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Research article

Dioscorea alata tuber proteome analysis shows over thirty dioscorin isoforms and novel tuber proteins

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ABSTRACT

In *Dioscorea*, dioscorin (31 kDa) is the major storage protein constituting 85% of the total tuber proteins. An integrated proteomic and biochemical approach was used to understand the physiological role of dioscorin in the two contrasting growth stages (germinating and mature tuber). HPLC analysis showed 3 fold reduction in mannitol and 12.88 and 1.24 fold increase in sucrose and maltose in the germinating tuber. A 1.8 and 3 fold increase in sucrose phosphate synthase and mannitol dehydrogenase activity respectively was observed in the germinating tuber while a 2 fold higher invertase probably lowers the sucrose accumulation in the mature tuber. SDS-PAGE and 2-D maps of the mature and germinating tubers confirmed depletion (more than 50%) of dioscorin on germination. Dioscorin was purified using ion exchange and gel filtration chromatography with 43.32 fold purification and 38.16 yield. Out of a trail of 35 spots at 31 kDa only 12 spots (identified as dioscorin isoforms) were present in the 2D gel of the purified fraction. To search for other tuber proteins besides dioscorin, the unbound fractions of DEAE column were analysed by 2DGE. DREB 1A, caffeic acid 3-O-methyltransferase and Rab-1 small GTP binding protein were identified perhaps for the first time in the *Dioscorea* proteome. The interactome analysis revealed these to be involved in oxidative stress, carotenoid synthesis and vesicular transport. This is perhaps the first attempt to identify tuber proteome (although limited) and to understand the physiological significance of these proteins.

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1. Introduction

Dioscorea (commonly called as yam), a tuberous crop common in Eastern Africa and Asia contributes to 6% of the world's tuber production (FAO 1994). It is regarded as a staple crop and serves as a nutritional supplement (Wanasundera and Ravindran, 1994). Potato, cassava, yam, taro and sweet potato are the major tuber crops cultivated in India, of which yams are the most understudied. *Dioscorea alata* (greater yam), *D. esculenta* (lesser yam) are the major cultivated yams in India (Edison et al., 2006). In China, yams have been traditionally used as a health food and herbal medicine (Liu et al., 2007).

The major storage protein of yam tuber, dioscorin accounts for 80–85% of the total soluble proteins (Harvey and Boulter, 1983). It exhibits carbonic anhydrase (CA), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and trypsin inhibitor (TIA) activities (Hou et al., 1999; Hou et al., 2000). CAs are zinc metalloenzymes that catalyze the interchange of CO_2 and HCO_3^-. In plants, these play an important role in photosynthesis and respiration (Trumper et al., 1994; Xue et al., 2012). There are at least four distinct CA subfamilies: α, β, γ and δ, with no significant amino acid sequence identities. It is interesting to note that the amino acid sequence of dioscorin is closer to the α-CAs of animals than to plant β-CAs (Hewett-Emmett and Tashian, 1996). On the other hand, DHAR and MDHAR are important enzymes in the ascorbate-glutathione cycle, an antioxidative system for protecting plants from the toxicity of reactive oxygen species (ROS) (Foyer and Noctor 2005, 2011; Gill and Tuteja, 2010). DHAR catalyzes the reduction of DHA to ascorbate (ASC) using reduced glutathione (GSH) as an electron donor (Hou et al., 1999; Yang et al., 2009). Recently, it was shown that CA, DHAR and MDHAR activities are important for germination in *D. alata* tuber (Sharma et al., 2016).

Hou et al. reported that dioscorins isolated from *Dioscorea batatas, D. alata* and *D. pseudojaponica* tubers exhibited both CA and TI activities. However, Gaidamashvili et al., 2004 showed that dioscorins DB2 and DB3 from *D. batatas* tubers did not exhibit CA or

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TI activity. Instead, DB3L exhibited unique maltose-binding lectin activity.

Although the functional properties of dioscorin have been explored to some extent, the heterogeneity of dioscorin protein still needs to be investigated. Thus, the objective of the present study was to (i) analyse the tuber proteome on 1D and 2D in order to know the dioscorin isoforms, to search for other proteins apart from dioscorin as very little information is available about these (ii) analyse the sugar mobilization during tuber germination and (iii) purify dioscorin for detail biochemical characterization. A cumulative data about the proteome & biochemical information could be beneficial for future studies of the tuber.

2. Methods

2.1. Plant material

D. alata tubers (purple yam, wild variety) were grown in the Botanical Garden of University of Delhi, Delhi, India in March and harvested in April when the root growth was initiated and was designated as germinating tuber The next harvesting was done in December when the tuber had matured and it was designated as mature tuber. The tubers were stored at 4 °C until used.

2.2. Purification of dioscorin

Dioscorin was purified following Harvey and Boulter, 1983 with slight modifications. In brief, 50 g of the tuber was peeled, cut into thin slices and homogenized in 50 mM Tris, pH 8.3 [1:3 (w/v)] containing 10 mM β-merceptoethanol. The homogenate was centrifuged at 12,500 g for 25 min at 4 °C (Allegra 64R, Beckman Coulter). The concentrated supernatant (4 mg/ml) was loaded on a DEAE-cellulose ion exchange column (1.5 × 8 cm) equilibrated with the same buffer and allowed to bind to the column for 4–5 h. The elution was done in the buffer containing 150 mM NaCl with a flow rate of 1 ml/min (Peristaltic pump, Amersham Biosciences). The protein concentration was measured using Bradford's method (Bradford, 1976) and also measuring the absorbance at 280 nm. The fractions with maximum activity and protein concentration were pooled and subjected to acetone precipitation. The pellet was dissolved in minimum volume of Tris buffer without β-mercaptoethanol (10 ml) and loaded on a sephadex G-100 column (1.5 × 5 cm) for size exclusion chromatography. All steps were performed at 4 °C. The proteins obtained after DEAE ion exchange column and gel filtration column were separated on a 15% SDS- PAGE gel (Laemmli, 1970). The gel was subjected to silver staining to visualize the polypeptides (Merril et al., 1983). The densitometric scanning of the SDS-PAGE gel was done using AphaEaseFC software.

2.3. Two dimensional gel electrophoresis

Two dimensional gel electrophoresis of the purified dioscorin was performed as described previously (O' Farrel, 1975; Sharma et al., 2016) In brief, 100 μg of protein was loaded by rehydration loading overnight on a 3–10 non-linear IPG strip and was subjected to isoelectric focussing using EttanIPGPhor isoelectric focussing system (G.E Healthcare). Strips equilibrated with 1% DTT and then with 2.5% iodoacetamide in an equilibration buffer containing 6 M urea, 50 mM Tris pH 8.8, 30% glycerol, 2% SDS, and 0.002% BPB were loaded on the top of a 15% SDS-PAGE gel. It was stained with colloidal CBB stain (20% ethanol [v/v], 1.6% [v/v] phosphoric acid, 8% [w/v] ammonium sulphate, 0.08% [w/v] CBB G-250) and analysed by ImageMaster2DPlatinum software (ver. 6.0, G. E. Healthcare) to calculate the molecular weight and pI of the spots.

2.4. Protein identification by MALDI-TOF-TOF

MALDI-TOF-ToF analysis was carried out using pre-calibrated ABI 4800 plus MALDI-TOF–TOF analyser (Applied Biosystem) at Advanced Instrument Research Facility, Jawaharlal Nehru University, New Delhi, India. The silver-stained spots were excised from the gel, destained, and subjected to in gel digestion using 20 ngTrypsin (Gold Mass Spectrometry grade, Promega, Madison, USA) overnight at 37 °C. Reconstituted peptides were spotted on a 384 well LC MALDI stainless steelplate. The spots were illuminated with laser intensity of 3500 and total 1200 spectra were recorded. The ions were searched against NCBInr database on MASCOT search engine. The search parameters were as follows: mass values-monoisotopic, protein mass-unrestricted, fixed modifications-carbamidomethylation, variable modification-methionine oxidation, peptide mass tolerance- ± 50 ppm, peptide charge state- 1+, and maximum trypsin missed cleavage- 1. Only significant hits, as identified by the MASCOT probability analysis ($p < 0.05$) were accepted.

2.5. Spectrophotometric determination of CA, DHAR, MDHAR, TI and α-amylase activities

CA, DHAR, MDHAR, TI, α-amylase and β-amylase were assayed in the purified dioscorin. CA activity was measured following (Verpoorte et al., 1967). In brief, 15 μl of the protein extract was added to 15 mM Tris sulphate buffer pH 7.5 and 3 mM p-nitrophenyl acetate in a reaction volume of 1 ml. CA activity was assayed by measuring the change in absorbance of p-nitrophenyl acetate at 348 nm using a UV- visible spectrophotometer (DU 700, Beckman Coulter) for 5 min. For DHAR activity 5 μg protein was added to 900 μl of the DHA solution (10 mg DHA was dissolved in 5 ml 100 mM phosphate buffer with pH 7). The increase in absorbance at 265 nm was recorded for 5 min (Trumper et al., 1994). The MDHAR activity was determined according to (Hossain et al., 1984), based on the inhibition of MDA-driven NADH oxidation at 340 nm. The reaction mixture contained 50 mM potassium phosphate buffer pH 7, 0.33 mM NADH, 3 mM ascorbic acid, 0.9 units of ascorbate oxidase and 10 μl of the protein in a final volume of 1 ml. Trypsin inhibitor activity was performed following (Lin and Chen, 1980) modified procedure. In brief, the sample assay was performed by adding 75 μl of the protein extract to 250 μl of 2% casein solution (previously boiled for 2 min) and 250 μl of trypsin solution (20 μg trypsin in 0.25 mM HCl). It was then incubated at 37 °C for 20 min for proteolytic digestion. The reaction was terminated with 750 μl of 10% trichloroacetic acid and allowed to stand for 1 h. The precipitate formed was removed by centrifugation. The concentration of TCA-soluble peptides with aromatic amino acids in the supernatants was determined by measuring the absorbance at 280 nm. The standard and control assays were performed without protein extract and trypsin solution respectively. The percentage inhibition was calculated using $[(A_{280}$ of standard + A_{280} of control)$-A_{280}$ of sample$]/A_{280}$ of standard × 100. The α-amylase and β-amylase activities were done according to (Bernfeld, 1955). Briefly to the enzyme solution (20 μl), 1% soluble starch (100 μl) dissolved in 20 mM sodium phosphate buffer containing 6.9 mM sodium chloride, pH 6.9 was added. To this 100 μl of colour reagent was added (prepared by mixing sodium potassium tartrate solution with 3, 5-Dinitrosalicylic acid) and the absorbance was read at 540 nm. Maltose (0.2%) was used as a standard.

2.6. Sugar extraction and analysis by HPLC

For extraction the ratio of tuber to solvent (methanol) was kept at 1:2 (wt/vol). The extract obtained was refluxed for 30 min

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