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PARTIAL PURIFICATION AND CHARACTERIZATION OF α-AMYLASES FROM *Abrus precatorius*, *Burnatia enneandra* AND *Cadaba farinosa*

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Abstract- Leaves of *Abrus precatorius,* tubers of *Burnatia enneandra* and stems of *Cadaba farinosa* are used in savannah regions of Cameroon in traditional food processing, particularly in sweetening and liquefaction of gruels. α-amylase was extracted and partially purified from these plants using conventional methods of protein purification including ammonium sulfate fractionation and two steps of gel filtration. Purification achieved 58, 61 and 46 fold respectively for *A. precatorius, B. enneandra* and *C. farinosa*. The purified enzymes were then characterized in terms of molecular weight, optimum pH and stability, optimum temperature and stability, K_m, V_{max} and metals ions effects. The optimum pH of enzymes varied from 6.0 for amylases from *B. enneandra* and *C. farinosa*, to 7.0 for amylase from *A. precatorius*; while the optimum temperature was 60°C for amylases from *A. precatorius* and *B. enneandra*, and 65°c for amylase from *C. farinosa*. The three enzymes displayed, respectively for *A. precatorius, B enneandra* and *C farinosa*, a molecular weight of 60, 65 and 48.5 kDa, K_m for hydrolyzing soluble starch of 3.25, 1.81 and 3.18 mg/ml, and strong individual activation by Ca^{2+}, Co^{2+} and Fe^{3+}. Li^{2+} appeared as a common activator for all the amylases, while Ag^{+}, Hg^{2+}, Zn^{2+} and Cu^{2+} act as common inhibitors.

Keywords- *Abrus precatorius, Burnatia enneandra*, *Cadaba farinosa*, α-amylase, purification, properties

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Introduction

α-Amylase (α-1-4 D-glucan glucanohydrolase EC 3.2.1.1) catalyzes endohydrolysis of α-1-4 glucosidic linkages in starch and any related polysaccharides to produce oligosaccharides and glucose. The functionality of this enzyme has, for centuries, been exploited in different food processing practices such as malting, brewery, gruel fluidification etc. [1]. This food processing demand of the enzyme has supported its extraction and purification from different vegetable foods, including tubers [2], leaves [3] and malted cereals [4,5]. Meanwhile, the need to characterize amylase content of plants still remains, regarding local endogenous uses of the enzyme. In this respect, some populations of sudano sahelian regions, particularly in Northern Cameroon, use leaves of *A. precatorius,* tubers of *B. enneandra* and stems of *C. farinosa* to liquefy and to sweeten cereal gruels. This practice results from scarcity of sugar in this region, due to the low economic status of these populations. Amylase is extracted from the plant through mashing, water extraction and filtration and the extract is mixed with cereal gruel. Glew *et al.* (2010) showed the rich protein and minerals (Ca, Fe, K, Na, Mg, Mn and Zn) potentials of the three plants [6] while Nso *et al.*(2013) evidenced their mashing and brewing potentials [7]. In addition, Nkengbeza and Nso [8] optimized the extraction of α-amylase from *C. farinosa* at 60 min. Out of these studies, no scientific work has been undertaken on the characterization of amylases from these plants.

The present paper aims then at purifying and characterizing amylases from *A: precatorius, B. enneandra* and *C: farinosa* in order to understand the logic of their uses in house liquefaction and sweetening of gruels.

Materials and Methods

Sample Preparation and Crude Enzyme Extraction

Fresh leaves of *A. precatorius*, tubers of *B. enneandra* and stems of *C: farinosa* were collected directly from the field at Kalfou (a town in the Northern region of Cameroon). The samples were sliced and crushed into a paste using a mortar with pestle, then used either for enzyme identification or for crude enzyme extraction. For identification of the nature of enzymes, the Megazyme kits, Ceralpha and Betamyl-3 (Megazyme International, Ireland) and the corresponding methods were used.

For crude enzyme extraction, cold phosphate buffer 50 mM at pH 6.0 (containing 5 mM of β-mercaptoethanol) was added to the paste, and the mixture was homogenized using a Waring Blender (Kenwood, 33 Bl 51, France). The homogenate was further clarified by centrifugation at 7500g for 30 min. The supernatant was dialyzed

against 50mM phosphate buffer, pH 6.0 for 12 hrs. with one change of buffer. The dialyzed sample was used as crude enzyme extract.

All the operations were performed at 4°C and all buffers contained 0.02% (w/v) sodium azide to prevent microbial growth.

Purification of the Extract

The crude enzyme extract was purified through ammonium sulfate precipitation and size exclusion chromatography using successively Sephadex G-100 and Sephadex G-200. The ammonium sulfate precipitation of proteins went through addition of salt in a range between 60% and 100% saturation (*A. precatorius*), 30% and 100% saturation (*B. enneandra*), 60% saturation (*C. farinosa*) and incubation at 4°C for 12 hrs., followed by centrifugation at 7500g for 15 min, and dialysis of the sediment for 24 hrs. with 2 change of buffer. The dialysate was then concentrated in a 20% solution of polyethylene glycol and filtered (0.45µm).

The concentrated extract was eluted on a Sephadex G-100 column (1 x 55 cm) using 50 mM phosphate buffer, pH 6.0, at a flow rate of 15 ml/h. Fractions of 3 ml each were collected and immediately stored in ice, and subjected to protein estimation and amylase assay. The last step of purification of the amylase was carried out by size exclusion column chromatography on Sephadex G- 200 (1 x 55 cm) in the same conditions as above. Protein estimation and amylase assay were done for each fraction. The fractions presenting the highest amylase activities were combined, concentrated and kept at 4°C for further analyses.

Enzyme Characterization

Protein Estimation and Enzyme Assay

Protein content was estimated by the method of Bradford [9] using bovine serum albumin as standard.

The determination of the nature of the amylases was done using the Megazyme kits: Ceralpha and Betamyl-3 (Megazyme International, Ireland). The Ceralpha method which uses a non-reducing-end blocked maltoheptaoside (BNPNPG7) as a substrate was used to determine the presence of α-amylase. Amylase HR reagent was incubated with appropriately diluted enzyme extract at 40°C for 10 min. The reaction was stopped by the addition of tri-sodium phosphate buffer pH 11. One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable α-glucosidase, required for the releasing of one micromole of *p* -nitrophenol from BPNPG7 in one minute under the defined assay conditions, and is termed as Ceralpha Unit. The β-amylase activity was measured by the specific 'Betamyl-3' substrate *p*- nitrophenyl-β-D-maltotriose (PNPβ-G3). Betamyl 3® reagent was incubated with appropriately diluted enzyme extract at 40ºC for 10 min. The reaction was stopped by the addition of Trizma base. One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable β-glucosidase, required to release one micromole of *p* -nitrophenol from PNPβ-G3 in one minute under the defined assay conditions, and is termed as Betamyl-3 ® Unit.

All along the purification and characterization process, α-amylase activity was assayed according to the procedure of Bernfeld [10]. Gelatinized soluble starch (1%) in 0.1 M phosphate buffer was incubated with appropriately diluted enzyme. The reaction was stopped by the addition of DNS reagent (1ml). One unit of enzyme activity was defined as µmol maltose released/min under the assay conditions. The specific activity was expressed as activity units /mg protein.

Purity and Molecular Weight

Purity and molecular weight of the purified enzymes were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using the Laemmli [11] gel method on 10% polyacrylamide slab gel containing 1% SDS. Gels were stained with Coomassie Brilliant Blue. Standard proteins used for calibration were: Trypsinogen, bovine pancreas (26 kDa); Glyceraldehyde-3-phosphate dehydrogenase(55 kDa); rabbit muscle (34 kDa); Ovalbumin, chicken egg (43 kDa); Glutamic dehydrogenase, bovine liver (55 kDa); Fructose-6-phosphate kinase (72 kDa); Phosphorylase B, rabbit muscle (95 kDa); β-Galactosidase, *E. coli* (130 kDa); Myosin porcine heart (170 kDa).

Optimum pH and Temperature

The optimum pH for the amylase activity was determined by assaying the activity at different pH values (2-11), using the following buffers: 0.1 M acetate buffer (pH 2.5-5.5), 0.1 M phosphate buffer (pH 6.0-7.5), 0.1 M Tris-HCl buffer (pH 8.0-9.0) and glycine-NaOH (pH 10-11). The optimum temperature for amylase activity was determined by assaying the enzyme at temperatures from 30 to 100°C.

pH and Heat Stability

Stability of the enzymes at different pH values was determined by measuring the residual activity after incubating the enzyme at a pH range of 2.5-11.0 for 3 h. Heat stability was measured by incubating the enzyme at a temperature range of 30-100°C, for 30 min. After heat treatment, the enzyme solution was cooled and the residual activity was determined.

Kinetic Constants

Kinetic parameters of the purified amylases, with soluble starch as substrate were determined. The Michaelis constant (K_m) and the maximum velocity (V_{max}), were determined from Lineweaver-Burk plots.

Metal Ion and EDTA Effects

The effects of various metal ions (Ca^{2+}, Mg^{2+}, Na^{+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Ag^{+}, Fe^{3+}, Co^{2+}, Li^{2+} et K^{+}, and Ba^{2+}) (1 mM) and EDTA (1 - 40 mM) on enzyme activity were determined by pre-incubating the enzyme with 0.5 ml of individual reagent for 20 min, followed by incubation with soluble starch under the standard assay conditions and then assaying the enzyme activity. The activity assayed in the absence of metal ions or reagents was considered as 100%.

Results and Discussion

Type of Amylases in Plants

The enzyme assays, using Megazyme kits, show that there is no β-amylase activity in all the plants. The amylolytic activity is due only to α-amylase [Table-1]. The tubers of *B. enneandra* have the highest α-amylase activity, followed by *A. precatorius* leaves, and finally *C. farinosa* stems.

Table 1- Activity (U/g) of amylolytic enzymes

	A. precatorius	*B. enneandra*	*C. farinosa*
α-amylase activity (U/g)	142.02 ± 6.31	229.34 ± 4.82	83.11 ± 3.64
β-amylase activity (U/g)	0	0	0

Purification of Amylase

From the elution profile, it is observed that the purified enzymes are eluted as a well resolved peak of amylase activity [Fig-1]. Each step of purification increases the specific activity of the enzymes and, at the end of chromatography on Sephadex G200, purification folds of 58, 61 and 44 are achieved respectively for *A. precatorius, B. enneandra* and *C. farinosa*, with corresponding specific activity of 965, 1540 and 224 Units /mg. The protein recovery of the three enzymes in these conditions are respectively 47, 45 and 55%. The purification data of α-amylase from the three plants are summarized in [Table-2].

Chromatography on Sephadex G200 appears as the most determining step for purification of the three enzymes. The protocol of purification used in this study allows a better purification fold for *B. enneandra* and a better yield for *C. farinosa.* This is confirmed by the homogeneous profile of the enzymes on SDS polyacrylamide gel electrophoresis, which displays a single protein band, with molecular weights of 60, 65 and 48.5 kDa, respectively for *A. precatorius, B. enneandra* and *C. farinosa* [Fig-2].

Optimum pH and Temperature

The suitability of an enzyme preparation for use in industry depends on its behavior as a function of pH and temperature. The purified enzymes display an optimum pH value of 7.0 for amylase from *A. precatorius*, and 6.0 for amylase from *B. enneandra* and *C. farinosa* [Fig-3]. The pH stability where the purified amylases conserve 100% of its original activity after 3 hrs. of incubation at 40°C, follows the same distribution, with a pH range of 6.0-8.5 for amylase from *A. precatorius,* while amylases from *B. enneandra* and *C. farinosa* are stable in a pH range of 4.0-7.0.

Regarding the temperature effect, the amylase activity increases and is stable with increasing temperature, up to an optimum activity at 60°C for enzyme from *A. precatorius* and *B. enneandra*, while *C. farinosa* amylase activity has its optimum temperature at 65°C [Fig-4]. The enzyme activity then declines above these optimum temperature values, indicating loss in the active conformation of the protein. Amylase from *C. farinosa* appears as the most thermostable, since it keeps almost 58% of its activity after 30 min incubation at 80°C, while amylase from *B. enneandra* keep only 10% keep only 10% of their activity in the same conditions.

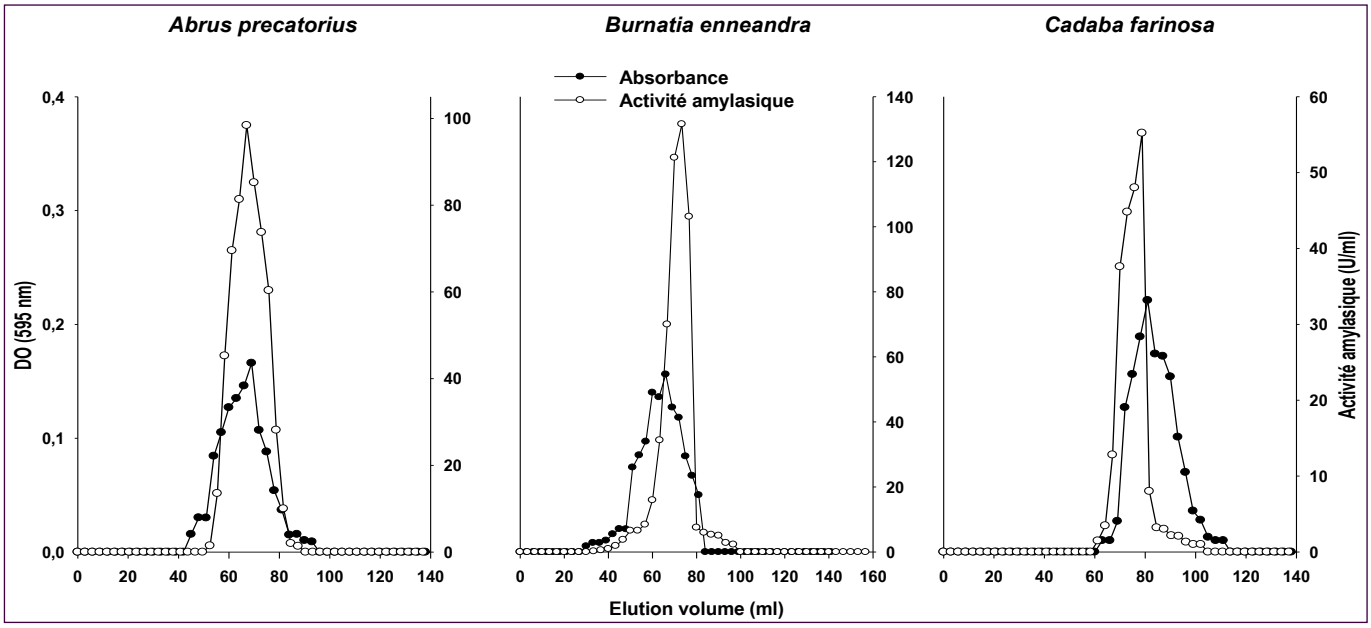

Fig. 1- Elution profile of Sephadex G200 chromatography

Table 2- Summary of purification of α-amylases from the plants.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
A. precatorius					
Crude extract	512	8548	16.69	1	100
Ammonium Sulfate precipitation	90	7804	86.75	5.2	91
Sephadex G100	23	6355	273.53	16.39	74
Sephadex G200	4	4038	965.06	57.82	47
B. enneandra					
Crude extract	1075	27347	25.43	1	100
Ammonium Sulfate precipitation	169	24791	147.01	5.78	91
Sephadex G100	57	19571	340.95	13.41	72
Sephadex G200	8	12193	1539.5	60.53	45
C. farinosa					
Crude extract	285	1441	5.06	1	100
Ammonium Sulfate precipitation	78	1364	17.4	3.44	95
Sephadex G100	20	1267[c]	64.29	12.7	88
Sephadex G200	4	788	224	44.26	55

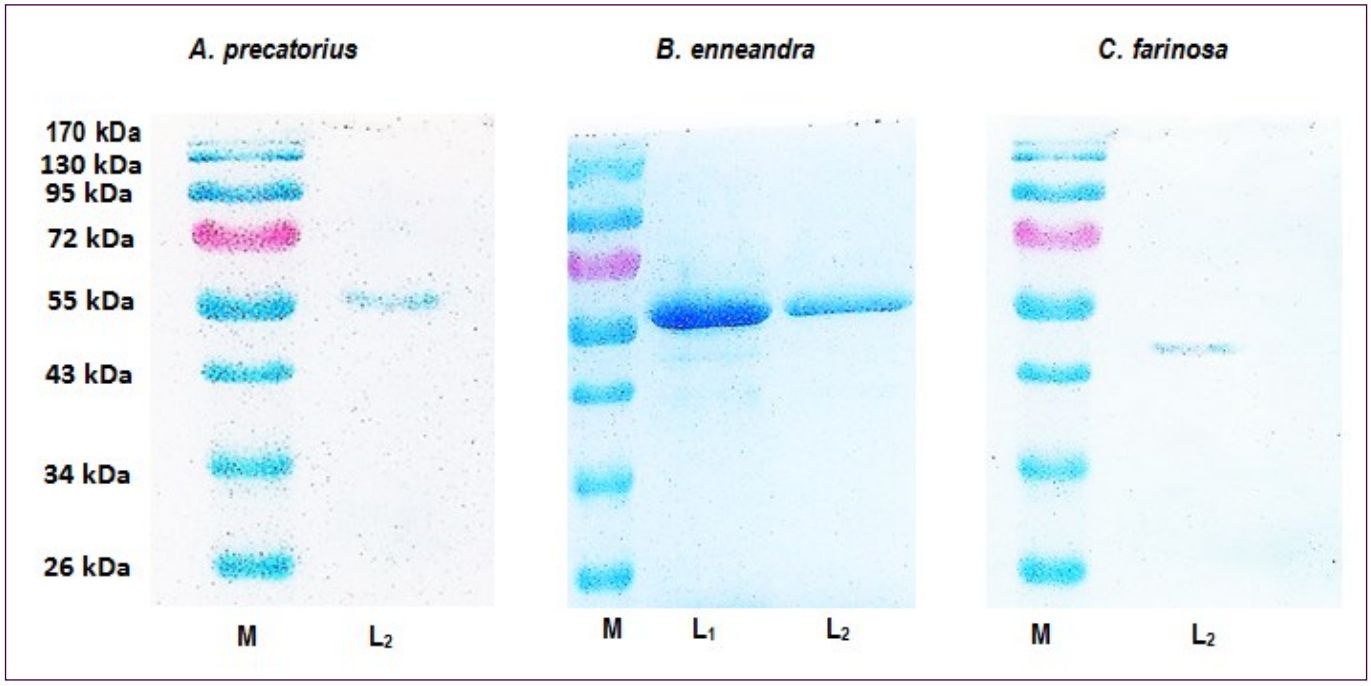

Fig. 2- Photographic SDS-PAGE patterns of the purified enzymes.

M: Marker proteins; L_1: Fraction from Sephadex G100 column; L_2: Fraction from Sephadex G200 column

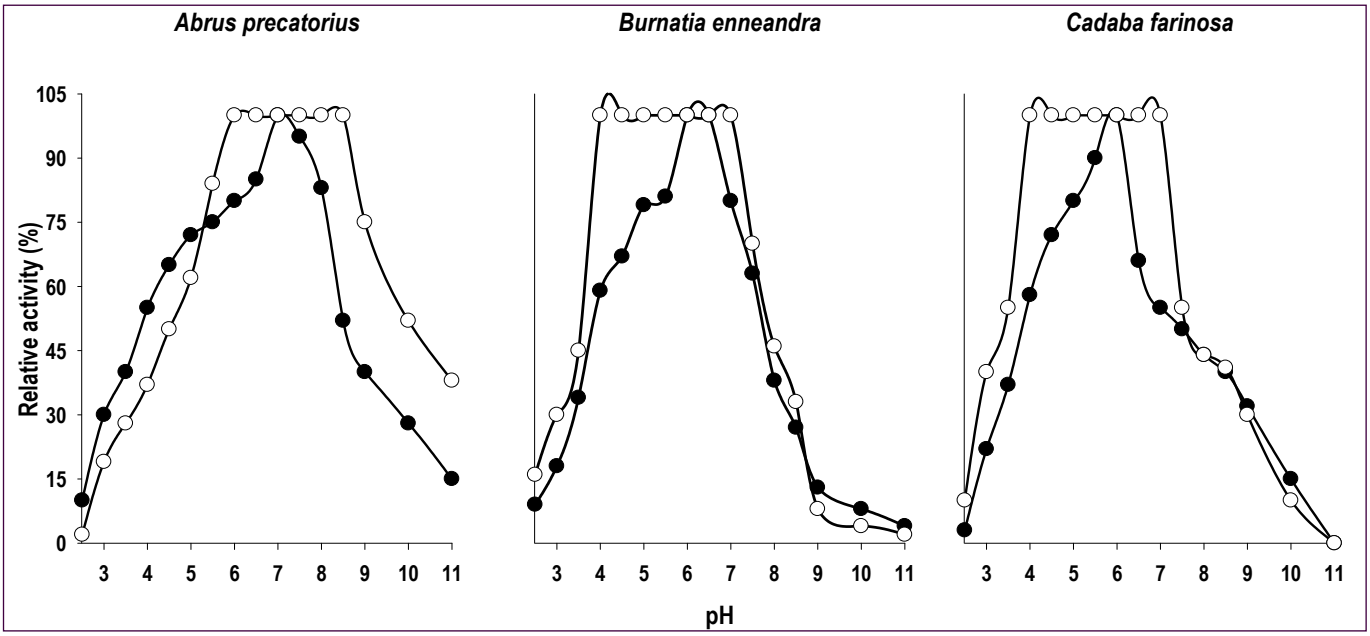

Fig. 3- Effect of pH on the activity (●) and the stability (○) of the enzymes

Kinetic Constants

Kinetic studies, carried out under standard conditions using soluble starch, display for amylase from *B. enneandra*, a K_m value lower than that of amylases from the two other plants [Table-3]. This indicates the high affinity of *B. enneandra* amylase for soluble starch, compared to the two other amylases. In this respect, *B. enneandra* appears more suitable for the liquefaction of gruel, justifying, in a certain extend the popular use of this plant by local populations in the savannah region of Cameroon. V_{max} values vary between 605.08 µmoles/min/mg for *B. enneandra* and 204.12 µmoles/min/mg for *C. farinosa*.

Metals Ions and EDTA Effects

Tests of various metal ions on the activity of enzymes display a differential activation/inhibition effect depending on the nature of enzyme, and the type and concentration of metal ion [Fig-5]; Li^{2+} appears as a common activator for all the three enzymes, while Ag^{+}, Hg^{2+}, Zn^{2+}, Fe^{3+} and Cu^{2+} are common inhibitor of all the enzymes. The α-amylases are known to be metalloenzymes. Ca^{2+} is known to be the frequent cofactor. Enhancement of amylase activity by Ca^{2+} ions is based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acids, which results in stabilization as well as maintenance of enzyme

conformation. In addition to this interaction, calcium is known to have a role in substrate binding [12]. In our case only amylase from *A. precatorius* is enhanced by Ca^{2+}, while, with amylases from *B. enneandra* and *C: farinosa*, Ca^{2+} appears as inhibitor, particularly when its concentration increases. This differentiation may be the consequence of metal competition or of difference in enzymes structure. In fact, other cations such as Co^{2+}, Mg^{2+} and Fe^{3+} can also act as activators for the α-amylases [2,4,13,14]. In this respect the activity of α-amylase from *B. enneandra* doubled in the presence of Co^{2+}, while *C: farinosa* amylase activity is enhanced in the presence of Fe^{3+}.

All the three amylases were almost inhibited by EDTA, indicating the metal ion requirement of this enzyme [Fig-6]. The EDTA acts by complexing the metal ions (cofactors) contained in enzymes [15]. These results confirm the metalloprotein structure of the three amylases.

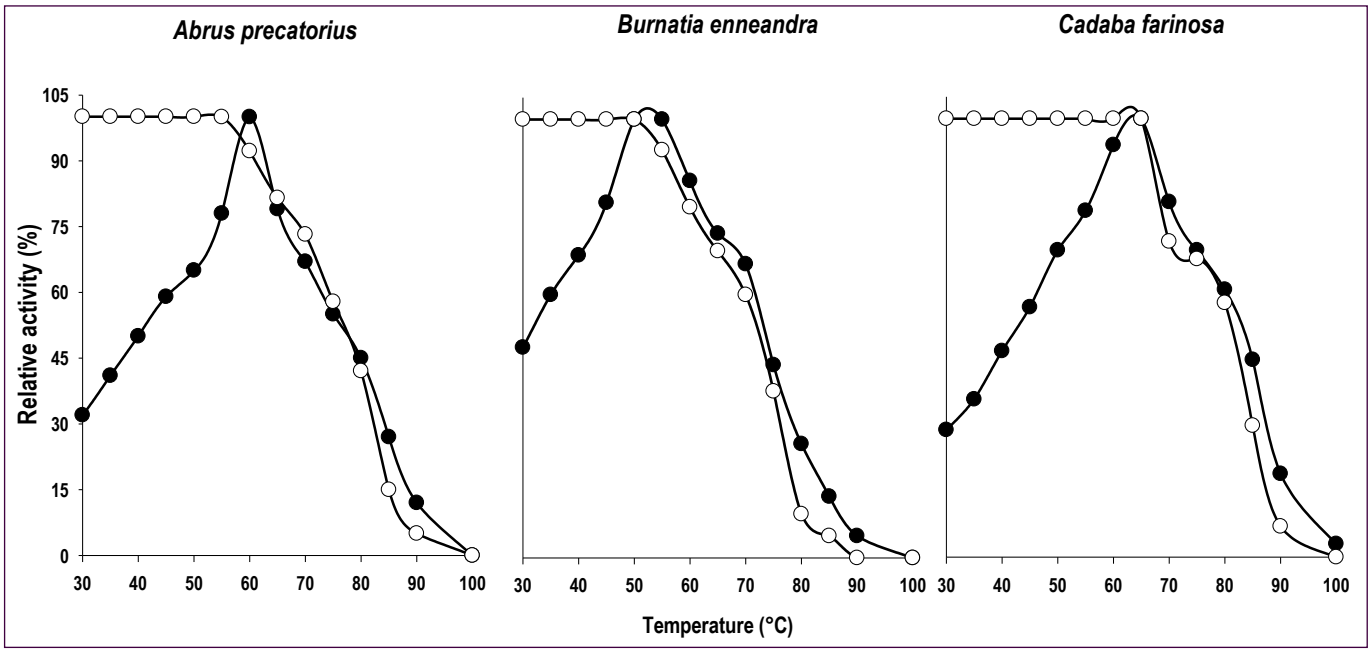

Fig. 4- Effect of temperature on activity (●) and stability (○) of the enzymes

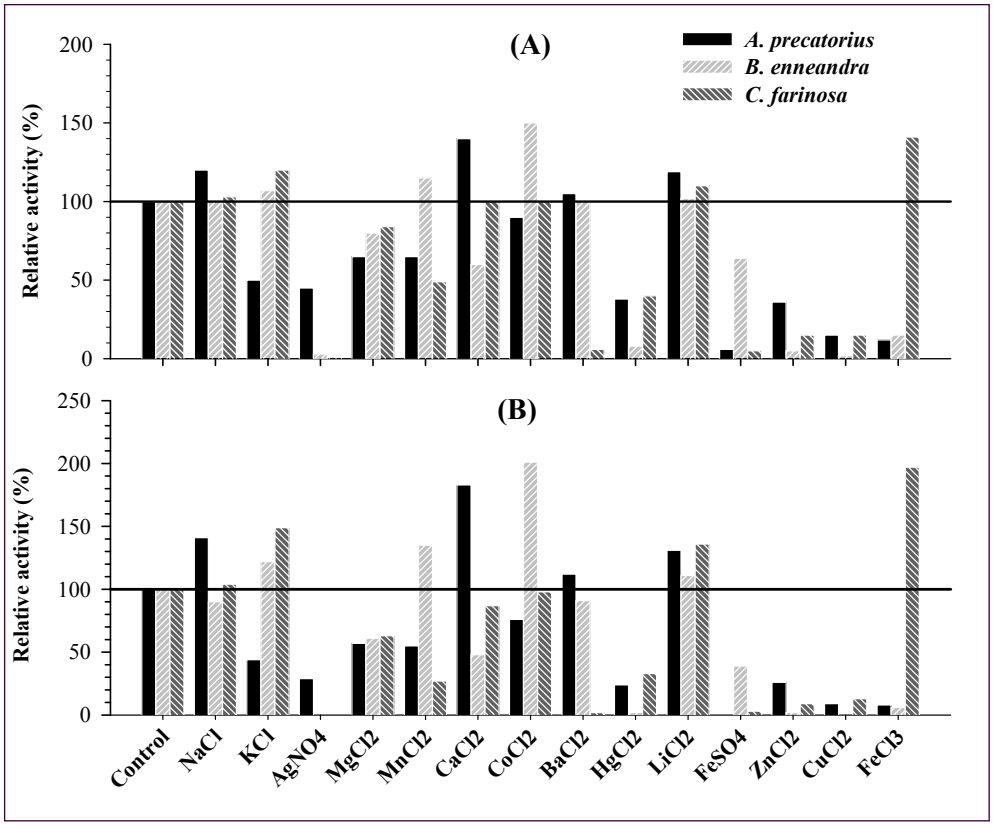

Fig. 5- Effect of metal ions [1mM (A) and 5 mM (B)] on the activity of amylases

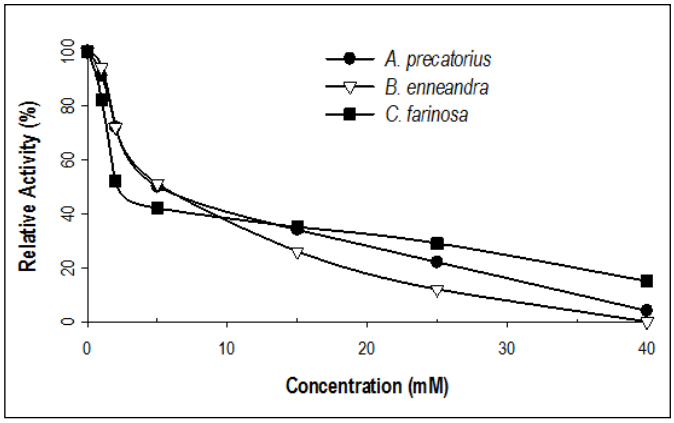

Fig. 6- Effect of EDTA on the activity of amylases

Table 3- Kinetic parameters of α-amylases

	A. precatorius	*B. enneandra*	*C. farinosa*
K_m (mg/ml)	3.25 ± 0.38	1.81 ± 0.10	3.18 ± 0.13
V_m (µmoles/min/mg)	260.94 ± 21.76	605.08 ± 14.88	204.12 ± 3.28

Conclusion

The amylolytic properties of *A. precatorius* leaves, *B. enneandra* tubers and *C. farinosa* stems are exclusively the result of a-amylases contained in these plants. These amylases are metalloenzymes which can be differentiated by their molecular weight, their pH and temperature activity and stability, the kinetics constants, and their metal cofactor. Appraisal of the composition and structure of the proteins constitute the next challenge for a better understanding and valorization of these plants and their amylases.

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Conflict of Interest: None declared.

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