Advanced Journal of Microbiology Research Vol. 2007
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Biochemical characterization of two-mannosidases from breadfruit (*Artocarpus communis*) seeds

Amedée Pascal Ahi[1], Jean Tia Gonnety[1], Betty Meuwiah Faulet[1], Lucien Patrice Kouamé[1] and Sébastien Niamké[2]*.

[1]Laboratoire de Biochimie et Technologie des Aliments de l'Unité de Formation et de Recherche en Sciences et Technologie des Aliments de l'Université d'Abobo-Adjamé, 02 BP 801 Abidjan 02, Côte d'Ivoire. [2]Laboratoire de Biotechnologies, Filière Biochimie-Microbiologie de l'Unité de Formation et de Recherche en Biosciences de l'Université de Cocody-Abidjan, 22 BP 582 Abidjan 22, Côte d'Ivoire.

Accepted 02 April, 2007

The importance of -mannosidases in glycoproteins processing and their application in food and pharmaceutical industry led us to further explore plant -mannosidases. Thus, two -mannosidases were purified from matured breadfruit (*Artocarpus communis*) seeds, by successive chromatography on Diethylaminoethyl-Sepharose CL-6B and Sephacryl S-200 HR to apparent homogeneity. The two isoenzymes named Ma and Mb had native molecular weights of approximately 75 and 60 kDa, respectively. Sodium Dodecyl Sulfate- polyacrylamide gel electrophoresis of these -mannosidases resolved a single protein band with molecular weights estimated to be 75 kDa for isoform Ma and 61 kDa for Mb. Breadfruit -mannosidases had optima pH (5.6) and temperature (60°C), and appeared to be stable in presence of some detergents such as Hexansulfonic acid sodium salt, Polyoxyethylen-9-lauryl ether, Nonidet P40, Triton X-100 as well as Ca^{2+} and Zn^{2+}. The effect of -mannosidase inhibitors on the two isoenzymes showed that swainsonine and 1,4- dideoxy-1,4-imino mannitol at 0.01 mM totally inhibited their hydrolytic activity, while kifunensine and deoxymannojirimycin at the same concentration had no effect on these enzymes. Substrate specificity tests revealed that the enzymes exerted only -mannosidase activity and cleaved - (1,2); -(1,3) and -(1,6) linked mannobiose. Since breadfruit seed -mannosidases were sensitive to furanose transition state analogs such as swainsonine and 1,4- dideoxy- 1,4- imino mannitol and showed broad substrate specificity, these enzymes would belong to class II -mannosidases.

Key words: -mannosidases, *Artocarpus communis*, breadfruit, purification, characterization.

INTRODUCTION

-mannosidases are ubiquitous in nature. They have been purified and well characterized from various plants, microbial and animal sources. These enzymes are an abundant constituent of plant hydrolytic system (Snaith, 1975; Forsee et al., 1989). They are accumulated in vacuoles and are thought to be involved in catabolism and turnover of N-linked glycoproteins (Pastuszak et al., 1990; Woo and Kimura, 2005).

Some previous studies showed that the levels of -mannosidases increased during seed germination and fruit ripening (Kestwal et al., 2007). This observation led to propose that these enzymes may be playing an important role during these processes by removing mannose residues from mannoglycans and thereby exposing the core cell wall glycan structures to facilite further hydrolytic attack (Gaikwad et al., 1995; Kishimoto et al., 2001).

-mannosidases have been classified into two independently derived groups, Class I and Class II, based on biochemical properties, substrate specificity, inhibitor profiles, and sequence alignments (Daniel et al., 1994; Moremen et al., 1994; Eades et al., 1998). The first group contains -1,2-mannosidases found in the endoplasmic reticulum (ER) and Golgi, including the ER Man_9-mannosidase, endomannosidase and Golgi mannosidase IA/IB. The second group of -mannosidases is more heterogeneous and contains the lysosomal mannosidases, Golgi mannosidase II and a distantly related group of en-

*Corresponding author. E-mail: niamkes@yahoo.fr. Fax: (225) 20 37 81 18. Tel : (225) 07 84 64 09.

enzymes, including the rat ER/cytosolic mannosidase (Bischoff et al., 1990), yeast vacuolar mannosidase (Yoshihisa and Anraku, 1989), and the *Aspergillus nidulans* Class II mannosidase (Eades et al., 1998). Regarding inhibitor profiles, -mannosidases susceptible to kifunensine (KIF) and deoxymannojirimycin (DMNJ) inhibition belong to class I. In contrast, those sensitive to swainsonine (SW) and 1,4-dideoxy 1,4-imino-D-mannitol (DIM) belong to class II mannosidases.

The mannosidases could find application in the food and pharmaceutical industry for production of fruit juices, degradation of plant material (Christgau et al., 1994), coffee extraction (McCleary, 1990) and for the manufacture of oligosaccharides (Akino et al., 1988) . Furthermore, mannosidases are applied in combination with xylanases in the pulp and paper industry to partially breakdown mannan and xylan in softwood pulps. This leads to a significant reduction in the amount of chemical required for bleaching (Viikari et al., 1994; Lahtinen et al., 1995).

In search for new sources of -mannosidase we investtigated breadfruit seeds, an available seeds rich in carbohydrate and high protein content, described as an important staple food of a high economic value (Soetjipto and Lubis, 1981). They form a portion of the diet in several tropical countries particularly West Indies, West Africa (Ghana, Sierra Leone and Nigeria) and Jamaïca (Purseglove, 1963; Dailziel, 1955) . In addition, more recently, some studies showed that -mannosidases activities increased during seeds germination and fruit maturation (Kestwal et al., 2007). It was thought important to look into properties of -mannosidases from breadfruit seeds where high activity of the enzymes has been noticed during the fruit maturation.

In this study, different chromatographic methods were employed to purify two -mannosidases from matured breadfruit seeds in order to classify them and find possible application in food and biotechnology industries.

MATERIALS AND METHODS

Materials

Breadfruit seeds were obtained locally in Côte d'Ivoire. *para*-Nitrophenyl- -D-Mannopyranoside (*p*NP- -Man), Mannose and Mannobiose containing disaccharides linked -D-Man*p*-(1,2)-D-Man*p*, -D-Man*p*-(1,3)-D-Man*p*, -D-Man*p*-(1,6)-D-Man*p*, Swainsonine (SW), 1,4-Dideoxy-1,4-iminomannitol (DIM) and Deoxymannojirimycin (DMNJ) were purchased from Sigma-Aldrich. Kifunensine (*Kitasatosporia kifunense*) (KIF), was obtained from Calbiochem. DEAE-Sepharose CL-6B and Sephacryl S-200 HR gels were provided from Pharmacia Biotech. Standard proteins were from Bio-Rad. Silicate gel 60 thin-layer chromatography (TLC) was purchased from Merck Darmstadt, Germany. All the other reagents used were of analytical grade.

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 7750 g for 30 min. The supernatant filtered through cotton was used as the crude extract and conserved at 4 °C.

Enzyme assay

Under the standard test conditions, -mannosidase activity was measured at 50°C for 15 min in 100 mM acetate buffer (pH 5.6) containing 1.5 mM *para*-nitrophenyl- -D-Mannopyranoside. After pre-warning the mixture at 50°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 *para*-nitrophenyl- -D-Mannopyranoside (*p*NP- -Man) was determined by measuring the liberated *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 mol of *p*NP released per min. Specific activity was expressed as the units of enzyme activity per mg of protein.

Protein estimation

Protein elution profiles from chromatographic columns were determined by absorbances measurement at 280 nm in spectrophotometer GENESIS and the concentration of purified enzyme was determined according to Lowry et al. (1951). Bovin Serum Albumine (BSA) was used as the standard protein.

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 cm x 6.0 cm) 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. The flow rate was 1ml/min and fractions of 3.0 ml were collected.

To the pooled active fractions, solid ammonium sulphate was slowly added to give a final concentration of 4.2 M (80 % saturation). The pellet obtained after centrifugation (7750 g) was resuspended in 1 ml of 20 mM sodium acetate buffer pH 4.6 and applied to Sephacryl S-200 HR column (1.6 x 65 cm) equilibrated with the same buffer. Fractions of 1ml were collected at a flow rate of 0.25 ml/min.

Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was carried out by the method of Laemmli (1970) 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 sample buffer without 2-mercaptoethanol and SDS.

Gels were stained with Coomassie brilliant blue R-250. The standard molecular weights (Bio-Rad) comprising myosine (200 kDa), -galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa) and ovalbumin (45.0 kDa) were used.

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 5.6) containing sodium azide 0.5 % (w/v) to estimate the molecular weight. Standard molecular weights used were -amylase (200,000 Da), BSA (66,000 Da), ovalbumin (45,000 Da) and cytochrome C (12,400 Da).

pH and temperature optima

The effect of pH on the enzyme (3.4 µg) activity was determined by measuring the hydrolysis of the substrate *para*-nitrophenyl - -D-Mannopyranoside in a series of buffers at various pH values ranging from pH 3.6 to 8.0. The buffers used were sodium acetate buffer (100 mM) from pH 3.6 to 5.6 and sodium phosphate buffer (100 mM) from pH 5.6 to 8.0. The pH values of each buffer were determined at 25°C.

The effect of temperature on -mannosidase (3.4 µg) activity was performed in 100 mM acetate buffer pH 5.6 over a temperature range of 30 to 80 °C using *p*NP- -Man (5 mM) under the standard test conditions. Activation energy was determined from an Arrhenius plot.

pH and temperature stabilities

The pH stability of each enzyme was studied in pH range 3.6 to 8.0 with 100 mM buffers. The buffers were the same as in the study of pH and temperature optima (above). After 2 h pre-incubation (3.4 µg of enzyme) at 37°C, aliquots were taken and immediately assayed for residual -mannosidase activity.

The thermal inactivation of the enzyme was determined at 37 and 60°C after exposure to each temperature for a period from 15 to 120 min. The enzyme (3.4 µg) was incubated in 100 mM acetate buffer (pH 5.6). Aliquots were withdrawn at intervals and immediately cooled.

Concerning thermal denaturation tests, the aliquots of enzyme (3.4 µg) were pre-incubated at different temperatures ranging from 30 to 80°C for 15 min.

Residual activities, determined in the three cases at 50°C under the standard test conditions, are expressed as percentage activity of zero-time control of untreated enzyme.

Effect of chemical agents

To determine the effect of various compounds (ions, detergents, dithiol-reducing agents and -mannosidase specific inhibitors) as possible activators or inhibitors of the purified -mannosidases (3.4 µg), enzyme solutions were pre-incubated at 37°C for 30 min with the compounds and then the activity was assayed. The substrate *p*NP- - Man (5 mM) was added to the medium and incubated at 50°C for 15 min. The residual activity was assayed as the standard conditions and expressed as percentage of zero-time control of untreated enzyme with chemical agents.

Substrate specificity and kinetic parameters determination

The substrate specificity of the -mannosidase activity was determined by incubating each enzyme with the substrates *para*-nitrophenyl- -D-Mannopyranoside, *para*-nitrophenyl- -D-Glucopyranoside, *para*-nitrophenyl- -L-Fucopyranoside, *para*-nitrophenyl- -D-Galactopyranoside, *para*-nitrophenyl- -D-Fucopyranoside, *para*-nitrophenyl- -D-Glucopyranoside, *para*-nitrophenyl- -D-Galactopyranoside, *para*-nitrophenyl- -D-Xylopyranoside (5 mM) at 50°C in 100 mM sodium acetate buffer (pH 5.6) for 15 min.

The kinetic parameters (K_M, V_{max} and V_{max} / K_M) were determined in 100 mM sodium acetate buffer (pH 5.6) at 50°C. The hydrolysis of synthetic substrates was quantified on the basis of released *p*NP, as the standard enzyme assay. K_M and V_{max} were determined from a Lineweaver-Burk plot using different concentrations of the substrate *para*-nitrophenyl- -D-Mannopyranoside.

Characterization of hydrolytic specificity

The hydrolytic specificity was determined by incubating 0.64 µg of purified enzyme preparation with 9 µl of 5 mM of -D-Man*p*-(1,2)-D-Man*p*, -D-Man*p*-(1,3)-D-Man*p* or -D- Man*p*-(1,6)-D-Man*p*, at pH 5.6 in 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.

RESULTS

Enzyme purification

The results of the purification of - mannosidases from breadfruit seeds are summarized in Table 1. Two isoenzymes were purified from the crude extract. The purification protocol involved two steps of chromatography for both enzymes (Figure 1).

One major peak of -mannosidase activity was resolved on the DEAE-Sepharose CL-6B column at 0.3 M NaCl concentration in 20 mM sodium acetate buffer (pH 4.6) and was designated M (Figure 1A). Pooled fractions of the peak of -mannosidase activity resolved on the DEAE-Sepharose CL-6B column step was applied to a Sephacryl S-200 HR gel (Figure 1B). Two peaks showing -mannosidase activities were eluted. The two isoforms were named Ma and Mb based on their distinct elution profile on Sephacryl S-200 HR gel. Their specific activeties were 2.97 and 0.65 UI/mg of protein, respectively. The purity of isoforms Ma and Mb increased 18 and 4 fold, respectively (Table 1).

Each -mannosidase isoenzyme (Ma and Mb) sho-wed a single protein band on native polyacrylamide gel electrophoresis staining with Coomassie brilliant blue R-250 (Figure 2).

Molecular weights estimation

The electrophoresis profiles (SDS-PAGE) for purified enzymes are depicted in Figure 3. After SDS - PAGE analysis under reducing conditions, each - mannosidase (Ma and Mb) showed a single protein band. Their relative molecular weights were estimated to be 75 and 61 kDa, respectively (Figure 3).

The two isoforms resolved by gel filtration chromato-

Table 1. Purification procedure of -mannosidases from breadfruit seeds.

Purification steps	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Yield (%)	Purification fold
Crude extract	1340	224.25	0.17	100	1
DEAE-Sepharose CL-6B					
-mannosidase M	24.66	8.26	0.33	3.60	2
Sephacryl-S 200 HR					
-mannosidase Ma	0.16	0.47	2.97	0.21	17.73
-mannosidase Mb	1.47	0.96	0.65	0.42	3.89

Values given are the average of at least three experiments

Table 2. Some physicochemical characteristics of -mannosidases from breadfruit seeds.

Physicochemical properties	Values	
	Ma	Mb
Optimum temperature (°C)	60.0	60.0
Optimum pH	5.6	5.6
pH stability	4.6-5.6	4.6-5.6
Molecular weight (kDa)		
SDS-PAGE	75.0	61.0
Gel filtration	74.0	60.0
Activation energy (kJ/mol)	35.6	36.2

Values given are the average of at least three experiments.

graphy, when individually subjected to gel permeation chromatography with TSK QC-PAK GFC 200 column, showed distinct separation, indicating different molecular weights of 74 and 60 kDa for Ma and Mb, respectively (Table 2).

Effect of pH and temperature

The effect of pH and temperature on the two –manno-sidases activities is shown in Table 2. Both enzymes were most active at pH 5.6 and 60°C. At 50°C, the two enzymes were stable over pH range 4.6 – 5.6 and their activation energy was 35.6 and 36.2 for Ma and Mb, respectively (Table 2).

The thermal inactivation studies indicated that at pH 5.6, the two -mannosidases remained almost stable for 120 min at 37°C (Figure 4). But at 60°C (optimum temperature), the two isoforms retained around 97 % of their activities for 15 min. Above this time of pre-incubation, -mannosidases (Ma and Mb) were less stable and lost 70 to 80% of their hydrolytic activities after 120 min of pre-incubation (Figure 4). The half-life of the two isoforms at 60°C was 60 min.

The thermal denaturation was investigated by incubating each enzyme at various temperatures for 15 min. The result shows that the enzymes were fairly stable at temperature up to 60°C. Above this temperature, their activities declined rapidly as the temperature increased, but the enzymes were not completely inactivated even at 80°C (Figure 5).

Effect of ions, reducing and detergent agents

The effect of chemical agents on the activity of breadfruit seeds -mannosidases (Ma and Mb) using *para*-nitro-phenyl- -D-mannopyranoside was examined (Table 3). The purified -mannosidases Ma and Mb were activated by Zn^{2+} (131 and 144.7%, respectively) and Ca^{2+} (217 and 135%, respectively). Only the activity of the isoform Ma was enhanced by Mg^{2+} (125 - 130%) and Triton X-100 (139 %). However, EDTA, Sr^{2+} and Cu^{2+} inhibited both -mannosidases at 5 mM. The other ions tested had little or no effect on isoforms Ma and Mb.

Table 4 shows the effect of reducing agents on the purified enzymes Ma and Mb. All the reducing agents tested were found to be inhibitory.

The influence of various detergents on the two -mannosidase activities was studied (Table 5). All the detergents tested stimulated Ma and Mb with exception of cationic detergents and SDS in contrast to Ma, Mb is sensitive to the same detergents except Lubrol Wx. At 1 % concentration, cationic detergents showed a little inhibition on Ma and Mb activities by 5 to 35 %. SDS was a strong inhibitor for the two enzymes.

Substrate specificity and kinetic properties

The purified -mannosidases were assayed for hydroly-tic activities against a variety of synthetic and natural substrates. No detectable activities towards *para*-nitro-phenyl-glycosides were observed except *para*-nitrophe-nyl- -D-mannopyranoside on which Linewea-ver-Burk-plot in range of concentration (1 mM to 6 mM) showed K_M and Vmax values of -mannnosidases (Table 6). Using *p*NP- -D-mannopyranoside, the K_M values were 2.54 mM and 2.44 mM for isoforms Ma and Mb, respectively.

On the other hand, linkage specificity of Ma and Mb was investigated with natural substrates such as disac-

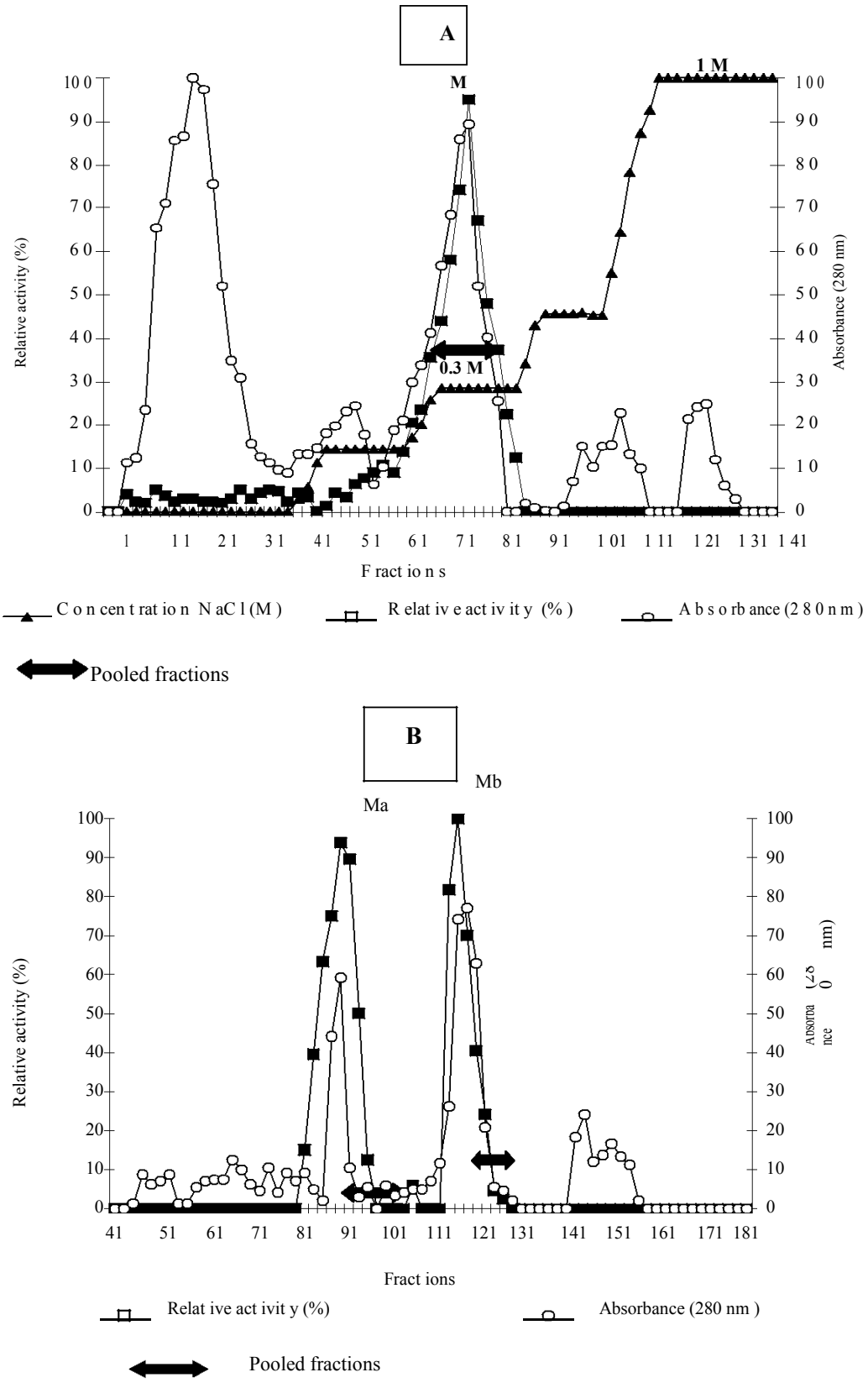

Figure 1. Purification of -mannosidases from breadfruit seeds. Enzyme activity was measured in acetate buffer pH 4.6 at 50°C using pNP- -Man as substrate. (A) Chromatogram of Anion-exchange chroma-tography on DEAE-Sepharose CL-6B. (B) Chromatogram of Gel filtration chromatography on Sephacryl S-200 HR column.

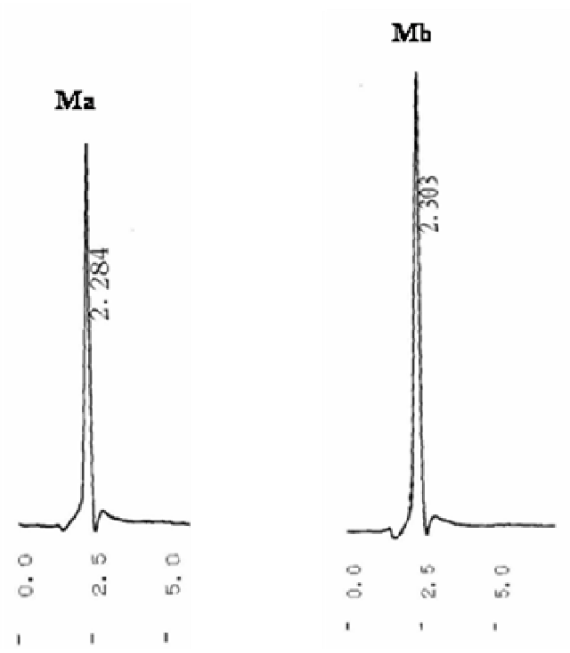

Figure 2. HPLC profiles of the purified -mannosidases from breadfruit seeds. The purified enzymes were applied to a gel TSK QC-PAK GFC 200 HPLC column equilibrated with 20 mM sodium acetate buffer (pH 5.6) -mannosidase Ma (5 µg); -mannosidase Mb (5 µg).

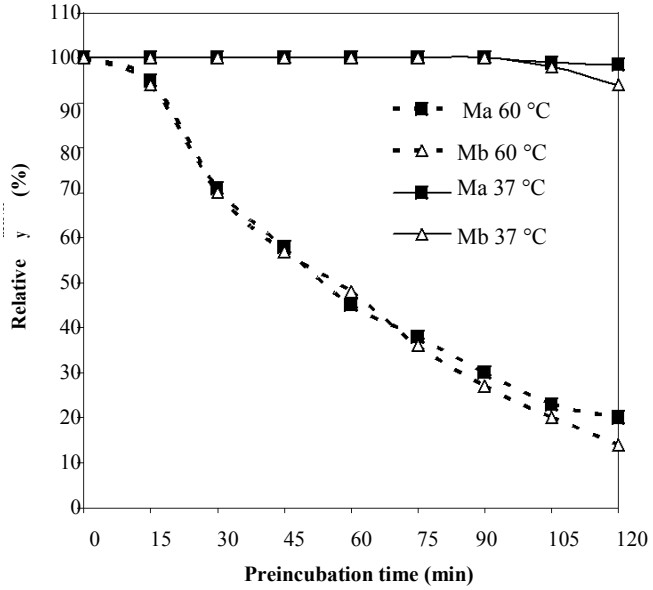

Figure 4. Thermal inactivation of -mannosidases from breadfruit seeds (3.4 µg). The enzymes were pre- incubated at 37 °C and 60°C in 100 mM sodium acetate buffer (pH 5.6). At the indicated times, aliquots were withdrawn and the residual activity was measured at 50°C under the standard test conditions. Values given are the average from at least three experiments.

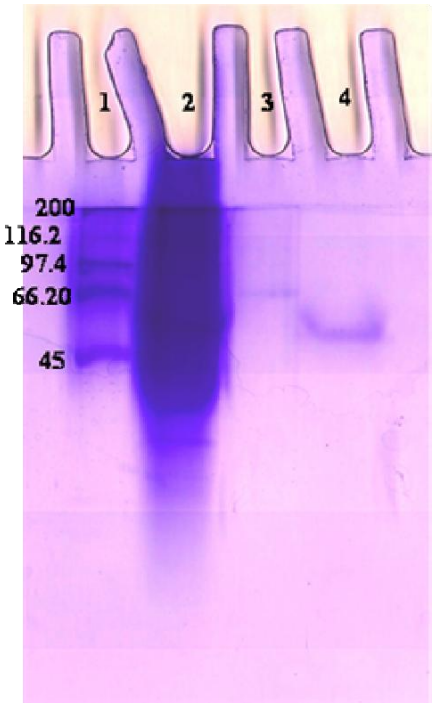

Figure 3. SDS -PAGE analysis of the purified -mannosidases from breadfruit seeds. The samples were loaded onto a 12 % gel. Lane 1, molecular weight markers; lane 2, crude extract of bread-fruit seeds; lane 3, -mannosidase Ma (8 µg); lane 4, –mannosi-dase Mb (8 µg).Numbers on the left indicate the molecular weight (kDa) of the markers.

charides 2-O- -D -mannopyranosyl-D-mannopyranoside (Man- -1,2-Man), 3-O- -D-mannopyranosyl-D-manno-pyranoside (Man- -1,3-Man) and 6-O- -D-mannopyra-nosyl-D-mannopyranoside (Man- -1,6-Man). Following incubation with Ma and Mb, released mannose was sepa-rated by TLC (Figure 6).

Both -mannosidases cleaved -D-Man*p*(1,6)-D-Man*p* and -D- Man*p*(1,2)-D- Man*p*. In addition, only Mb cleaved -(1,3)- Mannobiose.

Effect of -mannosidase inhibitors

The influence of -mannosidase inhibitors such as De-oxymannojirimycin (DMNJ); Kifunensine (KIF); Swain-sonine (SW) and 1, 4 - dideoxyimino - mannitol (DIM) on the enzymes activities is presented in Table 7.

Hydrolysis of *p* – NP - –D - Man by Ma and Mb was almost totally inhibited by SW and DIM (80 - 87%) at 0.01 mM. At 1 mM, the same inhibitors had strong inhi-bitory effect on both isoforms of -mannosidase from breadfruit seeds. The other inhibitors as KIF and DMNJ (0.01 mM) had not such effect. While, at 1 mM, – man-nosidases Ma and Mb was strongly inhibited by KIF and DMNJ.

DISCUSSION

In this work, purification of -mannosidases activity from breadfruit by two steps procedure involving anion exch-ange and size exclusion chromatography led to the sepa-

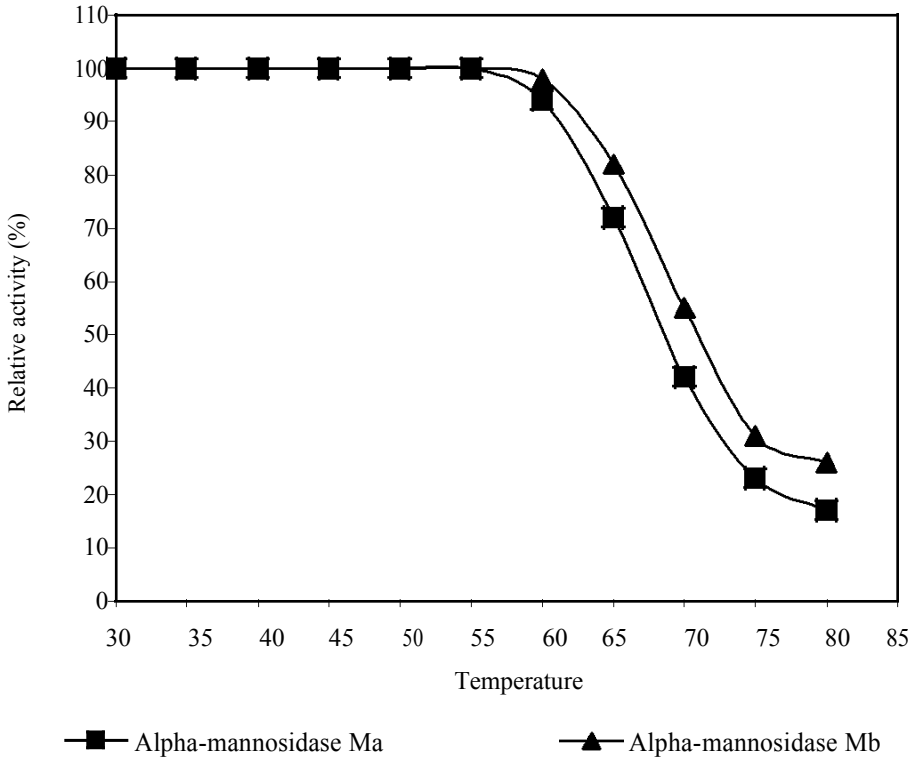

Figure 5. Temperature stability study of -mannosidases from breadfruit seeds (3.4 µg). The enzymes were pre-incubated at each temperature for 15 min. The remaining activity was measured at 50°C under the standard test conditions. The value at 30°C was taken as 100%. Values given are the average from at least three experiments.

Table 3. Effect of some ions and chelating agents on the activity of –mannosidases from breadfruit seeds.

Reagents	Concentration (mM)	Relative activity (%)	
		Ma	Mb
Control	0	100.0 (± 0.1)	100.0 (± 0.2)
Na^{+}	1	100.0 (± 0.2)	112.0 (± 0.3)
	5	119.0 (± 0.1)	116.0 (± 0.3)
Mg^{2+}	1	125.0 (± 0.3)	100.0 (± 0.2)
	5	130.0 ± 0.2)	100.0 (± 0.1)
Sr^{2+}	1	94.0 (± 0.1)	89.0 (± 0.3)
	5	79.2 (± 0.1)	67.3 (± 0.2)
Ca^{2+}	1	101.0 (± 0.2)	117.0 (± 0.2)
	5	217.0 (± 0.1)	135.0 (± 0.1)
Ba^{2+}	1	98.0 (± 0.2)	108.0 (± 0.3)
	5	104.0 (± 0.2)	136.7 (± 0.2)
Cu^{2+}	1	110.0 (± 0.3)	52.0 (± 0.2)
	5	90.0 (± 0.3)	23.2 (± 0.3)
Zn^{2+}	1	101.0 (± 0.2)	103.0 (± 0.1)
	5	131.0 (± 0.2)	144.7 (± 0.2)
EDTA	1	100.0 (± 0.3)	80.0 (± 0.3)
	5	89.0 (± 0.1)	72.0 (± 0.3)

Values given are the average of at least three experiments.

Table 4. Effect of some reducing agents on the activity of -mannosidases from breadfruit seeds.

Reducing agents	Concentration (% w/v)	Relative activity (%)	
		Ma	Mb
*p*CMB[a]	0.1	50.0 (± 0.2)	55.0 (± 0.3)
	1	8.0 (± 0.3)	10.0 (± 0.3)
DTNB[b]	0.1	9.0 (± 0.1)	17.0 (± 0.2)
	1	0 (± 0.1)	0 (± 0.1)
L-cystein	0.1	49.2 (± 0.1)	83.0 (± 0.1)
	1	23.2 (± 0.3)	13.0 (± 0.2)
-mercaptoethanol	0.1 (v/v)	60.0 (± 0.3)	80.0 (± 0.1)
	1 (v/v)	40.0 (± 0.2)	56.0 (± 0.2)

a: Sodium parachloromercuribenzoate. b: 5,5'-dithio-2,2'dinitro-dibenzoïc acid. Values given are the average of at least three experiments.

Table 5. Effect of detergents on the activity of -mannosidases from breadfruit seeds.

Detergents	Concentration (% w/v)	Relative activity (%)	
		Ma	Mb
Anionic			
SDS	0.1	36.0 (± 0.1)	0 (± 0.3)
1-Hexansulfonic acid sodium salt	0.1	100.0 (± 0.1)	100.0 (± 0.1)
	1	144.1 (± 0.2)	101.2 (± 0.3)
Polyoxyethylen-9-lauryl ether	0.1	100.0 (± 0.1)	100.0 (± 0.3)
	1	145.5 (± 0.2)	87.4 (± 0.1)
Cationic			
Tetradecyl trimethyl ammonium bromide	0.1	102.0 (± 0.2)	86.3 (± 0.1)
	1	94.8 (± 0.1)	64.9 (± 0.1)
Hexadecyl trimethyl ammonium bromide	0.1	100.0 (± 0.2)	100.0 (± 0.3)
	1	79.2 (± 0.1)	91.2 (± 0.3)
None ionic			
Nonidet P 40	0.1	100.0 (± 0.3)	100.0 (± 0.1)
	1	136.3 (± 0.1)	67.7 (± 0.2)
Triton X-100	0.1	110.0 (± 0.2)	100.0 (± 0.3)
	1	139.0 (± 0.1)	79.3 (± 0.2)
Lubrol Wx	0.1	106.0 (± 0.2)	105.0 (± 0.2)
	1	186.6 (± 0.1)	128.4 (± 0.3)

Values given are the average of at least three experiments.

Table 6. Kinetic parameters of -Mannosidases purified from breadfruit seeds.

-mannosidases	K_M	Vmax	Vmax/K_M
Ma	2.5	3.7	1.4
Mb	2.4	4.7	1.9

The Michaelis constants (K_M) and the maximum velocities (Vmax) are expressed as mM and units/mg of protein, respectively. *p*NP-α-Man was used as substrate. Values given are the average of at least three experiments.

with specific activities of 0.47 and 0.96 UI/mg of protein. Similar easy protocols were used during purification of mango and tomato -mannosidases (Suvarnalatha and Prabha, 1999; Yashoda et al., 2007).

The molecular weights of the purified enzymes were 75 and 61 kDa for isoforms Ma and Mb, respectively, by SDS-PAGE in reducing conditions. On the other hand, by gel filtration, both isoforms showed molecular weights of 75 for Ma and 60 kDa for Mb, suggesting that these -mannosidases have a monomeric structure. Except the rat liver and jack bean -mannosidases which are tetra-

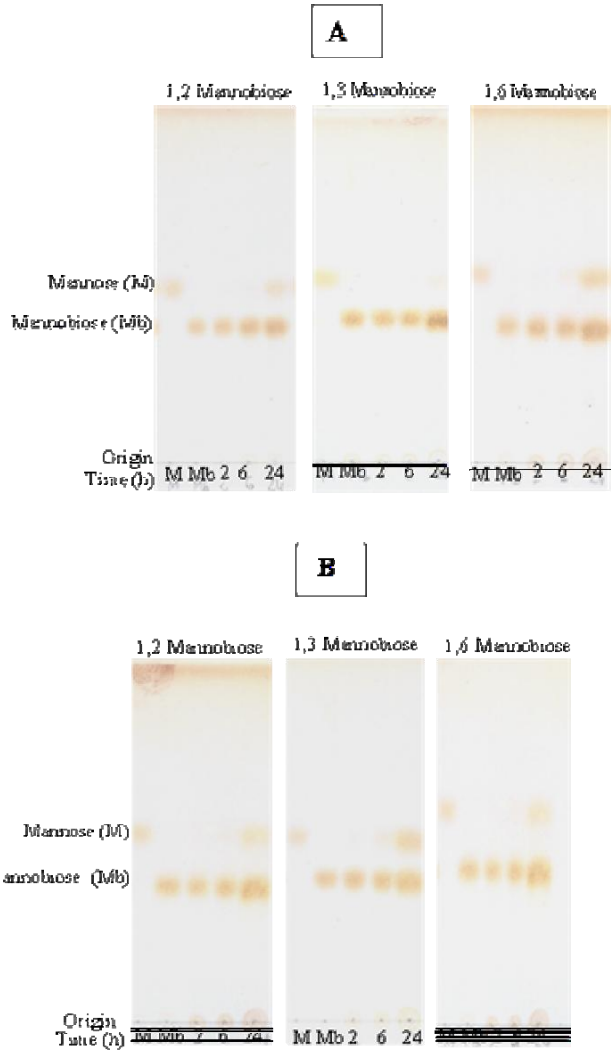

Figure 6. TLC plates showing the hydrolytic activity of the enzymes (Ma[A] and Mb[B]) extract towards differently linked disaccharides, monitored over 24 h incubation time at 37 °C, pH 5.6. Separation of hydrolyse products was realized with butanol-acetic acid-water (9:3.-75:2.25 v/v/v). The plates were developed with naphto-resorcinol in ethanol and H_2SO_4 20 % (v/v).The sugar spots were visualised at 105°C for 8 min.

meric protein with different subunits (Tulsiani et al., 1982; Kimura et al., 1999), most of -mannnosidases are monomeric polypeptides with molecular weights in the range of 51 - 73 kDa (Bischoff and Kornfeld, 1986). In other respects, breadfruit -mannosidases molecular weights are comparable to those found for others charac-terized glycolsidases from termite *Macrotermes subhyali-nus* (Kouamé et al., 2005; Kouamé, 2006).

The optimum pH of the enzymes was around 5.6 with *p*NP- -D-Man as a substrate, suggesting that they are kind of acidic glycosidases, such as several glycosidases (Faulet et al., 2006a, b) . Breadfruit - mannosidases displayed a better stability at pH ranging 4.6 and 6.0. So, a pH of 5.6 is a good compromise between the activity and stability of these enzymes to perform the natural substrates hydrolysis over a long time. These plant –mannosidases which activities are maximal at acidic region appear to be comparable to certain lysosomal –mannosidases in animals' cells. The pHs of these enzymes are within the range of those reported from cloned *Trichoderma* (Maras et al., 2000) and from *Ginkgo biloba* seeds (Woo et al., 2004).

-mannosidases Ma and Mb had an optimal temperature at 60°C and remained stable at the same temperature for 15 min of pre-incubation. Their thermal optimum and stability are similar to those from *Capsicum* (Priya-Sethu and Prabha, 1997).

The purified -mannosidases Ma and Mb were checked for other glycosidase activities with *p*NP-substrates such as - and -D-glucopyranoside, - and -D-gal-actoside, - and -D-fucopyranoside, and -and -D-xylopyranoside. These -mannosidases of breadfruit seeds did not show any activity with these substrates; this indicates that, the activity of these enzymes is restri-cted towards a substrate (*p*NP- -D-Man) normally acted upon by -mannosidase.

Using *p*NP- -D-Man as substrate, the kinetic parameters for purified -mannosidases from breadfruit was determined at pH 5.6 (100 mM sodium acetate). The K_M values of isoforms Ma and Mb were 2.54 and 2.44 mM, respectively, while for the bell-pepper enzyme, the K_M as 0.7 mM (Priya-Sethu and Prabha, 1997). Therefore, these -mannnosidases from breadfruit seeds had K_M values which are consistent with that from watermelon (Nakagawa et al., 1988).

Breadfruit -mannosidases activities on *p*NP- -D-Man were significantly enhanced by Ca^{2+} and Zn^{2+} but not Cu^{2+} and Sr^{2+}, although it has been reported that Zn^{2+} and Ca^{2+} ions often activate some plant acidic – mannosidases (Li and Li, 1972; Pastuszak et al., 1990) or some processing -mannosidases (Suzumilo et al., 1986; Kimura et al., 1991). Furthermore, the enzyme acti-vities were inhibited by addition of EDTA, like that from watermelon (Nakagawa et al., 1988). This result suggested that Ca^{2+} and Zn^{2+} are essential for hydrolytic activity themselves, or those ions regulate the enzyme activity during seeds maturation.

The two -mannosidases were sensitive to reducing agents. These chemicals must be eliminated after treatment of substrate when this needs to be reduced before its hydrolysis by enzymes.

-mannosidase Ma was activated by most detergents. Similar observations have been reported for rat liver -mannosidase where 1% of Triton X-100 and other detergents increased 3-4 fold this activity (Bonay et al., 1992). For these authors, this result suggests that, in the native enzyme structure, the catalytic domain is partially inaccessible to the substrate unless this -mannosidase integrity is disrupted by detergent to expose the active site. In contrast, Mb did not need high concentration of detergent for its catalytic activity. -mannosidase activities were

Table 7. Effect of specific inhibitors on the activity of-mannosidases from breadfruit seeds.

Inhibitors	Concentration (mM)	Relative activity (%)	
		Ma	Mb
Swainsonine	0.01	19.5 (± 0.1)	13.8 (± 0.2)
	0.1	10.2 (± 0.2)	6.2 (± 0.1)
	1	0 (± 0.3)	3.4 (± 0.2)
1,4 Dideoxy 1,4 Imino Mannitol	0.01	16.0 (± 0.2)	13.0 (± 0.2)
	0.1	13.0 (± 0.3)	3.0 (± 0.3)
	1	6.3 (± 0.2)	0 (± 0.1)
Kifunensine	0.01	89.1 (± 0.1)	100.0 (± 0.1)
	0.1	67.2 (± 0.2)	75.0 (± 0.2)
	1	45.0 (± 0.1)	36.8 (± 0.1)
Deoxymannojirimycin	0.01	97.0 (± 0.2)	100.0 (± 0.3)
	0 .1	54.6 (± 0.2)	87.5 (± 0.2)
	1	39.6 (± 0.1)	37.5 (± 0.3)

Values given are the average of at least three experiments.

determined in presence of specific inhibitors such as SW, DIM, KIF and DMNJ. The hydrolysis of *p*NP- -D-Man by either Ma or Mb was not blocked by KIF and DMNJ at low concentration, while, in the same condition, mannofuranose analogs SW and DIM blocked totally hydrolysis of the same substrate by both isoforms Ma and Mb. It has become apparent that DIM, an agent which has previously been used to inhibit a number of –mannosidases including those from jack bean and lysosomes (Elbein, 1991; De Gasperi et al., 1992; Daniel et al., 1994), has an effect opposite to that of KIF on the ER mannosidases and therefore can function as an additional valuable tool in distinguishing between –mannosidases. It's also well known that pka of SW is 7.4 and thus it would be fully ionized at pH 5.0 (Dorling et al., 1980), near optimum pH of breadfruit seed -mannosidases. This may account for the apparent specificity of SW for breadfruit seed –mannosidases. Indeed, the ionization of SW could improve its fixation in the catalytic centre of the enzymes. Using molecular models, it can be seen that the configuration of secondary hydroxyl groups of SW is identical to that in DIM (Cenci di Bello et al., 1989; Winchester et al., 1993) . This supports the similar effect of DIM on breadfruit seeds -mannosidases. Taken together, these results suggest that -mannosidases of breadfruit seeds belong to class II -mannosidases.

The hydrolysis of -mannosidase Ma for differently linked Mannobiose showed that this enzyme cleaved 1,2 and 1,6 linkages. On the other hand, the -mannosidase Mb did not have strict linkage specificity. These results suggest that, the isoforms would be involved in the degradation of oligomannose type free N-glycans which exhibit biological activity such as growth factors during the early development of plant (Preim et al., 1992), stimulation of fruit ripening (Priem and Gross, 1992) and suppressors of corresponding glycopeptide elicitors of stress responses (Basse and Boller, 1992). They could also find importance in combination with xylanase in pulp and paper industry. Similar observations have been reported by Viikari et al. (1994) in pulp and paper bleaching. The broad specificity of the breadfruit seeds -mannosidases implied their broad subcellular distribution (Bonay et al., 1992).

This study showed that the two – mannosidases purified from matured breadfruit seeds are class II -mannosidases since they are sensitive to furanose transition state analogs SW and DIM. This classification is supported by the broad substrate specificity, and implied their role in degradation of N-glycans in breadfruit seeds. These characteristics show the breadfruit –mannosidases importance in production of short sugar chains active biologically, and could find application in food and biotechnology industry.

REFERENCES

Akino T, Nakamura N, Horikoshi K (1988). Characterization of three -mannanases of an alkalophylic *Bacillus sp*. Agric. Biol. Chem. 52: 773-779.

Basse CW, Boller T (1992). Glycopeptide elicitors of stress responses in tomato cells. Plant Physiol. 98: 1239-1247.

Bischoff J, Kornfeld R (1986). The soluble form of rat liver -mannosidase is immunologically related to the endoplasmic reticulum membrane -mannosidase. J. Biol. Chem. 261:4758-4765.

Bischoff J, Moremen K, Lodish HF (1990). Isolation, characterization, and expression of cDNA encoding a rat liver endoplasmic reticulum -mannosidase. J. Biol. Chem. 265: 17110-17117.

Bonay P, Roth J, Huggues CR (1992). Subcellular distribution in rat liver of a novel broad –specificity (-1,2, -1,3 and -1,6) mannosidase active on oligomannose glycans. Eur. J. Biochem. 205: 399-407.

Cenci di Bello I, Fleet G, Nangoong SK, Tandano KI, Winchester B (1989). Structure-activity relationship of swainsonine. Inhibition of hu-

human -mannosidases by swainsonine analogs. Biochem. J. 259: 855-861.
Christgau S, Andersen LN, Kauppinen S, Heldt-hansen HP, Dalboege H (1994). Purified enzyme exhibiting mannanase activity; application in oil, paper, pulp, fruit and vegetable juice industry and carrageenan extraction. Patent Novo-Nordisk, 9425576 10 November 1994.
Dailziel JM (1955). African fruits. University of London Press. p. 450.
Daniel PF, Winchester B, Warren CD (1994). Mammalian -mannosidases multiple forms but a commun purpuse? Glycobiol. 4: 551-556.
De Gasperi R, Daniel PF, Warren CD (1992). A human lysosomal -mannosidase specific for the core of complex glycans. J. Biol. Chem. 267: 9706-9712.
Dorling PR, Huxtable CR, Colegate SM (1980). Inhibition of lysosomal -mannosidase by swainsonine, an indolizidine alkaloid isolated from *Swainsona canescens*. Biochem. J. 191: 649-665.
Eades CJ, Gilbert AM, Goodman CD, Hintz WE (1998). Identification and analysis of a class 2 -mannosidase from *Aspergillus nidulans*. Glycobiol. 8:17-33.
Elbein AD (1991). Glycosidase inhibitors: Inhibitors of N-linked oligosaccharide processing. FASEB J. 5: 3055-3063.
Faulet MB, Niamké S, Gonnety TJ, Kouamé LP (2006a). Purification and biochemical properties of a new thermostable xylanase from symbiotic fungus *Termitomyces sp*. Afri. J. Biotechnol. 5: 273-283.
Faulet MB, Niamké S, Gonnety TJ, Kouamé LP (2006b). Purification and biochemical characteristics of a new strictly specific endoxylanase from termite *Macrotermes subhyalinus* workers. Bull. Insect. 59:17-26.
Forsee WT, Palmer CF, Schutzbach JS (1989). Purification and characterization of an -1,2-mannosidase involved in processing asparagines-linked oligosaccharides. J. Biol. Chem. 264:3869-3876.
Gaikwad SM, Keskar SS, Khan MI (1995). Purification and characterization of -D-mannosidase from *Aspergillus sp*. Biochim. Biophys. Acta. 1250: 144-148.
Kestwal RM, Konozy EHE, Hsiao C-D, Roque-Barreira MC, Bhide SV (2007). Characterization of -mannosidase from *Erythrina indica* seeds and influence of endogenous lectin on its activity. Biochim. Biophys. Acta. 1770: 24-28.
Kimura Y, Hess D, Sturm A (1999). The N-glycans of jack bean -mannosidase structure, topology and function. Eur. J. Biochem. 264: 168-175.
Kimura Y, Yamaguchi D, Suehisa H, Takagi S (1991). In vitro hydrolysis of oligomannosidase-type sugar chains by an -1,2-mannosidase from microsomes of developing castor bean cotyledons. Biochem. Biophys. Acta. 1075: 6-11.
Kishimoto T, Hori H, Takano D, Nakano Y, Watanabe M, Mitsui T (2001). Rice -mannosidase digesting the high mannose glycopeptide of glutelin. Physiol. Planta. 112: 15-24.
Kouamé LP, Kouamé AF, Niamké LS, Faulet MB, Kamenan A (2005). Biochemical and catalytic properties of two -glycosidases purified from workers of the termite *Macrotermes subhyalinus* (Isoptera: Termitidae). Int. J. Trop. Insect. Sci. 25: 103-113.
Kouamé LP (2006). Identification de proteases du termite *Macrotermes subhylinus* (Rambour) et de son champignon symbiotique *Termitomyces sp*. Caractérisation des exo-glycosidases thermophiles ou possédant une bonne activité de transglycosylation. Thèse de Doctorat d'Etat, Université Abobo-Adjamé, Abidjan, Côte d'Ivoire. p. 210 .
Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London). 227: 658-685.
Lahtinen T, Kristoo P, Paloheimo M (1995). Mannanases in Softwood Kraft Pulp Bleaching. Abstract PG4-101, sixth International Conference on Biotechnology in the Pulp and Paper Industry, 11-15 June 1995, Vienna, Austria.
Li YT, Li SSC (1972). -mannosidase, -N-acetylhexoaminidases, and -galactosidase from jack bean meal. Methods Enzymol. 28:702-713.
Lowry OH, Rosebrough NJ, Farrra L, Randall RJ (1951). Protein measurement with Folin-phenol reagent. J. Biol. Chem. 193: 265-275.
Maras CN, Piens K, Claeyssens M, Martinet WD, Contreras H, Dewerte I, Penttillä M, Contreras R (2000). Molecular cloning and enzymatic characterization of a *Trichoderma reesei* 1,2- -D-mannosidase. J. Biotech. 77: 255-263.
McCleary BV (1990). Comparison of endolytic hydrolases that deploymerize 1,4- -D-mannan, 1,5- -L-arabinan and 1,4- -D-galaktan. ACS Symposium Series 460: 437-449.
Moremen KW, Trimble RB, Herscovics A (1994). Glycosidases of the asparagine-linked oligosaccharide processing pathway. Glycobiol. 4: 113-125.
Nakagawa H, Enomoto N, Asakawa M, Uda Y (1988). Purification and characterization of -mannosidase and -acetyl hexosaminidase from watermelon fruit. Agric. Biol. Chem. 52: 2223-2230.
Pastuszak I, Kaushal GP, Wall KA, Pan YT, Sturm A, Elbein AD (1990). Purification and properties of arylmannosidases from mung bean seedlings and soybean cells. Glycobiol. 1: 71-82.
Priem B, Gross KC (1992). Mannoxyl and xylosyl containing glycans promote tomato (*Lycopersicon esculentum* M.), Plant Physiol. 98: 399-401.
Priem B, Morvan H, Hafez MA, Morvan C (1992). Infuence of a plant Glycan of the Oligosaccharide Types on the Growth of Flax Plantlets. CR Academy of Sciences, Paris. 311: 411-416.
Priya Sethu KM, Prabha TN (1997). -D-mannosidase from *Capsicum annuum*. Phytochem. 44: 383-387.
Purseglove JW (1968). Tropical crops: Dicotyledons. Logmans, London. p. 719.
Snaith SM (1975). Characterization of jack-bean -D-mannosidase as a zinc metalloenzyme. Biochem. J. 147: 83-90.
Soetjipto NN, Lubis AS (1981). Vegetables: I.B.P.G.R. Secretariat, Rome. p. 330.
Suvarnalatha G, Prabha TN (1999). -mannosidase from *Lycopersicon esculentum* II. Phytochem. 50: 1111-1115.
Suzumilo T, Kaushal GP, Hori H, Elbein A (1986). Purification and properties of a glycoprotein processing -mannosidase from mung bean seedling. Plant Physiol. 81: 383-389.
Tulsiani DRP, Hubbard SC, Robbins PW, Touster O (1982). –mannosidases of rat liver Golgi membranes. J. Biol. Chem. 257: 3660-3668.
Viikari L, Kantelinen A, Sundquist J, Linko M (1994). Xylanases in bleaching: from an idea to the industry. FEMS Microbiol. Rev. 13: 335-350.
Winchester B, AL Daher S, Carpenter CN, Cenci di Bello I, Choi SS, Fairbanks JA, Fleet G (1993). The structural basis of the inhibition of human -mannosidases by azafuranose analogs of mannose. Biochem. J. 290: 743-749.
Woo KK, Kimura Y (2005). Regulation of substrate specificity of plant -mannosidase by cobalt ion: in vitro hydrolysis of high-mannose type N-glycans by Co^{2+} activated Ginkgo -mannosidase. Biosci. Biotechnol. Biochem. 69: 1111-1119.
Woo KK, Miyazaki M, Harra S, Kimura M (2004). Purification and characterization of Co(II)-sensitive -mannosidase from *Ginkgo biloba* seeds. Biosci. Biotechnol. Biochem. 68: 2547-2556.
Yashoda HM, Prabha TN, Tharanathan RN (2007). Mango ripening-role of carbohydrases in tissue softening. F. Chem. 102: 691-698.
Yoshihisa T, Anraku Y (1989). Nucleotide Sequence of AMSI. The structure gene of vacuolar -mannosidase of *Saccharomyces cerevisae*. Biochem. Biophys. Res. Commun. 163: 908-915.