

Use of Galactose Oxidase in Hydrazide-Capturing Technique to Isolate N-linked Glycoallergens from Olive Tree Pollen

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Abstract. In this work, a simple method hydrazide-capturing technique followed by MALDI MS and MS/MS analysis for characterization of major pollen allergens of *Olea europaea* pollen was exploited. The strategy developed has different stages: devising of sample preparation protocol to sequester glycoproteins from a crude extract, and allowing the identification of antigenic peptides, glycosylation sites and modified glycans by MALDI MS and MS/MS.

1. Introduction

Air pollutants can directly interact with pollen and other plant materials [1-5], modify the allergen content of pollen further influencing its immunostimulatory properties. Many allergens from pollens are N-glycosylated and it is reported that the N-glycans themselves does not represent the major epitopes for the antibodies. The antibodies probably recognize a conformation constructed by the peptide portion around the glycosylation site and the N-glycan [6]. Considering that immunostimulatory properties of glycoallergens are essentially determined by the peptide portion around the glycosylation site, the application of hydrazide-capturing techniques followed by MALDI MS/MS analysis offers significant opportunities to obtain precious information [7-9]. Mass spectrometry is a well-established method for the high-throughput detection and quantitative analysis [10-12] of metabolites [13-15], amino acids [16] and their synthetic analogues [17], and proteins [18-22]. MS analysis of crude extracts [23] (i.e. pollen, fruit etc.) has been found to be a useful and robust approach for the chemical fingerprinting when a screening of matrix is wanted [24, 25]. MALDI-TOF-MS has been reported to be a very useful analytical technique for allergens detection [26, 27]. The specificity of MS and MS/MS experiments enable the identification of biomarkers through the elucidation of their molecular structures [28]. In this work, a simple method hydrazide-capturing technique followed by MALDI MS and MS/MS analysis for characterization of major pollen allergens of *Olea europaea* pollen was exploited. The strategy developed has different stages: devising of sample preparation protocol to sequester glycoproteins from a crude extract, and allowing the identification of antigenic peptides, glycosylation sites and modified glycans by MALDI MS and MS/MS.

2. Materials and Methods

2.1. Materials



Bovine serum albumin (BSA, P02769), Fetuin from fetal calf serum (F3385), Ribonuclease B from bovine pancreas (RNase B, R1153), PNGase F Proteomics Grade (P7367), Trypsin from porcine pancreas (T6567), Galactose Oxidase from *Dactylium dendroides* (GAOX, G7400), Catalase from bovine liver (C1345) are purchased from Sigma Aldrich- Merck (Milano, Italy). Affi-Gel® Hz Hydrazide Gel is purchased from BIO RAD (Italy).

2.2. Preparation of standard protein mixture

Bovine serum albumin (P02769), Fetuin (F3385), and Ribonuclease B (R1153) are used as individual standards and also to prepare in-house proteins standard solution. Each protein is solubilized in a phosphate buffer (pH 6) at a concentration of 5ppm (1mg/200 μ L). Stock solution of proteins is prepared at concentrations of 5ppm (BSA: Fetuine: RNase B/1:1:1 molar ratio) in the same phosphate buffer.

2.3. Oxidation of glycoproteins

The oxidation is performed by using Galactose Oxidase from *Dactylium dendroides* (GAOX), 4U/mg of protein, in presence of 10 μ L di CuSO₄ (0.78 M) and a little amount of catalase. The oxidation is performed overnight in the dark glass vial, under magnetic stirring and at room temperature. Desalting step is subsequently performed by means of the SPE Strata C18-E column (55um, 70A, Phenomenex, USA). Condition step of the column is performed by washing twice with 4 ml of 0.1% TFA in 50% CH₃CN and then twice with 4 ml 0.1% TFA. Sample containing oxidized proteins is added with 3.5ml of TFA 0.1% and loaded onto conditioned SPE column. The device was washed three times with 2 mL of 0.1% TFA, while the proteins were eluted with 4mL of 80% CH₃CN with 0.1% TFA, 4mL of 50% CH₃CN with 0.1% TFA and 4mL of 0.1% TFA in order to collect twelve protein fractions. The collected fractions are unified and completely dried by Concentrator Plus system (Eppendorf, Germany) and suspended in 200 μ L of Affi-Gel coupling buffer, pH 5.5 (BIO RAD).

2.4. Coupling of oxidized glycoproteins to hydrazide resin

Affi-Gel Hz hydrazide gel is supplied in isopropanol, therefore 200 μ L of gel/isopropanol slurry is transferred into a new centrifuge tube. When the gel settled, the supernatant is removed and the resin is washed two times with 800 μ L of water and two times with 800 μ L of coupling buffer, pH 5.5. The coupling is performed using 200 μ L of oxidized proteins (pH 5.5) added with 10 μ L of SDS (10%). The coupling reaction is conducted overnight on a rocking platform at room temperature. After that coupling reaction is completed, the supernatant containing the uncoupled protein is removed and the resin is washed several times to remove the unreacted material using three times the following series of solutions: a) 500 μ L of NaCl (1.5 M); b) 500 μ L of CH₃CN (80%), c) 500 μ L of CH₃OH; d) 500 μ L H₂O; e) 500 μ L of NH₄HCO₃ (50mM). The last one step is performed with 200 μ L of NH₄HCO₃ (50mM), ideal condition for tryptic digestion. The digestion is performed overnight at 37°C, by adding 160 pmol of trypsin. The tryptic peptides are recovered and analyzed by MALDI MS and MS/MS.

2.5. Recovery of glycopeptides and glycan moieties

The resin coupled with glycopeptides is suspended in 200 μ L of NH₄HCO₃ (50mM) and added with 5 μ L of PNGase F (500U/ μ L). The de-glycosylation step is performed at 37 °C for 8h, the de-glycosylated peptides are recovered and analyzed by MALDI MS and MS/MS. Finally, the glycan moieties are removed from the resin with formic acid (0.1 M, 100 μ L) at 80°C for 1 hour. The supernatant together with the wash solution (500 μ L, CH₃CN: H₂O, 50:50, v: v) was concentrated in the Concentrator Plus system and analyzed by mass spectrometry.

2.6. Tree olea europaea pollen sample preparation

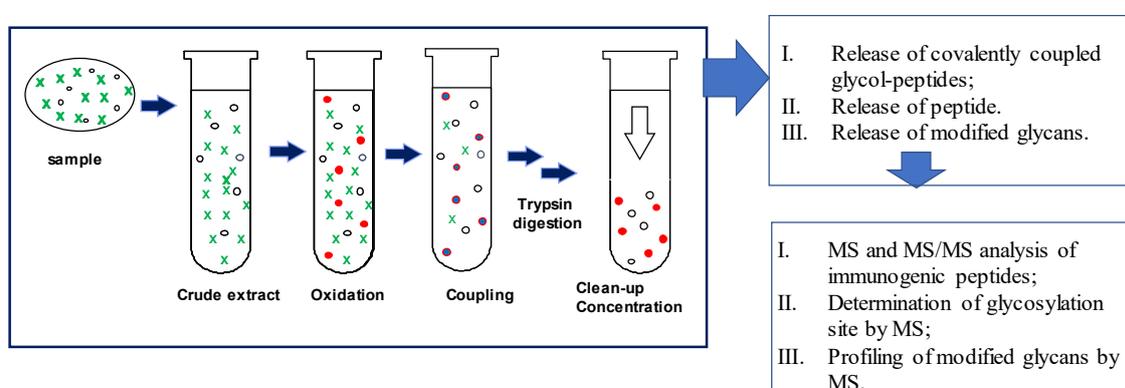
Tree Olea europaea pollen (2mg) are extracted with 400 μ L of phosphate buffer (pH 6) for 20 min at room temperature, as previously reported. The protein mixture is then treated as reported above for the oxidation, coupling and digestion steps.

2.7. Mass spectrometry analysis

A 1 μL portion of a premixed solution of each sample and α -CHCA ($\text{H}_2\text{O}/\text{CH}_3\text{CN}$, 40:60, v/v, 0.3% in TFA) was spotted on the matrix target, dried at room temperature, and directly analyzed by MALDI mass spectrometry. MALDI-time-of-flight (TOF) analyses are performed using a 5800 Proteomics Analyzer mass spectrometer, from ABI SCIEX (AB SCIEX, Darmstadt, Germany) in reflectron positive mode with a mass accuracy of 5 ppm. At least 4000 laser shots are typically accumulated with a laser pulse rate of 400 Hz in the MS mode, whereas in the MS/MS mode spectra up to 5000 laser shots are acquired and averaged with a pulse rate of 1000 Hz. CID (Collision Induced Dissociation) MS/MS experiments are performed at a collision energy of 1 kV, ambient air is used as collision gas at a pressure of 10^{-6} Torr. Proteins are identified by searching a comprehensive protein database using Mascot programs (www.matrixscience.com). All MS/MS searches were performed using an initial mass tolerance of 50 ppm with enzyme cleavage specificity.

3. Results and discussions

A schematic illustration of the adopted approach is shown in Scheme 1.



Scheme 1.

Hydrazide-capturing technique, consists in a chemical coupling between bead-immobilized hydrazide group and dialdehyde group derived from glycan moiety of glycoprotein. This approach requires that *cis*-diols on glycans are oxidized into aldehydes by sodium periodate, then covalently coupled with hydrazide immobilized to solid beads. This approach is selective to *cis*-diols on monosaccharides consisting of glycans, but is not promising for selective capturing of protein glycoforms having a specific glycan structure. The extension of hydrazide-capturing techniques to glycoallergens, belonging to antigenic plant complex type structures, and consisting of α 1–2 xylose and/or α 1–3 fucose residues requires the use of an alternative oxidant reagent. Here, we describe the oxidation of the primary alcohol on glycans with galactose oxidase (GOAX) to the corresponding aldehyde derivatives, which can be subsequently covalently bound to hydrazide beads. A protein standard mixture was tested in order to study the chemical reactivity of GOAX reagents and the recovery. Bound glycoproteins were trypsin-digested in situ, the nonglycosylated peptides are removed by washing, peptides are released by PNGase F and finally derivatized glycans are released by acidic hydrolysis (figure 1).

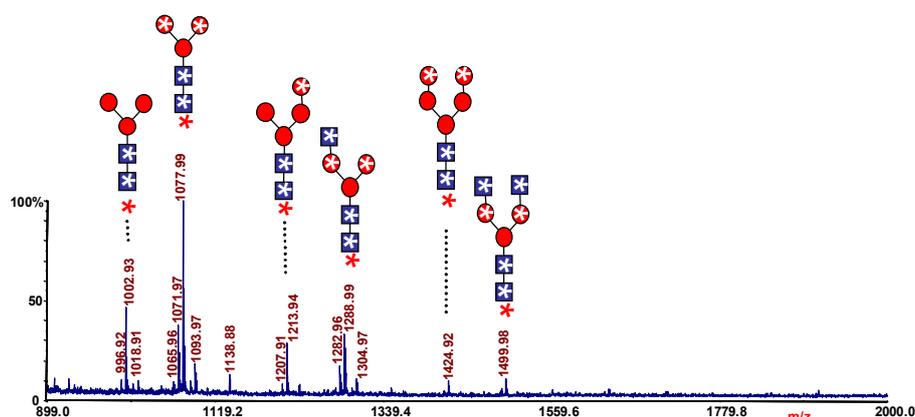


Figure 1. MALDI MS spectrum of derivatized glycans from standard protein mixture.

Covalently coupled glycopeptides were then released by PNGase F, and the resulting deglycopeptides are analyzed by mass spectrometry. The bound glycopeptides were released by acidic hydrolysis rather than enzymatic cleavage to analyze selectively glycopeptides. Finally, the methodology was extended to crude extract of olive tree pollen. The N-linked glycoallergens were selectively GOAX-oxidized, captured on hydrazide beads, trypsinized (figure 2A) and released by PNGase F (figure 2B). Tryptic peptides obtained by in situ digestion allowed the identification of the major pollen allergen (figure 2A).

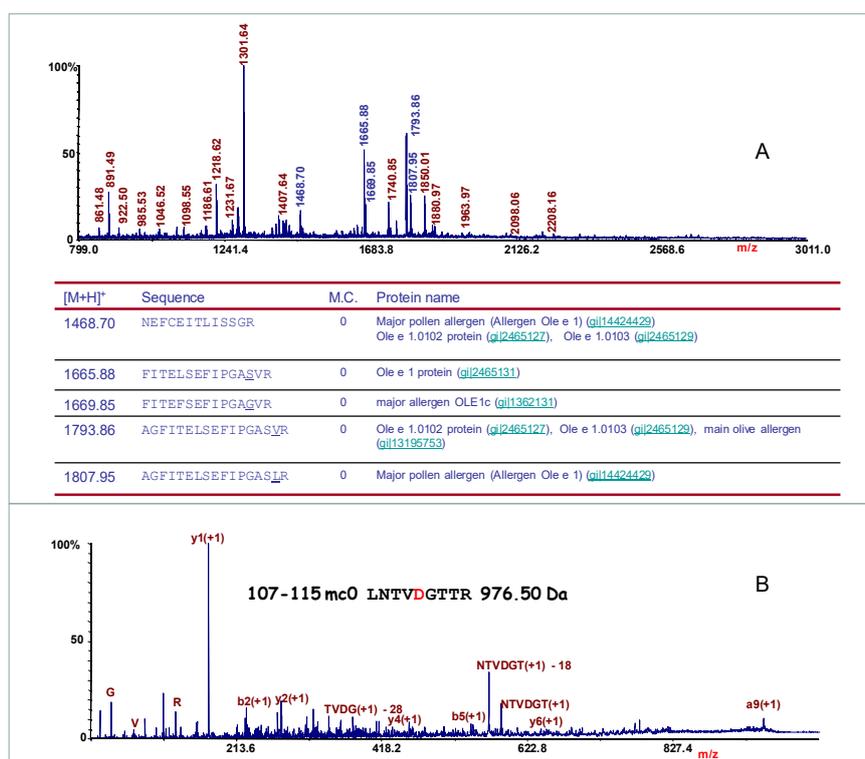


Figure 2. MALDI MS of tryptic peptides from captured major pollen allergen (A); MALDI MS/MS spectrum of peptide released by PNGases F (B).

The asparagine residue of the NX(S/T) consensus sequence of N-glycosides hydrazide-captured is converted into an aspartic acid (D) containing COOH functionality at side chain, as shown in figure 2B. Therefore, the PNGases F-mediated enzyme action allows the determination of glycation site.

4. Conclusions

In this paper, a hydrazide-capturing technique combined with GAOX strategy was developed to capture olive tree pollen glycoallergens, belonging to antigenic plant complex type structures. The developed strategy allowed the identification of antigen, the glycosilation site and glycans composition.

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