Supplementary Material

First proteome analysis of poplar-type propolis

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Materials and methods

Sample preparation

Raw propolis material was collected from beehives located in lowland parts of Croatia. These apiaries are surrounded by forests comprising primarily poplar and oak trees. Propolis was harvested from traps by cooling and scraping. The obtained material was refrigerated at -20 °C prior to preparation. Subsequently, all the propolis samples were cleaned and combined into a single composite sample weighing 1 kg, ground, and milled.

To obtain the protein fraction raw propolis sample was ground again to a fine powder using a mortar and pestle. 300 µL of a buffer (50 mM NH4HCO3, pH 7.8) containing 0.1% (v/v) Triton™ X-100 was added to 100 mg of propolis powder in a 2 mL polypropylene tube, and the sample was sonicated (SONOPULS mini20; Bandelin, Berlin, Germany) for 30 seconds

while kept cold in the cooling rack. It was then centrifuged at 12000 g for 10 minutes at 4 °C. 800 µL of cold acetone (-20 °C) was added to 200 µL of supernatant, and the proteins were left to precipitate overnight at -20 °C. Subsequently the sample was centrifuged at 12000 g for 20 min at 10 °C. The protein pellet was washed twice with cold acetone and dried in a vacuum centrifuge for 5 minutes at room temperature. The reconstituted proteins were subjected to in-solution trypsin digestion for 18 h at 37 °C.

LC-MS configuration

Tryptic peptides were separated on a Waters nanoAcquity UPLC system with flow rate 1 µL/min by using the nanoAcquity UPLC column BEH130 C_{18} (100 µm x 100 mm, 1.7 µm, Waters, Milford, MA, USA) and pre-column 2G-V/M Trap 5µm Symmetry C18 (180 µm x 20 mm, Waters, Milford, MA, USA) with flow rate 15 µL/min to desalt samples prior separation. A 65-min gradient was used for the separation of peptides (1-35% B in 40 min, 35-50% B in 3 min, 50-99% B in 14 min, 99-1% B in 3 min and 1% B for 5 min). Solvent A consisted of 0.1% formic acid in water (v/v) and solvent B of 0.1% formic acid in 95% acetonitrile (v/v). The sample injection volume was 2 µL. UPLC system was coupled to the ESI-qTOF SYNAPT G2-Si (resolution mode of operation) mass spectrometer (Waters, Milford, MA, USA). The instrument parameters were set using the Mass Lynx software version 4.1. SCN902 (Waters, Milford, MA, USA).

Accurate mass LC–MS data were collected in low-energy and elevated-energy (MS^E) modes of acquisition. Parameters were set as follows: positive ion mode, desolvation nitrogen flow 0.6 Bar with a temperature of 150 °C, the capillary voltage 4.3 kV, and the cone voltage 40 V. The spectral acquisition time was 1 s. MS acquisition was set to 50 – 3000 Da. In low energy MS mode, data were collected at a constant collision energy of 4 eV and in elevated energy MS mode, the collision energy was ramped from 20 to 55 eV during each 1 s data collection cycle. The mass accuracy of the raw data was corrected by infusing leucine enkephalin (1

ng/μL, 0.4 μL/min flow rate, 556.2771 Da $[M + H]^+$) into the mass spectrometer as a lock mass during sample analysis.

Accurate mass LC-MS/MS DDA data were obtained as follows. MS survey scans of 1 s duration with an interscan delay of 0.01 s were acquired. MS/MS data were obtained for up to five ions of charge 2, 3 or 4 detected in the survey scan. MS/MS was obtained at a scan rate of 1s with 0.015 s interscan delay and a collision energy ramp from 19 - 22 eV for the low mass and 51 to 69 eV for the high mass. A dynamic exclusion window was set to 60 s. The acquisition was switched from MS to MS/MS mode when the base peak intensity (BPI) exceeded a threshold of 1000 counts and returned to the MS mode when the TIC in the MS/MS channel exceeded 200000 counts or when 0.3 s were acquired.

Data analysis

The generated raw data were analyzed with ProteinLynx Global Server (PLGS; version 3.0.1) (Waters, Milford, MA, USA) on a computer workstation running Windows 7 64-bit. Data were searched against Uniprot databases of *Apis* and *Populus* including common mass spectrometry contaminant sequences at ≥95% confidence. Search settings included up to two missed cleavages and the methionine oxidation as variable modification. For DIA analysis, peak processing settings were a low energy threshold of 135 counts, an elevated energy threshold of 30 counts, and an intensity threshold of 750 counts. Peptide and fragment tolerances were set to automatic, a minimum of three fragment ion matches were required per peptide, and seven fragment ion matches with at least one peptide match were required per protein identification. The false discovery rate was set to 4%. For DDA searches, the software parameters were set as follows: Savitzky-Golay smoothing method was applied for filtering data noise with 2 smoothing iterations; fast deisotoping was performed; peptide tolerance was set to 30 ppm; fragment tolerance was set to 0.05 Da.