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Three α-amylases from malted finger millet (Ragi, *Eleusine coracana*, Indaf-15)—purification and partial characterization

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Abstract

Three α-amylases (E.C. 3.2.1.1) were purified to apparent homogeneity from 72 h finger millet 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 D.P. 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. K_m values of these amylases for various cereal starches varied between 0.59 and 1.43%. Carbodiimide (50 mM) and metal ions such as Al^{3+}, Fe^{2+}, and Hg^{2+} (5 mM) have completely inhibited these enzymes at 45° C. Amino acid analysis of these enzymes indicated high amounts of glycine which is an unusual feature of these enzymes.

Keywords: α-Amylases; Finger millet; Ragi; *Eleusine coracana*; Purification; Partial characterization

1. Introduction

Amylases (E.C: 3.2.1.0) are a class of hydrolases widely distributed in microbes, plants and animals. They can specifically cleave the O-glycosidic bonds in starch, a storage polysaccharide present in seeds, tubers etc. of various plants. Starch consists of two components, a linear glucose polymer, amylose which contains α-1, 4 linkages and a branched polymer, amylopectin in which linear chains of α-1, 4 residues are inter linked by α-1, 6 linkages. Starch depolymerization by amylases is the basis for several industrial processes such as the preparation of glucose syrups, bread making and brewing. Amylases are instrumental in starch digestion in animals resulting in the formation of sugars, which are subsequently used in various metabolic activities (Leloup et al., 1994).

Amylases can be classified into endo amylases, (α-amylases), exoamylases (β-amylase, glucoamylase) and debranching enzymes (pullulanase) based on their mode of action (Banks and Greenwood, 1975).

Cereal α-amylases play a very important role in the starch metabolism in developing as well as germinating cereals. These highly expressed enzymes are synthesized under the influence of plant growth hormones such as gibberellic acid (GA_3) and exist in multiple forms (Mitchell, 1972; MacGregor, 1977). The presence of α-amylase activity during malting of barley, wheat and oat seed maturation and germination has been extensively examined (Meredith and Jenkins, 1973; Hill and MacGregor, 1988).

Finger millet (*Eleusine coracana*) or ragi, an indigenous minor millet, rich in calcium and dietary fiber is extensively consumed by South Indian rural population and is used both in the native and processed (malted) forms (Subba Rao and Muralikrishna, 2001). Attempts are underway at CFTRI to use malted ragi in bread making, weaning and infant food formulations. Studies carried out on recently released finger millet variety (Indaf-15) indicated an array of carbohydrate degrading enzymes, out of which amylase was the most pre-

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ponderant in the malts (Nirmala et al., 2000). No systematic studies were carried out on the basic aspects of amylases from malted ragi with respect to their isolation, purification and characterization, hence the present study.

2. Results and discussion

2.1. Extraction of amylases

Ragi millet malted for 72 h was found to be the best as compared to 24, 48 and 96 h ragi malts with respect to the amylase activity (Nirmala et al., 2000). To achieve quantitative isolation of amylases, 72 h malt was extracted with phosphate buffer, repeatedly for three times. More than 90% of the activity was obtained in the first two extractions and subsequent extraction (3rd) did not yield any significant activity and accordingly the number of extractions were restricted to two (first extraction 235 units/g malt, second extraction 176 units/g malt).

In the present study 1% PVPP was added to the extractant to minimize the co-extraction of phenolic compounds present in ragi malt. The extractable phenolic compounds were not found to be having any effect on amylase activity (+ PVPP 220/g malt: PVPP 212/g malt).

Different concentrations of calcium acetate at different temperatures were employed in isolation of α-amylases from wheat (Kruger and Tkachuk, 1969, Warchaleswski and Tkachuk, 1978), barley (Greenwood and MacGregor, 1965), hard spring wheat (Marchylo et al., 1976; Tkachuk and Kruger, 1974). Tris–HCl buffer was employed for the extraction of α-amylases from malted barley and wheat (Zawistowski et al., 1988), whereas acetate buffer was employed for the extraction of amylases from immature barley and native wheat (Machiah and Vakil, 1984). Heat treatment of the cereal malt extracts is generally carried out at 70 °C to inactivate the associated β-amylase activity (Meredith and Jenkins, 1973), but, this step was avoided in the present study due to the very low level of β-amylase activity in ragi malt, which is in consonance with the one reported from immature barley (MacGregor et al., 1971).

2.2. Detection of amylases on PAGE

PAGE of the crude extract of ragi malt indicated the presence of three major amylases and several other non-amylolytic proteins (figures not shown). Seven α-amylase components were identified in pearl millet (Beleia and Varriano-Martson, 1981). Whereas, in wheat as many as 22 forms were identified (Marchylo et al., 1976). Genetic diversity is one of the main reasons suggested for multiple forms of cereal α-amylases (Ainsworth and Gale, 1987; Muthukrishnan and Chandra, 1988).

2.3. Purification of amylases

Crude phosphate buffer extract from ragi malt was subjected to acetone precipitation and separated into two fractions (0–20 and 20–75%). More than 80% of the activity is obtained from 20 to 75% fraction and was taken for further purification. Acetone was also used in fractionation of cereal amylases from immature barley, red spring wheat (Marchylo et al., 1976) with comparatively low yields than in the present study (47–58%).

Acetone fraction (20–75%) was fractionated on DEAE-Sephacel column (Fig. 1). This step effectively removed large amounts of unbound and contaminating proteins. The bound proteins were eluted with a linear gradient of NaCl (0–0.4 M), wherein major non-amylolytic proteins were eluted ahead of amylases. Amylases were separated into three main activity peaks and eluted from the column at 0.15 M (α-1), 0.19 M (α-2) and 0.23 M (α-3) NaCl concentrations. The recovery was found to be 11.6 (α-1) 4.7 (α-2) and 11% (α-3) with a fold purification of 8.0 (α-1), 11.3 (α-2) and 24 (α-3).

The DEAE-Sephacel purified amylases were loaded individually on Sephacryl S-200 column. Fig. 2a–c depicts the elution profiles of α-1, α-2 and α-3, respectively. α-1 from DEAE-Sephacel column was separated into three proteins out of which two were amylolytic peaks and were eluted at 38 ml ($\alpha\text{-}1_{(a)}$) and 77 ml ($\alpha\text{-}1_{(b)}$) respectively and the activity of the second peak ($\alpha\text{-}1_{(b)}$) constituted almost 70% of the activity loaded. The percentage recovery of $\alpha\text{-}1_{(b)}$ was 6.5 with a fold purification of 26, which was nearly 3.2 times more compared to DEAE-Sephacel column peak indicating effective removal of contaminating proteins on GPC. The activity peak eluted at 38 ml ($\alpha\text{-}1_{(a)}$) was found to be different from amylase α-1, α-2 and α-3 which are identified on PAGE by activity staining. $\alpha\text{-}1_{(a)}$ was not taken for further studies due to its low yield.

α-2 was separated from the two contaminating protein peaks or Sephacryl S-200. α-2 was found to be eluting slightly ahead of $\alpha\text{-}1_{(b)}$ approximately at 75 ml and its specific activity was increased to 17 fold with a recovery of 2.9%. This amylase was found to be a minor one compared to amylase $\alpha\text{-}1_{(b)}$ and α-3.

α-3 elution profile on Sephacryl S-200 is identical to that of $\alpha\text{-}1_{(b)}$ (elution volume 77) and was obtained in 9.6% yield with a fold purification of 31. This isoform was found to be the major one among the three amylases.

The overall scheme employed in the purification of amylases from ragi malt was summarized in Table 1. Sephadex G-100 gel permeation chromatography was employed in the separation of several α-amylases from

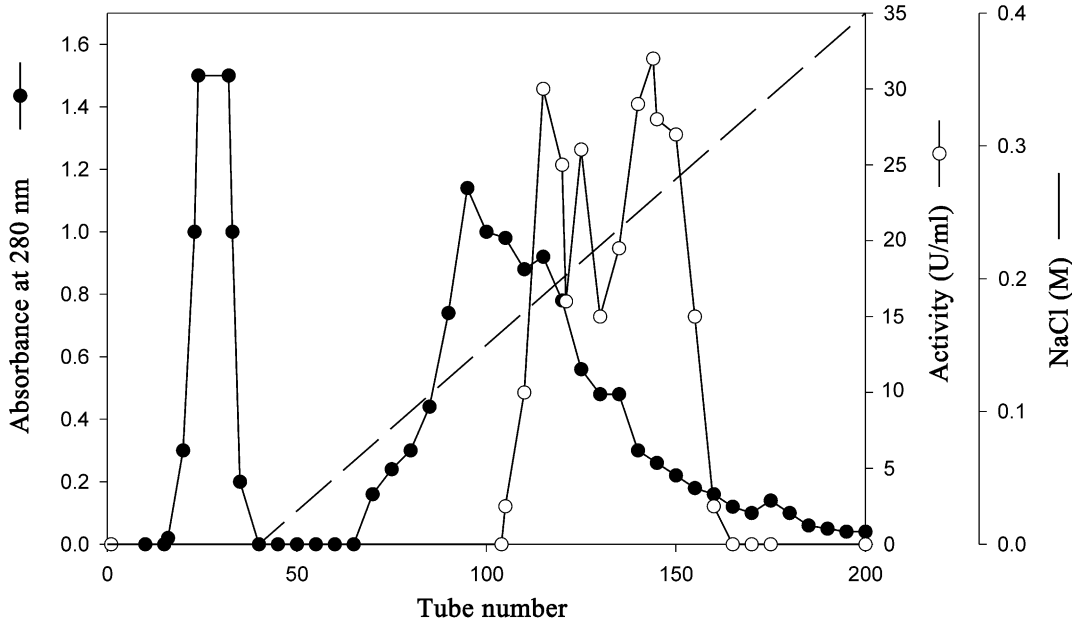

Fig. 1. Elution profile of ragi amylases (20–75% acetone fraction) on DEAE-Sephacel column.

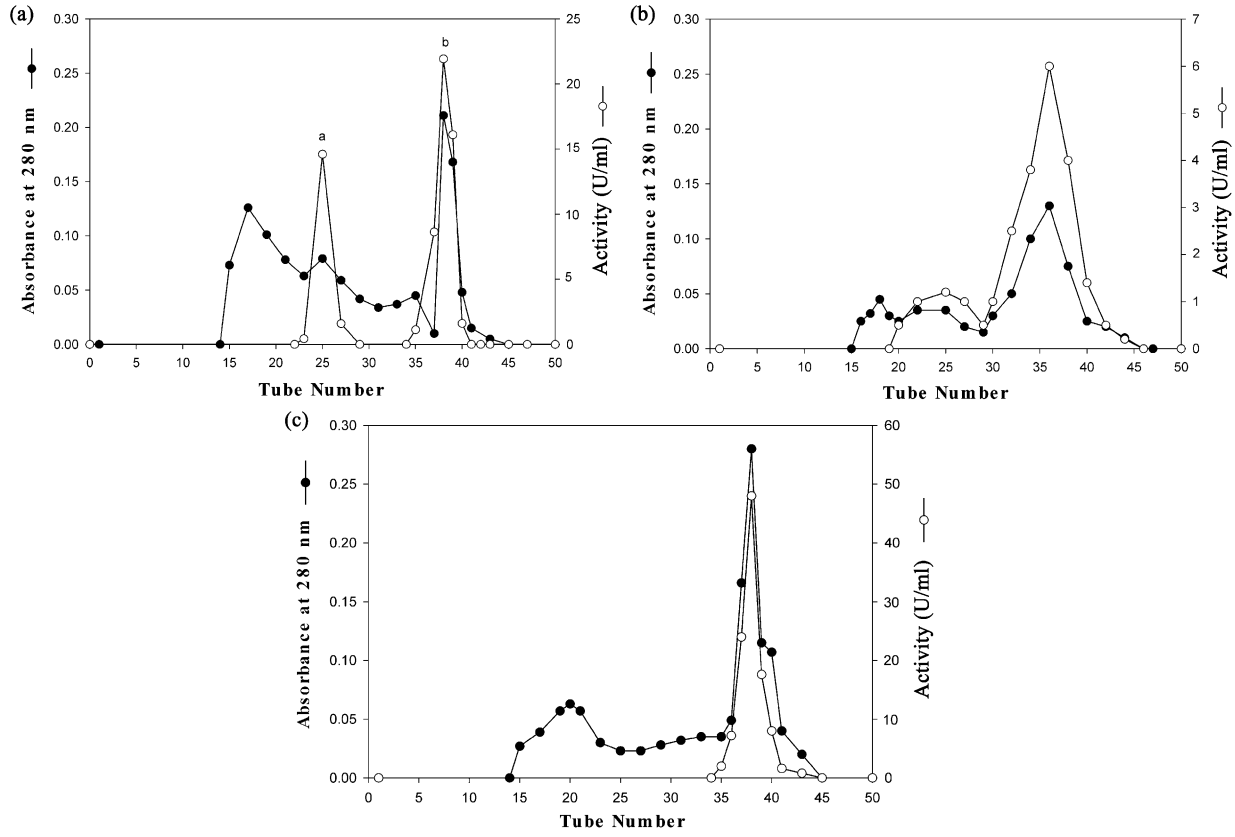

Fig. 2. (a) Elution profile of ragi amylase-1 (α-$1_{(b)}$), obtained from DEAE-Sephacel column on S-200. (b) Elution profile of ragi amylase-2 (α-2), obtained from DEAE-Sephacel column, on S-200. (c) Elution profile of ragi amylase-3 (α-3), obtained from DEAE-Sephacel column on S-200.

aleurone layer of barley (Bilderback, 1974). Two consecutive gel permeation chromatographies on Sepharose-CL-4B followed by Biogel P-100 were employed in the purification of α-amylases from germinating wheat (Machaiah and Vakil, 1984).

2.4. Criteria of purity

The purified amylases were passed through Sephadex G-75 to ascertain their purity and all the three amylases eluted as single symmetrical proteins and activity peaks

Table 1
Summary of the purification of amylases from malted ragi[a]

Step		Total activity[b] (U)	Total protein (mg)	Specific activity[c] (U/mg)	Fold purification	Percentage recovery
Crude		27 605	480	57.5	1	100
Acetone (20–75%)		21 808	153.6	142	2.47	79
DEAE- Sephacel	α-1	3200	7	457	7.95	11.6
	α-2	1300	2	650	11.3	4.7
	α-3	3045	2.2	1384	24	11
Sephacryl S-200	$\alpha\text{-}1_{(b)}$	1800	1.2	1500	26	6.5
	α-2	800	0.82	975	17	2.9
	α-3	2660	1.5	1773	31	9.63

[a] 100 g scale (values are average of three independent experiments).
[b] One unit is equivalent to 1 μmol of maltose released min^{-1}.
[c] Specific activity is expressed as 1 μmol of maltose released min^{-1} mg $protein^{-1}$.

indicating their apparent homogeneity on the basis of molecular weight (figure not shown).

Crude phosphate buffer extract, has many proteins and three amylases (figure not shown). Upon purification the amylases were separated from one another and other contaminating proteins and showed their apparent homogeneity with respect to protein (figure not shown) and activity staining (activity staining of purified amylase is given in Fig. 3).

PAGE was used extensively to determine the purity of the amylases from wheat (Kruger and Tkachuk, 1969), barley aleurone layers and endosperm (Bilderback, 1974), Durum wheat (Warchaleswiski and Tkachuk, 1978), immature hard spring wheat (Marchylo et al., 1976) and pearl millet (Belaia and VarrienoMarston, 1981).

2.5. Molecular weight

The native molecular weights of $\alpha\text{-}1_{(b)}$ and α-3 were found to be 22 kDa whereas α-2 had a molecular weight of 26 kDa as determined by GPC on Sephadex G-75 (figure not shown). However, the molecular weight of a denatured protein on SDS PAGE was found to be ~47 kDa (Fig. 4). The low molecular weight values obtained by GPC were not surprising as similar types of results were also obtained by earlier researchers (Tkachuk and Kruger, 1974) wherein they indicated the interaction of α-amylases with various gel matrices (Kruger and Lineback, 1987).

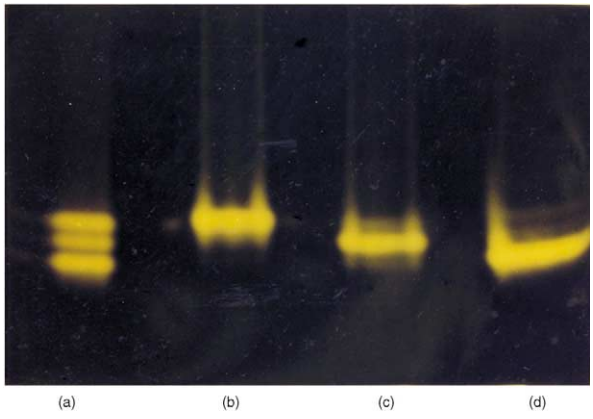

Fig. 3. PAGE of purified amylases from malted ragi-activity staining (a) Crude; (b) $\alpha\text{-}1_{(b)}$ (c) α-2; (d) α-3.

The α-amylase from malted wheat had a molecular weight of 23 kDa as determined by GPC whereas its molecular weight was found to be 42 kDa on SDS PAGE (Tkachuk and Kruger, 1974) and is comparable with the ones reported in the present study. In general, the molecular weight of malt cereal enzymes ranged from 42 to 46 kDa (Greenwood and Milne, 1965). However, there are exceptions wherein the molecular weight ranged from 20 to 57 kDa in the case of amylases isolated from wheat (Tkachuk and Kruger, 1974) barley (MacGregor et al., 1978) and immature wheat (Marchylo et al., 1976). Variation in molecular weight of several amylases determined by various methods should be viewed with caution as small molecular weight dextrins bound to the α-amylases may impart anomalous elution pattern in GPC (Marchylo et al., 1976). In addition, the molecular weight determined may vary according to the method employed (Tkachuk and Kruger, 1974).

2.6. Nature of action of amylases

The nature of action of purified amylases was determined by carrying out the digestion of soluble starch by $\alpha\text{-}1_{(b)}$, α-2 and α-3 analysis of the resultant products by PC, HPLC, optical rotation and rate of viscosity drop in soluble starch solution. The results obtained from PC and HPLC indicated preponderantly oligosaccharides varying in their degree of polymerisation (DP2–7) and quantity (figures not shown), and this pattern is a characteristic feature of hitherto known α-amylases. The optical rotation values observed during the starch

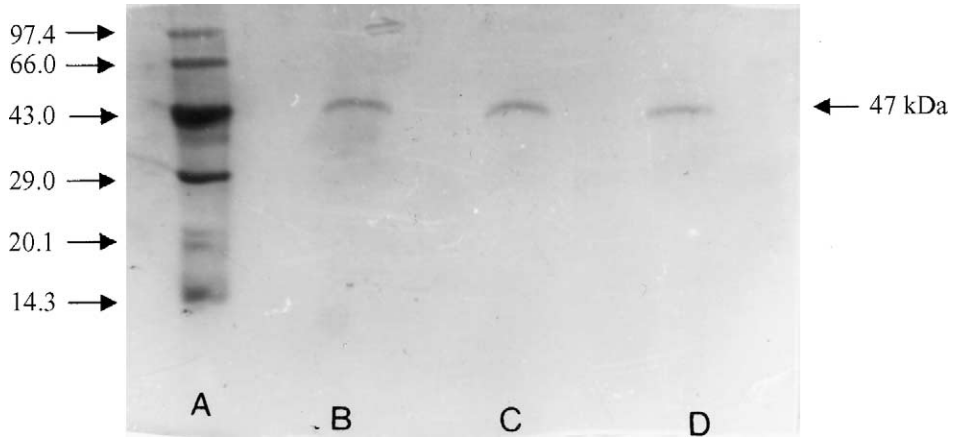

Fig. 4. SDS PAGE of purified amylases (A) molecular weight markers; (B) α-$1_{(b)}$ (C) α-2; (D) α-3 97.4 kDa phosphorylase b; 66.0 kDa bovine serum albumin; 43.0 kDa ovalbumin; 29.0 kDa carbonic anhydrase; 20.1 kDa trypsin inhibitor; 14.3 kDa lysozyme.

hydrolysis slightly increased and became constant during the course of reaction and were decreased upon alkalization $(\alpha)_D - 0.074 \rightarrow 0.060$ (α-$1_{(b)}$), $0.030 \rightarrow 0.024$ (α-2), $0.082 \rightarrow 0{,}074$ (α-3)}, indicating the α nature of α-$1_{(b)}$, α-2 and α-3 derived from ragi malt. In addition, a sharp decrease in viscosity (~60%) within 5 min of hydrolysis of soluble starch by ragi amylases further substantiated the α-nature of these enzymes (figures not shown).

Similar methods were used for the determination of the nature of α-amylases from various sources and their results are inconsonance with the present study. In contrast to α-amylases, β-amylases releases β-D-maltose during starch hydrolysis and the viscosity drop of starch is only marginal and the optical rotation values of the released product (β-D-maltose) is usually low.

2.7. pH Optima

α-$1_{(b)}$ and α-3 were found to have a pH optima of 5.0 which was less than that of α-2 (5.5). The activities of these amylases in alkaline pH are more than the ones observed in acidic pH (figures not shown). The activities of the α-$1_{(b)}$, α-2 and α-3 were decreased both in phosphate and Tris–HCl buffers at a higher pH compared to sodium acetate and sodium succinate buffer. However, the drop in activity in phosphate buffer was much more (α-$1_{(b)}$, 88%; α-2 64%; and α-3 92%) than in Tris–HCl buffer (α-$1_{(b)}$, 60%; α-2, 47%; α-3, 82%) indicating the better stability of these α-amylases in Tris–HCl buffer. At pH 4.0 the enzyme activities of α-$1_{(b)}$, α-2 and α-3 decreased drastically both in acetate as well as succinate buffer indicating their acid labile nature, a feature characteristics of several cereal α-amylases (Thoma et al., 1971). α-3 acts in a narrow range of pH compared to α-$1_{(b)}$ and α-2 and its activity was much less in alkaline pH. However, amylases from sorghum (Botes et al., 1967). Pearl millet (Beleia et al., 1981) and malted sorghum (Dube and Norden, 1961) showed low pH optima values (4.85) compared to the ragi amylases.

2.8. Temperature optima

To determine the temperature optima of ragi α-amylases, activities were determined at a temperature range of 30–70 °C, α-$1_{(b)}$ and α-3 were found to have a temperature optima of 45 °C and this was slightly less than that of α-2 (50 °C; figures not shown).

These values are comparable to the one reported for barley α-amylases (Greenwood and Milne, 1968a, b; Greenwood and MacGregor, 1965). However, slightly higher temperature optima was observed for α-amylase from immature barley (Marchylo et al., 1976); where as wheat α-amylase had a broad temperature optima of 40–50 °C (Tkachuk and Kruger, 1974).

2.9. Amino acid composition

The amino acid composition of α-$1_{(b)}$, α-2 and α-3 given in Table 2 indicated very high amount of glycine (α-$1_{(b)}$, 29%, α-2 25% and α-3 25%), which is substantially higher than the reported values for cereal α-amylases. The glutamic acid/glutamine and aspartic acid/aspargine ratios were found to be in the range of 4–8%. The percentage of hydrophobic amino acids was relatively high in α-$1_{(b)}$ compared to α-2 and α-3. Sulphur containing amino acids (methionine and cysteine) were found to be very less especially in α-3. The quantity of aromatic amino acids (tyrosine and phenyl alanine) ranged from 3 to 4%.

Amino acid composition has been determined for a few cereal amylases. In the case of sorghum α-amylase, aspartic acid content (13%) was found to be more than glycine (11%; Botes et al., 1967). Amino acid analysis of carboxy methylated α-amylases from wheat has shown the absence of sulphydryl groups. Wheat α-amylase

Table 2
Amino acid composition of finger millet α-amylases. (represented as % p moles)

Amino acid	α-$1_{(b)}$	α-2	α-3
Aspartic acid/aspargine	4.0	5.5	7.3
Glutamic acid/glutamine	5.4	8.0	8.4
Serine	6.0	11.0	10.4
Glycine	29	26.2	25.0
Histidine	1.0	2.2	2.0
Arginine	5.2	5.0	3.3
Threonine	3.0	4.0	6.3
Alanine	11.0	10.0	9.7
Proline	12.0	7.0	3.0
Tyrosine	1.0	1.0	3.2
Valine	4.3	5.5	6.0
Methionine	1.2	0.4	0.48
Cysteine	1.3	1.4	0.52
Isoleucine	2.6	3.0	3.5
Leucine	7.2	5.0	5.7
Phenyl alanine	2.0	1.8	2.7
Lysine	3.8	3.0	2.5

isoforms indicated quantitative similarity except in the content of arginine (Tkachuk and Kruger, 1974). Barley aleurone layer α-amylases showed a high content of glycine followed by aspartic acid, whereas the content of glutamic acid was considerably low. However, in barley aleurone layer α-amylase, histidine content was found to be very high compared to other cereal α-amylases (Rodaway, 1978). It can be concluded that amino acid composition of α-amylases vary from cereal to cereal and also in different parts of the same cereals.

2.10. Effect of metal ions

Various metal ions such as Ca^{2+}, Ba^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Al^{3+}, Fe^{2+} and Hg^{2+} at 5 mM concentration were tested for amylase activation/inhibition effect and the results are given in Table 3. Ca^{2+} and Ba^{2+} were found to have both activating and stabilizing effect as indicated by increased activity where as Co^{2+} and Mg^{2+} had negligible effect on activity. However, the inactivating effect of Cd^{2+}, Zn^{2+} and Cu^{2+} was found to be partial. Metals such as Al^{3+} Fe^{2+} and Hg^{2+} completely inactivated all the three amylases. The inactivation by these metals may be due to their binding to either catalytic residues or by replacing the Ca^{+2} from the substrate binding site of the enzyme. The cereal amylases are also inactivated by molybdate at a concentration of 10 mM where as cyanide and chloride did not have any visible effect in barley α-amylase (Greenwood and Milne, 1968a). Role of Ca^{2+} and Mg^{2+} in maintaining the stability and structure of the α-amylase is well documented (Parkin, 1993).

Table 3
Effect of metal ions (5 mM) on purified ragi amylases activity (represented as % activity)

Salts	α-$1_{(b)}$	α-2	α-3
Control	100	100	100
$CaCl_2$	115	108	125
$BaCl_2$	106	102	114
$CoCl_2$	100	98	95.8
$MgCl_2$	95	100	98
$CdCl_2$	57	86	62
$ZnCl_2$	30	26	20
$CuCl_2$	11.8	11.3	6.3
$AlCl_3$	0	0	0
$FeCl_2$	0	0	0
$HgCl_2$	0	0	0

Enhancement of amylase activity of Ca^{2+} ions is based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acids, which resulted in stabilization as well as maintenance of enzyme conformation. In addition, calcium is known to have a role in substrate binding (Sprinz, 1999). It has also been documented that binding of Ca^{2+} to amylase is preferred over other cations such as Mg^{2+} (Bush et al., 1989). Replacement of Ca^{2+} by Sr^{2+}, Na^{2+} and Ba^{2+} resulted in partial activation of barley α-amylases, however it is not comparable to the effect of Ca^{2+} on amylase activity.

2.11. Effect of specific reagents on amylase activity

Amylase activity was determined in the presence of chemicals such as PCMB, iodoacetamide and carbodimide at 45 °C and 50 mM concentration. The amylases were found to be completely inactivated by PCMB as well as carbodimide whereas iodoacetamide had minimal effect. The inhibition of carbodimide suggests the possible presence of acidic amino acids such as aspartic and glutamic acids at the active site. The pH optima value of these amylases (5.0–5.5) also supports this as the ionization values of acidic amino acids fall in this range.

2.12. Fluorescence spectra

The excitation maxima of purified amylases showed a range of 284–288 nm and their emission maxima was in a range of 332–336 nm. The fluorescence of proteins originates mainly due to the quenching of aromatic amino acids, especially tryptophan (figure not shown).

2.13. Effect of substrate concentration

Effect of different substrate concentration on the initial velocity was calculated and the kinetic constants K_m and V_{max} were calculated from the double reciprocal plots (LB plot, Lineweaver and Burk, 1934; figures not shown). The K_m value of α-$1_{(b)}$ for ragi, rice, wheat and maize starches varied between 0.59 and 1.0% and V_{max}

was found to be between 2381 and 2778 u/mg/min respectively. For α-2 K_m values ranged between 1.1 and 1.43% for various cereal starches where as the V_{max} values had a range of 1087–1390 u/mg/min. However, K_m values for α-3 were found to be very low for cereal starches ranging between 0.53 and 1.0% indicating its high substrate specificity compared to α-$1_{(b)}$ and α-2. The V_{max} values of α-3 for cereal starches ranged between 2778 and 2945 u/mg/min (Table 4).

It was observed that all the three amylase were found to have a high affinity towards its natural substrate i.e., ragi starch. The α-3 was found to be most efficient followed by α-$1_{(b)}$ and α-2. The affinity for cereal starches were found to be in the order of ragi > rice > wheat > maize for α-$1_{(b)}$ and α-3 whereas for α-2 it was in the order of ragi > wheat > rice > maize. All the three amylases were found to be have the least affinity towards maize starch as indicated by the high K_m values.

Limited information is available as far as the detailed kinetics and substrate specificity of cereal α-amylases are concerned. This is mainly due to differences in physiochemical properties of various starches based on their origin. The variation of the rate of hydrolysis with a varying concentration of gelatinized soluble starch was studied and K_m was calculated to be 1.57×10^2 mole glycosidic bonds per liter at pH 4.6 for sorghum malt α-amylase (Botes et al., 1966). The effect of substrate concentration was determined for the α-amylase components using reduced starch in 0.2 M sodium acetate, pH 5.5 containing 0.001 M $CaCl_2$ and the K_m was found to be 2.5×10^{-4} g/ml, 5.33×10^{-4} g/ml and 2.35×10^{-4} g/ml for α-amylase-1, amylase-2 and α-amylase-3 respectively as determined by the LB plot (Marchylo et al., 1976).

3. Conclusion

Above studies clearly indicated the presence of three individual α-amylases in the ragi malt, and can be purified to apparent homogeneity by three-step purification and were found to be monomeric with a molecular weight of ~45 kDa. Their amino acid analysis indicated high amounts of glycine, which is a special feature, not found in cereal α-amylases. Their pH and temperature optima are comparable with that of cereal amylases.

Table 4
Kinetic constants of ragi amylases for cereal starches

Starch	α-$1_{(b)}$		α-2		α-3	
	K_m[a]	V_{max}	K_m	V_{max}	K_m	V_{max}
Ragi	0.59	2381	1.1	1111	0.53	2778
Rice	0.71	2439	1.33	1190	0.58	2778
Wheat	0.83	2439	1.2	1389	0.67	2941
Maize	1.0	2778	1.43	1087	1.0	2941

[a] K_m (%), V_{max} (U/mg protein).

4. Experimental

4.1. General

DEAE-Sephacel is obtained from Pharmacia fine chemicals, Uppsala Sweden. Sephadex G-75; Sephacryl S-200; acrylamide; bis acrylamide, triethylamine, phenyl isothiocyanate; Tris TEMED, ammonium persulphate, protein molecular weight standards (GPC), amino acid standards, were from Sigma Chemical Company, St. Louis, USA. Protein molecular weight markers (SDS) obtained from Gene, Bangalore, India. Pico-Tag reverse phase column was obtained from waters associates Milford, Massachusetts, USA. All other chemicals used were of analytical grade.

4.2. Plant material

Finger millet (*Eleusine coracana*, Indaf-15) seeds were procured from V.C. Farm, University of Agricultural Science, Bangalore, located at Mandya, Karnataka, India.

4.3. 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).

4.4. Enzymes extraction and purification

Malted ragi flour 72 h; 100 g) was extracted with 0.05 M sodium phosphate buffer (1:4, pH 6.0, 400 ml) containing 1% PVPP for 2 h at 4 °C and supernatant was collected by centrifugation (6500 *g*, 4 °C) using refrigerated centrifuge, dialyzed against the extraction buffer and was precipitated with acetone (pre cooled at −10 °C for 12 h) at 4 °C up to 20% (V/V) saturation. The precipitate was removed by centrifugation and the supernatant was subjected to 75% acetone (v/v) precipitation. Precipitate obtained was redissolved in extraction buffer and loaded on to a DEAE-sephacel glass column (2×40 cm) pre-equilibriated with sodium phosphate buffer (500 ml, 20 mM, pH 6.8) at a flow rate of 12 ml/h and washed

with the same buffer to remove unbound proteins. A linear NaCl gradient (0–0.4 M) in equilibrating buffer was used to elute the bound components, which were collected (3 ml each) and monitored for protein (280 nm) as well as amylase activity. The three amylase activity peaks were labeled as α-1, α-2 and α-3 in order of their elution. α-1, α-2 and α-3 were concentrated and individually loaded on Sephacryl S-200 glass column [(1×100 cm), pre-equilibrated with sodium acetate buffer, 50 mM, pH 5.0] and fractions (1.5 ml) were collected, monitored for protein and amylase activity.

4.5. Enzyme assay

Amylase was assayed according to the procedure of Bernfeld (1955). Gelatinized soluble starch (1%, 1 ml) in sodium acetate buffer (50 mM, pH 5.0) was incubated with appropriately diluted enzyme (50 μl) at 45 °C for 30 min. The reaction was stopped by adding DNS reagent (1 ml). One unit of enzyme activity was defined as μ mol maltose equivalent released/min under the assay conditions. The specific activity was calculated as activity/mg protein.

4.6. Protein determination

Protein concentration was determined according to the dye binding method of Bradford (1976) with bovine serum albumin as standard.

4.7. Purity criteria

4.7.1. Polyacrylamide gel electrophoresis (PAGE)

PAGE (12.5%) under native conditions was carried out to evaluate the purity of amylases (Walker, 1996). Duplicate samples were run for simultaneous protein and enzyme staining. The gels were stained for protein with silver staining (Wray et al., 1981) and enzyme activity staining (Acevedo and Cardemil, 1997).

4.8. Amylase activity staining

The gel after electrophoresis was marked in gelatinized and cooled (R.T.) starch (2%) prepared in sodium acetate buffer (50 mM, pH 5.0) and incubated at 45 °C for 30 min. Subsequently the gel was stained with iodine reagent (3%). The amylase bands were visualized as transparent bands on a dark blue background.

4.9. Purity and molecular weight determination

The amylase activity peaks obtained from Sephacryl S-200 column chromatography were ascertained for their purity as well as for their apparent molecular weight an Sephadex G-75 (Whitakar, 1970) as well as on SDS-PAGE (Laemmli, 1970). BSA (66 kDa), Ovalbumin (45 kDa), carbonic anhydrase (29 kDa), lysozyme (14 kDa) Aprotinin (65 kDa) served as molecular weight standards an Sephadex G-75, where as phosphorylase-b (97.4 kDa), Bovine serum albumin (66 kDa), ovalbumin (43 kDa) carbonic anhydrase (28 kDa), soybean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa) were SDS-PAGE standards.

4.10. Nature of amylase

The nature of the amylases (α/β) were carried out by following methods.

4.10.1. Viscosity

Gelatinized soluble starch (1%, 15 ml) was incubated with purified amylases (500 μl) in an Ostwald viscometer at 45 °C for 30 min. The drop in viscosity was monitored at every 5 min. Viscosity of the gelatinized soluble starch before the enzyme addition was taken as 100% and relative viscosity was calculated at each time interval (Muralikrishna et al., 1987).

4.10.2. Separation and identification of products by paper chromatography

Descending paper chromatography on Whatman No. 1 was carried out to identity the nature of products using propanol: ethanol:water (7:1:2) solvent system and sugars were identified by spraying with aniline phthalate reagent (Patridge, 1949).

4.10.3. Separation and identification of products by HPLC

Soluble starch was gelatinized and subjected to purified amylases $\alpha\text{-}1_{(b)}$, α-2, α-3 digestion for 15′ and resultant solution was precipitated with 3 volumes of absolute ethanol and kept overnight at 4 °C and the undigested residual starch was separated by centrifugation at 5000 *g*. The supernatants were concentrated by vacuum evaporation and taken in ultra pure water, filtered through Millipore filter and analyzed by HPLC on μ-Bonda pack amino column using acetonitrile:water solvent system (70:30) as eluant, keeping flow rate as 1 ml/min. Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose served as standards (McGinnis and Fang, 1980).

4.10.4. Optical rotation

Optical rotation of the products was determined using polarimeter to know their configuration (α/β). The gelatinized soluble starch (1%, 2 ml) was incubated with purified amylases in 5 ml polarimeter tube at 27 °C and the change in optical rotation was monitored at every 10 min. When the polarimeter value reached constant, 10 mg of sodium carbonate were added and direction (+ or−) and magnitude of change in optical rotation were recorded. The optical rotation value of gelatinized

starch prior to the addition of enzyme was taken as the value at 0 min (Swain and Dekker, 1966).

4.11. Amino acid composition

The purified enzymes were hydrolyzed in vacuo at 110 °C in constant boiling HCl for 24 h using the Pico-Tag workstation. Amino acid analysis was performed by pre column derivatization using phenyl isothiocyanate. The phenyl thiocarbamoyl amino acids were analyzed by Rp HPLC (Bidlingmeyer et al., 1984).

4.12. Effect of pH

Amylases activities were determined at various pH values using different buffers such as sodium acetate and sodium succinate (pH 4.0–6.0), sodium phosphate (pH 6.0–7.0), Tris–HCl (pH 7.0–9.0) at 50 mM concentration at 45 °C. The maximum activity was taken as 100% and reactive activity plotted against different pH values.

4.13. Effect of temperature

Amylases activities were determined at a temperature range of 30–70 °C (with an interval of 5 °C) with 1% soluble starch as substrate in 50 mM sodium acetate buffer. The maximum activity was taken as 100% and relative activities were plotted against different temperatures.

4.14. Effect of metal ions

Purified amylases were incubated with 5 mM solution of salts of metal ions (chlorides of Ca^{2+}, Ba^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Al^{3+}, Hg^{2+}) and their activities were determined. The enzyme activities without metal ions was taken as 100% and relative activities determined in the presence of metal ions were calculated.

4.15. Effect of group specific reagents

The purified amylases were incubated with 50 mM of parachloromercuric benzoate, iodoacetamide and carbodiimide in sodium acetate buffer, pH, 5.0 at 45 °C for 15 min and the residual activities were estimated. The enzyme activities without these chemicals were taken as 100% and relative activities were calculated.

4.16. Fluorescence spectra

The excitation and emission spectra of the purified amylases were taken in an Aminco-Bowman spectrofluorimeter. The emission spectra were recorded after excitation of the sample. The relative fluorescences intensities of the enzymes were recorded (Suelter, 1985).

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