ABSTRACT

The local fungal strain of *Aspergillus niger* was grown under submerged growth conditions on 2% sucrose (w/v) at 30°C, pH 5.0 for the production of extra cellular invertases.. The flasks were inoculated with 1.84 mg cells ml^{-1}. After 120 hrs of incubation, 14.26 U mg^{-1} protein was produced. Crude invertase was purified to homogeneity level after subjecting it to ammonium sulfate precipitation, HiLoad anion exchange, hydrophobic interaction and gel filtration chromatography on Pharmacia FPLC unit. The four step purification protocol for invertase resulted into 7.3 fold purification. The recovery of purified invertase was 25 %. The precipitation of invertase was carried out by adding solid ammonium sulfate to achieve 85% saturation at 0°C. The onset of invertase precipitation occurred at 50%, while complete precipitation was observed at 85%. Purification was increased to about 3.6 fold after Hiload Q-sepharose column of FPLC. The invertase was eluted at 520 mM NaCl concentration. Partially purified invertase from Hiload column was then applied on phenyl superose column of HIC and the enzyme purification after this stage was 5.9 fold, whereas, invertase recovery was 36 %. The invertase was eluted at 850 mM ammonium sulphate concentration. The purified invertase after HIC was finally applied on gel filtration column of FPLC.

Various metal ions such as: Hg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+} and Fe^{3+} were tested to evaluate their effect on the activity of *Aspergillus niger* invertases. All metals significantly inhibited the invertase activity except $Ca^{2+,}$ which reasonably enhanced the enzyme activity. The Ca^{2+} ions showed about 2.5 fold activation at 1 and 2 mM $CaCl_2$, while the invertase activity was inhibited at 3 mM $CaCl_2$. Therefore, Ca^{2+} was selected to determine its effect on various physiochemical properties of the enzyme. Effect of $CaCl_2$ concentrations on temperature optimum and the activation energy (*E*a) for sucrose hydrolysis by invertase was determined by applying Arrhenius plot. The temperature optimum remained same at all $CaCl_2$ concentrations as compared to apo-enzyme (60°C) with the only exception that at 2.5mM $CaCl_2$ it was increased to 65°C. The *E*a for the formation of ES*-complex (sucrose hydrolysis) at all metal concentrations was decreased except at 1.5mM $CaCl_2$, it was slightly increased.

It was observed that invertase of *A. niger* worked optimally at pH 5.3 in the presence of 1.5mM $CaCl_2$, while metal free invertase i.e. apo-enzyme showed pH optima of 4.4. The p*K*a of active site residues was determined by applying Dixon plot. The pKa_1 and pKa_2 of ionizable groups of apo-invertase were 3.45 and 5.9, respectively, while metal treated invertase showed pKa_1 and pKa_2 of 3.45 and 6.4, respectively.

The k_{cat} and K_m of Apo-invertase was 1824 s^{-1} and 0.83% (w/v), respectively. Calcium ions activated the invertases of *A. niger* because the turn over (k_{cat}) was increased at all $CaCl_2$ concentrations. Maximum activation was observed at 2.0 mM $CaCl_2$. The Michaelis constant (K_m) value for apo-enzyme was lowest as compared to those in the presence of Ca^{2+}. The K_m value in the presence of 2.5 and 3 mM $CaCl_2$ was 1.25% (w/v), which was lowest as compared to other concentrations. The specificity constant for sucrose (k_{cat}/K_m) was increased in the presence of 2.0 mM $CaCl_2$, while at all other concentrations of $CaCl_2$ it was decreased as compared to apo-invertase.

Thermodynamic parameters for effect of Ca^{2+} on hydrolysis of sucrose by the invertases were also calculated. The free energy for activation of sucrose hydrolysis (ΔG^*) by the apo-invertase was 61.07 kJ mol^{-1}. The Ca^{2+} decreased ΔG^* at all metal concentrations and least value was found at 2.0 mM $CaCl_2$ which was equal to 58.08 kJ mol^{-1}. Enthalpy change for activation of substrate hydrolysis (ΔH^*) was lowest at 0.5 mM $CaCl_2$ (16.42 kJ mol^{-1}) as compared to apo-invertase and in the presence of other metal concentrations. The ΔS^* which is the entropy change for activation of substrate hydrolysis was highest for invertases bound with 1.5 mM $CaCl_2$ and was equal to -152.56 J mol^{-1} K^{-1}. The free energy change for activation of transition state formation (ΔG^*_{E-T}) was also calculated and the value in the presence of 2.0 mM $CaCl_2$ was lowest (-22.36 kJ mol^{-1}) as compared to apo and other metal concentrations. The free energy change for activation of enzyme substrate complex formation (ΔG^*_{E-S}) was lowest for apo-invertase (-0.5 kJ mol^{-1}). Among calcium ion treated invertases the value of ΔG^*_{E-S} in the presence of 2.5 and 3.0 mM $CaCl_2$ was lowest (0.62 kJ mol^{-1}).

Calcium ions activated catalysis of sucrose by invertases of *Aspergillus niger*. The Ca^{2+} ions showed activation at almost all concentrations. The double reciprocal plots between 1/*v* and 1/s at a series of fixed concentrations of activator (Ca^{2+}) intersected almost at the same point on +1/s axis (Fig-4.20A). Such type of system

occurs when the activation is of partially non-competitive type. The pattern of Dixon plots showed that up to 0.2 % (w/v) sucrose concentration, activation of the invertase by calcium ions was of partially noncompetitive type, which was confirmed from the values of kinetic constants (K_m^A, Ks^A, K_m^S and K_s^S). For such type of activation the $K_m^A = Ks^A$ and $K_m^S = K_s^S$ and it was found that the values of these constants were almost same. The K_m^S i.e. dissociation constant of sucrose from E already bound with A was 0.178 % (w/v) and K_s^S (dissociation constant of S from E) was 0.2 % (w/v). While, K_m^A (dissociation constant of A from E bound with S) was equal to 1.43 mM $CaCl_2$ and K_s^A i.e. dissociation constant of A from free E was 1.74 mM $CaCl_2$.

On the other hand at higher sucrose concentration the activation by Ca^{2+} ions was of partially uncompetitive type because according to Dixon and Webb for such type of activators the $K_m^A \neq Ks^A$ and $K_m^S \neq K_s^S$. The K_m^S was 4.6 % (w/v) and K_s^S was 10.0 % (w/v), while, K_m^A was equal to 1.34 mM $CaCl_2$ and K_s^A was 0.64 mM $CaCl_2$.

The energy of activation for irreversible thermal inactivation ($Ea_{(d)}$) of apo- and Ca^{2+} bound invertases was 26.35 and 27.5 kJ mol^{-1}, respectively. The chelation of metals from native enzyme resulted into a sharp decrease in half life. Surprisingly, Ca^{2+} bound invertase showed an increase in half life at 59°C, otherwise calcium binding showed destabilizing effect at all temperatures. The half life of apo- and Ca^{2+} bound invertases at 59°C was 24.3 and 50.4 min, respectively. The ΔH*, ΔG*, ΔS* of apo-invertase and Ca^{2+} bound invertase at 59°C were (23.59 kJ mol^{-1}, 102.73 kJ mol^{-1} and -238.37 J mol^{-1} k^{-1}) and (24.74 kJ mol^{-1}, 104.74 kJ mol^{-1} and -240.97 J mol^{-1} k^{-1}), respectively. The Apo-invertase showed a high temperature inactivation trend. The refolded apo-enzyme at 65°C showed a half life of 21.8 min.