Protein Expression and Purification 89 (2013) 7–15

Contents lists available at SciVerse ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Characterization of α-mannosidase from *Dolichos lablab* seeds: Partial amino acid sequencing and *N*-glycan analysis

B.S. Gnanesh Kumar [a], G. Pohlentz [b], M. Mormann [b], N. Siva Kumar [a,*]

[a] *Protein Biochemistry and Glycobiology Laboratory, Department of Biochemistry, University of Hyderabad, Hyderabad 500046, India*
[b] *Institute for Hygiene, Westfälische Wilhelms-University, Münster D-48149, Germany*

ARTICLE INFO

Article history:
Received 28 November 2012
and in revised form 4 February 2013
Available online 16 February 2013

Keywords:
De novo sequencing
Dolichos lablab
Glycosyl hydrolases
N-glycans
Mass spectrometry

ABSTRACT

α-Mannosidase is a key enzyme in processing and degradation of *N*-glycans in plants and animals. In the present study α-mannosidase from crude extracts of *Dolichos lablab* (Indian beans) has been purified by ammonium sulfate precipitation, anion exchange, galactose Sepharose, phenyl Sepharose, gel permeation and Con A Sepharose chromatography. The purified protein migrated as a single band corresponding to 116 kDa on SDS–PAGE under reducing conditions. The pH and temperature optima of α-mannosidase activity determined by use of *p*-nitrophenyl-α-D-mannopyranoside as substrate were found to be 5.0 and 60–65 °C, respectively. The K_M was 1.48 mM and swainsonine was a potent inhibitor of the enzyme with IC_{50} value 50–80 nM. Additionally, the *de novo* amino acid sequencing showed active site regions highly conserved among other plant acidic α-mannosidases and yielded sequence coverage of approximately 32.5%. *N*-glycopeptide analysis revealed the presence of paucimannosidic type structure in a conserved *N*-glycosylation site as well as at least one oligo mannosidic glycan at an undetermined site after ZIC-HILIC enrichment of proteolytic glycopeptides. The partial biochemical and molecular characterization of this enzyme reveals that it is a class II α-mannosidase from the glycosyl hydrolase family 38.
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Introduction

α-Mannosidases (EC 3.2.1.24) hydrolyse terminal α-mannosidic linkages from both the high mannose and complex type *N*-glycans present in glycoproteins. Based on sequence similarity and inhibition, they are broadly divided into two classes. Class I α-mannosidases belong to glycosyl hydrolase family 47 and are involved in processing of *N*-glycans and quality control. These are located in endoplasmic reticulum and Golgi bodies (Golgi mannosidase I :GMI)[1]. This enzyme has specificity to α1,2 linked mannose (EC 3.2.1.113) and is inhibited by kifunensine (KIF) and 1-deoxymannojirimycin (dMNJ)[1]. In plants, this enzyme has been purified from mung bean seedlings and in *Glycine max* α-mannosidase I gene was identified and expression studies revealed its sub cellular localization [2,3]. There are three subcategories of class II α-mannosidases belong to the glycosyl hydrolase family 38: the Golgi, the cytosolic and the lysosomal/vacuolar α-mannosidase. The Golgi α-mannosidase (GMII) is also involved in processing of *N*-glycans with specificity to α1,3-1,6 linked mannose (EC 3.2.1.114). This enzyme was purified to homogeneity from mung bean seedlings [4] and in *Arabidopsis thaliana* GMII gene was identified and characterized at the molecular level [5]. This class α-mannosidases are also involved in turnover or degradation of *N*-glycans present in cytosol and lysosomes of animals or protein storage vacuoles in plants [1,6]. These acidic α-mannosidases have a broad specificity for α1,2-, α1,3-, and α1,6-linked mannose residues and are inhibited by swainsonine [1].

Several vacuolar or acidic α-mannosidases of glycosyl hydrolase family 38 are purified and characterized from various legume and non-legume sources like jack bean [7], kidney bean [8], alfalfa [9], rice [10], babaco [11] and tomato [12]. These enzymes are initially synthesized as large polypeptides (~1000 amino acids) that undergo further cleavage yielding the mature functional proteins. The mature enzyme size differs among organisms [13]. The jack bean α-mannosidase is well characterized and widely used for the analysis of various glycoconjugates [14]. α-Mannosidases being glycoproteins, undergo post translation modifications such as *N*-glycosylation. Initial studies on the different α-mannosidases focused on their purification and biochemical characterization. With the onset of genome projects, several plant α-mannosidase primary sequences are deposited in the database (NCBI/UniProtKB) and all of them are known to have potential *N*-glycosylation sites

* Corresponding author. Fax: +91 40 23010120.
E-mail addresses: nsksl@uohyd.ernet.in, drnsk7@yahoo.co.in (N. Siva Kumar).

[1] *Abbreviations Used:* Con A Sepharose, Concanavalin A Sepharose; DTT, dithiothreitol; *D. lablab, Dolichos lablab;* dMNJ, 1-deoxymannojirimycin, ESI-Q-ToF MS, electrospray ionization quadrupole time of flight mass spectrometry, IC_{50}, half maximal inhibitory concentration, MWCO, molecular weight cut off; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ZIC-HILIC, zwitterionic hydrophilic interaction liquid chromatography.

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http://dx.doi.org/10.1016/j.pep.2013.02.004

(based on enzyme sequence annotation). Although a number of α-mannosidases have been characterized, only the *N*-glycans of jack bean α-mannosidase together with their site specificity has been determined [6]. For other plant α-mannosidases characterized, information on the nature and composition of *N*-glycans is limited.

We earlier purified lectins and glycosidases from *Dolichos lablab* (Indian beans) seeds and in a preliminary study α-mannosidase was partially characterized using conventional protein purification techniques from the seeds procured locally [15]. In a recent study a β-*N*-acetylhexosaminidase from *D. lablab* has been purified using specific seeds (KR 307) [16]. In the present study, we modified the purification protocol to purify the α-mannosidase from these seeds that involved also an affinity chromatographic step. Purified enzyme was biochemically characterized and partial amino acid sequences were generated from in-gel digestions of the enzyme using mass spectrometry. Further, analysis of *N*-glycopeptides derived from ingel proteolytic digests showed the presence of paucimannosidic type of *N*-glycans with site specificity.

Materials and methods

Materials

D. lablab seeds (KR-307) were purchased from Wipro seeds (India). Trypsin and chymotrypsin were obtained from Roche Diagnostics GmbH (Mannheim, Germany). Thermolysin, Sephacryl S-200, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl-α-D-mannopyranoside, methyl-α-D-mannopyranoside, phenyl Sepharose, divinyl sulfone, Sepharose 6B and Bradford reagent were purchased from Sigma–Aldrich. DE-52 anion exchanger was obtained from Whatmann. Con A Sepharose was obtained from GE Healthcare. Swainsonine and 1-deoxymannojirimycin were purchased from Carbosynth (Berkshire, UK). Precision plus protein dual color standards is from Bio-Rad. ZIC-HILIC ProteaTip, 10–200 μl, Sample Prep Kit from dichrom GmbH (Marl, Germany). Zip Tip C18 pipette tips and Amicon ultra centrifugal devices were purchased from Millipore (Billerica, USA). All solvents used were of HPLC grade purity.

Preparation of the crude extract and enzyme assay

Twenty five gram of the dehulled and delipidated seed powder was extracted overnight with 175 ml of 25 mM Tris buffered saline pH 7.4 (TBS) at 4 °C. The suspension was clarified by centrifugation (12,000 rpm for 30 min) and the supernatant was subjected to 0–60% ammonium sulfate fractionation. The precipitated protein was collected by centrifugation at 12,000 rpm and dialyzed against 0.05 M sodium acetate buffer pH 5 extensively with several changes. The pellet obtained during dialysis was discarded and the clear supernatant was dialyzed against 25 mM Tris–HCl buffer pH 7.4 overnight. The activities of α-mannosidase and β-*N*-acetyl hexosaminidase were assayed using *p*-nitrophenyl derivatives of the respective sugars. In a typical assay aliquots of the extracts were taken and the volume was finally made up to 400 μl with 25 mM sodium acetate buffer pH 5.0. To this 100 μl of 5 mM of corresponding substrate was added and incubated at 37 °C for 30 min. The reaction was stopped with 500 μl of 0.2 M glycine-NaOH buffer pH 10, and the color developed was measured at $\lambda = 405$ nm.

Ion exchange chromatography on DE-52 cellulose gel

DE-52 anion exchange gel was (10 × 2 cm) equilibrated with 25 mM Tris–HCl buffer pH 7.4. After applying the sample, the gel was washed extensively with the same buffer until the absorbance at $\lambda = 280$ nm was <0.05. Bound protein was eluted with 0.05, 0.1, 0.15 and 0.2 M sodium chloride in 25 mM Tris–HCl buffer pH 7.4. The maximum activity of enzyme was found in 0.05 and 0.1 M eluates. All fractions comprising α-mannosidase activity were pooled and concentrated by ammonium sulfate precipitation (80% saturation).

Chromatography on galactose sepharose and phenyl sepharose gels

In order to deplete the extracts from the galactose specific lectin, the concentrated sample was dissolved in 25 mM Tris–HCl buffer pH 7.4 containing 1.5 M ammonium sulfate and applied on a galactose Sepharose gel equilibrated with the same buffer. The unbound fraction containing the α-mannosidase was collected and directly applied on a phenyl Sepharose gel equilibrated with 25 mM Tris–HCl buffer pH 7.4 containing 1 M ammonium sulfate. The gel was washed extensively with the same buffer until the absorbance at $\lambda = 280$ nm was <0.05. Lower concentrations of ammonium sulfate in buffer did not release the enzyme completely. Therefore 25 mM Tris–HCl buffer pH 7.4 was used without ammonium sulfate to elute the enzyme. Protein content and α-mannosidase activity in fractions were measured at $\lambda = 280$ nm and 405 nm, respectively. Fractions containing α-mannosidase activity were pooled and concentrated using Amicon ultra centrifugal filter units with 30 kDa molecular weight cut off (MWCO).

Sephacryl S-200 gel permeation chromatography

The fraction containing α-mannosidase activity was applied on a Sephacryl S-200 gel permeation column (60 cm × 1.2 cm) equilibrated with TBS. Fractions of 1.0 ml were collected and enzyme activity was checked in all fractions. Fractions containing the α-mannosidase activity were pooled and concentrated using Amicon ultra centrifugal filter units with 30 kDa MWCO.

Concanavalin A Sepharose (Con A-Sepharose) chromatography

Con A-Sepharose chromatography (2 ml matrix) was carried out according to supplier's protocol. In brief, the sample was applied on Con A Sepharose which was equilibrated with 25 mM Tris–HCl pH 7.4 containing 250 μM $CaCl_2$, 250 μM $MnCl_2$, and 0.5 M NaCl. The column was washed by use of aqueous NaCl (0.75 M) and elution was first performed by using 0.1 M methyl α-D-mannopyranoside in 0.75 M NaCl. Owing to the poor elution of the bound enzyme at this concentration of ligand, concentration was raised to 0.3 M and incubated for 2 h. Subsequently, fractions were collected and the last step was performed several times in order to recover more bound enzyme. Fractions comprising α-mannosidase activity were pooled and concentrated by Amicon concentrator 10 kDa MWCO, further the salts and ligand concentrations were reduced by following desalting step according to manufacturer's protocol.

Protein estimation

Protein from the column fractions was measured by taking the absorbance at 280 nm. To precisely quantify the protein, estimation was carried out Bradford assay using BSA as standard [17].

SDS–PAGE

For the analysis of purified enzyme 10% SDS–PAGE was performed under reducing conditions [18]. The sample was heated in presence of DTT at 95 °C for 5 min. The gel was immersed for 30 min in coomassie solution (40% water, 50% methanol, 10% acetic acid, and 0.1% coomassie brilliant blue R 250). After this the staining solution was decanted and gels were immersed for 3 h in the

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