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RESEARCH ARTICLE

Purification and biochemical characterization of a new strictly α1,2-mannosidase from breadfruit (*Artocarpus communis*) seeds.

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Abstract

□-mannosidases importance in N-glycoproteins processing and degradation prompted us to search for a new source. Thus, one □-mannosidase was purified from matured breadfruit (*Artocarpus communis*) seeds, by successive chromatography on DEAE-Sepharose CL-6B, CM-Sepharose CL-6B and Phenyl-Sepharose HP to apparent homogeneity. The enzyme had native molecular weight of approximately 46 kDa. SDS-PAGE analysis resolved a single protein band with molecular weight estimated to 46 kDa. Breadfruit □-mannosidase had optimal pH (4.6) and temperature (55 °C). Its activity was enhanced in presence of 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 enzyme showed that swainsonine (SW) and 1,4-dideoxy-1,4-imino mannitol (DIM) inhibited it's hydrolytic activity at low concentrations, while kifunensine (KIF) and deoxymannojirimycin (DMNJ) had no significant effect at the same concentrations on this enzyme. Substrate specificity tests revealed that the enzyme exhibited only □1,2-mannosidase activity. Thus, breadfruit □-mannosidase may be a useful tool for applications requiring selective removal of mannose.

Since breadfruit seed □-mannosidase was not sensitive to KIF and cleaved only □-(1,2) linked mannobiose, this enzyme would belong to class I □-mannosidases and seems to be □1,2- mannosidase Kifunensine resistant-like.
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Introduction

In the enzyme Nomenclature Recommendations (NC-IUBMB, 1992), α-mannosidases are assigned the following five EC numbers: EC 3.2.1.24 to those that do not have strict linkage specificity, EC 3.2.1.77 to those acting on (1,2)- and (1,3)- linkages, EC 3.2.1.113 to those acting on (1,2)- only, EC 3.2.1.114 to those acting on (1,3)- and (1,6)- linkages and EC 3.2.1.137 to those acting on (1,2)- and (1,6)- linkages.

□-mannosidases have been also 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). Class I α1,2-mannosidases are calcium-dependent enzymes (Vallée et al., 2000) that cleave α1,2-linked mannose units with inversion of anomeric configuration (Lipari et al., 1995). The second group, termed Class II α-mannosidases, is more heterogeneous and contains □-mannosidases sensitive to mannofuranose analogs such as swainsonine (SW) and 1,4-dideoxy 1,4-imino-D-mannitol (DIM) that show broad substrate specificity.

The importance of these enzymes in the processing system glycoproteins is well known. *In vivo*, mannosidases are involved in processing complex-type oligosaccharides in mammalian cells, and mannans in yeasts (Kornfeld and Kornfeld, 1985; Moremen et al., 1994). In plant, acidic α-mannosidases have been considered to be involved in the degradation of N-glycosylated proteins in the vacuole or protein body, but not in the processing of N-glycans in the ER or Golgi apparatus (Woo et al., 2004; Woo and Kimura, 2005). As far as α1,2-mannosidases are concerned, these enzymes play an essential role in the early step of N-linked oligosaccharides maturation in mammalian cells (Schneikert and Herscovics, 1994). Several α1,2-mannosidases in the ER and Golgi of mammalian cells are involved in the processing of $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$, the substrate leading to the formation of hybrid and complex oligosaccharide structures (Scaman et al., 1996). However, the ER-resident enzyme removes a single mannose from the middle arm of $Man_9GlcNAc_2$ to form $Man_8GlcNAc_2$ isomer B (Byrd et al., 1982; Jelinek-Kelly et al., 1985; Gonzalez et al., 1999), whereas the Golgi enzymes remove all four α1,2-linked mannose units (Bause et al., 1992; Kawar et al., 2000).

In vitro, mannosidases are useful tools for enzymatic analysis of high-mannose oligosaccharide structures (Jacob and Scudder, 1994; Maruyama et al., 1994) and for oligosaccharide synthesis (Maitin et al., 2004; Athanasopoulos et al., 2004). In this respect, the strictly specific α1,2-mannosidase become useful for applications requiring selective removal of mannose units or selective synthesis of oligomannosides.

To date, many acidic α-mannosidases have been purified and characterized from seeds and fruits (Li and Li, 1972; Ohtani and Masaki, 1983; Nakagawa et al., 1988; Ogawa et al., 1990; Pastuszak et al., 1990; Kimura and Ohno, 1998), but few α1,2-mannosidases from plant have been reported. Therefore, we investigated plant α-mannosidases. In this study, the biochemical characteristics of a new strictly specific α1,2-mannosidase purified from breadfruit (*Artocarpus communis*) seeds are reported.

1. Materials and methods

1.1. 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-Man*p*-(1,2)-D-Man*p*, α-D-Man*p*-(1,3)-D-Man*p*, α-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 from Calbiochem. DEAE-Sepharose CL-6B, Sephacryl S-200 HR 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.

1.2. Enzyme extraction

Matured breadfruit seeds (20 g) were ground in a prechilled 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.

1.3. 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 *para*-nitrophenyl-α-D-Mannopyranoside. 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 *para*-nitrophenyl-α-D-Mannopyranoside 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.

1.4. 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 et al. (1951). BSA was used as the standard protein.

1.5. 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 □-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. □-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 satured 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.

1.6. 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 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 myosine (200 kDa), □-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa) and ovalbumin (45.0 kDa) were used.

1.7. 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).

1.8. pH and Temperature optima

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

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

1.9. pH and temperature stabilities

The pH stability of the □-mannosidase from breadfruit was studied in pH range 3.6 to 9.0 in 100 mM buffers. The buffers were the same as in the study of the pH and temperature optima (above). After 2 h pre-incubation 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 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, are expressed as percentage activity of zero-time control of untreated enzyme.

1.10. Effect of some 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 □-mannosidase, enzyme solutions were preincubated 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 37 °C for 15 min. The residual activity was assayed as the standard conditions.

1.11. Substrate specificity and kinetic parameters determination

The substrate specificity of the □-mannosidase activity was determined by incubating the purified 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 37 °C in 100 mM sodium acetate buffer (pH 4.6) for 15 min.

The kinetic parameters (K_M, V_{max} and V_{max} / K_M) were determined in 100 mM sodium acetate buffer (pH 4.6) at 37 °C. The hydrolysis of synthetic substrates was quantified on the basis of released *p*NP, as in the standard enzyme assay. K_M and V_{max} were determined from a Lineweaver-Burk plot using different concentrations of the substrate *p*NP-□-Man.

1.12. Characterization of the hydrolytic specificity

The hydrolytic specifity was determined by incubating 10 µl 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 4.6 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.

2. Results

2.1. Enzyme purification

The purification procedure of breadfruit □-mannosidase is summarized in table 1. After DEAE-Sepharose CL-6B of the crude enzyme solution, □-mannosidase activity was detected at the fractions eluted with the salt-free acetate buffer (unbound fraction). These fractions were pooled and further separated by Carboxyl-methyl-Sepharose. The breadfruit □-mannosidase activity was finally purified by Phenyl-Sepharose HP hydrophobic interaction chromatography as show in figure 1. One peak was observed on the chromatogram. Active fractions were collected as purified □-mannosidase and used for further characterization of the enzyme. The specific activity was 0.97 UI/mg of protein. The purity of the purified □-mannosidase increased by 6 fold (Table 1).
Breadfruit □-mannosidase showed a single protein band on native-polyacrylamide gel electrophoresis staining with Coomassie brilliant blue R-250 (Figure 2).

2.2. Molecular weight estimation

SDS-PAGE profile of the purified enzyme is depicted in figure 3. After SDS-PAGE analysis under reducing conditions, breadfruit □-mannosidase showed a single protein band. Its relative molecular weight was estimated to be 46 kDa (Figure 3).

Breadfruit □-mannosidase resolved by hydrophobic interaction chromatography, when subjected to gel permeation chromatography with TSK QC-PAK GFC 200 column showed that the molecular weight in the native state is 46 kDa (Table 2).

2.3. Effect of pH and temperature

The effect of pH and temperature on the purified □-mannosidase activity is shown in table 2. The enzyme was most active in acetate buffer than the other buffers (Data not shown) at pH 4.6 and 55 °C. At 37 °C, this enzyme was stable over pH range 4.6 – 5.6 (Table 2).

Thermal inactivation studies indicated that at pH 4.6, breadfruit □-mannosidase remained almost stable for 120 min at 37 °C (Figure 4). But at 60 °C, the enzyme retained around 80 % of its activity for 15 min. Above this pre-incubation time, this enzyme was less stable and lost 90 % of its hydrolytic activity after 120 min of pre-incubation (Figure 4). The half-life of breadfruit □-mannosidase at 60 °C was 25 min.

The thermal denaturation was investigated by incubation of the enzyme at various temperatures for 15 min. The result showed that this enzyme was fairly stable at temperature up to 55 °C. Above this temperature, its activity declined rapidly as the temperature increased, but the enzyme was not completely inactivated even at 80 °C (Figure 5).

2. 4. Substrate specificity and kinetic properties

The purified □-mannosidase was assayed for hydrolytic activity against a variety of synthetic and natural substrates. No detectable activities towards *para*-nitrophenyl-glycosides were observed except *para*-nitrophenyl-□□-D-mannopyranoside on which Lineweaver-Burk-plot in range of concentrations (1 to 6 mM) showed K_M and Vmax values of breadfruit □-mannnosidase of 1.91 mM and 2.1 Units/mg of protein, respectively. The catalytic efficiency ($Vmax/K_M$) determined, was 1.1.

On the other hand, linkage specificity of breadfruit □-mannosidase was investigated with natural substrates such as disaccharides 2-O-□□-D-mannopyranosyl-D-mannopyranoside (Man-□□-1,2-Man), 3-O-□□-D-mannopyranosyl-D-mannopyranoside (Man-□□-1,3-Man), 6-O-□□-D-mannopyranosyl-D-mannopyranoside (Man-□□-1,6-Man). Released mannose was separated by TLC (Figure 6). The purified □-mannosidase of *Artocarpus communis* cleaved only □-D-Man*p* (1,2)-D-Man*p*, and was found to be free to other mannobioses.

2.5. Effect of ions, reducing and detergent agents

The effect of some chemical agents on the activity of breadfruit seeds □-mannosidase using *para*-nitrophenyl-□-D-mannopyranoside as subtrate was examined (Table 3). The purified □-mannosidase was activated by 5 mM of Mg^{2+}, Zn^{2+} and Ca^{2+} in the order of 111 to 163 %. However, Sr^{2+}, Cu^{2+} and EDTA at 5 mM inhibited the breadfruit □-mannosidase. The other ions tested had little or no effect on the purified enzyme.

The table 4 shows the effect of reducing agents on the purified enzyme. With exception of DL-dithiothreitol which displayed a little stimulatory effect on the breadfruit □-mannosidase at 1 %, all the other reducing agents tested were found to be inhibitory.

The influence of various detergents on the breadfruit □-mannosidase activity was studied (Table 5). All the detergents tested stimulated the enzyme with exception of cationic detergents and SDS. At 1 % concentration, cationic detergents strongly inhibited this enzyme by more than 50 %. As far as concerning SDS, at 0.1 %, the enzyme lost all its hydrolytic activity.

2.6. Effect of □-mannosidase inhibitors

The influence of □-mannosidase inhibitors such as Deoxymannojirimycin (DMNJ), Kifunensine (KIF), Swainsonine (SW) and 1,4-Dideoxy-imino-mannitol (DIM) on the enzyme activity is presented in table 6. Hydrolysis of *p*NP-□-D-Man by the purified □-mannosidase was strongly inhibited by SW and DIM. The other inhibitors as KIF and DMNJ (0.01 mM) had not such effect. However at 1 mM, the enzyme□was inhibited by KIF and DMNJ (50 %).

Table 1: Summary of -mannosidase from breadfruit seeds purification. 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	2.73	4.28	0.63	1.20	3.80
Phenyl-Sepharose HP	0.60	0.61	0.97	0.30	5.81

Table 2: Some physicochemical characteristics of -mannosidase from breadfruit seeds.Values given are the averages of at least three experiments

Physicochemical properties	Values
Optimum temperature (°C)	55
Optimum pH	4.6
pH stability	4.6-5.6
Molecular weight (kDa)	
SDS-PAGE	46
Gel filtration	46
Activation energy (kJ/mol)	28.62

Table 3: Effect of some ions and chelating agent on the activity of -mannosidase from breadfruit seeds. Values given are the averages of at least three experiments.

Reagent	Concentration (mM)	Relative activity (%)
Control	0	100
Na^{+}	1	100
	5	100
Mg^{2+}	1	100
	5	111
Sr^{2+}	1	66.3
	5	32
Ca^{2+}	1	120
	5	163
Ba^{2+}	1	82
	5	53
Cu^{2+}	1	53
	5	33
Zn^{2+}	1	100
	5	146.66
EDTA	1	54
	5	9

Table 4: Effect of some reducing agents on the activity of -mannosidase from breadfruit seeds. Values given are the averages of at least three experiments.

Reducing agent	Concentration (% ; w/v)	Relative activity (%)
Control	0	100
DL-dithiothreitol	0.1	100
	1	142
*p*CMB	0.1	52
	1	7
DTNB	0.1	18
	1	0

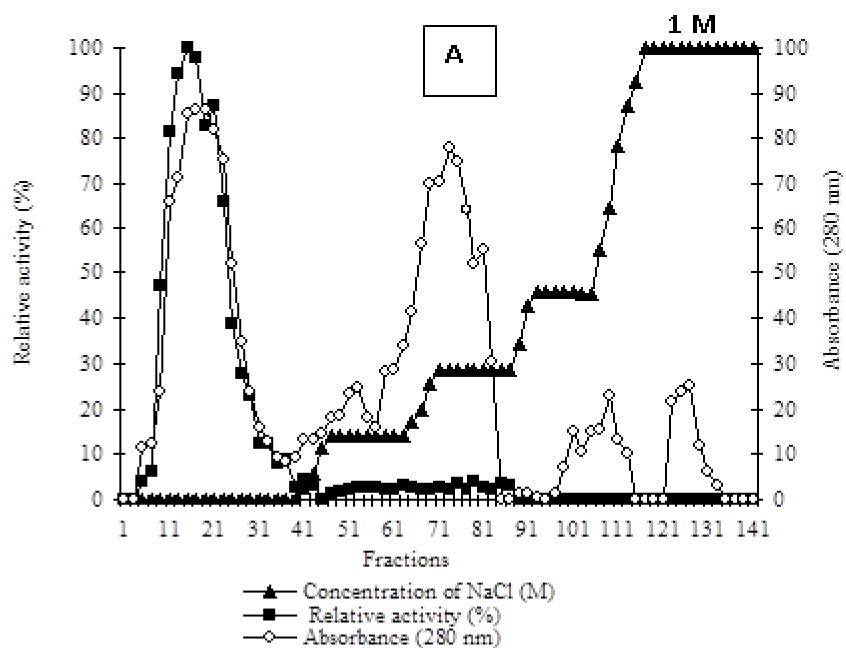
L-cystein	0.1	75
	1	10
β-mercaptoethanol	0.1 (v/v)	78
	1 (v/v)	52

Table 5: Effect of detergents on the activity of -mannosidase from breadfruit seeds. Values given are the averages of at least three experiments.

Detergent	Concentration (% ; w/v)	Relative activity (%)
Control	0	100
Anionic		
SDS	0.1	0
1-Hexanesulfonic acid sodium salt	0.1	102
	1	122
Polyoxyethylen-9-lauryl ether	0.1	125
	1	158
Cationic		
Tetradecyl trimethyl ammonium bromide	0.1	59
	1	52
Hexadecyl trimethyl ammonium bromide	0.1	79
	1	40
None ionic		
Nonidet P 40	0.1	100
	1	103
Triton X-100	0.1	100
	1	125
Lubrol Wx	0.1	110
	1	100

Table 6: Effect of specific inhibitors on the activity of -mannosidase from breadfruit seeds. Values given are the averages of at least three experiments.

Inhibitor	Concentration (mM)	Relative activity (%)
Control	0	100
Swainsonine (SW)	0.01	5
	0.1	0
	1	0
1,4 Dideoxy 1,4 Imino Mannitol (DIM)	0.01	38
	0.1	24
	1	18
Kifunensine (KIF)	0.01	96.44
	0.1	80
	1	55.44
Deoxymannojirimycin (DMNJ)	0.01	88.30
	0.1	81.75
	1	49.76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1 M
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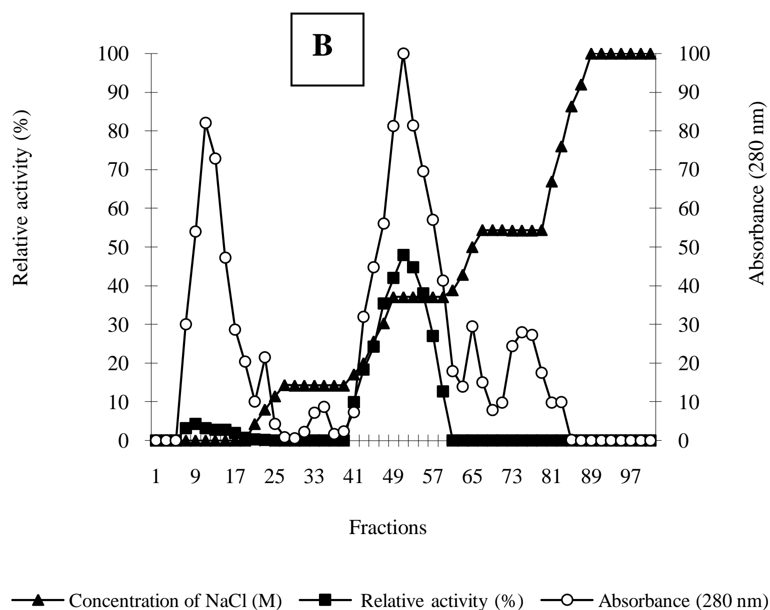
Relative activity (%)
Absorbance (280 nm)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Relative activity (%)
Absorbance (280 nm)

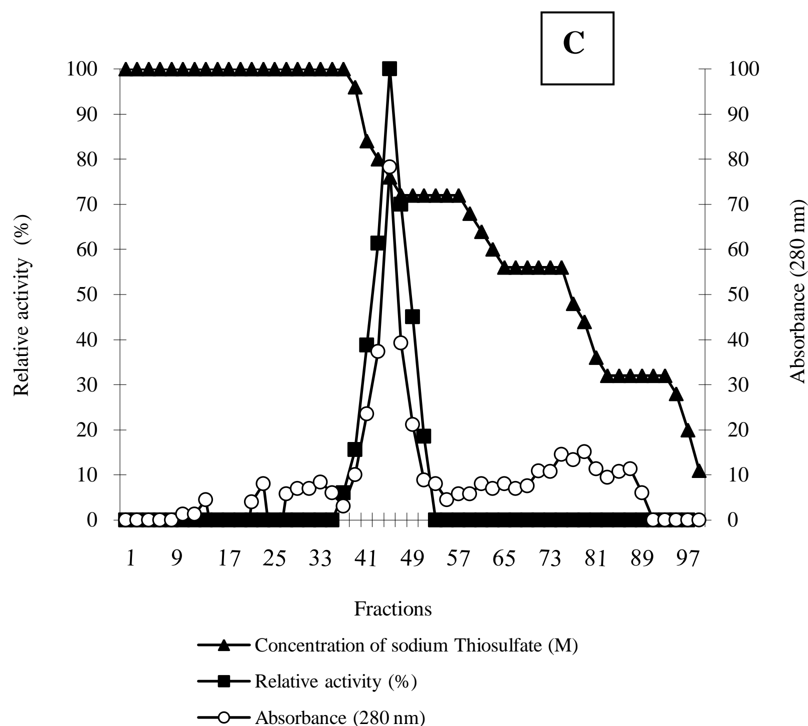

Fig. 1: Purification of -mannosidase from breadfruit seeds. The enzyme activity was measured in acetate buffer (pH 4.6) at 37 °C using *p*NP--Man as substrate. (A) Anion-exchange chromatography on DEAE-Sepharose CL-6B. (B) Cation-exchange chromatography on CM-Sepharose CL-6B. (C) Hydrophobic interaction chromatography on Phenyl-Sepharose HP column.

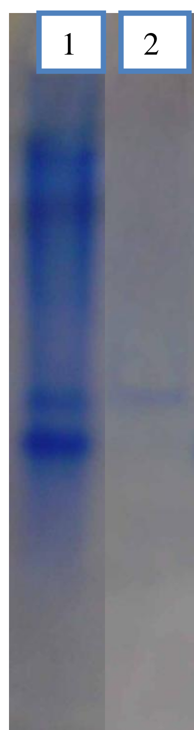

Fig. 2: Native-PAGE analysis of the purified -mannosidase from breadfruit seeds. The sample was loaded onto a 12 % gel. Lane 1, crude extract of breadfruit seeds. Lane 2, purified -mannosidase.

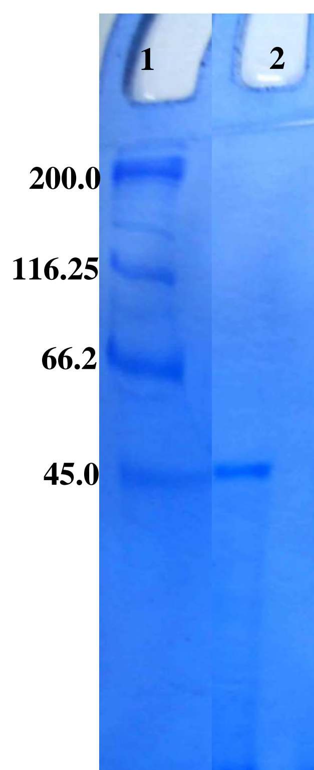

Fig. 3: SDS-PAGE analysis of the purified -mannosidase from breadfruit seeds. The sample was loaded onto a 12 % gel. Lane 1, molecular weight markers. Lane 2, purified -mannosidase. Numbers on the left indicate the molecular weight (kDa) of the makers.

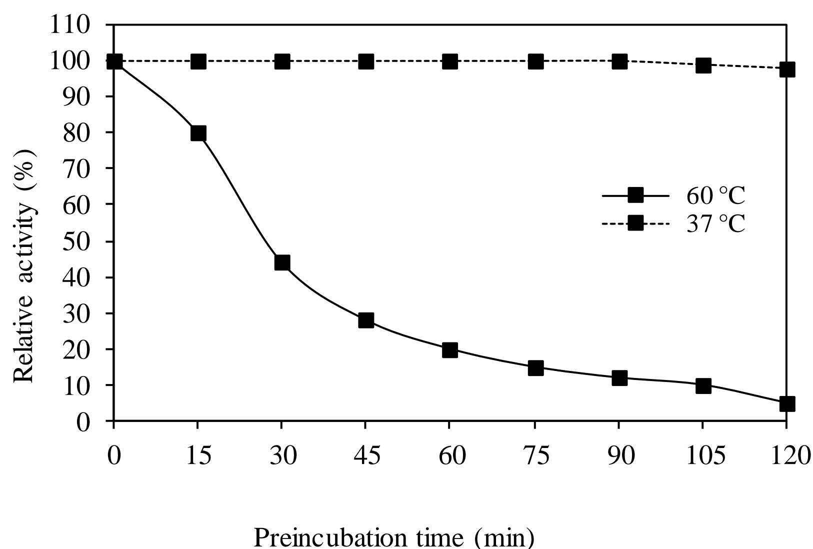

Fig. 4: Thermal inactivation of -mannosidase from breadfruit seeds. The enzyme was preincubated at 37 °C and 60 °C in 100 mM sodium acetate buffer (pH 4.6). At the indicated times, aliquots were withdrawn and the residual activity was measured at 37 °C under the standard test conditions. Values given are the averages from at least three experiments.

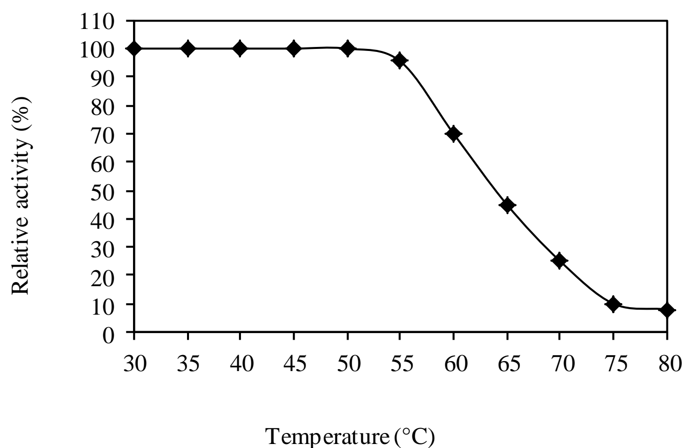

Fig. 5: Thermal denaturation of -mannosidase from breadfruit seeds. The enzyme was preincubated at each temperature for 15 min. The remaining activity was measured at 37 °C under the standard test conditions. Values given are the averages from at least three experiments.

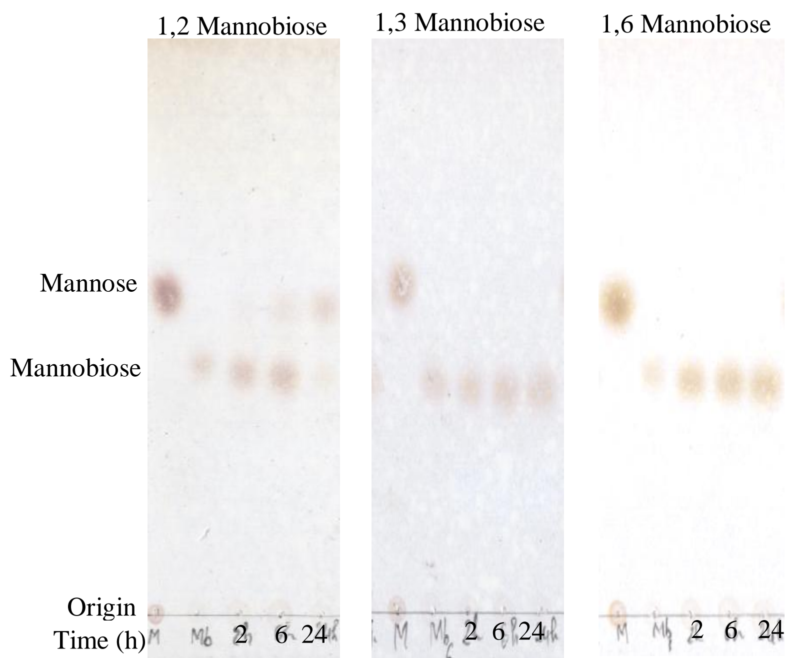

Fig. 6: TLC plates showing the hydrolytic activity of breadfruit -mannosidase towards differently linked disaccharides. It was monitored over 24 h incubation time at 37 °C and at pH 4.6, using butanol-acetic acid-water 9:3.75:2.25 (v/v/v). The plates were developed with naphto-resorcinol in ethanol and H_2SO_4 20 % (v/v). The sugar spots were visualised at 105 °C for 8 min.

3. Discussion

In the present work, we demonstrate that the crude extract of breadfruit seeds contains one □-mannosidase. The enzyme was purified to homogeneity by conventional chromatographies. During breadfruit □-mannosidase purification, three steps were used. The pigments, which are abundant in the crude extract, were almost completely removed during DEAE-Sepharose CL-6B step. The cation-exchange chromatography on CM-Sepharose CL-6B gel and hydrophobic interaction chromatography on Phenyl-Sepharose HP gel were crucial to separate the □-mannosidase from the other proteins and impurities of the crude extract. A Similar result concerning Phenyl-Sepharose gel has been reported for purification of the specific endopeptidase Thr-N from *Archachatina ventricosa* digestive juice (Niamké et al., 1999). As well, the same chromatography steps were used for purification of *Ginkgo biloba* seeds □-mannosidase (Woo et al., 2004). In contrary, only two steps of chromatography were used while purifying tomato and mango □-mannosidases (Suvarnalatha and Prabha, 1999; Yashoda et al., 2007).

The purity of purified □-mannosidase over the crude extract increased by 5.81 fold, with specific activity of 0.97 UI/mg of protein. The molecular weights of the purified enzyme by SDS-PAGE in reducing conditions, and by gel filtration were similar (46 kDa). This observation clearly demonstrates that this □-mannosidase functions in a monomeric structure. Except the rat liver and jack bean □-mannosidases which are tetrameric protein with different subunits (Tulsiani et al., 1982; Kimura et al., 1999), most of □-mannosidases are monomeric polypeptides with molecular weights in range of 51-73 kDa (Bischoff and Kornfeld, 1986). Although breadfruit □-mannosidase molecular weight is lower than those mentioned above, it's consistent with that from mango (Yashoda et al., 2007).

The activity of the enzyme was maximal in acidic region, with *p*NP-□-D-Man as substrat. So, it appears to be comparable to certain lysosomal □-mannosidases in animal cells. Breadfruit seeds □-mannosidase displayed a better stability at pH ranging 4.6 and 5.6. Therefore, the optimum pH is a good compromise between the activity and stability of this enzyme to perform the natural substrates hydrolysis over a long time. The pH stability of □-mannosidase from breadfruit seeds is in range of those reported for other mammalian and plant cells □-mannosidases (4.6-8.0) (Schweden and Bause, 1989; Weng and Spiro, 1993; Hossain et al., 2010; Kumar et al., 2013). The importance of the nature of the buffer should also be noted. The activity of the enzyme was higher in sodium acetate buffer than in citrate phosphate or sodium phosphate buffers. Similar behaviour has been reported for other glycosidases (Kouamé et al., 2001 and 2005; Faulet et al., 2006).

The purified □-mannosidase was checked for other glycosidase activities with some *p*NP-substrates. The results indicated that, the activity of the enzyme is restricted towards a substrate (*p*NP-□-D-Man) normally acted upon by □-mannosidases. Using the same substrate, the K_M value (1.91 mM) of the enzyme was comparable to those from *Candida albican* (Vazquez-Reyna et al., 1999) and from *Phaseolus vulgaris* (Paus, 1977), while the K_M value for bell-pepper □-mannosidase was 0.7 mM (Priya Sethu and Prabha, 1997).

Breadfruit □-mannosidase activity was enhanced by Ca^{2+} and Zn^{2+}. Similar observations have been reported for some plant acidic □-mannosidases (Li and Li, 1972; Pastuszak et al., 1990), and some processing □-mannosidases (Suzumilo et al., 1986; Kimura et al., 1991; Woo and Kimura, 2005). On the other hand, it has been noticed that such divalent ions can also influence enzyme specificity. Indeed, *Ginkgo biloba* □1,2-mannosidase, changed its own substrate specificity, and hydrolyse □1,3/1,6-mannosyl linkage in addition to the □1,2-mannosyl linkage, when 1 mM of Co^{2+} was added to the medium (Woo and Kimura, 2005). Furthermore, the enzyme activity was inhibited by addition of EDTA, like that from watermelon (Nakagawa et al., 1988). This result suggests that, ions would be implicated in enzyme catalytic site, and so, would be essential for hydrolytic activity itself, or these ions would regulate breadfruit □-mannosidase activity.

Breadfruit□□-mannosidase was sensitive to reducing agents (*p*CMB and DTNB), and some detergents (cationic and SDS). These chemicals must be eliminated after treatment of the substrate when this needs to be reduced before its hydrolysis by the enzyme, and must also be avoided when extracting or purifying this enzyme. A similar result has been reported for □-mannosidase from jack bean (Kimura et al., 1999) and the specific endoproteinase Thr-N from *Archachatina ventricosa* (Niamké et al., 2003).

The distinction between the breadfruit □-mannosidase and the other □-mannosidases characterized up to now made possible through their profile to□□-mannosidases inhibitors and substrat specificity. Indeed, SW and DIM (at low concentrations) inhibited the enzyme, while KIF and DMNJ had few or no significant effect, showing that breadfruit□□-mannosidase seems to be KIF-resistant. Concerning breadfruit □-mannosidase hydrolytic activity on mannobiose, the enzyme had □1,2-mannosidase-like activity. A similar result concerning plant □-mannosidases has been reported for □-mannosidase from *Ginkgo biloba* (Woo and Kimura, 2005). A part from *Ginkgo biloba* □□-mannosidase, this is the first plant □-mannosidase which displays such specificity. Based on the substrat specificity, the enzyme could belong to class I □1,2-mannosidases. In contrast, this enzyme was not fairly inhibited by KIF and DMNJ. Since our result formally established □□□1,2) linkage specificity for breadfruit □-mannosidase, this contradiction led us thought that this enzyme should be a class I □1,2-mannosidase, but KIF-resistant.

4. Conclusion

This study led to the isolation of one acidic □-mannosidase from breadfruit seeds. Since the enzyme cleaves only □□-(1,2) mannosyl linkage and seems to be KIF-resistant, it appears different from the other plant acidic □-mannosidases that belong to class I characterized up to now. The specificity of the enzyme suggests that it should be useful for applications requiring selective removal of mannose units and might prove to be a powerful tool for structural and functional analysis of high-mannose type N-glycan.

Acknowledgment

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