Nanoflow liquid chromatography and mass spectrometry analysis (nLC-MS)

Apidaecin 1 isoforms were identified and quantified according to procedure Danihlik et al. 2014 with minor modifications.

For identification and quantification of apidaecin 1 isoforms was used a system of nanoflow liquid capillary chromatograph (RSLCnano, Thermo, USA) connected to an ultra-high resolution Q-TOF mass spectrometer (UHR-QTOF maXis, Bruker Daltonik, Bremen, Germany) via nano-electrospray ion source (Captive Spray, Bruker Daltonik, Bremen, Germany). The system was controlled by CompassQTOF v 1.4 and HyStar v3.2 software (Bruker Daltonik, Bremen, Germany). Loading and separation of samples was carried out in a one-column setup (i. e. without any precolumn) with an analytical column (75 µm i. d. × 100 mm) ended with an IntegraFrit (New Objective, Inc., MA, USA) and packed with a C_4 sorbent (ReproSil-Gold C_4, 300Å, 5 µm, Dr. Maisch; Ammerbuch-Entringen, Germany) in length of 5 cm. Composition of mobile phases were as follows: mobile phase A consisted of 0.4% (v/v) formic acid (FA) and mobile phase B consisted of 0.4% (v/v) FA in 90% (v/v) acetonitrile. Dried samples were dissolved in 30 µL of 0.1% trifluoroacetic acid (TFA), vortexed, sonicated for 5 min and transferred into total recovery glass vials (Waters, USA). An aliquot of the sample (5 µL) was loaded and washed on the column using a loading mobile phase (2% FA) at flow 3 µl/min for 7 min. Retained peptides were eluted with 25-min fast gradient (all mobile phase changes were linear, 0 min, 2% B, 3 min, 8% B, 15 min, 65% B, 16 min, 85% B, 21 min, 85% B, 22 min, 2% B, 25 min, 2% B) at a flow rate of 300 nl/min. Each sample was injected twice to check the reproducibility of response during the process of electrospray ionization.

Full MS spectra were acquired in the positive MS mode in the range of m/z 350 – 1600 with a frequency of 0.5 s per MS. Prior to analysis, the mass spectrometer was always calibrated in the m/z range of 118-2722 (ESI-L TuneMix, Agilent Technologies Part. No: G1969-85000) and tuned for a minimal resolution of 25000 full width at half maximum (FWHM) using a calibration ion at m/z 922.0098. Other settings were as follows: Source (Capillary voltage 1200V, Dry gas 4 L/min, Dry temperature 130 °C). All raw data were processed using DataAnalysis software v 4.0 SP5 (Bruker Daltonik, Bremen, Germany). Targeted analytes (apidaecin 1 isoforms and the internal standard $[_{13}C^{6}{}_{15}N^{4}]$apidaecin 1A) were checked in a defined retention window of ± 0.5

min. Data analysis for analytes were carried out by extracting high-resolution accurate mass traces (± 0.005 Da) concerning the most dominating protonated ion charge state.

External calibration

For quantification of the apidaecin 1 isoforms level in the thorax samples, a calibration curve was generated using peptide mixture consisted of the standards of apidaecin 1A and [$_{13}C^{6}{}_{15}N^{4}$]apidaecin 1A diluted in a purified homogenate from freshly emerged bees in a concentration range of 0 to 5 pmol apidaecin 1A standard and 1 pmol of [$_{13}C^{6}{}_{15}N^{4}$]apidaecin 1A in the final volume of 30 µl of 0.1% (v/v) TFA. High-resolution extracted ion chromatograms (HR-EIC) were generated as a sum of the two most intense quadruply charged abundant isotopic peaks ($[M+4H]^{4+}$) ions of apidaecin 1A at m/z 527.80796 plus 528.05048 and of [$_{13}C^{6}{}_{15}N^{4}$]apidaecin 1A at m/z 530.30298 plus 530.55549. Areas under extracted ion chromatographic peaks were integrated to get total peak areas. Ratios of peak areas of apidaecin 1A *vs* [$_{13}C^{6}{}_{15}N^{4}$]apidaecin 1A were plotted against spiked amounts of apidaecin 1A (in pmol).

Quantification of apidaecin 1 isoforms in bee thoraces

Apidaecin 1 isoforms were isolated by the protocol based on weak cation exchange chromatography microcolumns (5 mg of Oasis® WCX 30 µm sorbent, Waters, USA) and analysed by nLC-high resolution MS. Collected raw data were processed in the DataAnalysis v 4.0 SP5 software as described above and the ratio of peak areas of apidaecins 1 vs [$_{13}C^{6}{}_{15}N^{4}$]apidaecin 1A was calculated. The content of apidaecin 1 isoforms in samples were estimated from the calibration curve and recalculated to original thorax samples. The diluting factor for apidaecin 1 isoforms content in homogenates and in hemolymph was 10 because from total 600 µl of homogenate 180 µl were used as an aliquot and lyophilized. Then the dried sample was reconstituted in 100 µl 5% FA and 33 µl was loaded onto the WCX-Tip microcolumn. Apidaecin 1 isoforms level in thorax homogenates was calculated according to the following equation: apidaecins 1 (ng) = quantity of apidaecin (pmol) × 10 (diluting factor) × 2107 (molecular weight of apidaecin 1 isoforms) × 0.001.