) was determined from Fig. 11.
\(t_{1/2}\) \(= (\text{half-life})\) \(= 0.693/K_d\)
\(\Delta H^* = E_a - RT\) \(E_a\) of Ca\(^{2+}\) bound amylase was 157.23 kJ mol\(^{-1}\) and was calculated from Fig. 13.
\(\Delta G^* = -RT \ln (K_d/k_bT)\)
\(\Delta S^* = (\Delta H^* - \Delta G^*)/T\)

Discussion

Amylases are called metaloenzymes and have at least one cation as necessary component for enzyme activity. Selection of cation is important for survival of enzyme during industrial applications at high temperature and pH.

Calculated as described (Siddiqui et al., 2000) and are presented in Table 1, 2 and 3. The energy of activation for irreversible thermal inactivation \(E_a\) of apo, Ca\(^{2+}\), Co\(^{2+}\) bound was 97.86, 157.23, 62.11 KJ mol\(^{-1}\), respectively, which was determined by Arrhenius plot. Co\(^{2+}\) bound enzyme showed least value of \(E_a\). The binding of the metals ions with α-amylase resulted in increased half life. Ca\(^{2+}\) showed increase in half life as compared to the apo enzyme. While the Co\(^{2+}\) showed decrease in half life as compared to the apo and calcium binding enzyme. The half life of apo, Ca\(^{2+}\), Co\(^{2+}\) bound α-amylase at 51°C was 131, 7001, and 98 min respectively. The \(\Delta G^*\), \(\Delta H^*\) and \(\Delta S^*\) of apo α-amylase, Ca\(^{2+}\), Co\(^{2+}\) bound α-amylase at 51°C were (104.72 KJmol\(^{-1}\), 95.17 KJmol\(^{-1}\), -29 Jmol\(^{-1}\)K\(^{-1}\)), (115.44 KJmol\(^{-1}\), 154.54 KJmol\(^{-1}\), 121 Jmol\(^{-1}\)K\(^{-1}\)), (103.94 KJmol\(^{-1}\), 59.42 KJmol\(^{-1}\), -137 Jmol\(^{-1}\)K\(^{-1}\)), respectively.

Fig. 9: Double reciprocal plot for the determination of effect of CaCl\(_2\) (6.0 mM) on kinetic constants \((V_{max} & K_m)\) for soluble starch hydrolysis by α-amylase of *A. oryzae*

Fluctuation (Zohra et al., 2014). Therefore, effect of various metals (CaCl\(_2\), CoCl\(_2\), MnCl\(_2\), FeCl\(_3\), AlCl\(_3\) and MgCl\(_2\)) was evaluated on α-amylase activity. Among all metals Ca\(^{2+}\) and Co\(^{2+}\) increased the enzyme activity while rest of the metals inhibited α-amylase activity at all concentrations. In accordance with the present study, another research work showed that different metals Fe\(^{3+}\), Cu\(^{2+}\), Zn\(^{2+}\) and Al\(^{3+}\)
strongly inhibit the α-amylase while Ca\(^{2+}\) stimulate the enzyme activity (Shafie et al., 2010). Furthermore, various concentrations of Ca\(^{2+}\) and Co\(^{2+}\) enhanced the enzyme activity to a certain level, after which a fall in activity was observed. Similarly, a significant stimulatory effect in enzyme activity was observed on addition of Co\(^{2+}\) with α-amylase by Prakash et al. (2011), whereas Afifi et al. (2008) did not observe any significant effect of this metal on enzyme activity. Moreover, in a study conducted by Zohra et al. (2014) it was declared that Ca\(^{2+}\) has stimulatory effect on α-amylase activity. Temperature has a significant role at industrial processes so stability of enzyme was checked under different temperature conditions on addition of Ca\(^{2+}\) and Co\(^{2+}\). The activation energy of apo-α-amylase determined by Arrhenius plot for the formation of enzyme–substrate complex was less than that of Ca\(^{2+}\) and Co\(^{2+}\). Contrary to the present study, Ahmed et al. (2008) observed the temperature optima range between 30-40°C for the alpha amylase produced from A. niger. Similarly activation energy (E\(_a\)) of free enzyme was 2.37 Kcal mol\(^{-1}\) which was higher than the immobilized enzyme by covalent binding or by ionic binding 1.05 and 1.59 Kcalmol\(^{-1}\) respectively (Ahmed et al., 2008), the difference in optimum temperature might be attributed to the strain difference and mutated strain used in present study may have different temperature requirements.

pH of the enzyme has enormous role in survival during industrial processes and most of native enzymes lose activity at higher pH values (Umbreen et al., 2013). α-amylase from Aspergillus oryzae showed lower optimum pH as compared to enzyme coupled with CaCl\(_2\) and CoCl\(_2\). So, the enzyme activity was observed to increase by coupling the enzyme with CaCl\(_2\) and CoCl\(_2\). In accordance with the present study, the production of extracellular thermostable α-amylase from moderate thermophilic Bacillus strain, isolated from fresh sheep’s milk showed maximal activity at pH 6.5 and stability was improved in the presence of Ca\(^{2+}\) ion (Konsula and Liakopoulou, 2004). Furthermore it was observed that calcium ions activated the α-amylase of A. oryzae and V\(_{\text{max}}\) was increased at all CaCl\(_2\) concentrations, while Cobalt ion resulted in decreased activity. Similarly, study conducted on the purification and characterization of alpha amylase from a bacterial strain of Bacillus licheniformis EMS-6 showed that enzyme was stable at the pH range 4.5-9.0 and optimum pH 7.0 (Haq et al., 2002).

It has been approved that metals binding with enzymes increases the protein stability. Moreover, the folded proteins are more stable by binding of the metals ions which is coordinated by donating of the lone pair from nitrogen and oxygen atoms. The present study showed that the enzyme was stable at optimum temperature but at high temperature the enzyme is denatured. Thermal denaturation of enzymes results in dissociation of subunit dissociation with an increased enthalpy (Umbreen et al., 2013) ultimately leading to increased entropy of activation. Increase in

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**Fig. 10:** Pseudo first order plots for irreversible thermal stability of A. oryzae apo-α-amylase

**Fig. 11:** Pseudo first order plots for the effect of Ca\(^{2+}\) on irreversible thermal stability of A. oryzae α-amylase

**Fig. 12:** Pseudo first order plots for the effect of Co\(^{2+}\) on irreversible thermal stability of A. oryzae α-amylase

**Fig. 13:** Arrhenius plots for the determination of E\(_a\)(d) of irreversible thermal inactivation of A. oryzae α-amylase
Gibb’s free energy ($\Delta G^*$) on addition of metals specially calcium ions showed that enzyme became more stable under high temperature as required by industrial processing. Furthermore negative value of enthalpy on addition of cobalt ion predicts less disorder in structure of enzyme so showing higher stability. In accordance with the present study Ikra-mul-haq et al. (2010) observed that thermodynamic parameters $E_a$, $\Delta S$, $\Delta G$ and $\Delta H$ for soluble starch hydrolysis of $\alpha$-amylase from $B$. licheniformis EMS-6 also increased.

**Conclusion**

In this research work, the effect of metals on kinetics of $\alpha$-amylase isolated from *Aspergillus oryzae* was evaluated. CaCl$_2$ activated the enzyme at all concentrations (1-8 mM) while other metals inhibited the enzyme activity. Ca$^{2+}$ ions improved the temperature optimum with 5°C increase as compared to the Co$^{2+}$ and apo enzyme. $E_a$ of Ca$^{2+}$ 27.2 K J mol$^{-1}$. Ca$^{2+}$ ions shifted the pH optimum toward basic site. Moreover the pka values confirm that the binding of Ca$^{2+}$ ions increased the specificity of soluble starch as compared to the apo enzyme. The Ca$^{2+}$ ions made the $\alpha$-amylase about 53 folds more stable as compared to the control (apo enzyme).

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**References**


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