Influence of different metals on the activation and inhibition of α-amylase from thermophilic *Bacillus firmus* KIBGE-IB28

Raheela Rahmat Zohra, Shah Ali Ul Qader*, Sidra Pervez and Afsheen Aman
The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, Karachi, Pakistan

Abstract: Thermophilic *Bacillus firmus* KIBGE-IB28 produced extracellular α-amylase at temperature 70°C. Enzyme was partially purified by ammonium sulfate precipitation with 42.80 fold purification and specific activity of 1889.6 U/mg. Effects of various metals on enzyme activity were determined and it was found that enzyme activity boosted significantly in presence of Ca^{2+}, K^{2+}, Ba^{2+}, Co^{2+} and Ni^{2+} whereas Zn^{2+}, Mg^{2+}, Na^{2+} and Cu^{2+} were found inhibitory at concentration 10mM.

Keywords: Metals, Inhibition, activation, amylase, *Bacillus firmus.*

INTRODUCTION

Amylases are amongst the major enzymes used in various industrial processes. α-amylases are ubiquitous in nature however, for the industrial production of enzyme microbial sources specifically *Bacillus* species are preferred over others due to cost effectiveness and ease of process modification and optimization (Pandey *et al*., 2000; Saha *et al*. 2014). α-amylase (EC 3.2.1.1: endo α-1-4 glucan-4-glucanohydolase) catalyzes the hydrolysis of α-1, 4 linkages in starch resulting in the formation of low molecular weight products of industrial importance(Gupta *et al*., 2003). The catalytic activity of enzyme is influenced by various substances that either activate or inhibit its activity. Metal ions in this regard play important role in the activation and stabilization of enzymes (Li *et al*., 2005). If metal ions are required by enzyme to maintain its stable, native state, it is referred to as metalloenzyme whereas if metal ions require only during catalytic activity enzymes are known as metal activated enzymes (Sudha, 2012). Most of the amylases are considered as metalloenzymes as they required calcium ions (Ca^{2+}) for their stability, activity and structural integrity.

MATERIALS AND METHODS

Production and partial purification of α-amylase
Bacillus firmus KIBGE-IB28 was used for the production of α-amylase. Overnight grown culture was inoculated in previously optimized sterile broth medium and incubated at 70°C for 24 hours. Cell free filtrate was obtained by centrifugation at 10,000 rpm for 10 mins at 4°C. Partially purified extra cellular enzyme was obtained by subjecting cell free filtrate to 40% ammonium sulfate precipitation. Precipitates containing α-amylase were collected by centrifugation at 10,000 rpm at 0°C for 20 mins. and dissolved in 50mM citrate phosphate buffer (pH 5.0) and stored at -18°C.

Enzyme assay and protein estimation
Enzyme activity was determined by taking 0.1ml of partially purified enzyme and 1.0ml substrate (1% soluble starch in 50mM citrate phosphate buffer, pH 5.0). The reaction tube was incubated at 50°C for 5 mins. Reaction was stopped by adding 1.0ml dinitro salicylic acid and tube was subjected to boiling for 5mins. Later 8.9 ml deionized water was added after cooling the tube. Amylase activity was determined by measuring the reducing sugar released from soluble starch (Miller, 1959). Unit amylase activity was defined as the amount of enzyme required for the formation of 1.0μmol of reducing sugar in 1.0 min at standard assay conditions.

The total protein was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

Effect of metal ions
Various cations were used to evaluate the stimulatory or inhibitory effects on enzyme activity. Chloride salts of different ions were used at the concentrations of 5mM and 10mM. Enzyme was mixed with different salt solutions in a ratio of 1:1 and kept at 30°C for 15 minutes and then enzyme assay was performed. Salts used for the study were: $BaCl_2$, $CoCl_2$, $MnCl_2$, $MgCl_2$, $ZnCl_2$, $CuCl_2$, $CaCl_2$, $CsCl_2$, NaCl, KCl, $NiCl_2$, $HgCl_2$, $FeCl_3$, $VoSO_4$ and $AlCl_3$. Activity of amylase in presence of salt was compared with the activity of purified enzyme in absence of salt, which act as control.

RESULTS

α-amylase from *Bacillus firmus* KIBGE-IB28 was partially purified up to 42.80 fold with specific activity of1889.6 U/mg (table 1).

Effect of various metal ions on enzyme activity
The effects of various metal ions on the activity of α-amylase produced by *Bacillus firmus* KIBGE-IB28 were investigated by incubating enzyme with metal ion

**Corresponding author:* e-mail: ali_kibge@yahoo.com

solutions of different concentrations. Metal ions were grouped as monovalent, divalent and trivalent cations and the effect of each was measured relatively to the activity of enzyme in absence of any metallic ion (table 2).

It was observed that between the two monovalent cations, K^+ at 5mM stimulated enzyme activity 2.35 times and at 10mM 15.88 times higher than Na^+ (fig. 1).

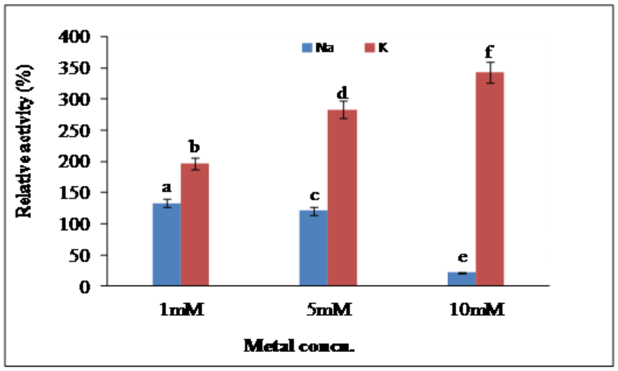

Fig. 1: Effect of monovalent cations on α-amylase activity. Symbols (means ±S.E., n=6) having similar letters are not significantly different from each other (Bonferroni test, P<0.05).

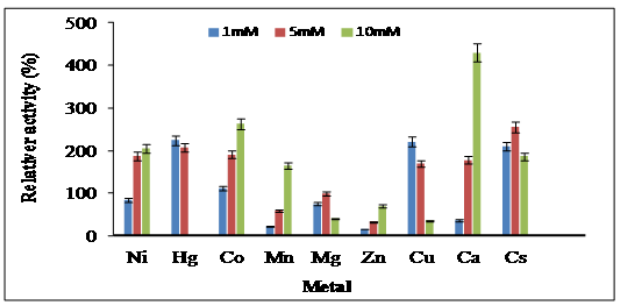

Fig. 2: Effect of divalent cations on α-amylase activity (means ± S.E., n=6)

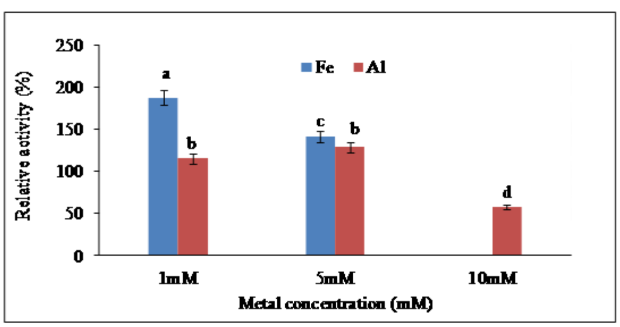

Fig. 3: Effect of trivalent cations on the activity of α-amylase. Symbols (means ± S.E., n=6) having similar letters are not significantly different from each other (Bonferroni test, P<0.05).

Amongst various divalent cations, Ca^{2+} showed highest stimulatory effect with 430% increased in enzyme activity. Increase in activity in the presence of 10mM Ca^{2+} is 4.3 times higher than Ba^{2+} (3.1 times), Co^{2+} (2.6 times) and Ni^{2+} (2.0 times) with reference to control having no ions treatment (fig. 2). At 5mM, all divalent cations induced stimulatory effect except Mn^{2+} and Zn^{2+}, however by increasing the concentration up to 10mM; Hg^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+} showed inhibitory effect.

Among Al^{3+} and Fe^{3+} as trivalent cations, Fe^{3+} ion showed relatively higher stimulation of enzyme in the presence of 1mM and 5mM Fe^{3+}. It was also observed that at a concentration of 10mM both the ions showed inhibitory activity (fig. 3).

DISCUSSION

α-amylase from *Bacillus firmus* KIBGE-IB28 showed stimulatory effect in presence of K^+. Increased in enzyme activity in presence of K^+ has also been reported by Aguilar *et al*. (2000) whereas both the stimulatory and inhibitory effect on amylase activity were reported by Bernharsdotter *et al*. (2005) and Burhan *et al*. (2003), respectively.

Generally amylases are recognized as metalloenzymes and contain at least one Ca^{2+} ion (Vallee *et al*., 1959) as an integral component for enzyme activity. Moreover, to enhance, stabilize and extend the half-life of enzyme, Ca^{2+} is the preferred cation. Thermostable α-amylases depict more thermo stability in the presence of Ca^{2+} (Vihinen and Mäntsälä, 1989; Robyt and French, 1963) which could be attributed to the salting out of hydrophobic residues in enzyme structure resulting in the formation of a more compact and heat resistant structure (Goyal *et al*., 2005). Current study deals with thermophilic *Bacillus* strain producing thermos table α-amylase and activated 4.3 times in presence of Ca^{2+}, these results are in according with the previous findings on stimulatory role of Ca^{2+} on thermostable α-amylases. However certain Ca^{2+} independent α-amylase have been reported from *Bacillus thermooleovorans* NP54 which produced thermostable enzyme without Ca^{2+} (Malhotra *et al*., 2000). Hg^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+} at concentration 10mM showed inhibitory effect on the activity of α-amylase from *Bacillus firmus* KIBGE-IB28. This decreased in metalloenzyme activity might be due to the competition between the protein associated and exogenous cations (Leveque *et al*., 2000). Krishnan and Chandra (1983) reported that *Bacillus licheniformis* has been strongly inhibited by Hg^{2+}, Cu^{2+} and $Zn2^+$ however its activity was stabilized in presence of Mg^{2+}.

The findings of Al^{3+} and Fe^{3+} are in contrary with the results obtained by Bano *et al*. (2009), in which the thermostable amylase was strongly inhibited by Al^{3+}; and by both Al^{3+} and Fe^{3+} (Aguilar *et al*. 2000; Hashim *et al*. 2005).

Table 1: Steps involved in the partial purification of α-amylase from *Bacillus firmus* KIBGE-IB28

Purification Steps	Enzyme activity (U)	Total Protein (mg)	Specific activity (U/mg)	Fold Purification	Yield (%)
Crude enzyme	281780	6381	44.15	1	100
Precipitation	152180	81.2	1874.14	42.44	54.0
Dialysis	142195	75.25	1889.6	42.80	50.46

Table 2: Various cations and relative activity of α-amylase from *Bacillus firmus* KIBGE-IB28

Metal Ions	Concentrations			Metal Ions	Concentrations		
	1mM	5Mm	10mM		1Mm	5mM	10mM
Ba	100	138.48	318.94	Zn	16.5	32.92	70.21
Co	112	191.13	263	Cu	221	169.78	35.1
Mn	23	58.26	165.2	Ca	36.4	177.68	430.0
Mg	75	100	40.5	K	196.4	283.44	343.04
Cs	210	255.55	186.3	Ni	84.32	187.95	204.74
Vo	192.5	152.41	75.61	Hg	224.61	206.9	0
Fe	187.46	140.56	0	Na	133	120.51	21.6
Al	114.62	128.2	56.71	Control	100	100	100

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