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Mass spectrometric characterization of isoform variants of peanut (*Arachis hypogaea*) stem lectin (SL-I)

*Praveen Agrawal, Saravanan Kumar, Hasi Rani Das**

Proteomics and Structural Biology Division, Institute of Genomics and Integrative Biology, Delhi University Campus, Mall Road, Delhi-110 007, India

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ABSTRACT

Matrix assisted laser desorption/ionization–time-of-flight (MALDI–TOF) mass spectrometric (MS) analysis of purified *Arachis hypogaea* stem lectin (SL-I) and its tryptic digests suggested it to be an isoformic glucose/mannose binding lectin. Two-dimensional gel electrophoresis of SL-I indicated six isoforms (A1–A6), which were confirmed by Western blotting and MALDI–TOF MS analysis. Comparative analysis of peptide mass spectra of the isoforms matched with *A. hypogaea* lectins with three different accession numbers (Q43376_ARAHY, Q43377_ARAHY, Q70DJ5_ARAHY). Tandem mass spectrometric (MS/MS) analysis of tryptic peptides revealed these to be isoformic variants with altered amino acid sequences. Among the peptides, the peptide T12 showed major variation. The ^{199}Val–Ser–Tyr–Asn^{202} sequence in peptide T12 of A1 and A2 was replaced by ^{199}Leu–Ser–His–Glu^{202} in A3 and A4 (T12′) while in A5 and A6 this sequence was ^{199}Val–Ser–Tyr–Val^{202} (T12″). Peptide T1 showed the presence of ^{10}Asn in the isoforms A1–A5 while in A6 this amino acid was replaced by ^{10}Lys (T1′). Overall amino acid sequence as identified by MS/MS showed a high degree of similarity between A1, A2 and among A3, A4, A5. Carbohydrate binding domain and adenine binding site seem to be conserved.

1. Introduction

Lectins are defined as carbohydrate binding proteins or glycoproteins of non-immune origin that are able to agglutinate cells or to precipitate carbohydrates, without having enzymatic activity [1,2]. Lectins are found in all types of living organisms, either in soluble or in membrane-bound form. They are structurally complex molecules with one or more carbohydrate-recognition domains [3]. Legume lectins are a large family of homologous proteins that possess a strong similarity at the level of their amino acid sequences and tertiary structures; however, their carbohydrate specificities and quaternary structures vary widely [4]. Leguminous plants are known to contain lectins abundantly in various organs such as seed, bark, stem, leaf, flower, and root. However, the vegetative tissue lectins are less characterized in comparison to their seed counterpart [5]. The lectins from different parts of plants are reported to exist as a highly complex mixture of isoforms as, the lectins of *Robinia pseudoacacia* [6], *Erythrina* genus [7], *Acacia constricta* [8], *Amaranthus leucocarpus* [9]. The presence of isoforms have high impact on the biological function of lectins as the existence of number of isoforms might offer an alternative strategy or evolutionary adaptation to recompense the lack of high specificity [10,11].

Peanut (*Arachis hypogaea*) vegetative tissue lectin SL-I is found to be present in all the tissues of the plant but

Abbreviations: 2-DE, Two-dimensional gel electrophoresis; *A. hypogaea*, *Arachis hypogaea*; MALDI–TOF MS, Matrix assisted laser desorption/ionization–time-of-flight mass spectrometry; PMF, Peptide mass fingerprinting.

* *Corresponding author*. Tel.: +91 11 27662581; fax: +91 11 27667471.
E-mail addresses: hasidas@yahoo.com, hdas@igib.res.in (H.R. Das).

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differentially expressed [12]. This lectin was found to have cytokinin/adenine binding site and was able to antagonize cytokinin function *in vitro* indicating an important physiological function of plant lectin [13]. The native purified SL-I protein was found to show broad band in SDS-PAGE and appeared to contain more than one molecular weight bands. We speculated that this observation could be due to the presence of different isomeric forms of SL-I.

The identification of isoforms is very helpful to understand their function and that needs a sensitive and specific analytical tool, as their identification relies on the observation of the local amino acid differences in protein sequence [14–16]. Protein isoforms may originate from alternative splicing of mRNA, single-point mutations, and co- or post-translational modifications such as N- or C-terminal truncation, glycosylation, phosphorylation, deamidation and proteolytic cleavages [17,18]. These modifications often introduce a variation in the molecular mass and net charge of the protein. In order to confirm the presence of isoforms, purified protein was analyzed using bottom-up approaches, i.e. separation of proteins by two-dimensional gel electrophoresis (2-DE) and protein identification by matrix assisted laser desorption/ionization–time-of-flight MS (MALDI–TOF MS) of proteolytic peptides.

Here, we report the presence of six different isoforms of *A. hypogaea* stem lectin (SL-I) and their partial amino acid sequence generated using mass spectrometry. Mass spectra and amino acid sequence analysis of the tryptic peptides established the existence of amino acid substitutions among various isoforms. However, molecular modeling studies revealed no change in carbohydrate binding and adenine binding domain among the isoforms.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *A. hypogaea*, cultivar ICGS-1, a nodulating variety of peanut were obtained from the Indian Agricultural Research Institute, New Delhi, India. Prior to germination, seeds were scarified by treatment with 95% ethanol and rinsed thoroughly with sterile distilled water. The seeds were then imbibed in sterile distilled water for 3 to 4 h at room temperature. Treated seeds were placed onto sealed petri-plates on moist filter paper at room temperature in the dark for 48 h. Germinated seedlings were transferred to 0.8% solidified agar and were grown under 16 h day/8 h night cycle. Developing shoots were collected after 14th day, weighed, used immediately or frozen in liquid nitrogen.

2.2. Purification of the lectin

Stem lectin (SL-I) was purified as described previously [19] by affinity chromatography using Sephadex G-50 beads as matrix (Sigma chemicals co. St. Louis). Five micrograms of purified protein was subjected to 12% SDS-PAGE [20]. Gel was stained with Coomassie blue R250 for 1 h and was destained in destaining solution containing 45% methanol, 5% acetic acid. Protein band was cut, washed with MQ water and stored at 4 °C.

2.3. 2-Dimensional gel electrophoresis and immunoblotting

For the first-dimensional separation, the sample (10 μg of protein) was loaded onto a 7-cm IPG linear strip (pI 4–7; Bio-Rad laboratories, USA) and isoelectric focusing (IEF) was performed according to the manufacturer's manual with slight modifications. Briefly, the voltage was linearly increased from 250 to 4000 V for 3 h and it was kept constant at 4000 V for about 2.5 h. Strip was then incubated in an equilibration buffer (50 mM Tris–HCl, pH 8.8, 7 M urea, 2 M thiourea, 20% glycerol, 2% SDS, 65 mM DTT, and a trace of bromophenol blue) for 15 min, followed by an additional 15 min incubation using a fresh equilibration buffer supplemented with 135 mM iodoacetamide (instead of DTT). The second-dimensional separation was carried out on 12% SDS polyacrylamide gel (7 cm, 1 mm) following the method of Laemmli. For Western blotting after completion of 2-DE proteins were electroblotted on to a nitrocellulose (NC) membrane (Millipore Corporation, USA) and blot was probed with anti-SL-I antibody as described earlier [21]. Briefly, the separated polypeptides were electrotransferred to NC membrane for 1 h at 100 V in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. After transfer, the membrane was blocked with phosphate buffered saline (20 mM sodium phosphate buffer, pH 6.8, 0.15 M NaCl, pH 7.2, PBS) containing 1% bovine serum albumin and 0.1% (v/v) Tween-20 for 1 h. The membrane was then incubated for 2 h with anti-SL-I antibody (raised against the purified SL-I lectin in rabbit). The membrane was then washed with PBST buffer thrice and incubated with anti-rabbit IgG horseradish peroxidase conjugated (1:1000 diluted in PBS containing 0.25% BSA) at room temperature for 1 h. After extensive washing with PBST buffer, the blot was exposed to peroxidase substrate (25 ml PBS containing 15 mg of 4-chloronapthol dissolved in 5 ml methanol with 0.1% hydrogen peroxide).

2.4. Enzymatic in-gel digestion and peptide extraction

The spots representing protein of interest were excised from Coomassie stained 2-D gels and subjected to in-gel tryptic digestion according to standard protocol with minor modifications [22]. Briefly, the excised gel pieces were washed with 100 μl destaining solution containing 1:1100 mM ammonium bicarbonate (NH_4HCO_3) and 100% acetonitrile (ACN) till the gel pieces appeared colorless and then dehydrated in ACN. Reduction of protein was carried out in 100 μl of 10 mM DTT in 50 mM NH_4HCO_3 at 56 °C for 45 min. Alkylation of protein was done in 100 μl of 55 mM iodoacetamide (IAA in 50 mM NH_4HCO_3). Gel pieces were then destained in 100 μl of solution containing 1:1 ratio of 50 mM NH_4HCO_3 and 100% ACN. Final dehydration was done in 100% ACN for 15 min. Gel pieces were then centrifuged, supernatant discarded and were completely dried in speed vac (Thermo savant, USA) for 20 min. Trypsin solution (20 ng/μl) (proteomic grade, Sigma chemicals co. St. Louis) was prepared in 25 mM NH_4HCO_3 and 10 μl of this solution was added to each Eppendorf tube, kept at 4 °C for 30 min for absorption. This was followed by further addition of 10 μl of 25 mM NH_4HCO_3 solution. The samples were kept for digestion at 37 °C for 16 h. After digestion, samples were centrifuged and supernatants containing peptides were collected and were transferred to the fresh microfuge tubes rinsed with 100% ACN. Ten microliters of peptide extraction