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STUDIES ON THE EFFECT OF METAL IONS, PROTEIN DENATURANTS AND TEMPERATURE, ON α- AMYLASE ACTIVITY IN *OPUNTIA VULGARIS*

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The existence of two isoforms T_{50} & T_{90} of amylase enzyme activity having a temperature optimum of 50 °C and 90°C respectively has been detected in the cladode of *Opuntia vulgaris*. The activity of the T_{90} isoform was found to be stabilized by Mg^{2+}, Ca^{2+} & Na^{2+}. The enzyme was strongly inhibited by Hg^{2+}, Zn^{2+}, Cu^{2+} and EDTA, but less affected by Ni^{2+} and Mn^{2+}. The enzyme showed relatively high thermostability and retained about 40% of its original activity after heating at 100 °C for 10 minutes.

Keywords : Amylase; Isoforms; Starch; Thermostability.

Introduction

Thermostable amylases of microbial origin are used in the liquefaction of starch, allowing the efficient production of dextrose[1]. The enzymatic hydrolysis of starch is catalysed by α - amylases (EC 3.2.1.1), glucoamylases (EC 3.2.1.3), β-amylases (EC 3.2.1.2) and debranching enzymes such as pullulanases (EC 3.2.1.41). Amylases are used widely in technical applications, such as bread making, production of glucose, fructose syrups and fuel ethanol from starches, and desizing of textiles and paper. The advantages in using the thermostable amylses in industrial processes include the decreased risk of contamination and decreased cost of external cooling. It has been reported that carbon sources greatly affect the production of thermophilic amylases[2-3]. Of carbohydrates used, starch is demonstrated to be a good carbon source for the synthesis of amylase[4]. So far several thermostable amylases have been isolated and characterized[5-6], most of them require additional Ca^{2+} for their thermostability. Present studies determined the presence of an amylase enzyme activity in *Opuntia vulgaris,* a xerophyte, that had a pH optimum of 8.5 and was active at higher temperature (>50 °C). This enzyme activity was further stabilized by the presence of specific mono and divalent cations. The industrial importance of the amylase enzyme activity makes the identification and characterization of this thermostable plant enzyme species, an interesting achievement.

Materials and Methods

Chemicals- Ammonium sulfate, Phenylmethanesulphonyl fluoride (PMSF), and Dowex-1 were obtained from Sgma Chemicals, St. Louis, Mo, USA. All the other chemicals used were of analytical grade obtained from manufacturers in India. Glass distilled water was used for the preparation of all reagents.

Enzyme Assay - α - Amylase activity was assayed using a previously described method[7], by measuring the amount of reducing sugar released during the reaction employing starch as the substrate. The reaction mixture contained 1.0 ml soluble starch 1.075%, 1.5 ml, 0.1 M KH_2PO_4/ Na_2HPO_4 buffer, pH 7.0, and 100µ l of the enzyme extract. The reaction was incubated for 30 min, stopped by the addition of 0.5 ml 2/3 N H_2SO_4 and 0.5 ml 10% sodium tungstate, and centrifuged at 5000 x g for 15 min in a refrigerated centrifuge, 1.0 ml of supernatant was taken and 1.0 ml of alkaline copper reagent was added to the reaction sample and the sample was heated in a boiling water bath for 6 min. 1.0 ml of phosphomolybdic acid was then added and incubation of the sample continued in water bath for an additional 2 min. The blue colour developed in the sample was read at 650 nm employing a UV-visible single beam spectrophotometer.

Enzyme fractionation - Opuntia vulgaris cladode homogenate 20% (w/v) was prepared in 0.1 M KH_2PO_4/Na_2HPO_4 buffer, pH 7.0, containing 1 mM PMSF. The extract

was filtered through cheese cloth and the filtrate was centrifuged at 10,000 x g for 15 min in a refrigerated centrifuge to obtain a clear supernatant that was used as the enzyme extract.

Fractionation of the amylase activity contained in the cladode extract was attempted using solid ammonium sulfate precipitation of soluble proteins. The ammonium sulfate 80% precipitated proteins were collected by centrifugation of the sample at 10,000 x g for 45 min using a refrigerated centrifuge. The precipitate obtained was re-dissolved in 0.1 M KH_2PO_4/ Na_2HPO_4 buffer, pH 7.0, and dialyzed for 18 h against the same buffer at room temperature, with three changes of the dialysate. The dialyzed protein sample was then taken for ion exchange chromatography.

Ion-exchange chromatography of the precipitated proteins was carried out employing a column (28 cm x 0.83 cm) of Dowex-1 anion exchange resin at 27 °C, equilibrated with 0.1 M KH_2PO_4/Na_2HPO_4 buffer, pH 7.0. A volume of 6 ml of the dialyzed samle was applied to the resin and fractions of 3 ml were collected manually. At the end of sample application, column wash was started with the equilibrating buffer, and continued until the absorbance of the wash fractions showed < 0.05 The adsorbed proteins were then eluted using a linear gradient of NaCl (0-1.0 M) in the wash buffer. Protein content of each fraction was monitored at A_{280} employing a UV-vis single beam spectrophotometer. The presence of amylase activity in each fraction was also detected by the assay as described earlier. Protein peak fractions containing amylase activity eluted at by NaCl and exhibiting a temperature optimum of 90 °C (T_{90}) were taken for further studies using divalent cations.

Effect of metal ions on enzyme activity - The metal ions were used at 1, 5 and 10 mM concentration in independent reactions and the assay of enzyme activity was carried out employing mono and divalent cations in their chloride forms at the optimum temperature of 90 °C in KH_2PO_4/Na_2PHO_4 buffer at pH 7.0, incubating the samples each time for 30 min. The activity of amylase assayed in the absence of metal ions served as the control.

Effect of protein denaturants on enzyme activity- The influence of protein denaturants on the amylase enzyme activity was carried out using phosphate buffer at the optimum temperture of 90 °C. The protein denaturants were used in different concentration and the enzyme assay carried out as described in materials and methods.

Thermo stability of α-amylase from Opuntia vulgaris - The thermo stability of T_{90} isoform was carried out using KH_2PO_4/Na_2HPO_4 buffer at pH 7.0, in different temperature at different time intervals (0-30 min), and the residual activity was measured.

Results and Discussion

The temperature profile of alpha amylase activity yielded two temperature optima, one at 50 °C (T_{50}), and the other at 90 °C (T_{90}), results not shown. The T_{90} isoform was partially purified by the ion exchange column chromatography which was taken for further studies. Among the various cations used (Fig. 1), Hg^{2+}, Zn^{2+} and Cu^{2+} were found to be strongly inhibitory for the amylase activity[8-9]; Ni^{2+}, Fe^{2+} and Mn^{2+} inhibited the amylase activity to a lesser extent. The enzyme was sensitive to EDTA inhibition[10], and not stimulated by Ca^{2+}. SDS decreased the activity of the enzyme and urea had no significant influence on the enzyme (Fig. 2).

Although Ca^{2+} is important for the amylases obtained from mesophilic sources[11-12], Ca^{2+} modified the amylase activity negatively with increase in concentration of the cation compared to its control value in *Opuntia vulgaris*. The presence of Na^+ and Mg^{2+} always showed an augmentation effect. The thermostability of the T_{90} isform was carried out at different temperatures and the residual activity was measured. Fig. 3 shows the influence of temperature on enzyme activity. The enzyme

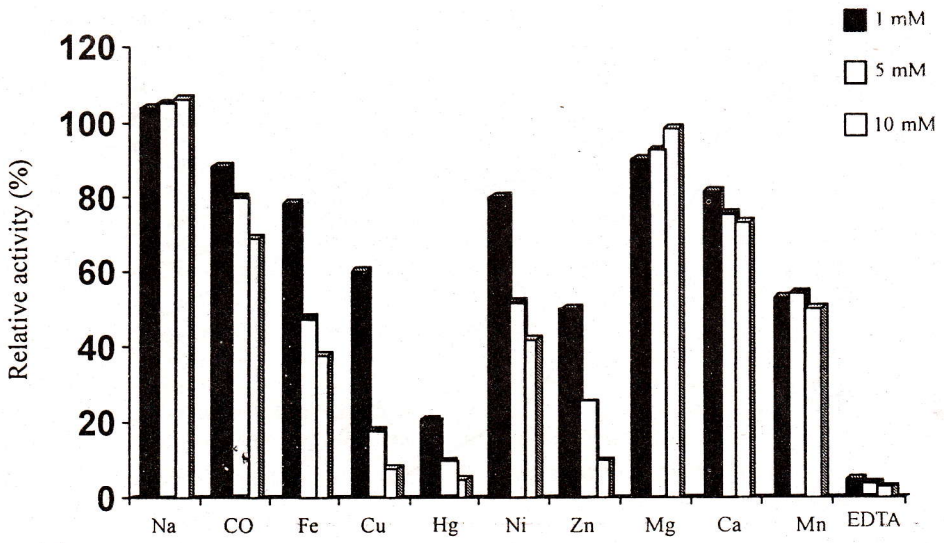

Fig.1. Influence of various metal ions and EDTA on T_{90} isoform amylase at 90 °C. The activity of enzyme assayed in the absence of metal ion and inhibitor which serve as a control was taken as 100%. All the metal ions were added as chloride salts.

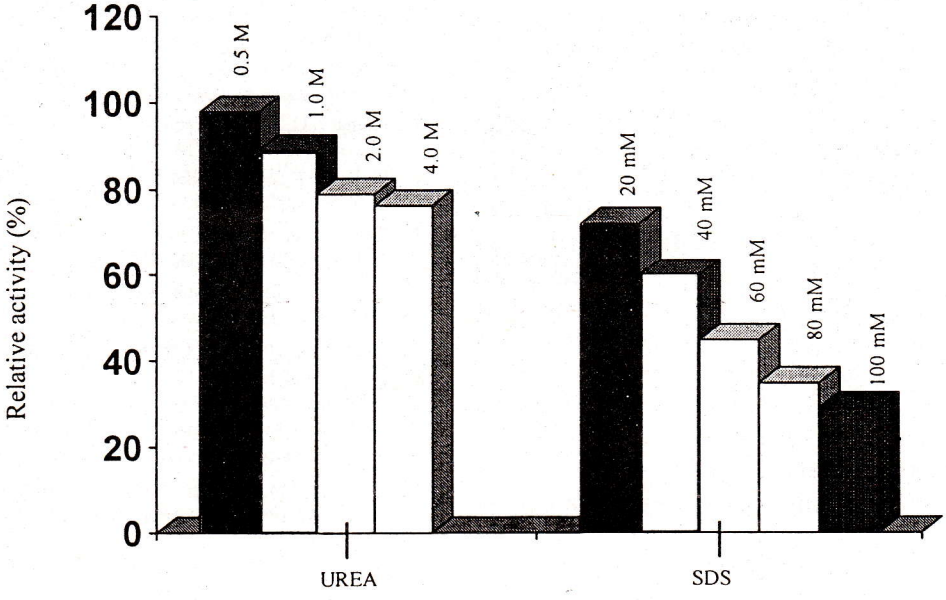

Fig.2. Influence of protein denaturants on enzyme activity.

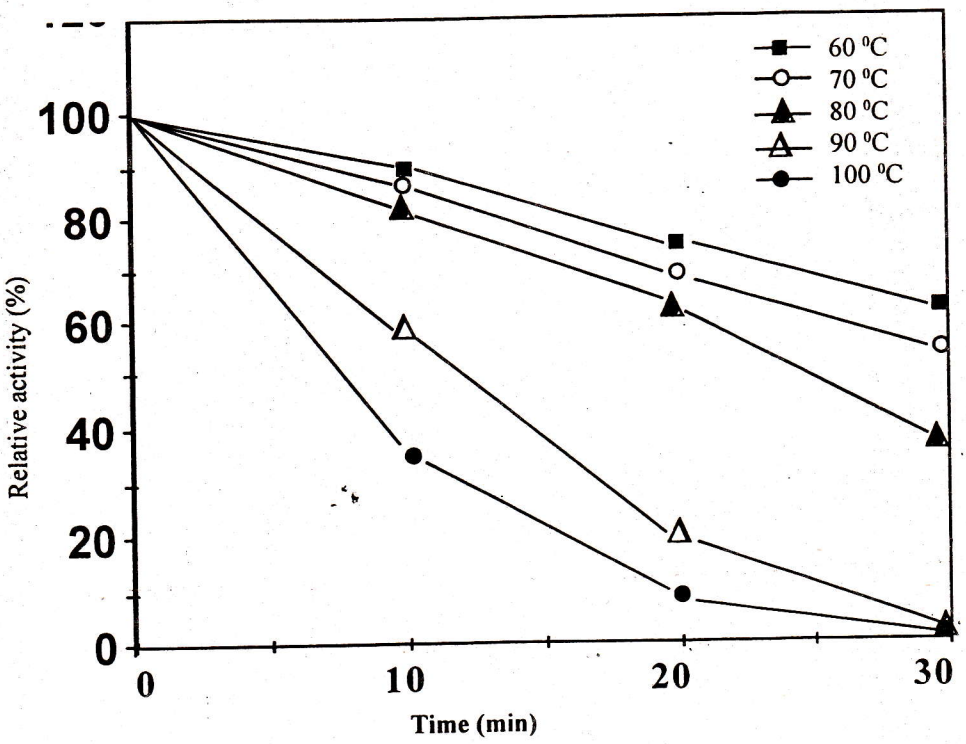

Fig.3. Thermostability of α-amylase. The T_{90} isoform in the absence of substrate was incubated at various temperatures for 0, 10, 20 and 30 min, and the residual soluble starch digesting activity was assayed as described in materials and methods.

showed relatively high thermostability and retained about 40% of its origianal activity after heating at 100 °C for 10 minutes.

The role of α - amylase as a starch degrading enzyme is well documented in literature. The presence of distinct isoforms of amylase activity was observed and they were separable due to differences in their molecular surface charges, as weakly and strongly anionic species. The amylase enzyme activity in *Opuntia vulgaris* is demonstrated to be influenced by divalent cation concentration in a temperature dependent manner. The divalent metal ion effects observed in these studies seem to offer an additional adaptive strategy for some plants like *Opuntia vulgaris*, for sustaining at higher environmental temperature.

Reference

1. Fogarthy W M and Kelly C T 1980, In : *Microbial Enzymes and Bioconversions*, Rose A H (eds), Academic Press, New York pp. 115-169.
2. Srivastava R A K, and Baruah J N 1986, *Appl. Environ. Microbial* **52** 179-184
3. Kumar S U, Rehana F, and Nand K 1990, *Enzyme Microb. Technol.* **12** 714 - 716
4. Welker N E, and Campbell LL 1963, *J. Bacteriaol.* **86** 681 - 686
5. Koch R, Spreinat A, Lemke K, and Antranikian G 1991, *Arch. Microbiol.* **155** 572 - 578
6. Dong G, Vieille C, Savchenko A, and Zeikus, J G 1997, *Appl. Environ. Microbiol.* **63** 3569-3576
7. Henry R J, Chiamori N 1960. *Clin. Chem.* **6** 434
8. Lin L L Tsau, M R, and Chu W S 1996, *Biotechnol. Appl. Biochem.* **24** 101-107
9. Vihinen M and Mantsala P 1990, *Biotechnol. Appl. Biochem.* **12** 427-435
10. Long-Liu Lin, Charng-Cherng Chyau and Wen-Hwei Hsu 1998. *Biotechnol. Appl. Biochem.* **28** 61-68
11. Hsiu J, Fischer E H and Stein E A 1964, *Biochemistry.* **3** 61-66
12. Vallee B L, Stein E A, Sumerwell W N and Fischer E H 1959, *J. Biol. Chem.* **234** 2901-2905