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Structural characterization of the N-glycosylation of individual soybean β-conglycinin subunits

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ABSTRACT

Soybean (*Glycine max*) 7S β-conglycinin is a seed storage protein consisting of homo- and hetero-trimers of three subunits, namely α (~67 kDa), α′ (~71 kDa), and β (~50 kDa), non-covalently associated. The N-glycans released from the whole β-conglycinin have been already characterized by ^{1}H NMR some decades ago. Nevertheless, the actual glycosylation of the potential sites and the glycoforms of the individual subunits have not been specifically investigated so far. In this study, up-to-date chromatographic, electrophoretic and mass spectrometric strategies have been combined to achieve the structural characterization of the glycoforms of the three individual β-conglycinin subunits. Glycosylation sites were assigned by analyzing the tryptic glycopeptides of the isolated subunits. Underivatized N-glycans were purified with a two-step clean-up, consisting in sequential reversed-phase and activated porous graphitized carbon micro-chromatography, and profiled by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS).

1. Introduction

Soy proteins have various functional properties and high nutritional value. For this reason, since October 1999 the USA Food and Drug Administration (FDA) has allowed manufacturers to claim decreased cardiovascular risk for soy protein-rich foods. Two major components, glycinin and β-conglycinin account for approximately 70% of the proteins in soybean seed.

β-Conglycinin is a trimeric protein consisting of three subunits, α (~67 kDa), α′ (~71 kDa), and β (~50 kDa), which are the products of a multigene family. Individual subunits can be combined forming randomly assorted homotrimers or heterotrimers [1]. The three subunits have a common core region (420–440 residues), while α and α′ contain extension regions (α′, 141 and α, 125 residues). The α, α′, and β subunits have potential Asn-Xaa-Ser/Thr (Xaa is any amino acid except Pro) consensus triplets of N-glycosylation (α, Asn199 and Asn 455; α′, Asn215 and Asn471; and β, Asn328) [2] and actually contains N-linked carbohydrates moieties representing 5% in weight of the protein complex [3].

Previous reports have associated to the β-conglycinin glycopeptides growth inhibitory effect on pathogens such as *Escherichia coli* and *Salmonella* both in cell cultures [4,5] and in vivo [6]. On the other hand all three subunits of β-conglycinin can potentially elicit IgE-mediated food allergies [7]. Glycans can enhance the allergenic potential of β-conglycinin subunits either because of carbohydrate epitopic structures [8] or indirectly by reducing the protein digestibility [9,10].

In plants, glycoprotein N-glycans can have paucimannosidic or high-mannose structures and, rather rarely, can be of the complex type, which generally are uncharged and non-sialylated. The IgE-binding carbohydrates are often N-glycans carrying core xylose and/or core α1,3-fucose [8]. Fig. 1 shows the structure of N-glycans released from non purified soybean storage glycoproteins [11], also including xylose and fucose containing N-glycans. Several decades ago, it has been suggested that N-linked 7S glycans are formed by 77% mannose and 23% *N*-acetylglucosamine [3]. The structure of the glycans has been inferred by analysis of the Asn-carbohydrate moieties released from pronase digestion of the 7S protein complex, but the direct structural characterization of both glycosylation sites and glycans of the individual subunits is still missing. NMR based

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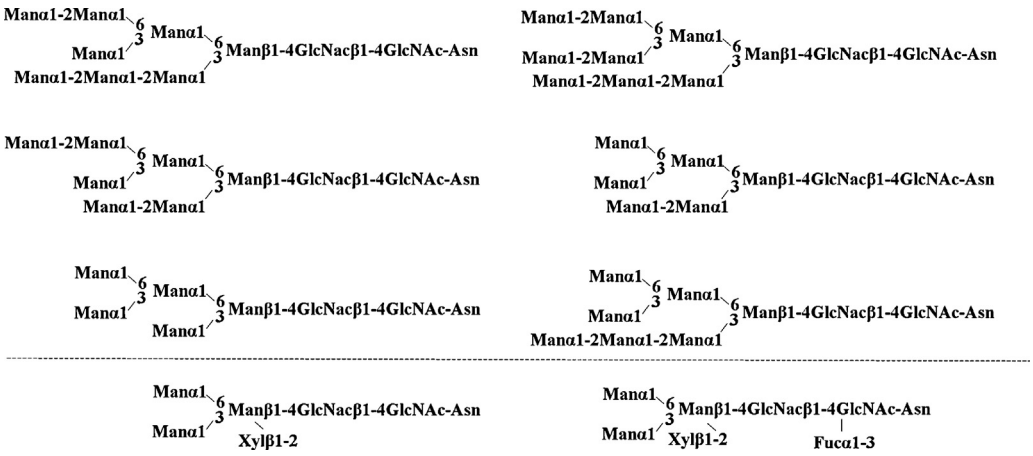

Fig. 1. Proposed structures of N-glycans released from unfractionated soybean storage proteins [11,12].

investigations, carried out on the glycans released form the entire protein complex, confirmed that the N-glycans of β-conglycinin are high-mannose type carbohydrate chains possessing a defined branching pattern [12].

The present research was aimed to achieve the structural characterization of glycostructures of the individual β-conglycinin subunits by combining multiplexed chromatographic and mass spectrometry (MS) up-to-date strategies. To the best of our knowledge, no previous investigations have specifically targeted the structural determination of the β-conglycinin glycoforms for each of the glycosylation sites of the three subunits. The assignment of the glycan structures to the three subunits required a previous complete isolation of the proteins chains. Generally speaking, due to the microheterogeneity introduced by glycans, a single purification step can be insufficient to isolate a glycoprotein with its varying glycoforms, particularly when multi-glycosylated proteins are to be analyzed. In this work the subunits of β-conglycinin were isolated from the isoelectric precipitate of soybean proteins using a sequential reversed-phase/SDS-PAGE electrophoresis method. Following in gel trypsinolysis, β-conglycinin subunits were characterized by MS at the level of glycopeptides as well as, after enzymatic release and a two-step micro-scale chromatography clean-up, at the level of glycans. The combined use of well-established, up-to-date techniques provides an example of a glycoproteomic strategy that can be designed as a function of the analytical inquiry and proposes a protocol of general applicability to characterize multi-subunits glycoproteins in plant seeds.

2. Materials and methods

2.1. Materials

Soy protein isolate was obtained from the Manuel Riesgo Company (Madrid, Spain). Guanidine·HCl, Tris base, dithiothreitol (DTT), α-cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid and sinapinic acid were from Sigma–Aldrich (St. Louis, MO, USA). Ammonium bicarbonate (AMBIC), trifluoroacetic acid (TFA) and HPLC-grade solvents were purchased from Carlo Erba (Milan, Italy). All the chemicals and solvents were of analytical or higher grade.

2.2. Isoelectric precipitation of β-conglycinin

Soybean β-conglycinin was separated from total soy protein by isoelectric precipitation following the method of Nagano et al. [13] with little modifications. Briefly, 20 g of soy protein isolate were diluted 1:15 (m/v) in milli-Q water, the pH was adjusted at 7.5 with 2 M NaOH. The resulting slurry was stirred for 1 h at room temperature and then centrifuged at room temperature (9000 $g \times 30$ min). Sodium bisulfite was added to the obtained supernatant (0.98 g/L supernatant), the pH was adjusted to 6.4 with 2 M HCl, and the mixture was kept in an ice cold bath overnight. The insoluble fraction was removed by centrifugation at 4 °C (6.500 $g \times 20$ min). NaCl was added to the obtained supernatant (up to 0.25 M) and the pH was adjusted to 5.0 with 2 M HCl. This mixture was stirred in an ice-cold bath for 1 h and centrifuged at 4 °C (9.000 $g \times 30$ min). The resulting supernatant was diluted 1:2 (v/v) with ice-cold milli-Q water and the pH adjusted at 4.8 with 2 M HCl, and then centrifuged at 4 °C (6.500 $g \times 20$ min). The β-conglycinin enriched pellet was washed twice with distilled water, freeze-dried and stored at 20 °C until HPLC purification.

2.3. HPLC purification

The 7S-enriched precipitate was dissolved at the 2 mg/mL concentration in a denaturing/reducing buffer (6 M guanidine HCl, 0.05 M Tris, 1 mM EDTA di-sodium salt, 10 mM DTT, pH 8.0) and heated 15 min at 56 °C. Proteins were fractionated by Reversed Phase (RP)-HPLC using an HP 1100 Agilent Technology modular system (Palo Alto, CA, USA) equipped with a Vydac (Hesperia, CA, USA) C4 column (214TP54, 5 μm, 250 mm × 2.1 mm i.d.) thermostatted at 37 °C. After 10 min of isocratic elution at 25% solvent B (0.1% TFA in acetonitrile, v/v) a 25–70% linear gradient of solvent B over 60 min was applied at a flow rate of 0.2 mL/min, followed by a 70–100% gradient of solvent B over 10 min. Solvent A was 0.1% TFA in water (v/v). For each analysis, approximately 100 μg of the protein solution were injected. Column effluents were monitored by UV detection at 220 and 280 nm. Protein fractions were manually collected, concentrated in a Speed-Vac, quantified with the Bradford assay and finally lyophilized. For the purpose of the identification, the protein peaks were hydrolyzed 6 h at 37 °C with trypsin in 50 mM NH_4HCO_3, pH 7.8, prior to freeze-drying and MALDI-TOF-based peptide mass fingerprinting (PMF).

2.4. Sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE)

Due to partial co-elution of the 7S subunits, an aliquot of the RP-HPLC purified proteins was further separated by monodimensional (1D)-SDS-PAGE, using the mini-Protean-tetra system (Bio-Rad, Hercules, CA, USA). The proteins were dissolved in SDS sample buffer (0.5 M Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 20 mM dithiothreitol, 0.05% bromphenol blue), boiled for 4 min, and ~8 μg of each HPLC fraction were applied to precast polyacrylamide