Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Polymers 54 (2003) 43–50
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

# Properties of three purified α-amylases from malted finger millet (Ragi, *Eleusine coracana*, Indaf-15)☆

M. Nirmala[1], G. Muralikrishna*

*Department of Biochemistry & Nutrition, Central Food Technological Research Institute, Mysore 570 013, India*

Received 24 March 2003; accepted 7 April 2003

**Abstract**

Purified α-amylases from ragi malt (designated as α-$1_{(b)}$, α-2 and α-3), were found to be completely inactivated below pH 4.0 but found to be comparatively more stable in alkaline pH. These iso-enzymes were inactivated around 70 °C. $CaCl_2$ (5–7.5 mM) was enhancing their thermal stability and also found to be an activator. Citric and oxalic acids were inhibiting these enzymes completely between 10 and 12.5 mM concentrations, respectively. EDTA was found to be a competitive inhibitor of these enzymes at micro molar concentrations and inhibition was temperature dependent.
© 2003 Elsevier Ltd. All rights reserved.

*Keywords:* α-amylases; Finger millet; Properties; Ragi

## 1. Introduction

α-amylase (α → 1-4 D-glucano-4-glucan hydrolase, E.C 3.2.1.1) is a key enzyme hydrolyzing reserve starch in the endosperm of germinating cereals (Machaiah & Vakil, 1984). It is synthesized denovo in the aleurone layers in response to gibberellic acid (Akazawa, Mitsui, & Hayashi, 1988). Amylases were purified from various cereals such as wheat, barley, maize, rice and millets such as sorghum and bajra and characterized with respect to various parameters such as pH and temperature optima and stability, effect of activators, stabilizers and inhibitors on their activity (Kruger & Lineback, 1987; MacGregor, 1983) and these properties are exploited for their use in brewing and bread making industries (Leloup, Colonna, & Buleon, 1994).

Finger millet (*Eleusine coracana*) popularly known as Ragi in India is used for geriatric, infant and health foods both in native and malted conditions due to its low cost and presence of high amylolytic activity in its malt. Ragi is contemplated as possible substitute for barley in bread making and brewing industry in India. So it is found warranted to study the nature of the amylases, their purification and characterization. Accordingly a study was initiated on ragi malt amylases and three α-amylases were purified to apparent homogeneity from 72 h malt by three step purification via fractional acetone precipitation, DEAE-Sephacel ion exchange and Sephacryl S-200 gel permeation chromatographies with a recovery of 6.5, 2.9, 9.6% and fold purification of 26, 17 and 31, respectively.

α-Nature of these amylases was identified by their ability to rapidly reduce the viscosity of starch solution and also in liberating oligosaccharides of higher DP (as determined by paper as well as high performance liquid chromatographies) from soluble starch and were accordingly designated as amylases α-$1_{(b)}$, α-2 and α-3, respectively. These amylases, having a molecular weight of 45 ± 2 kDa were found to be monomeric. The pH and temperature optima of these α-amylases were found to be in the range of 5.0–5.5 and 45–50 °C, respectively. Amino acid analysis of these enzymes indicated high amounts of glycine which is an unusual feature of these enzymes (Nirmala & Muralikrishna, 2003).

In this communication, we report the stability studies of these purified α-amylases with respect to their pH, temperature, effect of calcium chloride, EDTA, citric and

*Abbreviations:* EDTA, ethylene diamino tetra acidic acid; PCMB, *para*-chloro mercuric benzoate.

☆ Presented in the International conference on New Horizons in Biotechnology held at April 18–21, 2001 held at Trivandrum, Kerala, India.

* Corresponding author. Tel.: +91-821-514876; fax: +91-821-517233.
*E-mail address:* gmk@cscftri.ren.nic.in (G. Muralikrishna).
[1] Present address: Department of Biochemistry and Molecular Biology, Indiana-Purdue University, Indiana Polis, USA.

0144-8617/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0144-8617(03)00151-6

oxalic acids. This information will have a bearing on their potential use in the bread making and brewing industries.

## 2. Experimental

### 2.1. Materials

Finger millet (Indaf-15) seeds were procured from V.C. Farm, University of Agricultural Sciences, Bangalore, located at Mandya, Karnataka and used for the entire studies.

### 2.2. Malting of ragi

Ragi seeds (100 g) were cleaned, and steeped for 24 h and germinated under controlled conditions on moist cloth at 25 °C in a BOD incubator up to 72 h. Germinated seeds were taken and dried at 50 °C in an air oven for 12 h and vegetative growth portions were removed by gentle manual brushing. Devegetated seeds were weighed, powdered and used for the extraction of amylases (Nirmala et al., 2000).

### 2.3. Isolation and purification of amylases

#### 2.3.1. Isolation

Total amylases were extracted from 72 h malted with sodium phosphate buffer (50 mM, pH. 6.0) at 4 °C (Nirmala & Muralikrishna, 2003).

#### 2.3.2. Purification of amylases

Purification of amylases was carried out as described earlier (Nirmala & Muralikrishna, 2003).

### 2.4. Criteria for purity of amylases

Criteria of purity of amylases was determined on sepadex-G-75, (Nirmala & Muralikrishna, 2003) and by performing PAGE-electrophoresis (Walker, 1996) as well as SDS-PAGE-electrophoresis (Laemmli, 1970). Amylases activity staining was carried out according to the standard procedure (Acevedo & Cardemil, 1997).

### 2.5. General methods

#### 2.5.1. Enzyme/protein assay

Amylases (Bernfeld, 1955) and protein (Bradford, 1976) assays were carried out taking maltose and Bovine serum albumin, respectively.

### 2.6. pH stability

Stability of amylases was determined using different buffers such as (ranging in their pH values from 2.0 to 9.0) phthalate–HCl (pH 2.0–3.0), sodium acetate (pH 4.0–5.0), sodium phosphate (pH 7.0–8.0), Tris–HCl (pH 8.0–9.0) at 50 mM concentration. Purified amylases were incubated in different buffers at room temperature and 4 °C and the residual activities were measured at different time intervals. The pH stability at extreme pH values was cross checked by using buffers such as glycine–HCl (pH 2.0–3.0) and borate (pH 8.0–9.0). The original activity was taken as 100% and relative activity was plotted against different time intervals.

### 2.7. Thermal stability

Purified amylases in sodium acetate buffer (50 mM, pH 5.0) were incubated at a temperature range of 30–75 °C for 15 min. The residual activity was estimated taking original activity as control (100%) and relative activity was plotted against different temperatures.

### 2.8. Effect of inhibitors

The inhibitors such as EDTA (2.5–25 μM), citric acid (2.5–12.5 mM), oxalic acid (2.5– 12.5 mM) were incubated with purified amylases at 45 °C for 15 min and residual activity was measured. The enzyme activities without inhibitor was taken as 100% and relative activity was plotted against different inhibitor concentrations.

### 2.9. Effect of $CaCl_2$ on thermal stability of amylases

Purified amylases were incubated with different concentration of $CaCl_2$ (2.5–12.5 mM) at 75 °C for 15 min sodium acetate buffer, (50 mM, pH 5.0) and the activities in the presence of $CaCl_2$ were plotted as relative activities taking the original activity as 100%.

## 3. Results and discussion

### 3.1. pH stability

The stability of $\alpha$-$1_{(b)}$, $\alpha$-2 and $\alpha$-3 was tested after their pre incubation at different pHs for varying time intervals followed by determining the residual activity. Buffers such as phthalate (pH 2.0–3.0), acetate (pH 4.0–6.0), sodium phosphate (pH 7.0–8.0), Tris–HCl (pH 8.0–9.0) were tried at 50 mM concentration to maintain the buffering capability and to check the effect of buffer components on the stability of these enzymes. $\alpha$-$1_{(b)}$, $\alpha$-2 and $\alpha$3 were found to be stable in the pH range of 5.0–9.0 and retained almost 70% activity after 7 h of incubation (at pH 9.0). However, the activity of these amylases decreased drastically at pH 3.0 after pre incubation for 15 min ($\alpha$-3) and 45 min ($\alpha$-$1_{(b)}$ and $\alpha$-2) (Figs. 1a, 2a, 3a).

Buffers such as glycine–HCl (pH 2.0–3.0) and borate (pH 8.0–9.0) were used and %activity retained after different time intervals is shown in Figs. 1b, 2b, 3b. Even though the enzymes were losing their activity at pH 3.0 as discussed earlier, they retained 60% activity in glycine–HCl buffer indicating

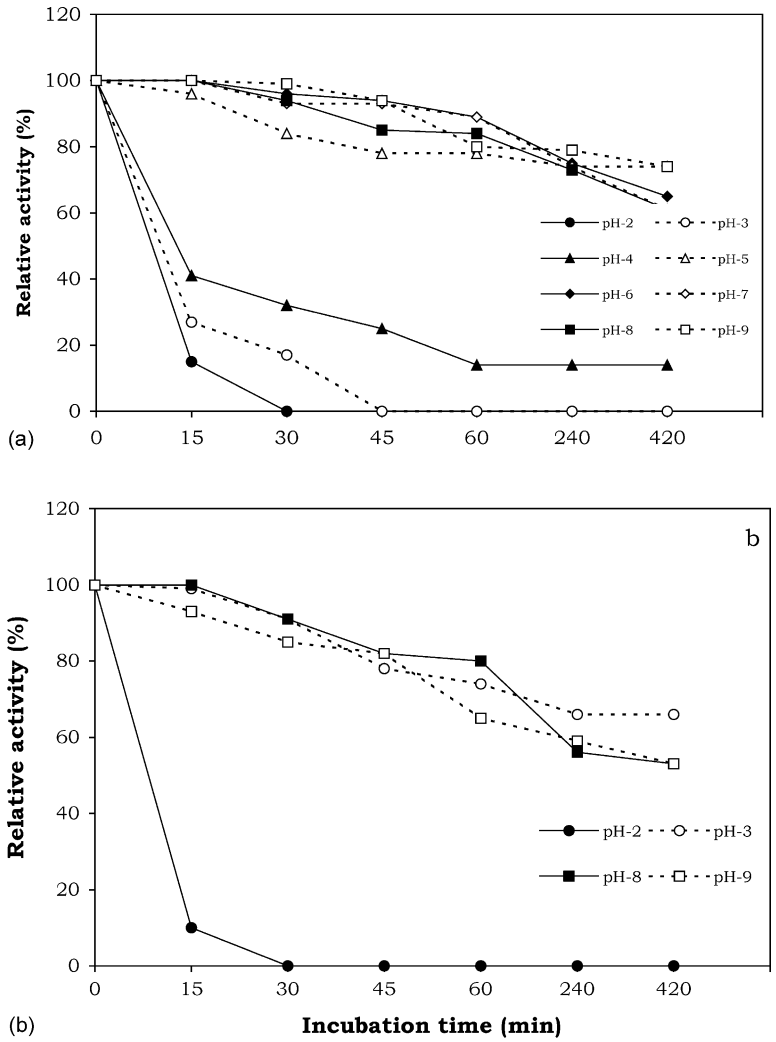


Fig. 1. Effect of pH on the stability of purified α-$1_{(b)}$. (a) pH 2.0–3.0: phthalate–HCl; pH 4.0–6.0: sodium acetate; pH 7.0–8.0: sodium phosphate; pH 8.0–9.0: Tris–HCl. (b) pH 2.0–3.0: glycine–HCl; pH 8.0–9.0: borate (relative activity is expressed by taking the average value of three independent experiments).

the stabilizing effect of glycine at lower pH. This may be perhaps due to the protection of active site residues from the surrounding environment through electrostatic interactions. All the three enzymes were also found to be stable even in alkaline pH range as indicated by the residual activity in borate buffer (40–50%). These results pertaining to pH stability are in agreement with the published literature (Kruger & Lineback, 1987).

α-amylases from immature red spring wheat was found to be stable in a pH range of 4.5–8.0 up to 30 min. However, it lost its activity completely below pH 2.0 within 2 min (Marchylo, Kruger, & Irvine, 1976). α-amylase from barley and its malt were found to be stable between pH 4.0 and 6.0 but lost the same completely on either side of this pH. α-Amylase isolated from malted barley was unusually stable at pH 3.6, retaining around 85% activity, whereas the amylase-2 from the same source lost its activity after pre incubation for 15 min. This clearly indicates the different pH stabilities of amylases isolated from the same source.

### 3.2. Thermal stability

The thermal stability of α-$1_{(b)}$, α-2 and α-3 were carried out by pre incubating them at different temperatures (30–75 °C) and measuring the residual activities after 15 min (Fig. 4). α-$1_{(b)}$ and α-3 were found to be more thermostable than α-2 as indicated by the residual activity. More than 85% of the activity was retained at 55 °C after 15 min pre incubation with respect to α-$1_{(b)}$ and α-3 whereas α-2 lost its activity completely. At 50 °C, α-2 could retain only 25% of its activity irrespective of its optimum temperature. However, at 75 °C, all the three were completely inactivated. This is in good agreement with the findings on barley malt α-amylase-1 (Bertoft, tfolk, & Kulp, 1984) whose temperature stability was better than the heat labile α-amylase-2 (MacGregor, 1978).

However, the α-amylase from wheat was stable up to 60 °C and started denaturing at 70 °C and retaining 15% of its activity after 15 min (Tkachuk & Kruger, 1974).

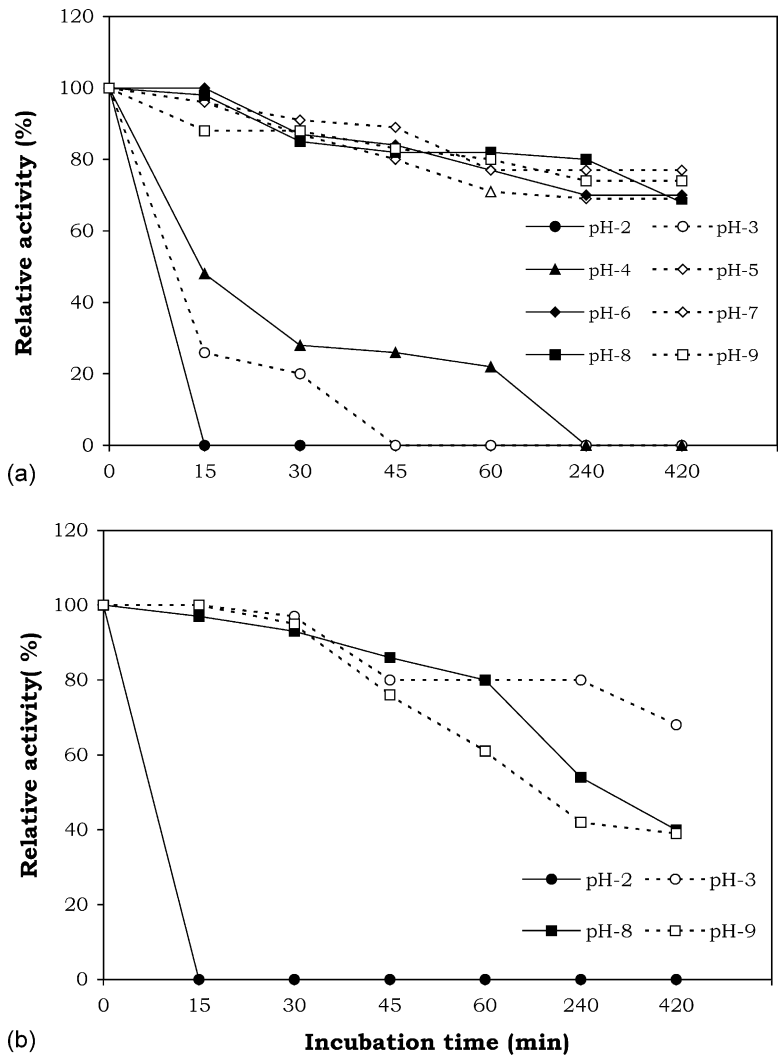


Fig. 2. Effect of pH on the stability of purified α-2. (a) pH 2.0–3.0: phthalate–HCl; pH 4.0–6.0: sodium acetate; pH 7.0–8.0: sodium phosphate; pH 8.0–9.0: Tris–HCl. (b) pH 2.0–3.0: glycine–HCl; 8.0–9.0: borate (relative activity is expressed by taking the average value of three independent experiments).

### 3.3. Effect of $CaCl_2$ on the thermal stability

Calcium chloride is added to stabilize several cereal α-amylases. To check this, purified ragi amylases were incubated with different concentrations of $CaCl_2$ (2.5–12.5 mM) at 75 °C for 15 min and residual activity was determined. This condition was chosen because all the three amylases were found to be losing their activity completely at this temperature. In case of α-1 and α-2, 7.5 mM $CaCl_2$ was found to stabilize the enzymes to the maximum extent (α-$1_{(b)}$, 31%; α-2, 35%) compared to the other concentrations, whereas α-3 was stabilized at 5 mM concentration where in 45% activity was retained. At higher concentration of $CaCl_2$ (10 and 12.5 mM) the stabilization is less pronounced (Fig. 5).

Studies on barley malt isoenzymes with respect to stabilization by $CaCl_2$ at 70 °C have shown (Bertoft et al., 1984) that a similar differential effect on their activity as observed in the present study. $Ca^{2+}$ was found to stabilize α-amylases from barley and its malt at lower pH as well as at higher temperatures (Greenwood & MacGregor, 1965). Differential $CaCl_2$ affinity was observed in the case of barley isoenzymes (Bush, Sticher, Hwystee, Wegner, & Jones, 1989). Exogenous addition of $CaCl_2$ was found to retard the thermal inactivation of cereal α-amylases as observed in the case of barley α-amylase (Greenwood & Milne, 1968a,b). Similarly, the loss of activity in the case of barley α-amylase was circumvented by the exogenous addition of $CaCl_2$ (Greenwood & Milne, 1968a,b).

### 3.4. Effect of citric and oxalic acids

The effect of citric/oxalic acids on the activities of α-$1_{(b)}$, α-2 and α-3 were tested in a concentration range of 0–12.5 mM. Citric acid inhibited all the three enzymes to 100% extent at 10 mM concentration, whereas oxalic acid had a similar effect at 12.5 mM concentration (Fig. 6).

The effect of oxalic/citric acids on α-3, was less pronounced compared to α-2 and α-$1_{(b)}$, whereas α-$1_{(b)}$ was more susceptible to oxalic/citric acids inhibition

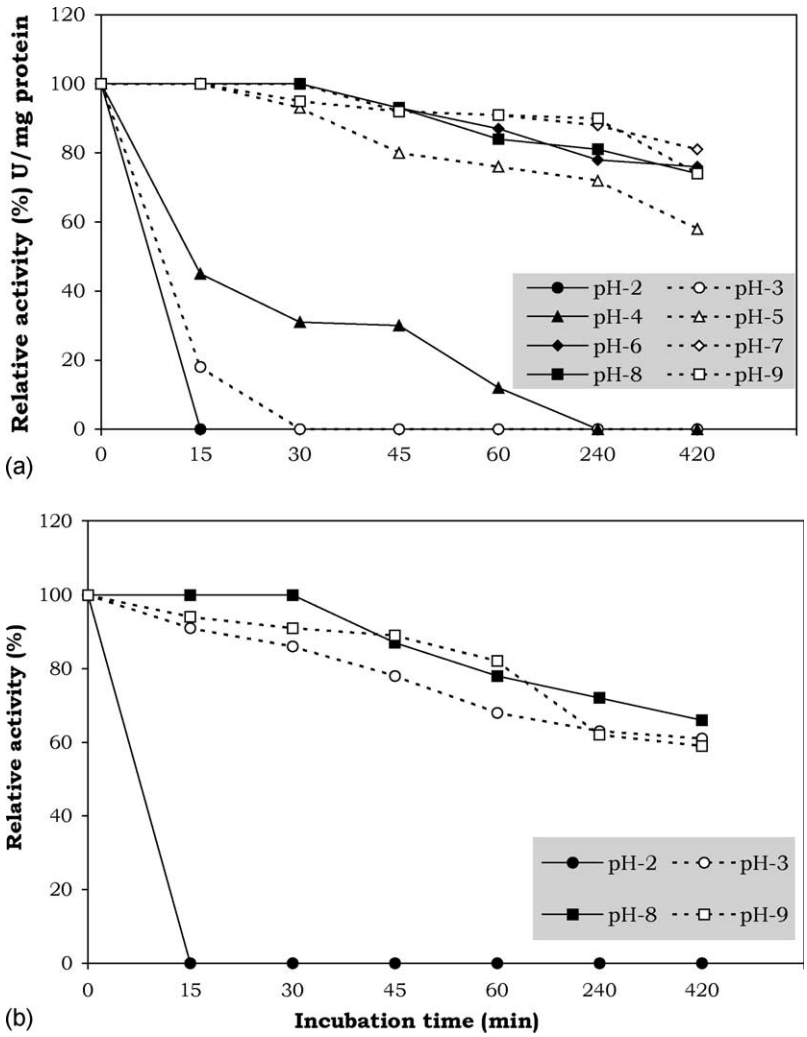


Fig. 3. Effect of pH on the stability of purified α-3. (a) pH 2.0–3.0: phthalate–HCl; pH 4.0–6.0: sodium acetate; pH 7.0–8.0: sodium phosphate; pH 8.0–9.0: Tris–HCl. (b) pH 2.0–3.0: glycine–HCl; pH 8.0–9.0: borate (relative activity is expressed by taking the average value of three independent experiments).

compared to α-2. 50% inhibition was observed at 3.6 mM (α-$1_{(b)}$), 3.7 mM (α-2) and 5.48 mM (α-3) concentrations of citric acid. Similarly the concentration of oxalic acid for 50% inhibition for α-$1_{(b)}$, α-2 and α-3 were 3.3, 3.7 and 7.0 mM, respectively.

Carboxylic acids such as oxalic and citric acids inhibit α-amylases by selective removal of $Ca^{2+}$ present in the enzyme. The differential inhibition effect of oxalic/citric acids on the activities of ragi amylases on the extent of $Ca^{2+}$ (strong/weak) binding to these enzymes and this has been implicated in the increased resistance of α-3.

### 3.5. Effect of EDTA

The effect of various concentrations of EDTA on the activities of α-$1_{(b)}$, α-2 and α-3 were tried at different experimental condition, i.e. at (a) 27 °C (b) 45 °C and (c) in the presence of substrate at 45 °C and the results are depicted in Fig. 7. At 45 °C, α-$1_{(b)}$, α-2 and α-3 were found

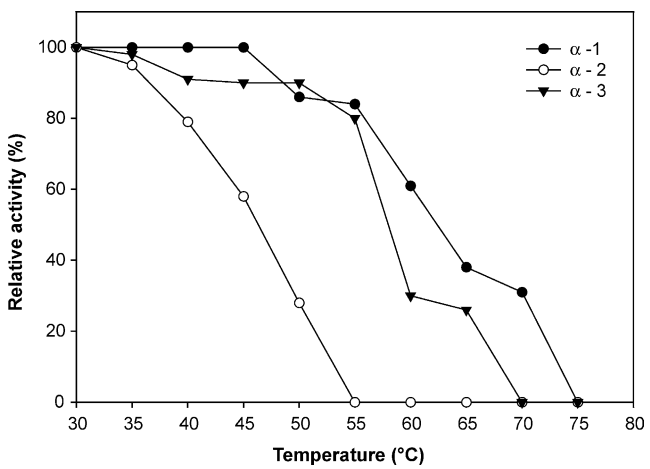


Fig. 4. Effect of temperature on the stabilities of purified ragi amylases (relative activity is expressed by taking the average value of three independent experiments).

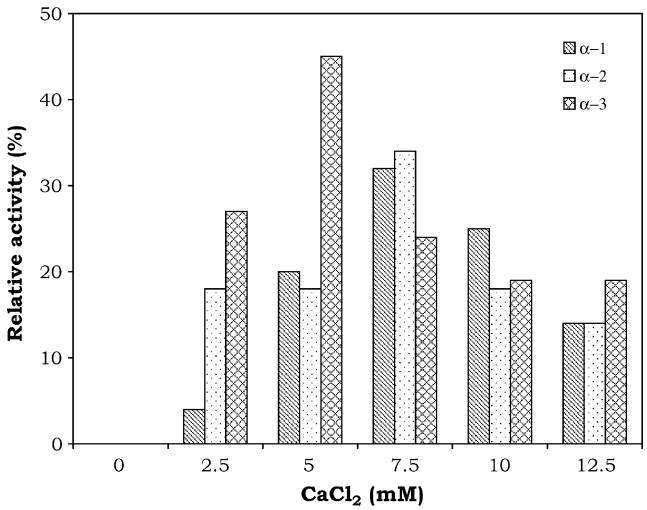


Fig. 5. Effect of $CaCl_2$ on the thermal stabilities of purified ragi amylases (relative activity is expressed by taking the average value of three independent experiments).

to lose 95, 86 and 89% activities, respectively, at 12.5 mM concentration of EDTA, whereas the inhibition was only 30, 55 and 25% for α-$1_{(b)}$, α-2 and α-3, respectively, at 27 °C. Temperature was found to have profound effect on inhibition. At higher temperature, i.e. at 45 °C, the chelating effect of EDTA was much more pronounced compared to the lower temperature (27 °C).

However, in the presence of substrate (soluble starch), the inhibition of EDTA was substantially less (~10%) in all the three amylases. This can be due to the preferential and rapid formation of substrate–enzyme complex over enzyme–inhibitor complex indicating this to be competitive type of inhibition. Once substrate enzyme complex is formed, the bound $Ca^{2+}$ is no more accessible to EDTA and hence resulting in very poor inhibition. From these results it can be proposed that bound calcium is involved in substrate binding.

Cereal α-amylases are known to be metallo enzymes containing at least one $Ca^{2+}$ per molecule (Janeck & Belaz,

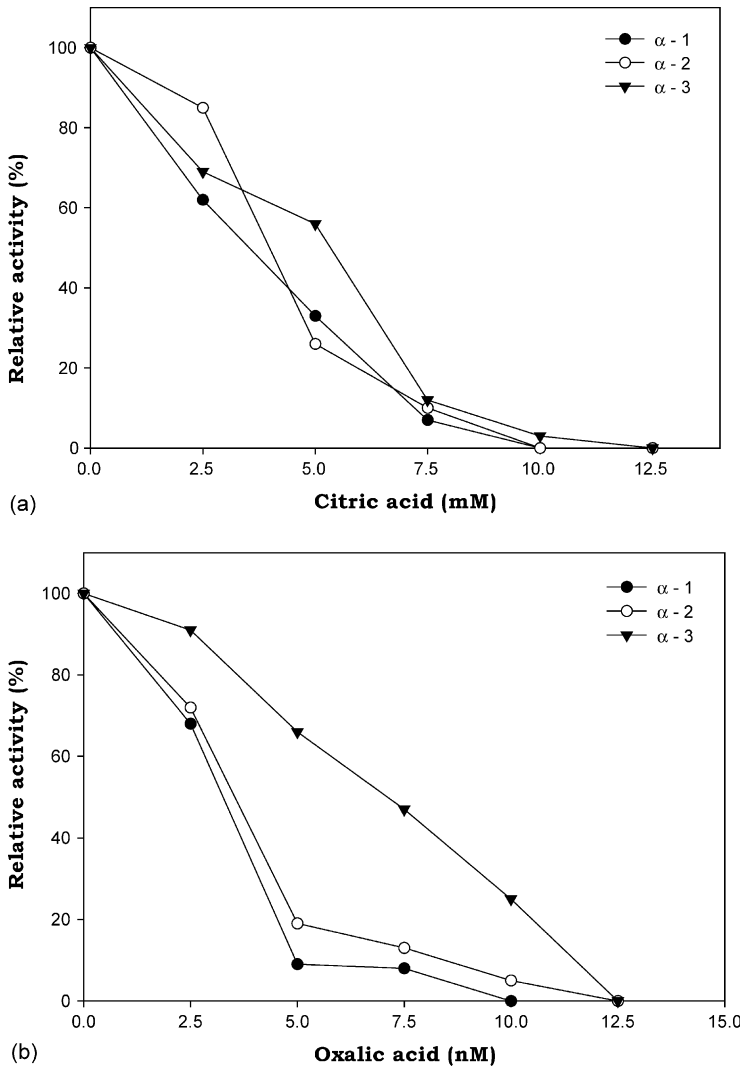


Fig. 6. Effect of (a) citric and (b) oxalic acids on the activities of purified ragi amylases (relative activity is expressed by taking the average value of three independent experiments).

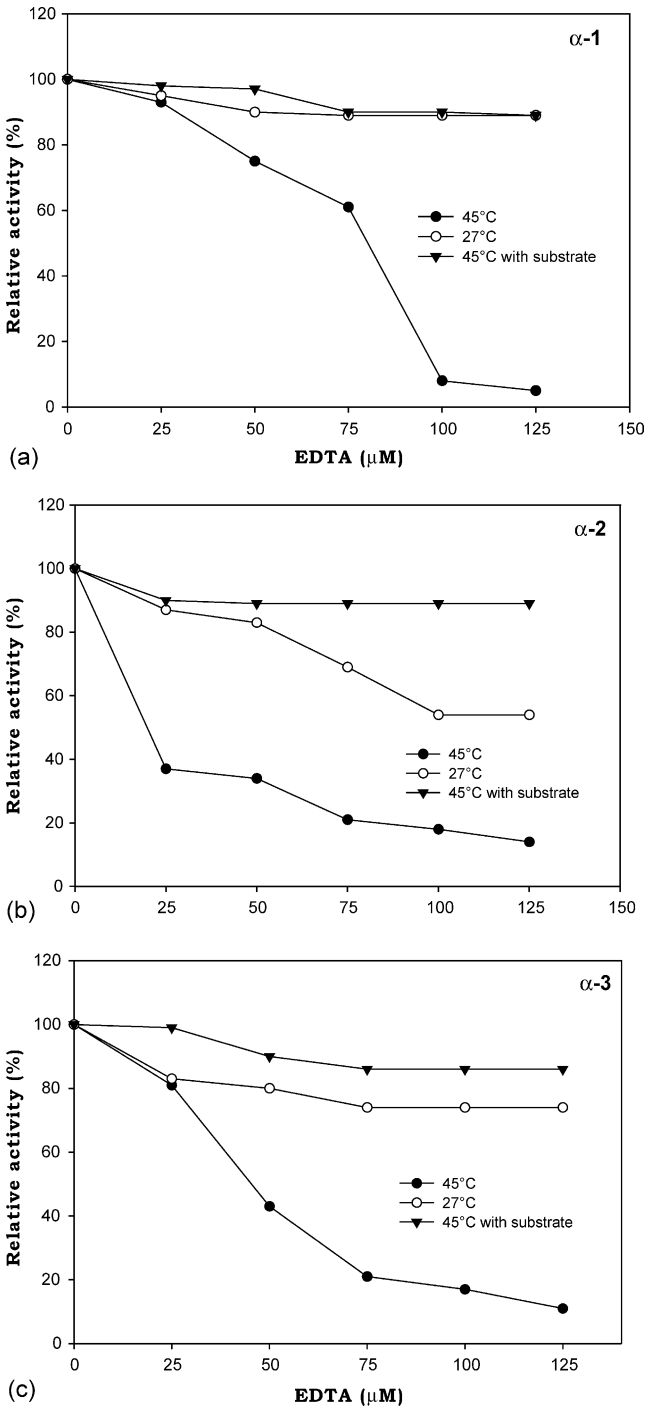


Fig. 7. Effect of EDTA on the activities of purified ragi amylases α-$1_{(b)}$, α-2 and α-3 (relative activity is expressed by taking the average value of three independent experiments).

1992) and its number may go up to 10 (Vihinen & Mantsala, 1989). It was suggested that $Ca^{2+}$ ion appears to stabilize the active site cleft by inducing an ionic bridge between the domains. All the plant α-amylases appear to contain loosely bound $Ca^{2+}$ compared to microbial enzymes and its removal results in both irreversible as well as reversible inactivation resulting in the loss of thermal stability (Thoma, Sparlin, & Dygert, 1971). Removal of $Ca^{2+}$ from α-amylases does not result in significant changes in their hydrodynamic properties but may result in minor conformational changes. $Ca^{2+}$ stabilizes the compact architecture of the enzyme and helps to maintain its active confirmation (Fischer & Stein, 1960).

The effect of temperature on the inhibition of barley α-amylase isoforms was studied in the presence of 10 mM EDTA and found to be having a marked effect on their stability. Barley α-amylase-1 was inhibited at all temperature tested whereas barley α-amylase-2 did not loose its activity at 45 °C in the presence of EDTA, however, amylase-1 lost 40% of the original activity at this temperature (Bertoft et al., 1984). This clearly indicated the effect of temperature on the metal chelating effect of EDTA. Thus, the data presented in the present study is in agreement with the published literature (MacGregor, 1983).

## 4. Conclusions

The purified ragi α-amylases were more or less completely inactivated below pH 4.0 and above 70 °C. $CaCl_2$ (5–7.5 mM) was enhancing their thermal stability and also found to an activator. α-$1_{(b)}$ was more susceptible to organic acids compared to α-2 and α-3. EDTA was found to be inhibiting these enzymes at micro molar concentration and inhibition was temperature dependant and higher at elevated temperature. However, in the presence of substrate, the inhibition was minimal (10%).

## Acknowledgements

Authors thank Dr V. Prakash, Director CFTRI for his keen interest in the work and encouragement. MN thank the council of scientific and industrial research (CSIR New Delhi) for the grant of research fellowship.

## References

Acevedo, E., & Cardemil, L. (1997). Regulation of α-amylase isoenzymes expression in *Araucaria araucona* by gibberellic and abscisic acids. *Phytochemistry*, *44*, 1401–1405.

Akazawa, T., Mitsui, T., & Hayashi, M. (1988). Recent progress in α-amylase biosynthesis. In J. Preiss (Ed.), (*Vol. 14*) (pp. 465–492). *Biochemistry of plants*.

Bernfeld, P. (1955). Amylases α and β. In S. P. Colowick, & N. O. Kalpan (Eds.), (*Vol. 1*) (pp. 149–158). *Methods in enzymology*, New York: Academic Press.

Bertoft, E., tfolk, C., & Kulp, S. E. (1984). Effect of pH, temperature and calcium ions on barley malt α-amylase isoenzymes. *Journal of Institute of Brewing*, *90*, 298–302.

Bradford, M. M. (1976). A rapid sensitive method of quantitation micro gram quantities of proteins utilizing the principles of protein—dye binding. *Analytical Biochemistry*, *72*, 248–254.

Bush, D. S., Sticher, L., Hwystee, R. V., Wegner, D., & Jones, R. L. (1989). The calcium requirement for stability and enzymatic activity of two

isoforms of barley aleuron α-amylase. *Journal of Biological Chemistry*, *264*, 19392–19398.

Fischer, E. H. & Stein, E. (1960). In B. D. Boyer, H. Lardy, & K. Myrbak (Eds.), (2nd ed.) (*Vol. 4*) (pp. 313–343). *The enzymes*.

Greenwood, C. T., & MacGregor, A. W. (1965). Isolation of α-amylase from barley and malted barley and a study of their properties and action patterns of the enzymes. *Journal of Institute of Brewing*, *71*, 405–417.

Greenwood, C. T., & Milne, E. A. (1968a). Studies on starch degrading enzymes. Part VII. Properties and action pattern of the α-amylase from barley, oats, rye and wheat. *Die starke*, *20*, 101–107.

Greenwood, C. T., & Milne, E. A. (1968b). Studies on starch degrading enzymes. Part VIII. A comparison of α-amylases from different sources their properties and action pattern. *Die starke*, *20*, 139–150.

Janeck, S., & Belaz, S. (1992). α-amylases and approaches leading to their enhanced stability. *FEBS Letters*, *304*, 1–3.

Kruger, J. E., & Lineback, D. R. (1987). Carbohydrate degrading enzymes in cereals. In J. E. Kruger, D. R. Lineback, & C. E. Stautter (Eds.), *Enzymes and their role in the cereal technology* (pp. 117–139). St Paul, Minnesota, USA: AACC.

Laemmli, U. K. (1970). Cleavage of structural protein during the assembly of the head of bacterio phase T4. *Nature*, *227*, 680–685.

Leloup, V., Colonna, P., & Buleon, A. (1994). Enzymatic processing of carbohydrates. In B. Godon (Ed.), *Bioconversion of cereal products* (p. pp) New York: VCH Publishers.

MacGregor, A. W. (1978). α-amylase from malted barley physical properties and action pattern on amylose. *Cereal Chemistry*, *55*, 754–765.

MacGregor, A. W. (1983). In J. Daussant, J. Mosse, & S. Vaughan (Eds.), *Seed proteins* (pp. 1–28). New York: Academic press.

Machaiah, J. P., & Vakil, U. K. (1984). Isolation and partial characterization of α-amylase components evolved during early wheat germination. *Journal of Bioscience*, *6*, 47–59.

Marchylo, B., Kruger, J. E., & Irvine, G. N. (1976). α-amylase from immature red spring wheat. I. Purification and some chemical and physical properties. *Cereal Chemistry*, *53*, 157–173.

Nirmala, M., Subba Rao, M. V. S. S. T., Muralikrishna, G., (2000). Carbohydrates and their degrading enzymes from native & malted finger millet (ragi, Eleusine coracana, Indaf–15). *Food Chemistry*, *69*, 175–180.

Nirmala, M., & Muralikrishna, G. (2003). Three α-amylases from malted finger millet (Ragi, *Eleusine coracana*, Indaf-15): purification and partial characterization. *Phytochemistry*, *62*, 21–30.

Thoma, J. A., Sparlin, J. E., & Dygert, S. (1971). Plant and animal amylases. In P. Boyer (Ed.), (*Vol. 5*) (pp. 115–189). *The enzymes*, New York: Academic press.

Tkachuk, R., & Kruger, J. E. (1974). Wheat α-amylases. II. Physical characterization. *Cereal Chemistry*, *51*, 508–529.

Vihinen, M., & Mantsala, P. (1989). Microbial amylolytic enzymes. *Critical Review in Biochemistry and Molecular Biology*, *24*, 329–348.

Walker, J. M. (1996). Non denaturing polyacrylamide gel electrophoresis of proteins. In J. M. Walker (Ed.), *Protein protocols* (pp. 51–55). Totowa, NJ: Human Press.