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Purification and characterization of a class II α-Mannosidase from *Moringa oleifera* seed kernels

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Abstract α-Mannosidase (EC. 3.2.1.114) belonging to class II glycosyl hydrolase family 38 was purified from *Moringa oleifera* seeds to apparent homogeneity by conventional protein purification methods followed by affinity chromatography on Con A Sepharose and size exclusion chromatography. The purified enzyme is a glycoprotein with 9.3 % carbohydrate and exhibited a native molecular mass of 240 kDa, comprising two heterogeneous subunits with molecular masses of 66 kDa (α-larger subunit) and 55 kDa (β-smaller subunit). Among both the subunits only larger subunit stained for carbohydrate with periodic acid Schiff's staining. The optimum temperature and pH for purified enzyme was 50 °C and pH 5.0, respectively. The enzyme was stable within the pH range of 3.0–7.0. The enzyme was inhibited by EDTA, Hg^{2+}, Ag^{2+}, and Cu^{2+}. The activity lost by EDTA was completely regained by addition of Zn^{2+}. The purified enzyme was characterized in terms of the kinetic parameters K_m (1.6 mM) and V_{max} (2.2 U/mg) using *para*-nitrophenyl-α-D-mannopyranoside as substrate. The enzyme was very strongly inhibited by swainsonine (SW) at 1 μM concentration a class II α-Mannosidase inhibitor, but not by deoxymannojirimycin (DMNJ). Chemical modification studies revealed involvement of tryptophan at active site. The inhibition by SW and requirement of the Zn^{2+} as a metal ion suggested that the enzyme belongs to class II α-Mannosidase.

Keywords *Moringa oleifera* · α-Mannosidase · Con A Sepharose 4B gel · DE-52 (DiEthyl cellulose) · PAS Staining

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Abbreviations

PKM_1	Periyakulam$_1$
DE-52	DiEthyl cellulose-52
Con A Sepharose	Concanavalin A Sepharose
SW	Swainsonine
DMNJ	Deoxymannojirimycin
Glu2SI	Glucosyl Salicilyl Imine
GSI	Galactosyl Salicilyl Imine
GNI	Galactosyl Naphthyl Imine
Glu2NI	Glucosyl Naphthyl Imine
MNI	Mannosyl Naphthyl Imine

Introduction

α-Mannosidases (E.C.3.2.1.24) are widely distributed in animals, plants, microorganisms and the enzyme is an abundant constituent of the plant hydrolytic system [1]. They play a key role in biosynthesis and the turnover of *N*-glycans by hydrolysing terminal α-mannosidic linkages which are involved in important biological activities in the cells [2]. α-Mannosidases have been classified into two independent groups, Class I and Class II, based on the biochemical properties, substrate specificity, inhibitor profiles, catalytic mechanism and characteristic regions of conserved amino acid sequences and sequence alignments. Class I α-Mannosidase belong to glycosyl hydrolase family 47 and are involved in processing (biosynthesis) of *N*-glycans by cleaving specifically α1, 2-mannose residue. These are inhibited by kifunensine (KIF) and 1-deoxymannojirimycin (DMNJ). This enzyme is also involved in processing (degradation) of *N*-glycans by cleaving α1,2-,α1,3-, and α1,6- mannose residue and is inhibited by swainsonine (SW). In a recent study the *Dolichos lablab* enzyme was purified and characterized [3].

To date, several vacuolar α-Mannosidases have been purified and characterized from various legume and non legume sources like *Canavalia ensiformis* [4], *Phaseolus vulgaris* [5], *Medicago sativa L* [6] *Indian lablab* beans [7], *Carica papaya* [8], *Oryza sativa* [9], *Triticale* [10], *Erythrina indica* [11], *Artocarpus communis* [12]. Among these the enzyme from the seeds of *Canavalia ensiformis* (jack bean) has been extensively characterized. The Jack bean α-Mannosidase was isolated by chromatography on concanavalin A, the lectin from the same plant, without involving its sugar binding site [13]. Jack bean α-Mannosidase is a tetrameric protein, with two different subunits (66 kDa and 44 kDa), in which the larger subunit is glycosylated and it is zinc containing metalloprotein [14]. Furthermore, the formation of oligomeric structure with all the subunits present was found to be essential for the enzyme activity that is involved in the degradation of the *N*-glycoproteins.

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-Mannosidase. The metal can be chelated using EDTA with loss of enzyme activity. The activity is regained by supplementing zinc externally[15]. The involvement of the tryptophan residues at the active site was previously demonstrated in *Phaseolus vulgaris* (pinto beans) and Jack bean α-Mannosidase [16].

It has been shown that the levels of the α-Mannosidase increase during seed germination and fruit ripening suggesting its role in removing mannose residues from mannoglycans from the cell wall glycans [17]. The enzyme has been used for the structural elucidation of glycoproteins and glycolipids. In particular, the α-Mannosidase from jack bean whose properties have been extensively characterized has generally been employed in determining the glycan structure [18]. Furthermore, studies were carried out for designing potential glycosidase inhibitors derived from simple carbohydrates. It gives important information regarding the involvement of aromatic, imine and carbohydrate moieties of these inhibitors in effective inhibition [19].

In a recent study a class II α-Mannosidase belonging to glycosyl hydrolase family 38 from *Dolichos lablab* seeds has been studied for its amino acid sequence and *N*-glycan structure [3]. More recently, *de novo* sequencing of Jack bean α-Mannosidase *N*-glycan was done and revealed its high mannose oligosaccharides structure. It has focused on the proximal cysteines which are present in all the acidic α-Mannosidases reported so far in eukaryotes [20]. As part of a general program of work in our laboratory on exploring the non-legume seeds for characterization of biologically important proteins such as lectins and glycosidases, we earlier purified and characterized an acidic α-galactosidase from the *Moringa oleifera* seeds [21]. During the course of these studies, it was found that these seeds also contain significant levels of α-Mannosidase activity. In the present work we describe the purification and biochemical characterization of α-Mannosidase from these seeds.

Materials and methods

Materials

Moringa oleifera seeds (PKM_1) variety was obtained from the local market. *para*-nitrophenyl-α-D-mannopyranoside, other *para*-nitrophenyl-α-D-glycosides, methyl-α-D-mannopyranoside, Sephacryl S-200 HR and phenyl-Sepharose CL-6B were purchased from Sigma-Aldrich, Con A Sepharose 4B gel supplied by GE Healthcare, DE-52 anion exchanger was obtained from Whatman, ready to use standard protein molecular weight marker mixture for SDS-PAGE was obtained from Fermentas. All other chemicals and reagents were of analytical grade and procured from reputed firms.

Enzyme assays

Under the standard test conditions, α-Mannosidase activity was measured by the release of *para*-nitrophenol from the chromogenic substrate *para*-nitrophenyl-α-D-mannopyranoside (*p*NP-α-D-Man) 5 mM, stock. An assay mixture (500 μL) consisting of a 100 mM acetate buffer pH 5.0, 100 μL of a 5 mM *para*-nitrophenyl-α-D-mannopyranoside (1 mM final concentration) and the enzyme solution, was incubated at 37 °C for 30 min. The control contained all reactants except the enzyme. The reaction was stopped after 30 min of incubation by addition of 1 M Na_2CO_3 buffer pH 10.0 to the reaction mixture. Liberated *p*-nitrophenol was measured spectrophotometerically at 405 nm. One unit of enzyme activity was defined as amount of enzyme hydrolyzing 1 μmol of substrate per min. The specific activity of the enzyme was expressed as units per mg protein (IU/mg). All assays were performed in triplicate and results were recorded as the mean of these experiments.

Extraction of *M.oleifera* α-Mannosidase from seeds

Unless otherwise stated, all the purification steps were carried out at 4 °C. Protein was monitored in the column fractions of various chromatographic steps by measuring the A_{280} in a spectrophotometer. Enzyme activities were also monitored as described above.

Seeds of *M.oleifera* (PKM_1) were deshelled just before the extraction and the kernel was ground using a kitchen blender. Seed powder obtained was defatted using chilled acetone, after removal of acetone by centrifugation the solids were air dried at room temperature. Total protein from 50 g of seed powder was extracted overnight with ten volumes of 25 mM Tris–HCl buffer pH 7.4. After extraction the homogenate was

centrifuged at 16,000 x g for 30 min. The pellet was discarded and the supernatant designated as crude extract was subjected to 0–40 % ammonium sulfate precipitation. After collecting the proteins precipitated by centrifugation as described above, to the supernatant was added ammonium sulfate to 80 % saturation. The fraction of 40–80 % precipitate which is found to be rich in α-Mannosidase activity was dissolved in 25 mM Tris–HCl pH 7.4 and dialyzed against the same buffer.

Anion exchange chromatography on DE-52

The dialyzed sample obtained above was applied on to the DE-52 cellulose gel (4×9 cm) that has been previously equilibrated with column buffer (25 mM Tris–HCl pH 7.4). The unbound proteins were removed from the column by washing with five column volumes of the column buffer. The absorbed proteins were then eluted using stepwise gradient of NaCl from 50 to 200 mM NaCl in the same buffer. Fractions (1 mL each) were collected at a flow rate of 60 mL/h and assayed for the enzyme activity. The active fractions containing α-Mannosidase were pooled and concentrated by Amicon concentrator.

Hydrophobic interaction chromatography using phenyl-Sepharose CL-6B

The pooled active fraction obtained above was made to 1.0 M ammonium sulfate and applied on a phenyl-Sepharose CL-6B column (1.5×5 cm) previously equilibrated with 25 mM Tris–HCl pH 7.4 containing 1 M $(NH_4)_2SO_4$. The column was washed with equilibration buffer extensively until no protein is seen in the washings and the bound proteins were then eluted with 25 mM Tris–HCl pH 7.4. Fractions of 1 mL were collected at a flow rate of 1 mL/min and active fractions were pooled together. The pooled fractions were concentrated and were dialyzed against 25 mM Tris–HCl buffer pH 7.4.

Affinity purification on Con-A Sepharose 4B column

The concentrated sample obtained above was applied on to a Con A-Sepharose 4B column (5 ml) previously equilibrated with 25 mM Tris–HCl pH 7.4 containing 0.5 M NaCl (Equilibration buffer). After washing the unbound protein bound α-Mannosidase was eluted with 0.4 M methyl-α-D-mannopyranoside in the same equilibration buffer at 10 mL/h. The elution of protein was monitored by checking absorbance at 280 nm as well as by checking the enzyme activity.

Gel exclusion chromatography on Sephacryl S-200 column

The α-Mannosidase activity rich fractions from the Con A gel were concentrated using centricon and applied onto a Sephacryl S-200 HR column (1×70 cm) previously equilibrated with 25 mM Tris–HCl pH 7.4, containing 150 mM NaCl. The protein was eluted at a flow rate of 10 mL/h. Fractions of 1 ml were collected. The fractions that contain α-Mannosidase activity were pooled and concentrated using Millipore Centriplus YM-30. This enzyme was used in all the studies.

Protein and carbohydrate estimation

Protein concentrations were determined by using commercially available Bradford dye reagent (from Sigma Aldrich), using BSA as the standard [22]. The neutral sugar content of the purified enzyme preparation was determined by phenol sulfuric acid method of Dubois *et al.,* [23] using glucose as standard.

Native molecular weight determination

Native molecular weight of purified *M.oleifera* was determined using Sephacryl S-200 HR column (1×70 cm) size exclusion chromatography, according to the method described by Andrews [24]. Before loading the protein sample the column was calibrated with proteins of known molecular weight *viz*., Catalase (250 kDa), Alcohol Dehydrogenase (150 kDa), Phosphorylase (96 kDa), BSA (66 kDa). The protein was eluted at a flow rate of 10 mL/h. Fractions of 1 ml was collected. The protein elution profile was monitored by absorbance by A_{280}. The graph was plotted as log MW *versus* V_e/V_o. Where: V_0 - Void volume, Ve - Protein elution volume.

Electrophoretic analysis

To check the homogeneity and determine subunit molecular weight, the purified enzyme was analyzed using SDS-PAGE (stacking gel 5 % and separating gel 10 %) under reducing and nonreducing conditions [25], using Fermentas unstained markers as standards. The gels were stained with Coomassie Brilliant Blue R-250. Native-PAGE was performed under non denaturing and nonreducing conditions.

Periodic acid Schiff's staining (PAS)

To determine the carbohydrate nature of the protein separated on the gel, periodic acid – Schiff's staining was carried out following the method of Zacharius *et al.,* [26], with minor modifications. SDS-PAGE gel was stained in 1 % periodic acid in 3 % acetic acid for 1 h. The gel was washed for 1 h with water and stained with Schiff's reagent for 30 min in dark. It was then destained with 10 % acetic acid, finally scanned and stored in 3 % acetic acid.

Effect of pH and pH stability

The effect of pH on enzyme activity was determined at 37 °C within a pH range of 2 to 8, using 0.1 M Citrate buffer (pH 2–3), 0.1 M Sodium acetate (pH 4–5), 0.1 M Sodium phosphate (pH 6–7), 0.1 M Tris–HCl (pH 8). Stability of the enzyme at various pH values is determined by incubating the enzyme in different buffers in the pH range from 2 to 8 at 37 °C for 12 h. After incubation the residual enzyme activity was subsequently assayed using standard assay conditions.

Effect of temperature and thermal stability

Determination of optimum temperature for the *M.oleifera* α-Mannosidase was performed with *para*-nitrophenyl-α-D-mannopyranoside (5 mM) in 100 mM Sodium acetate buffer pH 5.0 using incubation temperatures in the range of 30 °C to 90 °C. Thermal stability was determined by incubating the enzyme at 50 °C, 60 °C, 70 °C, 80 °C for 60 min, an aliquot was drawn at regular intervals and immediately cooled. The residual enzyme activity was determined by standard assay method.

Effects of EDTA and metal ions

The effect of EDTA and various divalent metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, CO^{2+}, Cu^{2+}, Hg^{2+}, Ag^{2+}, Fe^{2+}) on the activity of the α-Mannosidase was carried according to Bloom *et al.,* [15]. The effect of different metal ions on α-Mannosidase activity was tested both with and without removal of preexisting ions by EDTA treatment. After EDTA treatment the enzyme was dialyzed against Sodium acetate buffer pH 5.0. The effect of metal is tested by pre-incubating the enzyme in 100 mM Sodium acetate buffer pH 5.0 containing 1 mM concentration of each metal ion for 1 h prior to addition of the substrate (for EDTA 10 mM), and the residual enzyme activity was assayed by standard method. The enzyme activity without any metal ion is considered as a control (100 %).

Substrate specificity

The relative substrate specificity of α-Mannosidase towards various synthetic substrates *para*-nitrophenyl-α-D-mannopyranoside, *para*-nitrophenyl-α-D-glucopyranoside, *para*-nitrophenyl-α-D-fucopyranoside, *para*-nitrophenyl-α-D-galactopyranoside was determined. Substrates were prepared in 100 mM sodium acetate buffer pH 5.0 at final concentration of 5 mM (stock solution). The reaction was carried out using fixed concentration of enzyme and substrate under standard assay conditions. Relative activity on various substrates is expressed as percentage of the activity calculated with *para*-nitrophenyl-α-D-mannopyranoside as a substrate (100 %) with which enzyme showed maximum activity.

Determination of Kinetic parameters

The Michaelis–Menten kinetic parameters (K_m and V_{max}) were determined by incubating the enzyme at optimum temperature/pH with different concentrations of the substrate.

Chemical modification of tryptophan residues using *N*-bromosuccinimide (NBS)

Chemical modification studies were carried out to get information about the involvement of tryptophan in the active site of the enzyme. The tryptophan modifier, NBS 10 mM, (prepared in 100 mM sodium acetate buffer pH 5.0 was added in increments of 2 μL each time to the purified enzyme (0.3 mg/mL). After addition of this the residual enzyme activity was determined using the standard enzyme assay. The changes associated with the steady-state fluorescence of α-Mannosidase due to NBS modification was monitored both in presence and absence of substrate (methyl-α-D-mannopyranoside) using Perkin Elmer LS 55 fluorescence spectrophotometer, excitation at 280 nm and emission spectra was recorded in the range of 310–450 nm. The fluorescence spectra were measured at room temperature with a 1-cm path length cell. The monochromator slit width was kept at 1.5 nm in excitation and emission measurements.

Effect of Mannosidase specific inhibitors on the activity of α-Mannosidase from *Moringa oleifera* seeds

To determine whether the purified *M.oleifera* α-Mannosidase belongs to class I or II Mannosidase, specific inhibitors Deoxymannojirimycin (DMNJ) specific for class-I and Swainsonine (SW) specific for class-II were used. The purified enzyme was incubated with these for 30 min and the residual enzyme activity was determined using standard assay.

Inhibitory studies using transition state analogs

Preparation of various synthetic glycoconjugates (inhibitors) and performing the enzyme assay was as described earlier Kumar *et.al*, [19] using C1-/C2-aromatic-imino-glyco-conjugates of D-galactose, D-mannose and D-glucose (Glu2SI-Glucosyl Salicilyl Imine, GSI-Galactosyl Salicilyl Imine, GNI-Galactosyl Naphthyl Imine, Glu2NI-Glucosyl Naphthyl Imine, MNI-Mannosyl Naphthyl Imine), taking 50 μL of diluted purified enzyme with increasing amounts of the glycosidase inhibitor (*i.e.*; from 0 to 3 mM final conc.) and incubating at 37 °C for 20 min. Enzyme assay was performed under standard assay condition. The activity without the inhibitor was considered as 100 % and the remaining activities at each concentration of inhibitor were determined with reference to this value.

Results

Purification of α-Mannosidase

The ammonium sulfate precipitated protein obtained in the 40–80 % saturation was subjected to ion exchange chromatography when the α-Mannosidase was completely bound on the gel and could be eluted using different concentrations of the NaCl. As shown in Fig. 1a, much of the activity was recovered in the 100 mM NaCl which was pooled, concentrated and subjected to phenyl Sepharose chromatography as described under methods. After elution of the enzyme from this gel with 25 mM Tris–HCl buffer pH 7.4, the protein was passed through Con A Sepharose gel and the bound enzyme could be eluted completely with 0.4 M methyl-α-D-mannopyranoside in 25 mM Tris–HCl pH 7.4 containing 0.5 M NaCl (this concentration was used to ensure complete desorption of the bound enzyme). These eluates were pooled, concentrated and applied on a Sephacryl S-200 gel and the enzyme eluted as a single peak from this gel (Fig. 1b). Purification of the enzyme is summarized in Table 1. It is apparent that nearly 36.6 fold purification with 6.2 % yield was obtained through this purification process.

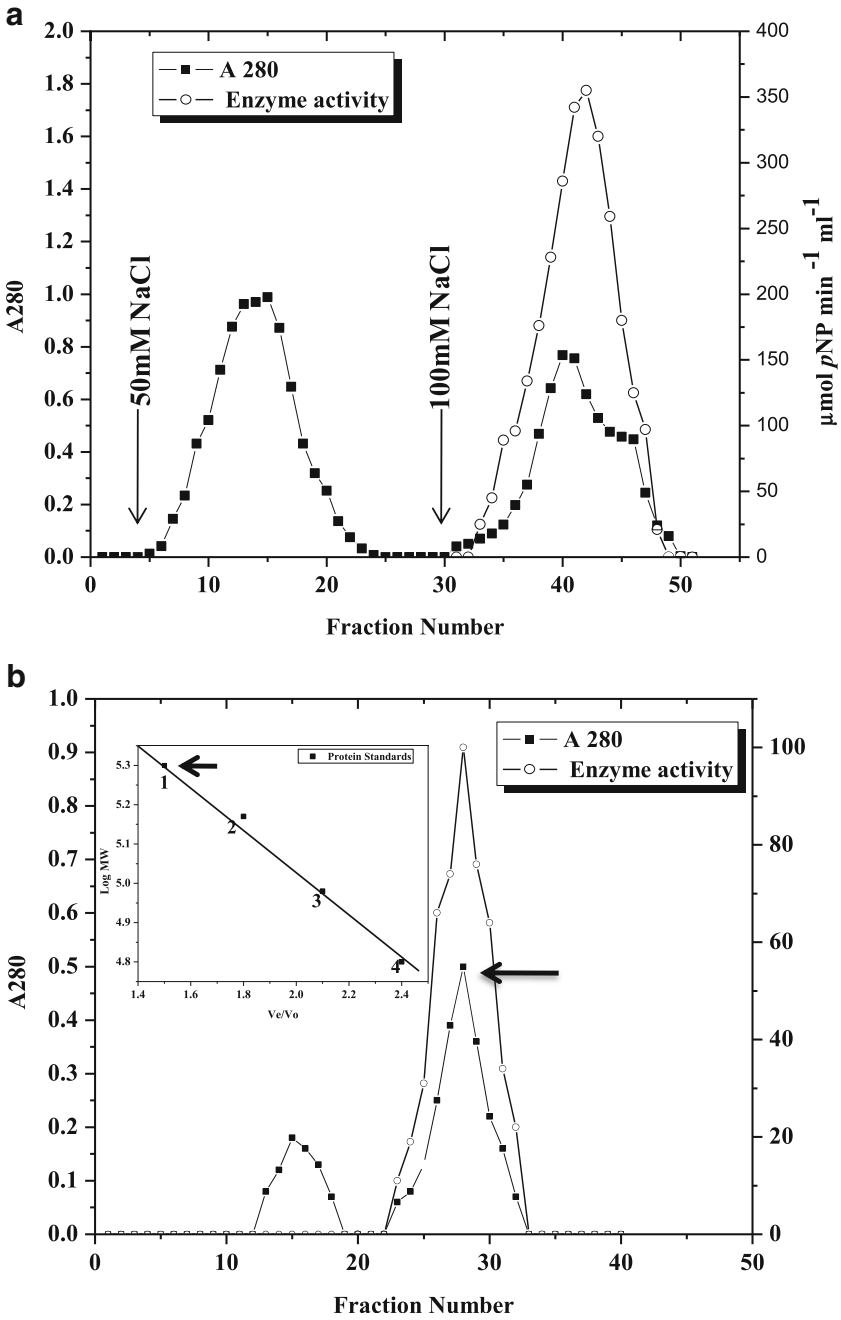

Fig. 1 **a & b**. Elution profile of *M.oleifera* α-Mannosidase on DE-52 column (**a**), Gel filtration and molecular weight determination using S-200(**b**): **a**. Fractions of 1 mL were collected and monitored absorbance at 280 nm. *Arrow* indicates point at which 50 mM and 100 mM NaCl is applied. **b**. The protein was eluted at 10 mL/h with 25 mM Tris–HCl buffer pH 7.4 containing 150 mM NaCl. The eluted fractions were checked for the enzyme activity. *Arrow* indicates the point where *M.oleifera* α-Mannosidase was eluted

Table 1 Purification of α-Mannosidase from *Moringa oleifera* Seeds

Purification step	Total Protein (mg)	Total activity [b] (units)	Specific activity [c]	Yield (%)	Fold purification
Crude extract [a]	1440	1435	0.99	100	1
40–80 % Ammonium sulfate	520	1224	2.3	85.2	2.3
DE-52 cellulose	120	846	7	58.5	7.07
phenyl-Sepharose CL-6B	15	425	28.3	29.6	28.5
Con-A Sepharose	7	220	31.4	15.3	31.7
Sephacryl- S 200	2.5	90	36	6.2	36.6

[a] 50 g of defatted *Moringa oleifera* seed kernel powder was used at a time and the purification in different chromatographic steps was carried out batch wise. Proteins were measured in chromatographic fractions using A280

[b] One unit of enzyme activity was defined as amount of enzyme hydrolyzing 1 μmol of *para*-nitrophenol from *pNP*-G per min at pH 5.0 and 37 °C

[c] The specific activity of the enzyme was expressed as units per mg protein. (U/mg of protein)

Molecular properties of the *M.oleifera* α-Mannosidase

The purified native *M.oleifera* α-Mannosidase exhibited a molecular mass of ~230–240 kDa on S-200 gel filtration [inset Fig. 1b]. In 10 % SDS-PAGE, the enzyme dissociated into two subunits with molecular masses of 66 kDa (α-larger subunit) and 55 kDa (β-smaller subunit), under both reducing and non-reducing conditions [Fig. 2a]. An additional band at 116 kDa was also observed which has been discussed in the later section. When the SDS-PAGE gel was stained for carbohydrate, using periodic acid Schiff's reagent only the larger subunit was found to be glycosylated [Fig. 2b]. Additionally the activity assay for the purified enzyme in a native PAGE confirms the protein purified is the α-Mannosidase [Fig. 2c]. The purified α-Mannosidase was found to contain 9.3 % carbohydrate as estimated by phenol sulphuric method.

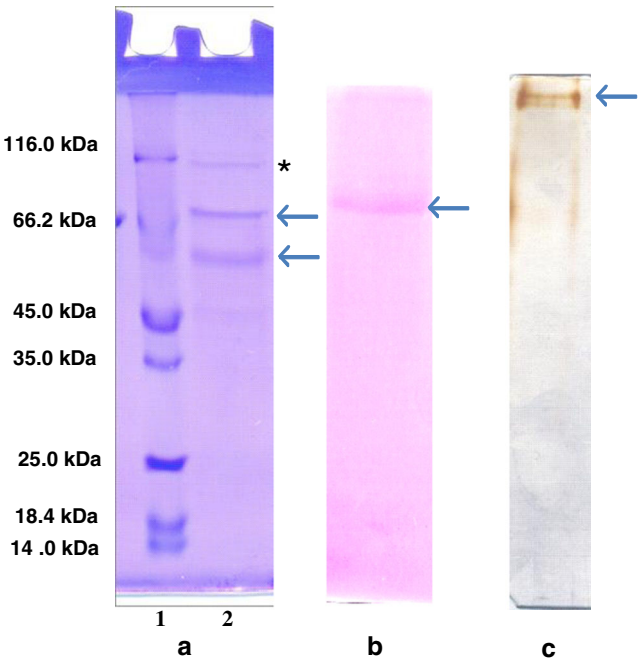

Fig. 2 **a**, **b** & **c**. 10 % SDS-PAGE (**a**), Periodic acid Schiffs (PAS) staining (**b**) and Native PAGE (**c**) of *M.oleifera* α-Mannosidase: **a.** 10 % SDS-PAGE of purified *Moringa oleifera* α-Mannosidase: Lane 1: Standard molecular weight markers, Lane 2: Purified α-Mannosidase under non-reducing conditions. The standard protein molecular weight markers used were; β- galactosidase (166.0 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), lactate degyhydrogenase (35.0 kDa), REase Bsp 981 (25 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa). **b** The same gel was run in duplicates under reducing conditions and stained with Schiffs reagent (PAS staining). Arrow corresponds to the larger subunit (66 kDa) protein in the SDS-PAGE. **c.** Native-PAGE of the purified *M.oleifera* α-Mannosidase used for the activity staining. *Asterisk* indicates possible 116 kDa precursor form of Mannosidase containing mannose binding domains. (As identified with MALDI-TOF; MS/MS analysis)

Biochemical properties of *M.oleifera* α-Mannosidase

The purified *M.oleifera* α-Mannosidase showed optimal enzyme activity at pH 5.0 [Fig. 3a] and stability from pH 3 to 7 with more than 80 % of the enzyme activity remained at this pH, after 12 h of incubation [Fig. 3b]. The temperature optimum of this enzyme is at 50 °C [Fig. 3c]. More than 80 % of activity is seen from 40 to 60 °C. Thermo stability of the enzyme was examined by incubation of the enzyme at various temperatures and the residual enzymatic activity was measured at regular intervals of time for 60 min. As it can be observed from [Fig. 3d], the result indicates more than 70 % of the activity is retained after incubation at 50 °C for 60 min. Enzyme completely lost its activity at 80 °C after 1 h of incubation and only 26 %, and 12 % activity is remained at 60 and 70 °C, respectively.

Effect of EDTA and various metal ions

The effect of different divalent cations and EDTA on α-Mannosidase activity was tested both with and without removal of pre-existing ions by EDTA treatment. The results are summarized in Table 2. EDTA treatment leads to 56 % loss of the activity after 1 h of incubation in the acidic medium. Prolonged incubation leads to complete loss of the activity. This activity is completely restored by addition of Zn^{2+} only in

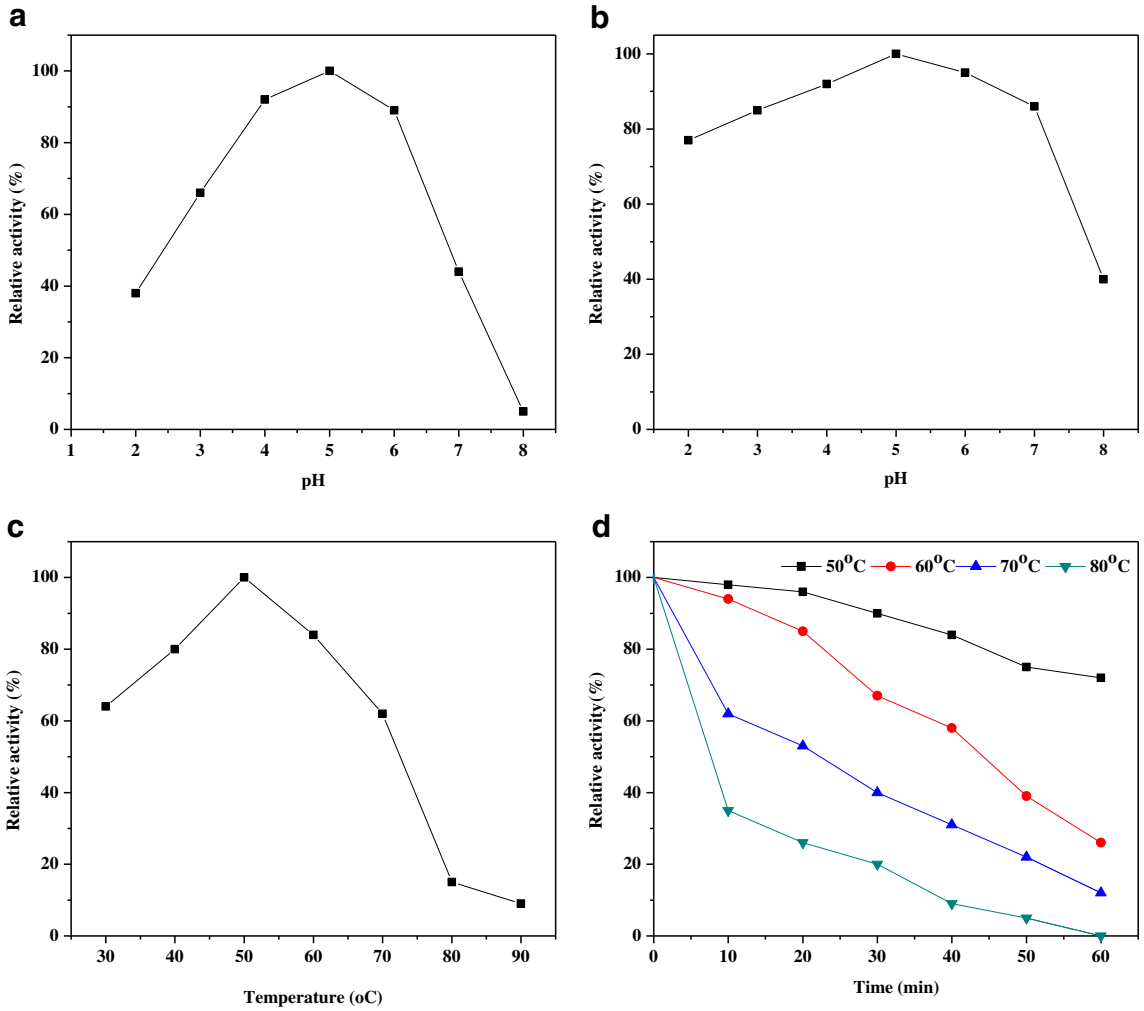

Fig. 3 a, b, c & d. Characterization of the enzymatic properties of purified α-Mannosidase from *Moringa oleifera*. (**a**) Effect of pH on the α-Mannosidase activity of *M. oleifera* was determined at 37 °C in buffers ranging from pH 2.0 to 8.0. The value obtained at pH 5.0 were α-Mannosidase activity is maximum was taken as 100 %. (**b**) pH stability of *M.oleifera* α-Mannosidase was measured after pre-incubation of the enzyme at 37 °C for 12 h in buffers ranging from pH 2.0 to 8.0. The activity of an untreated enzyme sample at pH 5.0 was defined as 100 %. (**c**) Effect of temperature on α-Mannosidase activity was determined in 100 mM NaOAc buffer (pH 5.0) at 30–90 °C. The value obtained at 50 °C was taken as 100 %. (**d**) Thermo stability of α-Mannosidase was determined by measuring α-Mannosidase activity under standard assay conditions after pre-incubation of the enzyme in 100 mM NaOAc buffer (pH 5.0) at 50, 60, 70 and 80 °C for various periods. The activity of an unheated enzyme sample was defined as 100 %

EDTA treated samples. In presence of Cu^{2+}, Hg^{2+} and Ag^{2+} 97 %, 90 % and 92 % of the enzyme activity was lost in non-EDTA treated samples. However, other metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} did not show any significant loss of activity.

Substrate specificity

The specificity of purified α-Mannosidase was ascertained as it cleaved only the *para*-nitrophenyl-α-D-mannopyranoside while it had no effect on other glycosidase substrates. The K_m and V_{max} values were measured using this substrate and was found to be 1.6 mM and 2.2 U/mg, respectively.

Chemical modification studies

The α-Mannosidase activity was completely inhibited at 10 mM concentration of NBS. Modification of tryptophan residues by NBS resulted in total quenching of fluorescence. Modification of tryptophan residues by NBS also associated with the blue shift quenching of the fluorescence spectrum [Fig. 4a]. However, in presence of substrate (methyl-α-D-mannopyranoside) total quenching was protected [Fig. 4b].

Effect of Mannosidase specific inhibitors

The effect of Mannosidase specific inhibitors such as deoxymannojirimycin (DMNJ) and swainsonine (SW) on enzyme activity is shown in Table 3. The enzyme activity

Table 2 Effect of divalent metal ions and EDTA on the *Moringa oleifera* α-mannosidase activity

Effector Agent	Relative Activity (%)	
	Non-EDTA treated	EDTA treated
Control	100	100
EDTA [a]	44	———
Ca^{2+}	97	96
Mg^{2+}	92	81
Mn^{2+}	86	83
Zn^{2+}	101	203
Fe^{2+}	65	78
Cu^{2+}	3	0
Co^{2+}	36	45
Hg^{2+}	10	0
Ag^{2+}	8	0

[a] EDTA concentration 10 mM, all other metal ions 1 mM, untreated enzyme without metal ion is taken as control 100 %

was completely inhibited by SW at 0.001 mM (1 μM) concentration. Whereas, DMNJ at the same concentration did not influence the enzyme activity. At 0.5 mM concentration of DMNJ showed only 31 % inhibition.

Inhibition by glycoconjugates

The inhibition results of the glycoconjugates are shown in Fig. 5a. Among the number of inhibitors tested for their inhibitory effect on enzyme activity, the naphthylidene glycoconjugates of mannose, glucose and galactose exhibit 100 % inhibition at 1.0 mM, 2.0 mM, and 2.0 mM concentration, respectively. The salicylidene-conjugates of the same sugars exhibit 100 % at 3.0 mM concentration. Concentration at which a 50 % inhibition of enzyme (IC_{50}) is obtained by glyco-conjugates is shown in Fig. 5b.

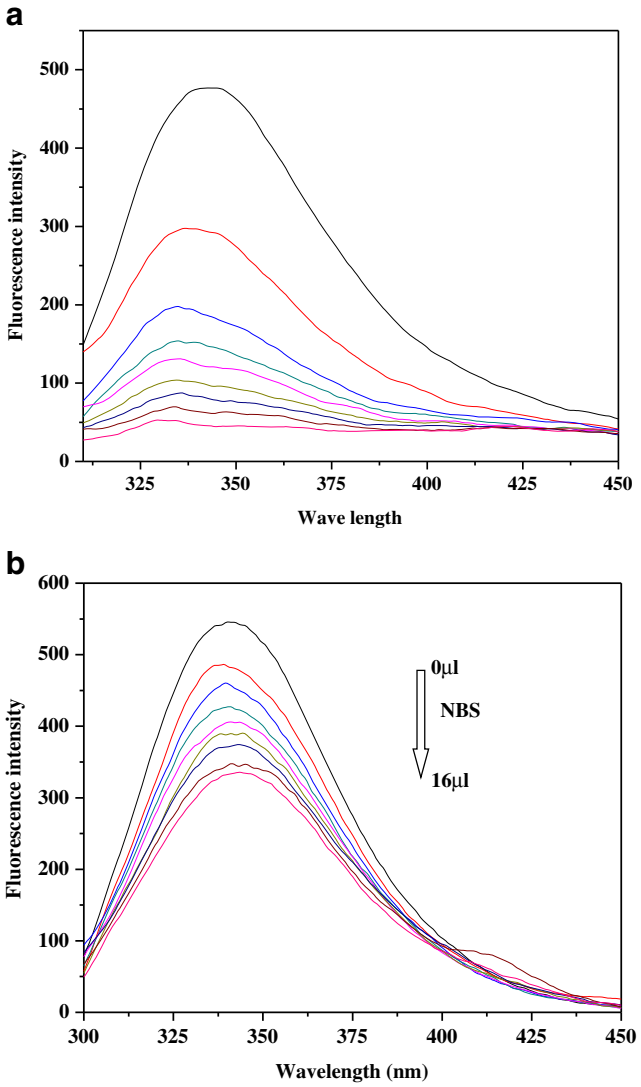

Fig. 4 a & b. Influence of NBS on *Moringa oleifera* α-Mannosidase: Fluorescence spectra of *M.oleifera* in absence (**a**) and presence (**b**) of substrate. The Fluorescence was recorded by excitation at 280 where, maximum emission was observed and emission was recorded in the range of 310–450 nm

Discussion

In the frame work of our interest in the isolation, purification and biochemical characterization of biologically important proteins with a long term objective to study their structure-function relationships, we have recently characterized a coagulant protein and α-galactosidase from the seeds of the non-legume plant *M.oleifera* [21]. The present manuscript describes the first isolation, purification and biochemical characterization of a class II α-Mannosidase from the seed kernels. The ammonium sulfate fractionation 0–40 % allowed separation of the bulk of the small cationic proteins from the seed extract and the 40–80 % fraction contained glycosidases among other proteins [27]. The α-Mannosidase activity from this was partially purified by ion exchange chromatography where the enzyme activity could be eluted with 100 mM NaCl and this is relatively free of other glycosidases. Subsequent hydrophobic chromatography revealed strong binding of the Mannosidase to the gel, consistent with our earlier observations for other glycosidase. Further binding of the enzyme on Con A-Sepharose gel and its specific elution with high sugar concentration reveals not only its glycoprotein nature but its specificity towards Con A. The single protein peak eluted from the gel filtration column with overlapping enzyme activity strongly suggests its homogeneous nature. This further allowed us to determine the native molecular mass of the enzyme to be ~230 kDa. Native PAGE analysis of the enzyme revealed that it is homogeneous and the band exhibited

Table 3 Effect of Mannosidase specific inhibitors on the activity of α-Mannosidases from *Moringa oleifera* seeds

Inhibitors	Concentration(mM)	Relative activity (%)
Control	0	100 %
Swainsonine (Class II)	0.001	5 %
	0.01	ND
	0.1	
	0.5	
Deoxymannojirimycin (Class I)	0.001	100
	0.01	99
	0.1	78
	0.5	69

Deoxymannojirimycin a class I and Swainsonine a class II inhibitor is used at the concentration of 1 μM

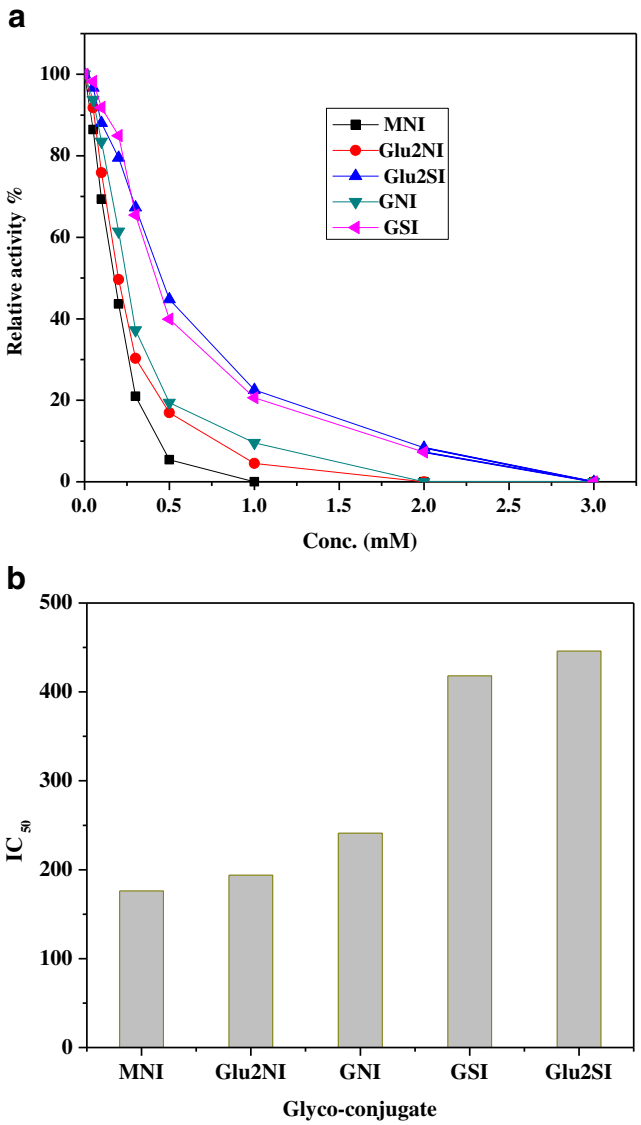

Fig. 5 Enzyme inhibition using Glyco-conjugates (**a**), IC_{50} values (**b**): (**a**) C1-/C2-aromatic-imino-glyco-conjugates of D-galactose, D-mannose and D-glucose: The activity without the inhibitor was considered as a 100 %. The relative activity at each concentration was measured. **b** IC_{50} values of these glyco-conjugates

enzyme activity. However on SDS-PAGE under reducing and non-reducing conditions the purified enzyme dissociated into two major subunits of molecular mass 66 kDa and 55 kDa, suggesting this enzyme to be tetrameric. Additionally a protein band was also seen at 116 kDa which is discussed below. The general subunit pattern of the purified enzyme closely resembles that of Jack bean and tomato α-Mannosidase. However, the subunit masses differ from these species. Jack bean enzyme has two subunits of 66 kDa and 44 kDa, whereas, tomato enzyme has subunits of 70 kDa and 47 kDa respectively [1, 2]. Under both reducing and non-reducing conditions the enzyme showed similar band pattern suggesting the interaction between the subunits to be non-covalent which is a common feature as reported earlier [14]. Monomeric forms of Mannosidase were also observed in some plant species like *Artocarpus communis* seeds (Isoform I-75 kDa; II-61 kDa), *Erythrina indica* seeds (127 kDa) [12, 17]. However *Ginko biloba* seeds α-Mannosidase is 340 kDa in the native form and subunit of 120 kDa [28]. In closely related members of *M.oleifera* order like tropical fruit babaco (brassicales) oligomeric form of α-Mannosidase was observed [15]. *Medicago sativa* and *Prunus serotina* Ehrh enzyme [6, 29] also has four subunits with different molecular masses. *M.oleifera* is a glycoprotein as detected by periodic acid-Schiff staining. This was further confirmed to contain 9.3 % carbohydrate. This carbohydrate content is more than that of Mannosidase from *Erythrina indica* seeds [17]. *M.oleifera* α-Mannosidase when stained with PAS staining, it was observed that only the larger subunit got stained with the Schiffs reagent indicating, larger subunit is glycosylated, it is also observed with jack bean α-Mannosidase [14]. The purity of the α-Mannosidase was verified by native PAGE (silver staining) and showed one homogeneous band. The enzymatic identity of this band was verified by cutting identical unstained lanes of the same gel, equivalent to band in Fig. 2c, into small pieces and performing activity assays. By relating the presence of activity with the respective position on the gel, the band responsible for the α-Mannosidase activity could be identified. SDS-PAGE of the purified enzyme showed one additional band at 116 kDa region, which was consistently seen in different batches of the purified enzyme. This protein band when subjected to MALDI analysis for the identification of the protein, it was confirmed to be a 116 kDa protein containing a mannose binding domain in it [results not shown]. This results guide to interesting findings which are reported on class II α-Mannosidase. In humans lysosomal α-Mannosidase the initial enzyme is synthesized as a polypeptide of 110 kDa that is subsequently processed into two subunits of 40–46 kDa and 63–67 kDa, which then constitute

the native protein (molecular mass, 210 kDa). The rat Golgi α-Mannosidase II is a dimer composed of 124 kDa subunits. Treatment with chymotrypsin causes limited proteolysis to give a dimer of 110 kDa subunits that retains full activity. Some studies reported the possibility that the jack bean α-Mannosidase is also synthesized as a polypeptide chain of 110 kDa, which forms a dimer. The fragments of mass 44 kDa and 66 kDa, observed by SDS-PAGE, could be due to limited proteolysis of the protein, which is still able to maintain its integrity unless exposed to denaturing conditions [30]. More recent studies on the *Dolichos lablab* enzyme from our laboratory revealed the subunit molecular mass of the protein to be 116 kDa in SDS-PAGE [3].

The optimum pH of *M.oleifera* α-Mannosidase enzyme was around pH 5.0 when tested with *p*ara-nitrophenyl-α-D-mannopyranoside as a substrate which is similar to those of other plant α-Mannosidases reported [2, 12]. Some enzymes have pH optimum between the range of pH 4.0–5.0 [9]. The optimal pH for the almond α-Mannosidase was around pH 3.8 [18]. The enzyme was active at acidic pH and activity decreased as the pH approached the alkaline range pH 8.0. This is within the range of reported values of α-Mannosidase from tropical fruit *Babaco* and *Capsicum* [15, 31]. It is interesting to note that the activity of the glycosidases is high at acidic pH and the pH that naturally exists in the protein bodies of the seeds where these enzymes are co-localized with storage proteins, lectins. This suggests possible *in vivo* physiological significance of their co-localizations that may be important during seed growth and development. The optimum temperature of this α-Mannosidase was found to be 50 °C, above this temperature, enzyme activity declined rapidly as the temperature increased, but the enzymes were not completely inactivated even at 80 °C after 30 min of incubation. Further the thermal stability was studied by incubating the enzyme at different temperatures for 60 min. The enzyme is quite stable to 50 °C for 60 min with more than 75 % of the activity retained. Even when it was incubated at 70 °C for 40 min 30 % of the original activity remained. This high temperature stability might possibly be due to the tetrameric nature of the enzyme as well as its glycoprotein nature.

Among several metal ions incubated with the EDTA treated sample, only Zn (II) is reported to restore the total activity of the enzyme. The study of inactivation of α-Mannosidase by EDTA and its reversion leads to the conclusion that α-Mannosidase is a zinc-containing metalloprotein, as observed for other α-Mannosidase from various plant species [15]. The role played by Zn^{2+} in the enzymatic reaction has not yet been elucidated. The zinc atoms may be involved in subunit interactions as has been shown for some enzymes. Cu (II), Hg (II) and Ag (II) are potent inhibitors of some plant α-Mannosidase [28], whereas Mn (II), Mg (II) and Ca (II) have no effect on the α-Mannosidase.

The effect of divalent cations is a useful parameter to distinguish among different α-Mannosidases. Members of family 47 can be stimulated by Ca^{2+} ions whereas activity of class II α-Mannosidase of family 38 exhibit diverse forms of metal ion dependency, cadmium activates the α-Mannosidase activity in *T.maritima* [32], Co (II) is the preferred cofactor for the α-Mannosidase from insect and *Bacillus sp.* [33], while the activity of the enzymes from jack bean require Zn (II) ions [34]. The anionic detergent like SDS showed complete inhibition on the enzyme activity at 1 mM concentration that can be attributed for the partial or complete disruption of the higher order structures of the enzyme. About 73 % of the original activity remained at 10 mM concentration of mannose, being a product analog it showed inhibition at higher concentration.

The purified α-Mannosidase was free of any other contaminating glycosidases. Preliminary experiments were done to know the involvement of tryptophan in the active site of the enzyme as tryptophan residues are essential for substrate binding in many glycosidases, including lysozyme, glucoamylase, cellulase and xylanase. Modification of the enzyme by NBS resulted in complete loss of activity suggesting the role of tryptophan in the catalytic activity of the enzyme. When the enzyme was incubated along with the substrate quenching of fluorescence is incomplete suggesting the role of tryptophan in the active site of *M.oleifera* α-Mannosidase. Some studies showed that addition of a substrate like *para*-nitrophenyl-α-D-mannopyranoside to the enzyme prior to NBS treatment protected the enzyme. In jack bean 4 tryptophan residues are present per enzyme monomer that is involved in enzyme activity. In *Canavalia ensiformis* and *Erythrina indica* seeds in addition to some amino acids, tryptophan residues present at the catalytic site have been shown to be essential for enzyme activity [16, 17].

We checked the effect of Mannosidase specific inhibitors such as SW, DMNJ on the α-Mannosidase activity. The activity was totally lost in presence of SW a furanose analogue at very low concentrations. While, at the same concentration DMNJ a pyranose analogue didn't show any effect. Studies have shown that at pH 5.0 the optimum pH of breadfruit α-Mannosidase, SW binds strongly to the catalytic centre of the enzyme due to ionization of the SW which, in turn acts as the strong inhibitor of the enzyme [12]. Furthermore, the α-Mannosidase purified from seeds of *M.oleifera* is a class II α-Mannosidase since it is sensitive to furanose transition state analog SW. Similar results were also observed for the enzyme from rice, bread fruit α-Mannosidase and Jack bean [9, 34]. The inhibition of enzyme activity caused by various synthetic glycoconjugates revealed that naphthylidene-conjugates were more potent inhibitors then the salicylidene-conjugates of the simple sugars. These results indicate that the naphthylidene-conjugates have more affinity to bind the enzyme and cause the inhibition. Similar results were reported with the Jack bean

α-Mannosidase [19]. It is interesting to note that the two Mannosidases purified from different plant families, legume (*Canavalia ensiformis*) and non-legume (*M.oleifera*) exhibit similar sugar binding properties suggesting that they have possibly similar amino acid sequence that permit efficient binding of bulky groups in synthetic glycoconjugates. These results state that they are highly conserved among different families of the plant kingdom.

In summary, this is the first report describing the purification, biochemical properties of an α-Mannosidase from the *Moringa oleifera* seeds. Several lines of evidence suggest that it is a class II enzyme belonging to GH family 38. First, it binds strongly to phenyl Sepharose gel and Con A Sepharose gel. Second, its native molecular mass is >2,00,000 and is possibly tetrameric in nature and is a glycoprotein. Third, it is a metallo enzyme and is thermo stable and tryptophan is involved in the active site. Fourth, it is specifically inhibited by Swainsonine and by the synthetic glycoconjugates, naphathalidine glycoconjugates of mannose, galactose and glucose were potent inhibitors of enzyme activity. This study provided the basic information of this newly purified enzyme and to advance our knowledge on the structure and function of this enzyme it is essential to obtain the sequence information that would further establish the relatedness of this enzyme to other enzymes reported and also explore the application of this enzyme in food and biotechnology which is the future direction of our work.

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