Purification and biochemical characterization of a class II α-mannosidase from breadfruit (*Artocarpus communis*) seeds

Amedee P. Ahi, Fankroma M. T. Kone*, Assoi Y. D. Yapi, S. Dabonne and Lucien P. Kouame

Abstract— An α-mannosidase (EC 3.2.1.137) from breadfruit (*Artocarpus communis*) seeds was purified to apparent homogeneity by anion-exchange, cation-exchange and hydrophobic interaction chromatographies. The purified enzyme named *M1A* exhibited a molecular weight of 45 kDa and appears to be a monomer. The apparent K_M value, pH and temperature optima of breadfruit α-mannosidase activity determined by use of *para*-nitrophenyl-α-D-mannopyranoside as substrate were found to be 1.38 mM, 5.6 and 60 °C, respectively. The enzyme was stable at pH ranging from 4.6-5.6 and remained fully stable at 37 °C, but it lost about 85 % of its activity after 1 h at 60 °C. Mg^{2+}, Ca^{2+} and Zn^{2+} enhanced whereas Sr^{2+}, Ba^{2+}, Cu^{2+}, EDTA, TTAB, HTAB and SDS inhibited its hydrolytic activity. Most of reducing agents tested have a strong inhibitor effect on α-mannosidase activity, except for DL-dithiothreitol. Swainsonine and 1,4-Dideoxyimino-mannitol (a class II α-mannosidase inhibitors) were found to be potent inhibitors of breadfruit α-mannosidase at 0.01 mM, while Kifunensine and Deoxymannojirimycin had no effect. Substrate specificity tests revealed that the enzyme exerted only α-mannosidase activity and digested mannobiose such as Man-α-1,2-Man and Man-α-1,6-Man, but not Man-α-1,3-Man. Based on its characteristics, this enzyme is an α-1,2-1,6-mannosidase which would belong to class II α-mannosidases.

Index Terms— α-Mannosidase, breadfruit seeds, *Artocarpus communis*, purification, biochemical characterization.

1 INTRODUCTION

Alpha-mannosidases are widely distributed and have been isolated from plants, animals, microorganisms **[1-3]**.

They play an essential role in the processing of *N*-glycans to complex and hybrid oligosaccharides by hydrolysing specific α-linked mannose residues. α-Mannosidases involved in *N*-glycans processing have been classified into two distinct groups, class I and class II, with different substrate specificities, intracellular locations, sizes, cations requirements, sensitivities to plant alkaloid inhibitors and amino acid sequence alignments **[4, 5]**.

Class I α-mannosidases belong to family 47 in the glycosyl hydrolase classification **[6]** and are inhibited by the azapyranose analogues of mannose, such as Kifunensine (KIF) and 1-Deoxymannojirimycin (DMNJ) **[7]**. These are involved in processing of *N*-glycans by cleaving specifically α-1,2 linked mannose residues. In contrast, those sensitive to Swainsonine (SW) and 1,4-Dideoxyiminomannitol (DIM), which are azafuranose analogues of mannose, belong to class II α-mannosidases **[8]**. These α-mannosidases belong to glycosyl hydrolase family 38 and are involved in *N*-glycans processing by cleaving α-1,2; α-1,3 and α-1,6 linked mannose residues.

Apart from the vital role in glycoproteins synthesis and degradation, α-mannosidases are currently an important therapeutic target for the development of anti-cancer agents **[9]**. The major potential application of α-mannosidases involves the strategies for treating the lysosomal storage disorder α-mannosidosis by enzyme replacement therapy. Indeed, α-mannosidosis results from deficient activity of α-mannosidase. The disease is characterized by massive intracellular accumulation of mannose-rich oligosaccharide, that is oligosaccharides carrying α-1,2; α-1,3 and α-1,6 mannosyl residues at the non-reducing termini **[10, 11]**. In this respect, the broad specific α-mannosidases becomes useful for application requiring removal of all α-mannosyl linkages. It is suggested that α-mannosidase and *N*-acetyl-β-hexosaminidase be used synergistically in the medical treatment of fungal diseases **[12]**. Furthermore, in vitro, α-mannosidases are useful tools for enzymatic analysis of high mannose oligosaccharide structures **[13]** and for oligosaccharide synthesis **[14]**.

Plant α-mannosidases generally require metal ions for their activity, zinc was found to be more common divalent metal ion required for class II α-mannosidases. The metal can be chelated using EDTA with loss of enzyme activity. The activity is regained by supplementing zinc externally **[15, 16]**.

To date, several α-mannosidases from various sources of legume and non-legume have been purified and characterized: *Canavalia ensiformis*, *Phaseolus vulgaris*, *Medicago sativa L.*, *Carica papaya*, *Indian lablab* beans, *Oryza sativa*, *Triticale*, *Erythrina indica*, *Artocarpus communis* **[16]**.

More recently, some studies showed that α-mannosidases activities increase during seeds germination and fruit ripening suggesting its role in removing mannose residues from mannoglycans from the cell wall glycans **[17]**. In our laboratory, we will focus to view into properties of these enzymes where high activity has been noticed during the fruit maturation, in order to classify them and find possible application in food and biotechnology industries.

In particular, we earlier purified and characterized four α-mannosidases from breadfruit *Artocarpus communis* seeds **[2, 18]**. But only three of them have already been published: two iso-enzymes named *M2A* and *M2B* **[2]**; and one strictly α-

*Corresponding author:
Departement of Food Science and Technology, University Nangui Abrogoua, Laboratory of Biocatalysis and Bioprocessing, 02 BP 801 Abidjan 02, Côte d'Ivoire
E-mail: fankrom@yahoo.fr

mannosidase named *M1B* **[18].** This paper described the purification and biochemical characterization of the new α-mannosidase (*M1A*) with dual specificity from these seeds. These data should provide some basic knowledge for additional studies on the enzymatic potential of these α-mannosidases in processing and degradation pathways of *N*-glycans.

2 Materials and Methods

2.1 Materials

Breadfruit seeds were obtained locally in Cote d'Ivoire. *para*-Nitrophenyl-α-D-mannopyranoside (*p*NP-α-Man), mannose and mannobiose containing disaccharides linked α-D-mannopyranosyl-(1,2)-D-mannopyranoside (Man-α-1,2-Man), α-D-mannopyranosyl-(1,3)-D-mannopyranoside (Man-α-1,3-Man), α-D-mannopyranosyl-(1,6)-D-mannopyranoside (Man-α-1,6-Man), Swainsonine (SW), 1,4-Dideoxyiminomannitol (DIM) and 1-Deoxymannojirimycin (DMNJ) were purchased from Sigma-Aldrich. Kifunensine (*Kitasatosporia kifunense*) (KIF) was from Calbiochem. DEAE-Sepharose CL-6B, CM-Sepharose CL-6B and Phenyl-Sepharose HP provided from Pharmacia Biotech. Standard proteins were obtained from Bio-Rad. Silicate gel 60 thin-layer chromatography (TLC) was purchased from Merck. All the other reagents used were of analytical grade.

2.2 Enzyme extraction

Matured breadfruit seeds (20 g) were ground in a pre-chilled mortar in 30 ml of 20 mM sodium acetate buffer (pH 4.6) containing NaCl 0.9 % (w/v). The homogenate was subjected to sonication using a TRANSSONIC T420 for 10 min and then centrifuged at 6,000 rpm for 30 min. The supernatant filtered through cotton was used as the crude extract and conserved at 4 °C.

2.3 Enzyme assay

Under the standard test conditions, α-mannosidase activity was measured at 37 °C for 15 min in 100 mM acetate buffer (pH 4.6) containing 1.5 mM *p*NP-α-Man. After pre-warning the mixture at 37 °C for 5 min, the reaction was initiated by adding the enzyme solution. The final volume was 250 µl and the reaction was stopped by adding 2 ml of sodium carbonate 2 % (w/v). Enzyme activity towards 1.5 mM of *p*NP-α-Man was determined by measuring the released *para*-nitrophenol (*p*NP) at 410 nm using a spectrophotometer GENESIS. *p*NP was used as standard. Under the above experimental conditions, one unit of enzyme activity was defined as 1 µmol of *p*NP released per min. Specific activity was expressed as the units of enzyme activity per mg of protein.

2.4 Protein estimation

Protein elution profiles from chromatographic columns were determined spectrophotometrically by absorbance measurement at 280 nm and the concentration of the purified enzyme was determined according to Lowry method **[19]**. Bovin Serum Albumin (BSA) was used as the standard protein.

2.5 Enzyme purification

All the purification procedure was carried out in cold room. The crude extract from matured breadfruit seeds was loaded onto a DEAE-Sepharose CL-6B column (2.6 x 6.0) equilibrated with 20 mM sodium acetate buffer (pH 4.6). Unbound proteins were removed by washing the gel with two bed volumes of equilibration buffer. Bound proteins were then eluted over stepwise gradient (0.1; 0.3; 0.5 and 1 M) NaCl in 20 mM sodium acetate buffer (pH 4.6) at a flow rate of 1 ml/min and fractions of 3.0 ml were collected. Pooled unbound α-mannosidase activity was loaded onto a CM-Sepharose CL-6B column (2.6 x 4.0) equilibrated with 20 mM sodium acetate buffer (pH 4.6). The column was washed with the same buffer at flow rate of 1 ml/min. α-Mannosidase activity was eluted with a stepwise salt gradient (0.1; 0.3; 0.5 and 1 M) NaCl in 20 mM sodium acetate buffer (pH 4.6). Fractions of 2.0 ml were collected. The pooled active fractions were saturated to a final concentration of 1.7 M sodium thiosulfate and then applied on a Phenyl-Sepharose HP column (1.4 x 4.6) previously equilibrated with 20 mM sodium acetate buffer (pH 4.6) containing 1.7 M sodium thiosulfate. The column was washed with equilibration buffer and the proteins retained were then eluted with a reverse stepwise gradient of sodium thiosulfate concentrations (from 1.7 to 0 M) in the same sodium acetate buffer at a flow rate of 0.33 ml/min. Fractions of 1 ml were collected. The active fractions pooled were dialyzed overnight against 20 mM sodium acetate buffer (pH 4.6) and constituted the purified enzyme.

2.6 Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was carried out by the Laemmli method **[20]** on 12 % (w/v) acrylamide gels under denaturing and non-denaturing conditions. In denaturing conditions, samples were incubated for 5 min at 100 °C with SDS-PAGE sample buffer containing 2-mercaptoethanol. Concerning non-denaturing conditions, samples were mixed just before running with the sample buffer without 2-mercaptoethanol and SDS. Gels were stained with Coomassie brilliant blue R-250. The molecular-weight standard makers (Bio-Rad) comprising myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa) and ovalbumin (45.0 kDa) were used.

2.7 Native molecular-weight determination

The purified enzyme was applied to a gel TSK QC-PAK GFC 200 HPLC column equilibrated with 20 mM sodium acetate buffer (pH 4.6) containing sodium azide 0.5 % (w/v) to estimate the molecular-weight. Molecular-weight standards used were β-amylase (200,000 Da), BSA (66,000 Da), ovalbumin (45,000 Da) and cytochrome C (12,400 Da).

2.8 pH and temperature optima

The effect of pH on the enzyme activity was determined by measuring the hydrolysis of *p*NP-α-Man in a series of buffers (100 mM) at various pH values (3.6 to 8.0). Buffers used were sodium acetate buffer (pH 3.6 to 5.6), sodium phosphate buffer (pH 5.6 to 8.0), Tris-HCl buffer (pH 7.0-9.0) and citrate phosphate buffer (pH 3.6-7.0). The pH values of each buffer were determined at 25 °C.

The effect of temperature on α-mannosidase activity was performed in 100 mM acetate buffer (pH 4.6) over a temperature range of 30 to 80 °C by using *p*NP-α-Man (5 mM) as substrate under the standard test conditions.

2.9 pH and temperature stabilities

The pH stability of the α-mannosidase from breadfruit was studied in pH range 3.6 to 8.0 in 100 mM buffers. Buffers used were the same as in the study of pH and temperature optima (above). After 1 h preincubation at 37 °C, aliquots were taken and immediately assayed for residual α-mannosidase activity. The thermal inactivation of the enzyme was determined at 37 and 60 °C after exposure to each temperature for a period from 1 to 120 min. The enzyme was incubated in 100 mM acetate buffer (pH 4.6). Aliquots were withdrawn at intervals and immediately cooled. Concerning thermal denaturation tests, aliquots of the enzyme were pre-incubated at different temperatures ranging from 30 to 80 °C for 15 min. Residual activities, determined at 37 °C under the standard test conditions, were expressed as percentage activity of zero-time control of untreated enzyme.

2.10 Effet of some chemical agents

To determine the effect of various compounds (ions, detergents, dithiol-reducing agents and α-mannosidase specific inhibitors) as possible activators or inhibitors of the purified α-mannosidase, enzyme solutions were preincubated at 37 °C for 30 min with different concentration of the compounds and then the activity was assayed. The substrate *p*NP-α-Man (5 mM) was added to the medium and incubated at 37 °C for 15 min. The residual activity was assayed as the standard conditions.

2.11 Sustrate specificity and kinetic parameters determination

Substrate specificity of the purified α-mannosidase was determined by incubating the purified enzyme with the substrates *p*NP-α-D-mannopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-α-D-fucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-β-D-xylopyranoside (5 mM) at 37 °C in 100 mM sodium acetate buffer (pH 4.6) for 15 min. The hydrolysis of synthetic substrates was quantified on the basis of released *p*NP, as in the standard enzyme assay. The kinetic parameters (K_M, V_{max} and V_{max}/K_M) were determined in 100 mM sodium acetate buffer (pH 4.6) at 37 °C. K_M and V_{max} were determined from a Lineweaver-Burk plot using different concentrations of the substrate *p*NP-α-Man.

2.12 Characterization of the hydrolytic specificity

The hydrolytic specificity was determined by incubating 10 µl of purified enzyme preparation with 9 µl of 5 mM of α-D-mannopyranosyl-(1,2)-D-mannopyranoside (Man-α-1,2-Man), α-D-mannopyranosyl-(1,3)-D-mannopyranoside (Man-α-1,3-Man) or α-D-mannopyranosyl-(1,6)-D-mannopyranoside (Man-α-1,6-Man), at pH 4.6 in 100 mM sodium acetate buffer at 37 °C for up to 24 h. Samples (3 µl) were removed at regular time intervals and applied to TLC plates to monitor the hydrolysis of differently linked disaccharides. The TLC plates were run with butanol-acetic acid-water 9:3.75:2.25 (v/v/v) and developed with naphto-resorcinol in ethanol and H_2SO_4 20 % (v/v). The sugar spots were visualised at 105 °C for 8 min.

3 Results and Discussion

3.1 Enzyme purification

The purification procedure of breadfruit α-mannosidase involved three steps including anion-exchange, cation-exchange and hydrophobic interaction chromatographies as summarized in Table 1.

Table 1
Purification procedure of α-mannosidase from breadfruit (*Artocarpus communis*) seeds. Values given are the averages of at least three experiments

Purification steps	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Yield (%)	Purification factor
Crude extract	1340	224.25	0.17	100	1
DEAE-Sepharose CL-6B	121.60	383.03	0.31	54	1.89
CM-Sepharose CL-6B	25.84	17.26	1.49	11.5	8.89
Phenyl-Sepharose HP	5.15	2.02	2.55	2.30	15.22

After DEAE-Sepharose CL-6B of the crude enzyme solution, two peaks of α-mannosidase activity were detected, when washing the column with 20 mM sodium acetate buffer (pH 4.6) and at 0.3 M NaCl concentration and, they were designated α-mannosidase *M1* and α-mannosidase *M2*, respectively. The α-mannosidase *M2* has already been published **[2]** and was found to be two iso-enzymes (*M2A* and *M2B*) after purification. Pooled fractions of α-mannosidase *M1* were further subjected to a CM-Sepharose CL-6B gel. Two peaks of protein, named *M1A* and *M1B*, containing α-mannosidase activity were eluted. In this work, we will focus on α-mannosidase *M1A* because α-mannosidase *M1B* was previously published **[18]**. The breadfruit *Artocarpus communis* α-mannosidase (*M1A*) was finally purified by using an ultimate hydrophobic chromatography on Phenyl-Sepharose HP, as show in Fig. 1. A single peak of α-mannosidase activity was resolved at 0.7 M sodium thiosulfate and the enzyme present in this peak was enriched 15.22-fold with 2.30 % yield. This low yield could be due to several fractionation steps used. However, this yield was higher than those obtained for other α-mannosidases already described **[2, 10, 18]**. Similar easy protocols were previously used during purification of mango α-mannosidase **[21]**. The specific activity of α-mannosidase was 2.55 UI/mg of protein (Table 1). This value is higher than values reported for two *A. communis* α-mannosidases (0.97 and 0.65 UI/mg for *M1B* and *M2B*, respectively) **[2, 18]** and for thermophilic (*Rhynchophorus palmarum*) α-mannosidase (1.71 UI/mg) **[3]**. Furthermore, it is comparable to breadfruit (*A. communis*) α-mannosidase *M2A* (2.97 UI/mg) and lower to three mesophilic (*R. palmarum*) α-mannosidases (3.17-12.22 UI/mg) **[10]**.

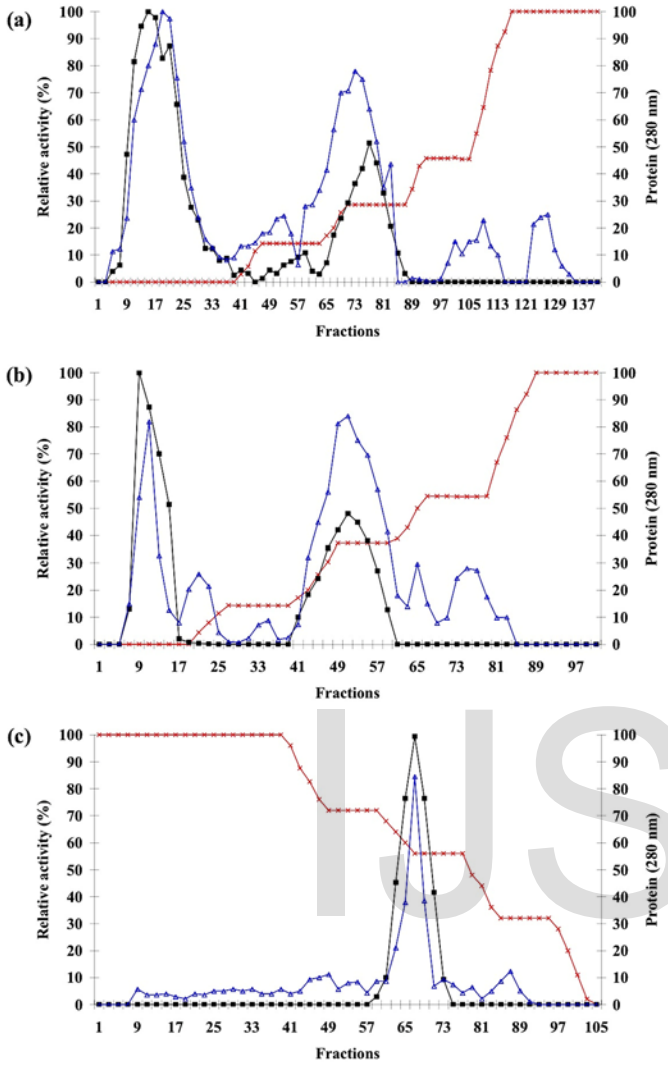

Fig. 1. Chromatographic profiles of α-mannosidase from breadfruit (*Artocarpus communis*) seeds. The enzyme activity was measured in 100 mM acetate buffer (pH 4.6) at 37 °C using *p*NP-α-D-mannopyranoside as substrate. (a) Anion-exchange chromatography on DEAE-Sepharose CL-6B. (b) Cation-exchange chromatography on CM-Sepharose CL-6B. (c) Hydrophobic interaction chromatography on Phenyl-Sepharose HP. α-Mannosidase activity (■), chloride sodium or sodium thiosulfate (×) and protein contents (▲)

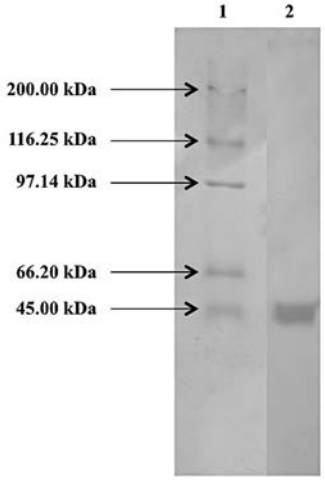

Fig. 2. Polyacrylamide gel electrophoresis in denaturing conditions of the purified α-mannosidase from breadfruit (*Artocarpus communis*) seeds. Lane 1, molecular weight markers; lane 2, purified enzyme

Table 2
Some physicochemical characteristics of α-mannosidase from breadfruit (*Artocarpus communis*) seeds. Values given are the averages of at least three experiments

Physicochemical properties	Values
Optimum temperature (°C)	60
Temperature coefficient (Q_{10})	1.44
Activation energy (kJ.mol^{-1})	29.26
Optimum pH	5.6
pH stability	4.6 - 5.6
Molecular weight (kDa)	
SDS-PAGE	45
Gel filtration	45
K_M (mM)	1.38
V_{max} (Units/mg of protein)	3.35
V_{max} / K_M (Units/mg.mM)	2.43

3.2 Molecular weights estimation

The purity of breadfruit (*A. communis*) α-mannosidase was verified by SDS-PAGE analysis and its relative molecular weight was estimated to be 45 kDa under reducing conditions and by gel filtration (Fig. 2, Table 2), suggesting that this α-mannosidase have a monomeric structure. Except the rat liver and jack bean α-mannosidases, which are tetrameric proteins with four subunits **[22, 23]**, most of α-mannosidases are monomeric **[24]**.

3.3 pH and temperature dependences

The optimum values of pH and temperature for studying α-mannosidase activity are represented in Table 2. The enzyme activity was maximal at 60 °C in 20 mM sodium acetate buffer pH 5.6. The optimum temperature of breadfruit (*A. communis*) α-mannosidase is in good agreement with those of the majority of α-mannosidases and other glycosidases purified from plants **[15]**, microbials **[25]** and insects **[26]** which are mostly mesophilic. Running biotechnological processes at a high temperature has many advantages. High temperature has a significant effect on bioavailability and solubility, and is accompanied by a decrease in viscosity and an increase in the

diffusion coefficient of organic compounds. Consequently, higher reaction rates due to smaller boundary layers are expected **[3]**. The optimum pH (5.6) obtained for the breadfruit (*A. communis*) α-mannosidase was similar to those of two α-mannosidases from the same species **[2]**. This value was higher to literature values (pH 4.3-5.0) reported for others α-mannosidases **[3, 16, 27, 28]**.

The purified breadfruit α-mannosidase showed best stability over pH values ranging from 4.6 to 5.6 for 60 min at 37 °C (Table 2). Therefore, the optimum pH is a good compromise between activity and stability of the enzyme to perform hydrolysis of natural substrates and biosynthesis reaction over a long time. This plant α-mannosidase which activity is maximal at acidic region appear to be comparable to the majority of insect larvae e.g. *Rhagium inquisitor* and *Rhynchosciara americana* **[29, 30]**, which are unlike α-mannosidases from *Drosophila melanogaster* **[31]** and *Spodoptera frugiperda* **[32]** that require a near neutral pH.

The thermal denaturation was investigated by incubation of breadfruit α-mannosidase at various temperatures for 15 min. The result showed that this enzyme was fairly stable at temperature up to 60 °C. Above this temperature, its activity declined as the temperature increased (Fig. 3).

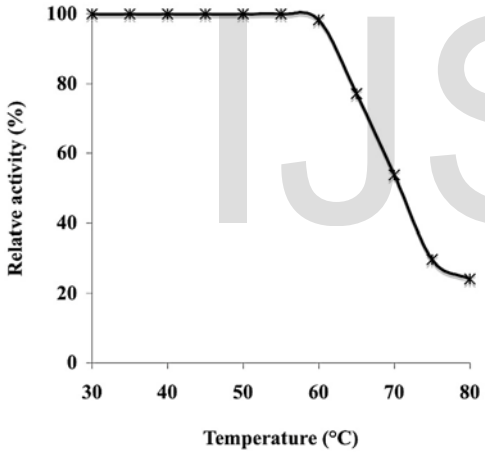

Fig. 3. Thermal denaturation of α-mannosidase from breadfruit (*Artocarpus communis*) seeds.

The thermal inactivation study at pH 4.6 indicated that, α-mannosidase activity from breadfruit remained fully stable for 120 min at 37 °C. At its optimum temperature (60 °C), this activity was less stable, showing half-life (50 % of activity) around 40 min. However, it lost about 85 % after 120 min of preincubation (Fig. 4). The stability of the breadfruit (*A. communis*) α-mannosidase (pH stability and thermostability) means it is suitable for use as an industrial biocatalyst **[33]**.

Value of temperature coefficient (Q_{10}) calculated between 35 and 45 °C was 1.44 for purified breadfruit α-mannosidase (Table 2). From Arrhenius plot (data not shown), its activation energy was found to be 29.26 kJ.mol^{-1} (Table 2). Values of activation energy indicate the relative tendency of a failure mechanism to be accelerated by temperature. In this respect, the breadfruit (*A. communis*) α-mannosidase should be top-grade tools for various catalyzing reactions since it is well known that enzymes are biocatalysts that speed up chemical reactions by lowering the required activation energy.

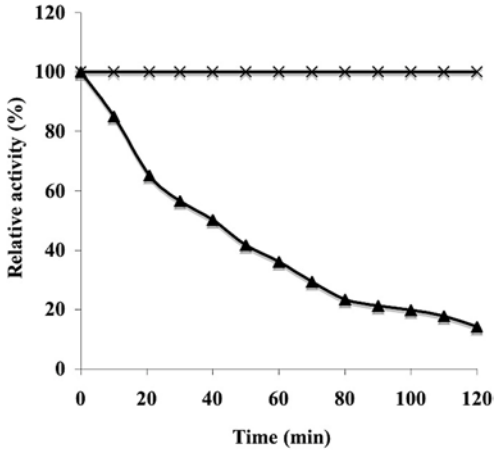

Fig. 4. Thermal inactivation of α-mannosidase from breadfruit (*Artocarpus communis*) seeds. Inactivation of the enzyme at 37 °C (×) and at 60 °C (▲).

3.4 Effect of metal ions, chelating and reducing agents and detergents

The effect of selective inhibitors or activators on the activity of breadfruit (*A. communis*) seeds α-mannosidase using *p*NP-α-D-mannopyranoside was examined. As regards the influence of cations, the results presented in Table 3 showed that Mg^{2+}, Ca^{2+} and Zn^{2+} activated the purified α-mannosidase, whereas Sr^{2+}, Ba^{2+}, Cu^{2+} and EDTA acts as inhibitors. EDTA and Cu^{2+} inhibited the activity by 70 and 75%, respectively. However, the α-mannosidase activity was not affect by Na^{+}. It should be noted that at 5 mM concentration, activation and/or inhibition of the enzyme activity was more pronounced. These results suggested that Mg^{2+}, Ca^{2+} and Zn^{2+} are essential for hydrolytic activity themselves, or those ions regulate the enzyme activity during seeds maturation. The metal ion Zn^{2+} has been identified to be present and essential at the active site of *Drosophila melanogaster* class II Golgi α-mannosidase **[34]** and other α-mannosidases **[16, 35]**. It has been reported that Ca^{2+} and Zn^{2+} ions often activate some plant acidic α-mannosidases **[36]** or some processing α-mannosidases **[37]**. Inhibition by EDTA (cations chelator) suggests that the breadfruit α-mannosidase requires divalent metal cations to be fully active. This result is similar to those for α-mannosidases from rice *Oryza sativa* dry seeds **[35]**, *R. palmarum* larvae **[3]** and *Moringa oleifera* seed kernels **[16]**.

Table 3
Effect of some ions and chelating agent on α-mannosidase activity from breadfruit (*Artocarpus communis*) seeds. Values given are the averages of at least three experiments

Reagent	Concentration (mM)	Relative activity (%)
None	0	100
Na^+	1	100
	5	100
Mg^{2+}	1	104
	5	151
Sr^{2+}	1	82.72
	5	42.75
Ca^{2+}	1	114.40
	5	167.71
Ba^{2+}	1	83.14
	5	38.99
Cu^{2+}	1	58
	5	25
Zn^{2+}	1	102
	5	125
EDTA	1	60
	5	31

EDTA: Ethylene diamine tetraacetic acid

Table 4 shows the effect of reducing agents on the purified breadfruit α-mannosidase. Except DL-dithiothreitol that displayed activation on α-mannosidase activity, all the other reducing agents tested were found to be inhibitory, suggesting the involvement of sulfhydryl groups in catalytic activity. These chemical agents must be eliminated after treatment of substrate when this needs to be reduced before its hydrolysis by enzymes, and also must be avoided when extracting or purifying this enzyme. Similar observations have been reported for α-mannosidase from jack bean **[22]** and for three extracellular α-mannosidases purified from the digestive fluid of oil palm weevil larvae *R. palmarum* **[10]**. The sensitivity of these enzymes in the presence of sulfhydryl-specific agents led us to assume that -SH groups participate probably in catalysis activities of the enzymes.

The influence of various detergents on breadfruit α-mannosidase activity showed that all detergents tested stimulated the enzyme with exception of cationic detergents (TTAB and HTAB) and SDS (Table 5). Indeed, cationic detergents displayed a slight inhibitory effect (5-20 % inhibition) on enzyme activity, when SDS has a strong inhibitory effect (100 % inhibition). Similar observations have been reported for α-mannosidase from the seeds extract of the blocky-fruited cultivar of *Lagenaria siceraria*, where 1 % of SDS displayed up to 98 % inhibition on enzyme activity **[28]**. Bonay et al. **[38]** showed that 1 % of Triton X-100 and other detergents increased 3-4 fold the activity of rat liver α-mannosidase. For these authors, this result suggests that, in the native enzyme structure, the catalytic domain is partially inaccessible to the substrate unless this α-mannosidase integrity is disrupted by detergent to expose the active site.

Table 4
Effect of some reducing agents on α-mannosidase activity from breadfruit (*Artocarpus communis*) seeds. Values given are the averages of at least three experiments

Reducing agent	Concentration (% ; w/v)	Relative activity (%)
None	0	100
DL-dithiothreitol	0.1	111
	1	155
*p*CMB	0.1	55
	1	12
DTNB	0.1	10
	1	0
L-cystein	0.1	80.51
	1	0
β-mercaptoethanol	0.1 (v/v)	87
	1 (v/v)	61

pCMB: sodium parachloromercuribenzoate; DTNB: 5,5'-dithio-2,2' dinitrodibenzoïc acid

Table 5
Effect of detergents on α-mannosidase activity from breadfruit (*Artocarpus communis*) seeds. Values given are the averages of at least three experiments

	Detergent	Concentration (% ; w/v)	Relative activity (%)
	None	0	100.0
Anionic	SDS	0.1	0
	HSAS	0.1	99.9
		1	100.0
	P9LE	0.1	101.0
		1	117.0
Cationic	TTAB	0.1	95.7
		1	81.8
	HTAB	0.1	92.0
		1	79.1
None ionic	Nonidet P 40	0.1	100.0
		1	109.0
	Triton X-100	0.1	101.0
		1	118.7
	Lubrol Wx	0.1	101.0
		1	117.7

SDS: Sodium Dodecyl Sulphate ; HSAS: 1-Hexanesulfonic acid sodium salt ; P9LE: Polyoxyethylen 9 Lauryl Ether ; TTAB: Tetradecyl Trimethyl Ammonium Bromide ; HTAB: Hexadecyl Trimethyl Ammonium Bromide

3.5 Substrate specificity and kinetic properties

The purified breadfruit α-mannosidase was assayed for hydrolytic activity against a variety of synthetic and natural substrates. The enzyme specifically hydrolyzed *p*NP-α-D-

mannopyranoside (*p*NP-α-Man) on which Lineweaver-Burk-plot in range of concentrations (1 to 6 mM) showed K_M and V_{max} values of breadfruit α-mannnosidase of 1.38 mM and 3.35 Units/mg of protein, respectively (Table 2). These values were comparable to values as reported in literature **[16, 27, 35]**. The catalytic efficiency of breadfruit α-mannosidase (2.43 Units/mg.mM), given by the V_{max}/K_M ratio is higher than those obtained for two α-mannosidases from *A. communis* (1.4-1.9 Units/mg.mM) **[2]**. Activities for other *p*NP-glycosides (*p*NP-α-D-glucopyranoside, *p*NP-α-D-fucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-β-D-xylopyranoside) were not detectable.

On the other hand, linkage specificity of breadfruit α-mannosidase was investigated with natural substrates such as disaccharides α-D-mannopyranosyl-(1,2)-D-mannopyranoside (Man-α-1,2-Man), α-D-mannopyranosyl-(1,3)-D-mannopyranoside (Man-α-1,3-Man) and α-D-mannopyranosyl-(1,6)-D-mannopyranoside (Man-α-1,6-Man). The hydrolysis product released by α-mannosidase was mainly the mannose and was analyzed by TLC (Fig. 5). The result showed that the purified (*A. communis*) α-mannosidase displayed a broad substrate specificity since it cleaves α-1,2 and α-1,6 linkages of mannobiose. This enzyme appears to be different from the three other breadfruit (*A. communis*) α-mannosidases since *M2A* and *M2B* hydrolyzed α-1,2; α-1,3 and α-1,6 linkages, and *M1B* only α-1,2 linkages of mannose residues **[2, 18]**.

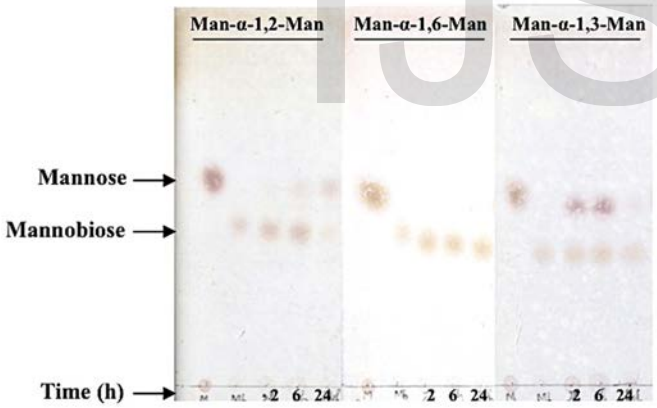

Fig. 5. TLC patterns and product distributions obtained using α-mannosidase from breadfruit (*Artocarpus communis*) seeds to catalyze different linked mannobioses (Man-α-1,2-Man: α-D-mannopyranosyl-(1,2)-D-mannopyranoside; Man-α-1,3-Man: α-D-mannopyranosyl-(1,3)-D-mannopyranoside; Man-α-1,6-Man: α-D-mannopyranosyl-(1,6)-D-mannopyranoside) at 37 °C in 100 mM sodium acetate buffer (pH 4.6)

3.6 Effect of α-mannosidase inhibitors

The influence of α-mannosidase inhibitors such as Swainsonine (SW), 1,4-Dideoxyimino-mannitol (DIM), Kifunensine (KIF) and 1-Deoxymannojirimycin (DMNJ) on the enzyme activity is presented in Table 6. Hydrolysis of *p*NP-α-Man by the purified breadfruit (*A. communis*) α-mannosidase was strongly inhibited by SW and DIM, whereas KIF and DMNJ had few or no significant effect at low concentration. However at 1 mM concentration, the enzyme was inhibited by KIF and DMNJ (~50 %). SW has been identified to be a potent inhibitor of α-mannosidase from *Dolichos lablab* seeds **[27]**. Using molecular models, it can be seen that the configuration of secondary hydroxyl groups of SW is identical to that in DIM **[8].** This observation supports the similar effect of DIM on the breadfruit α-mannosidase. However, the moderate effect of DIM may be explained by the accessibility of these compounds to the catalytic centre. DMNJ and KIF, both pyranose analogues, are normally not known to inhibit class II α-mannosidases **[5, 39]**. Tejavath and Nadimpalli **[16]** showed that α-mannosidase from *M. oleifera* seed kernels was very strongly inhibited by SW, but not by DMNJ and required the Zn^{2+} as a metal ion suggesting that his enzyme belongs to class II α-mannosidases. Taken together, these results suggest that the breadfruit (*A. communis*) α-mannosidase purified in this study belong to class II α-mannosidases.

Table 6
Effect of specific inhibitors on α-mannosidase activity from breadfruit (*Artocarpus communis*) seeds. Values given are the averages of at least three experiments

Inhibitor	Concentration (mM)	Relative activity (%)
None	0	100
Swainsonine (SW)	0.01	0
	0.1	0
	1	0
1,4-Dideoxyimino-mannitol (DIM)	0.01	42
	0.1	18
	1	6.34
Kifunensine (KIF)	0.01	100
	0.1	95
	1	58.90
Deoxymannojirimycin (DMNJ)	0.01	98.08
	0.1	89.23
	1	56.81

4 Conclusion

The present study showed that the α-mannosidase purified from matured breadfruit (*A. communis*) seeds is an α-1,2-1,6-mannosidase (EC 3.2.1.137) belonging to class II α-mannosidases since it is sensitive to azafuranose analogues SW and DIM. This classification is supported by the dual sub-

strate specificity, and implied their role in degradation of *N*-glycans in breadfruit seeds. Regarding the dual specificity for α-1,2 and α-1,6-linked mannoses, the purified (*A. communis*) α-mannosidase appears different from other plant mesophilic α-mannosidases belonging to class II characterized up to now. The interesting biochemical characteristics of this breadfruit α-mannosidase could be used in food industry and biotechnological applications.

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