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N-glycan analysis of mannose/glucose specific lectin from *Dolichos lablab* seeds

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ABSTRACT

An affinity purified mannose/glucose specific lectin from the seeds of *Dolichos lablab* (Indian bean/lablab bean) resolves into five subunits upon SDS-PAGE in the range of M_r 12–20 kDa. Partial *de novo* sequencing of subunits resulted in 88% and 73% sequence coverage for α and β subunits of the cDNA derived FRIL (Flt3 receptor interacting lectin) sequence, respectively and suggested that four bands correspond to the α-subunits while the band of lowest molecular mass is designated as β. It was proposed in an earlier study on FRIL that the difference in molecular mass of α-subunits is due to differences in C-terminal processing and differential *N*-glycosylation *i.e.* numbers of *N*-glycans present (Colucci et al., 1999). Thus, differential *N*-glycosylation of the purified mannose/glucose specific lectin was unravelled by in-gel trypsin/chymotrypsin digestion of the α-subunits followed by desalting and ZIC-HILIC enrichment of *N*-glycopeptides. Subsequently, analyses by nano electrospray ionisation quadrupole time of flight mass spectrometry and low-energy collision-induced dissociation experiments revealed the presence of a typical paucimannose type *N*-glycan ($Man_2(Xyl)GlcNAc_2(Fuc)$) in α subunits 2–4.

1. Introduction

N-Glycosylation is a common post translational modification of proteins in eukaryotes which occurs through the action of a series of glycosyl transferases and glycosyl hydrolases present in endoplasmic reticulum and Golgi apparatus. Even though the pathway is similar in plants and animals the extent of modification of *N*-glycan structures differs. While high-mannose type *N*-glycans are identical in structure in plants and animals, complex type *N*-glycans from plants lack terminal *N*-acetylglucosamine, galactose and sialic acid. Furthermore, several plant *N*-glycans specifically comprise β1,2-xylose and α1,3-fucose within the pentasaccharide core which is designated as paucimannose type [1,2].

Lectins are a class of carbohydrate binding proteins and are widely used in glycoconjugate analysis. Most of them undergo *N*-glycosylation as any other plant glycoproteins and structural analysis of *N*-glycans from several legume and non-legume lectins have been reported for soybean lectin [3], *Erythrina cristagalli* seed lectin [4], ricin [5], phytohemagglutinin (PHA) [6], jacalin [7], *Vatairea macrocarpa* seed lectin [8] *etc.* It has been found that majority of them possess mainly paucimannose type and fewer high mannose *N*-glycan structures.

The legume crop *Dolichos lablab* in India is grown as two varieties; *D. lablab* var. *lignosus* (field bean) and *D. lablab* var. *typicus* (Indian bean/lablab bean) [9] and both have been shown to contain lectins with two distinct sugar specificities in seeds, mannose/glucose specific [10] and galactose specific [11,12]. The mannose/glucose specific lectin purified from field bean comprised of two subunits with molecular mass of 15 kDa and 12 kDa and the complete amino acid sequence of both subunits has been deduced (UniProtKB: P38662) [13]. But the affinity purified mannose/glucose specific lectin from lablab bean resolved into five subunits with molecular mass ranging from 12 to 20 kDa upon SDS-PAGE. Strikingly, this pattern has also been identified by other researchers working on hyacinth bean lectin [14,15] which was also shown to be important in preservation of hematopoietic progenitors in suspension culture by interacting with Flt3 receptor and

Abbreviations: CID, collision-induced dissociation; *D. lablab*, *Dolichos lablab*; ESI-Q-TOF MS, electrospray ionisation quadrupole time of flight mass spectrometry; FRIL, Flt3 receptor interacting lectin; MALDI-TOF MS, matrix assisted laser desorption/ionization time of flight mass spectrometry; PMF, peptide mass fingerprint; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ZIC-HILIC, zwitter ionic hydrophilic interaction liquid chromatography.

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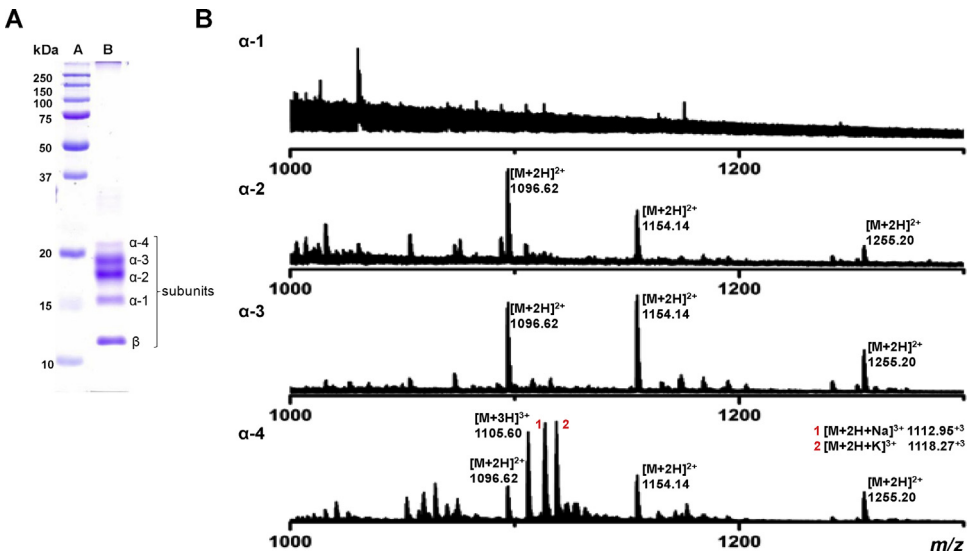

Fig. 1. (A) 12% SDS-PAGE analysis of mannose/glucose lectin purified from lablab bean. Lane A. Protein molecular weight marker. Lane B. Purified lectin. (B) NanoESI Q-TOF mass spectra of ZIC-HILIC enriched *N*-glycopeptides after in-gel trypsin/chymotrypsin digestion of the α-subunits.

thus designated as FRIL [15]. The N-terminal sequence of the FRIL subunits confirmed that the four subunits are α (α-1–4) and one subunit is β and cDNA derived protein sequence showed significant sequence identity (78%) with that of field bean lectin [13]. Further studies concluded that the different polypeptide masses of the α-subunits might be due to differential C-terminal processing of the corresponding propeptide or to differential *N*-glycosylation of subunits as there was a potential *N*-glycosylation site at Asn_7 of α 2–4 subunits while α-1 lacks the N-terminal sequence from aa_{1-8} present in the other subunits. The β subunit also lacks an *N*-glycosylation site [15]. However, no efforts were made for structural analysis of *N*-glycans in the subunits α 2–4.

The elucidation of glycan structures of proteins would provide information on understanding the role of glycosylation and useful means towards understanding their physiological significance. In the present study we investigated the *N*-glycans structure of a mannose/glucose specific lectin purified from lablab bean. The *N*-glycopeptides derived from in-gel proteolytic digestion of each α-subunit followed by ZIC-HILIC enrichment were subjected to mass spectrometric analysis which revealed the site- and subunit-specific *N*-glycosylation.

2. Methodology

2.1. Purification of the lectin from lablab bean

The lectin was purified from dehulled and delipidated lablab bean seed flour (KR 307 seeds procured from Wipro seeds, Hyderabad, India) following the protocol described previously [10] with minor modifications. The total proteins were extracted overnight in 200 mL 25 mM Tris–HCl buffer (pH 7.4), containing 150 mM NaCl (TBS, buffer A) and the extract obtained after centrifugation was saturated to 60% with solid ammonium sulfate and stirred for 3 h. The pellet obtained after centrifugation was dialysed extensively against sodium acetate buffer pH 5.0. The precipitate obtained after dialysis was discarded and the clear supernatant was dialysed against buffer A. The retentate was applied on a Sepharose–mannose affinity gel equilibrated with buffer A. After extensive washing with buffer A, bound protein was eluted with 0.25 M glucose in buffer A. The protein containing fractions were pooled, concentrated using an Amicon ultra filtration unit and applied on a Sephacryl S-200 gel filtration column to remove any possible aggregates or contaminating protein. The fractions with positive hemagglutination activity were pooled and the purity of protein was checked by performing 12% SDS-PAGE under reducing conditions [16].

2.2. In-gel digestion

The protein bands were subjected to in-gel digestion according to Shevchenko et al. [17]. Subsequent to destaining of gel pieces with 100 mM ammonium bicarbonate/acetonitrile (ACN) (1:1, v/v) and treating with ACN, sequence grade trypsin (Roche Diagnostics GmbH, Mannheim, Germany) in 25 mM ammonium bicarbonate buffer at a concentration of 25 ng/μL was added to dried gel pieces and incubated on an ice bath for 30 min in order to saturate the gel pieces with protease solution. Then additional buffer was added and incubation continued on ice bath for another 90 min. The gel pieces were covered with 25 mM ammonium bicarbonate buffer and incubated overnight at 37 °C. Proteolytic peptides were extracted by adding extraction buffer (5% formic acid/ACN, 1:2, v/v) to each tube and incubating for 15 min at 37 °C in a shaker. The supernatant was collected into a fresh tube and dried *in vacuo* and reconstituted in aqueous 0.1% trifluoroacetic acid (TFA) for desalting using C_{18} ZipTip (Millipore, Billerica, USA). For desalting, the ZipTip pipette tips were equilibrated five times with wetting solution (pure ACN) and five times with equilibrating solution (0.1% TFA in water). The digests reconstituted in 0.1% TFA were loaded onto the tips by 10 aspiration/ejection cycles. After washing five to six times with washing solution (0.1% TFA and 5% methanol in water) the peptides were eluted six to seven times with elution solution (50% ACN and 0.1% TFA) and finally dried *in vacuo*.

2.3. In-gel digestion of protein bands to obtain N-glycopeptides

N-glycopeptides were prepared as follows: subsequent to destaining of gel pieces with 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v) and treating with ACN, a 1:1 mixture of sequence grade trypsin (0.1 μg/μL) and chymotrypsin (0.1 μg/μL) (Roche Diagnostics GmbH, Mannheim, Germany) was added to the dried gel pieces and further processed as described in Section 2.2. After desalting of the extracted (glyco)peptides by use of C_{18} ZipTip (Section 2.2), the *N*-glycopeptides were enriched as follows: the ZipTip eluates were dissolved in ACN/H_2O + formic acid (80/20 + 2,