Asian Journal of Chemistry; Vol. 24, No. 10 (2012), 4305-4308

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Study of Various Factors Affecting Thermal Stability of Xylanase

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(*Received*: 27 June 2011; *Accepted*: 2 May 2012) AJC-11377

In this work, we have studied the factors affecting the thermal stability of xylanase. Metals like (Al^{3+}, Cu^{2+}, Hg^{2+}, Li^{+}, Mn^{2+}) with a concentration of 5 mM were applied to determine their effect on irreversible thermal stability of xylanase. Native xylanase was near about completely destroyed at 75 ºC with half life of 19.8 min. Attachment of Al^{3+} slightly stabilized the enzyme with half life 46.5 min at 75 ºC. Cu^{2+} does not stabilize or destabilize the enzyme. Attachment of Hg^{2+} stabilized the enzyme at 55 ºC but stability goes on decreasing with the increase in temperature. Li^{+} and Mn^{2+} stabilized the enzyme slightly with half life 82.82 and 25.9 min, respectively. Al^{3+} and Hg^{2+} were the best metals due to their stabilizing effect. These metals stabilized the enzyme at 55 ºC with 1888.3 min and 1039.0 $t_{1/2}$. Thermodynamic studies also supported the Al^{3+} and Hg^{2+} for their stabilizing capabilities. ΔG value of native enzyme was 97 kJ/mol at 55 ºC and ΔH was 119.18 kJ/mol, while ΔG and ΔH with Al^{3+} coordination was 102.57 and 169.11 kJ/mol at 55 ºC.

Key Words: Thermal stability, Reversible and irreversible, Destabilization temperature, Half life of enzyme activity.

INTRODUCTION

Xylanases (endo β-1,4-xylanases) cleave the xylan backbone into smaller ligosaccharides. They are the key enzymes for xylan degradation and differ in their specificities toward the xylan polymer. Many cleave only at unsubstituted regions whereas others have a requirement for side chains in the vicinity of the cleaved bonds. Most xylanases are also active on xylo-oligomers of degree of polymerization greater than to showing increasing affinity for xylo-oligomers of increasing length. Xylanases are endo type enzymes that hydrolyze internal linkages in xylan and act by a random attack mechanism yielding a mixture of xylo-oligosaccharides from the polymers.

The activity of xylanase was estimated using xylan as the substrate. One unit of enzyme activity is defined as the amount of enzyme required to produce one μmol of reducing sugar per minute under assay conditions. The amount of reducing sugar was estimated using the dinitrosalicyclic acid method[1].

Xylanase activity is increased seven-fold at alkaline pH in the presence of glycine and its pH optimum is shifted from pH 7 to 8 without using any protein engineering techniques. Analysis of the steady-state kinetics revealed that glycine in the reaction mixture increases the K_m and K_{cat} values of the enzyme. Chemoaffinity labeling and studies using glycine esters indicate an involvement of the carboxylate ion of glycine in enhancing xylanase catalytic activity. A novel possible mechanism for the glycine assisted catalytic action of xylanase is proposed[2].

The study of the extra cellular cellulolytic and hemicellulolytic enzymes showed that *T. arantiacus* is more xylanolytic than cellulolytic. The highest levels of enzymes were produced in corncob, grasses and corn straw. All the enzymes were stable at room temperature by 24 h over a broad pH range (3.0-9.0) and also stable at 60 ºC for 1 h. The optimum pH and temperature for xylanase and CMCase were 5.0-5.5 and 5.0 and 75 ºC, respectively. The microorganism grew quickly in stationary, simple and low cost medium. The secreted extra cellular enzymes presented properties that match with those frequently required in industrial environment.

Solubilization of lignin and carbohydrates from the lignin-holocellulose structure of fragments, producing reducing sugars and soluble lignin in the supernatant. Furthermore, the pre-treatment by enzyme enhanced the delignification in the subsequent alkaline scouring process and increased the lightness of the substrate[3].

Composite film production based on cotton stalk xylan was studied and the mechanical and physical properties of the films formed were investigated. Xylan and lignin were separated from cellulose by alkali extraction and, then, lignin was removed using ethanol washing. Self-supporting continuous films could not be produced using pure cotton stalk xylan.

However, film formation was achieved using 8-14 % (w/w) xylan without complete removal of lignin during xylan isolation. Keeping about 1 % lignin in xylan (w/w) was determined to be sufficient for film formation. Films were produced by casting the film-forming solutions, followed by solvent evaporation in a temperature (20 ºC) and relative humidity (40 %) controlled environment. The elastic modulus and hypothetical coating strength of the films obtained by using 8 % xylan were significantly different from the ones containing 10-14 % xylan. The water vapour transfer rates (WVTR) decreased with increasing xylan concentration, which made the films thicker. The glycerol addition as an additional plasticizer,resulting in more stretchable films having higher water vapour transfer rates and lower water solubility values. As a result, film production was successfully achieved from xylan, which was extracted from an agricultural waste (cotton stalk) and the film-forming effect of lignin on pure xylan has been demonstrated[4].

The role of xylan in spruce Kraft pulp fibers was investigated by selectively removing the pulp fibre xylan and also by sorbing xylan onto the pulp fibers. The effects of xylan removal and sorption on fibre properties were measured and the chemical composition of the fibres and also that of the selectively removed xylans was analyzed. According to the results the xylanase could act on both sorbed and native xylan located on accessible fibre surfaces[5].

Xylan was found to affect the strength properties of hand sheets. The location and the charge of xylan had a considerable impact on the formation of interfibre bonds. Scott Bond-values correlated with the amount of surface xylan on fibre surfaces, whereas tensile strength was affected by the total amount of xylan and particularly by the total charge of the fibres. The fracture energy was determined by the combined effect of interfibre bonding ability and effective fibre length[6].

EXPERIMENTAL

Pre-treatment of lignocellulosic substrate: Lignocellulosic substrate was cotton stalk ground into small particles to increase surface area for effective and efficient result. This cotton stalk was pretreated with 2 % NaOH and H_3PO_4 in the ratio of 1:20 (w/v) for 2 h at room temperature. The treated sample was thoroughly washed with water in order to remove alkali and acid to neutralize and then dried at 50 ºC in an oven to obtain a constant weight.

Effect of cofactors or activators on Xylanase activity: Xylanase activity was checked at pH-8 with different activators to see extent of enhancement in xylanase activity. Some cofactors were used like cobalt chloride ($CoCl_2$), zinc chloride ($ZnCl_2$) and EDTA *etc*.

One gram treated substrate was taken in four flasks separately. Then 2 cm^3 enzyme solution was added. Separately 25 cm^3 of buffer of pH-8 was added in each flask. 1 M solution of zinc chloride, cobalt chloride, EDTA and calcium chloride was prepared by one by one. Now 2 cm^3 of $ZnCl_2$ solution was added in flask 1, 2 cm^3 of $CoCl_2$ solution in flask 2, 2 cm^3 of $CaCl_2$ solution in flask 3 and 2 cm^3 of EDTA solution in flask 4. These 4 flasks were placed in an oven for 3, 6 and 9 h at 50 ºC for incubation. After particular time interval, titration of each solution was carried against 0.06 M Benedict solution to calculate reducing sugar and to see enhancement by different co-factors.

Xylanase assay: 0.2 mL of xylanase was taken in 10 different Ependorfs. These Ependorfs were incubated at 55, 65 and 75 ºC in shaking incubator. After every 5 min one Ependorfs was taken out. After that all these Ependorfs were cooled in ice for 0.5 h.

0.5 mL of appropriately diluted xylanase was taken in 10 test tubes. 1 mL of citrate phosphate buffer was added in each test tube. 0.5 mL of xylan was added in each test tube except control. These test tubes were incubated for 5 min in shaking water bath at 50 ºC. 1 mL of DNS was added in each test tube. These test tubes were boiled for 5 min in boiling water. After boiling test tubes were cooled in cold water. These solutions were centrifuged for 5 min in micro centrifuge. OD's was taken by UV-visible spectro photo meter. With the help of OD's, thermodynamic parameters like ΔG, ΔH and ΔS was calculated.

RESULTS AND DISCUSSION

To calculate various parameters for thermal denaturation of xylanase the enzyme was incubated at different temperatures and assayed. Arrhenius approach was applied to calculate K_d (denaturation constant). It was found that with increase in temperature the K_d was also increasing. Acticvation energy for denaturation (E_{ad}) was 121.91 kJ/mol, calculated by ploting estimated valued of K_d against different temperatures (Fig. 1). With help of this data all the thermodynamic parameters were calculated.

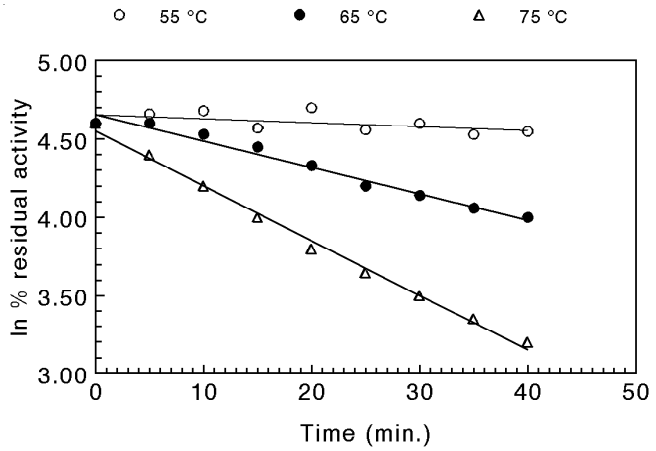

Fig. 1. Irreversible thermal denaturation of native xylanase

Effect of Al^{3+} ions on irreversible thermal denaturation: To calculate various parameters for thermal denaturation of xylanase the enzyme was incubated at different temperatures and assayed. Arrhenius approach was applied to calculate K_d (denaturation constant). For this percentage residual activity of xylanase was plotted against time of incubation at different temperature (Fig. 2). It was found that with increase in temperature the K_d was also increasing. Activation energy for denaturation (E_{ad}) was 171.84 kJ/mol, calculated by plotting estimated valued of K_d against different temperatures. With the increase in temperature ΔG^* value first decrease then increase. With the help of this data all the thermodynamic parameters were calculated (Table-1).

TABLE-1
KINETIC AND THERMODYNAMIC PARAMETERS FOR IRREVERSIBLE THERMAL DENATURATION OF XYLANASE IN THE PRESENCE OF Al^{3+}

Temp. (ºC)	Temp. (K)	K_d (min^{-1})	$t_{1/2}$ (min)	ΔH* (kJ mol^{-1})	ΔG* (kJ mol^{-1})	ΔS* (J mol^{-1} K^{-1})
55	328	0.000367	1888.3	169.11	102.57	202.86
65	338	0.006967	99.5	169.03	96.44	214.76
75	348	0.014900	46.5	168.95	97.68	204.79

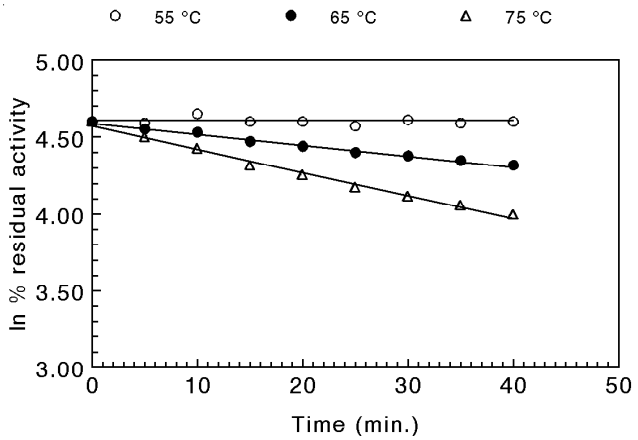

Fig. 2. Effect of Al^{3+} on irreversible thermal denaturation of xylanase

Effect of Cu^{2+} ions on irreversible thermal denaturation: To calculate various parameters for thermal denaturation of xylanase the enzyme was incubated at different temperatures in the presence of Cu^{2+} and assayed. Arrhenius approach was applied to calculate K_d (denaturation constant). For this percentage residual activity of xylanase was plotted against time of incubation at different temperature (Fig. 3). It was found that with increase in temperature the K_d was also increasing. Activation energy for denaturation (E_{ad}) was 130.65 kJ/mol, calculated by ploting estimated valued of K_d against different temperatures. With the increase in temperature ΔG* value first decrease then increase. With help of this data all the thermodynamic parameters were calculated (Table-2).

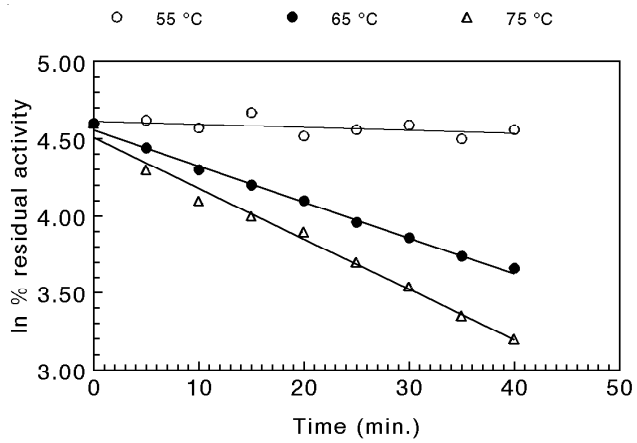

Fig. 3. Effect of Cu^{2+} on irreversible thermal denaturation of xylanase

Effect of Hg^{2+} ions on irreversible thermal denaturation: To calculate various parameters for thermal denaturation of xylanase the enzyme was incubated at different temperatures in the presence of Cu^{2+} and assayed. Arrhenius approach was applied to calculate K_d (denaturation constant). It was found that with increase in temperature the K_d was also increasing. Activation energy for denaturation (E_{ad}) was 183.79 kJ/mol, calculated by plotting estimated valued of K_d against different temperatures. With the increase in temperature ΔG* value is gradually decrease.

Effect of Li^+ ion on irreversible thermal denaturation: To calculate various parameters for thermal denaturation of xylanase the enzyme was incubated at different temperatures in the presence of Li^+ and assayed. Arrhenius approach was applied to calculate K_d (denaturation constant). It was found that with increase in temperature the K_d was also increasing. Activation energy for denaturation (E_{ad}) was 55.55 kJ/mol, calculated by plotting estimated valued of K_d against different temperatures. With the increase in temperature ΔG* value is first increase then decrease.

Effect of Mn^{2+} ions on irreversible thermal denaturation: To calculate various parameters for thermal denaturation of xylanase the enzyme was incubated at different temperatures in the presence of Mn^{2+} and assayed. Arrhenius approach was applied to calculate K_d (denaturation constant). For this percentage residual activity of xylanase was plotted against time of incubation at different temperature. It was found that with increase in temperature the K_d was also increasing. Acticvation energy for denaturation (E_{ad}) was 92.03 kJ/mol, calculated by ploting estimated valued of K_d against different temperatures. With the increase in temperature ΔG* value is first increase then decrease.

Conclusion

It is observed that $t_{1/2}$ (half life) goes on decreasing by increasing temperature of native xylanase. $t_{1/2}$ decreased from 273.91-19.8 min, when temperature increased from 55-75 ºC. ΔG values first decreased then slightly increased by heating from 55-75 ºC of native xylanase. E_{ad} value for native enzyme was 121.91 kJ/mol. In case of coordination bonding of Al^{3+} with xylanase, $t_{1/2}$ goes on decreasing as in case of native xylanase from 1888.3-46.5 min, when temperature was increased from 55-75 ºC. ΔG values are slightly increased as compared to native xylanase, but goes on decreasing when

TABLE-2
KINETIC AND THERMODYNAMIC PARAMETERS FOR IRREVERSIBLE THERMAL DENATURATION OF XYLANASE IN THE PRESENCE OF Cu^{2+}

Temp. (ºC)	Temp. (K)	K_d (min^{-1})	$t_{1/2}$ (min)	ΔH* (kJ mol^{-1})	ΔG* (kJ mol^{-1})	ΔS* (J mol^{-1} K^{-1})
55	328	0.001967	35.23	127.92	97.73	92.04
65	338	0.023267	29.80	127.84	93.68	101.06
75	348	0.032900	21.10	127.76	95.37	93.07

temperature increased as compared to native xylanase. It means Al^{3+} binding stabilize the xylanase.

ACKNOWLEDGEMENTS

This work is a part of Ph.D. research work of Muhammad Suleman. The authors acknowledged the support of Higher Education Commission of Pakistan.

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