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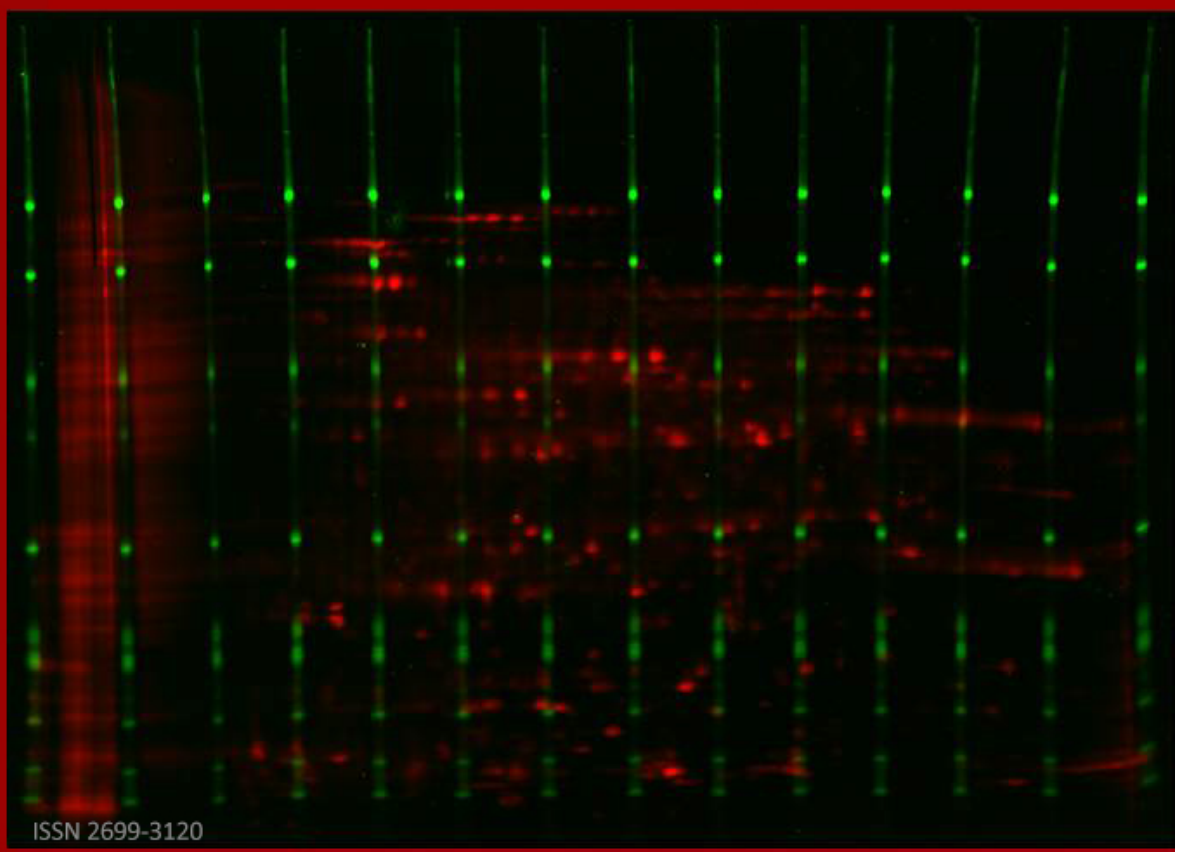

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Cover image
Mercator gel (run by D. Ackermann at CUP) representing the award-winning CoFGE technology for standardized gel electrophoresis

Protocol

Target analysis for the adipokinetic hormone of *Daphnia*

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Abstract

Members of the red pigment-concentrating hormone (RPCH)/adipokinetic hormone (AKH) peptide family are involved in many physiological, developmental and behavioral processes in insects, and pigment translocation in decapod crustaceans. In the branchiopod *Daphnia pulex*, a preprohormone for RPCH/AKH is encoded in the genome and the mature peptide, Dappu-RPCH (pQVNFSTSW-amide) is predicted from the preprohormone. Here, we have developed a method to measure the peptide in hemolymph of *Daphnia*. It uses reversed-phase liquid chromatography and high-resolution mass spectrometry of the singly- and the doubly-charged peptide ions, respectively, and their gas phase fragmentation for target identification. Pilot studies were conducted with synthetic Dappu-RPCH and the method was sensitive at the low fmol level without biological matrix. When hemolymph samples were however spiked with known amounts of the synthetic peptide, much less peptide was recovered and detected. Thus far, with our method of peptide purification, the endogenous peptide was not detectable in hemolymph of *Daphnia* where AKH titres of 1 fmol/µl hemolymph are expected.

Introduction

In insects, neuropeptides of the red pigment-concentrating hormone/adipokinetic hormone (RPCH/AKH) family mobilize stored metabolites, such as lipids, carbohydrates and proline, for energy-driven processes [1, 2]. RPCH/AKH peptides are, thus, essential for insect metabolism and play an important role in many physiological, developmental and behavioral processes [2]. The peptides are synthesized in the corpora cardiaca and released into the hemolymph. They bind to G protein-coupled receptors (GPCRs) on the membrane of their target cells and thereby initiate a signal transduction cascade that results in mobilization of the stored metabolic fuel. In decapod crustaceans, on the other hand, members of this peptide family are synthesized in the X-organ and released from the sinus gland into the hemolymph. Binding of the neuropeptide to its GPCR results in the concentration of pigments (especially red pigment) in the integument and the retina, hence the name “red pigment-concentrating hormone” [3].
Members of the RPCH/AKH peptide family show a number of conserved features, such as blocked termini (a pyroglutamic acid residue at the N-terminus and an amidated C-terminus), an aromatic amino acid in positions 4 (Phe) and 8 (Trp), and a chain length of 8 to 10 amino acids. The RPCH/AKH is proteolytically generated from a larger precursor, a preprohormone that characteristically consists of a signal peptide, the unmodified RPCH/AKH peptide and a peptide referred to as the RPCH/AKH precursor-related peptide (Fig. 1). [4]
The genome of the branchiopod crustacean, *Daphnia pulex*, predicts the preprohormone of a putative RPCH/AKH as an octapeptide with a primary amino acid peptide sequence of QVNFSTSW (scaffold 129; JGI_V11_114396: wfleabase.org, Fig. 1). Post-translational modification of this peptide (typically) will generate the mature RPCH/AKH sequence of pQVNFSTSW-amide, code-named Dappu-RPCH [5, 6]. In *Daphnia magna*, the homologous preprohormone is processed to the same mature octapeptide (Fig. 1).

>JGI_V11_114396
MANHRILILTLLMIGLASAQVNFSTSWGKRSPSTSTKAAEPPSAPSYRQNFHSKKVEPGT
LETLPNNQHLPESFDTVSSTIYDDAEEQRISISLPSPCLSILKSLLLVNQIVEFKNSPLD
GRMHRFKIENLFPLPNRTCRLYIRR*

>Dapma7bEVm008604t1
MMKVRKEFCRLKIIGNSFITTSRSYSINVFVSPSSSPLRMDVANVRVLIVMLLVIGLASA
QVNFSTSWGKRSPSVLSAAPASSAQGSYLALRQKLHSKKEPGMESQHNVLPDSDSGAQTV
YDEAEDQRTSGLLPSPCLSLLKVLMLVNQIAETELQKVEKYDRQQQ

Figure 1: *D. pulex* RPCH/AKH precursor deduced from the JGI gene prediction model (located on gene scaffold 129; wfleabase.org, top sequence). *D. magna* RPCH/AKH precursor deduced from the Evidential Gene Database (arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/Proteins, bottom sequence). The predicted signal peptide is shown in blue, the RPCH/AKH in red (underlined), and the precursor-related peptide in green.

The Dappu-RPCH preprohormone and the GPCR for Dappu-RPCH was cloned from *D. pulex* and confirmed to be a signaling system via an *in vitro* receptor assay, in which the role of each amino acid residue of the ligand was evaluated for its function and its binding characteristics to the Dappu-RPCH receptor [7]. The function of Dappu-RPCH in *D. pulex* is not known, and while

it does not affect integumental pigment concentration in decapod crustaceans, it causes lipid mobilization in a bioassay with insects [5]. One step towards determining the functional role of the peptide in *Daphnia* is to quantify the concentration of Dappu-RPCH in the hemolymph following various physiological triggers, and to correlate the release of the peptide from its (as yet unknown) site of synthesis with a change in the transcription rate of the ligand and its receptor in the water flea. In order to measure the titre of Dappu-RPCH in *Daphnia* hemolymph, we set up a target analysis method using reversed-phase liquid chromatography coupled to high-resolution mass spectrometry (LC-MS). The larger species *D. magna* was used for the experiments for more effective hemolymph collection.

Experimental

MS method

The peptide Dappu-RPCH was custom-synthesized by Dr. Kevin D. Clark (Department of Entomology, University of Georgia, USA). It was dissolved in 50% aqueous solvent (5% acetonitrile (ACN), 0.1% formic acid (FA)) and 50% methanol (MeOH) at 4 pmol/µl. This solution (0.2 µl) was used for LC-MS method optimization. Experiments were performed with Synapt G2 Si ion mobility mass spectrometer with an M-Class UPLC (trap column V/M Symmetry C18, 100 Å, 5 µm, 180 µm x 20 mm; reversed phase column HSS T3, 1.8 µm, 75 µm x 200 mm; Waters Corp., Manchester, UK). A 30 min gradient from 30% to 40% solvent B (ACN, 0.1% FA) was used for separation at a flow rate of 300 nl/min. Solvent A was water containing 0.1% FA. The doubly and the singly-charged peptide ions, respectively, were selected for target MS/MS (*m/z* 475.72, collision energy ramp 6-7 V; *m/z* 950.43, collision energy ramp 18-27 V; scan time 0.2 s).

Animals and hemolymph collection

D. magna, a clonal line originally obtained from Staatliches Umweltamt Münster, Germany, were raised at 20°C at normoxic conditions in Elendt-medium [8] (Sigma-Aldrich, Merck, Darmstadt, Germany) and a light:dark cycle of 16:8 h. Animals were fed with *Desmodesmus subspicatus* (SAG Göttingen) daily. *D. magna* yielded up to 0.5 µl hemolymph per animal. Hemolymph was taken from adult females with a fine capillary after perforating the carapace in the heart region with a needle. The hemolymph of ca. 100 animals was collected on ice. It was centrifuged for 10 min at 14000 g and the supernatant was frozen at -80°C until further use.

Dappu-RPCH recovery experiments

Different concentrations of the synthetic peptide were tested ranging between 1 and 100 pmol. The synthetic peptide (e.g. 10 pmol) was spiked into 10 µl hemolymph and into 100 µl methanol for control purposes. The solution was filtered (3.000 Da cut-off centrifugal filter units, Amicon Ultra 0.5 ml, Merck Millipore, Tullagreen, IRL) at 14000 g for 30 min to remove larger compounds. The filters were rinsed three times with 250 µl MeOH and centrifuged for maximal recovery of Dappu-RPCH. The filtrates were pooled, dried using a speedvac and dissolved in 10 µl 50% aqueous solvent (5% ACN, 0.1% FA) and 50% MeOH for MS analysis.

Preparation for analysis of endogenous Dappu-RPCH

Whole animal extracts and hemolymph were used for detection of the intrinsic peptide. Animals were starved for 0, 24, 48 or 72 h before bleeding them to trigger release of Dappu-RPCH into the hemolymph. A volume of 10 µl hemolymph was added to 100 µl MeOH and filtered as above. Extracts of whole animals, which included the synthesizing tissues, were prepared from about 350 individuals (0.7 g) in 1.4 ml MeOH with a Teflon© pestle (Sigma Aldrich). The solution was filtered as described above but dissolved in 30 µl solvent, because it was brownish and showed a precipitate.

Results

The synthetic peptide Dappu-RPCH showed a major peak for the singly-charged ion and a low-abundant doubly-charged ion (Fig. 2). Target traces for both the singly and the doubly-charged MS/MS-scans were programmed for best identification and prominent ion traces (*m/z* 711.30, 159.09, respectively) were extracted for visualization (Fig. 3, 4). The method was sensitive at the low fmol level (20-50 fmol).

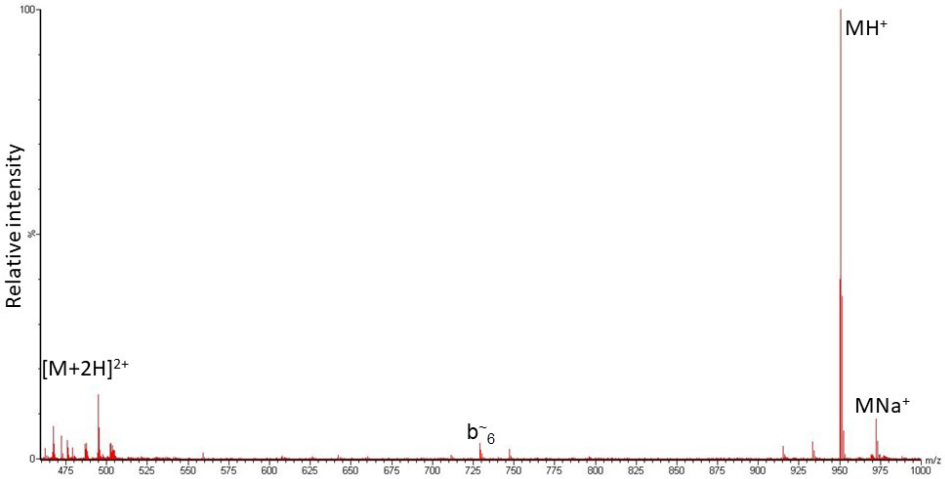

Figure 2: LC-MS/MS of Dappu- RPCH, overview scan. The singly-charged ion was predominantly observed. Few ion-source fragments such as $b^{\sim}_6$ were detected.

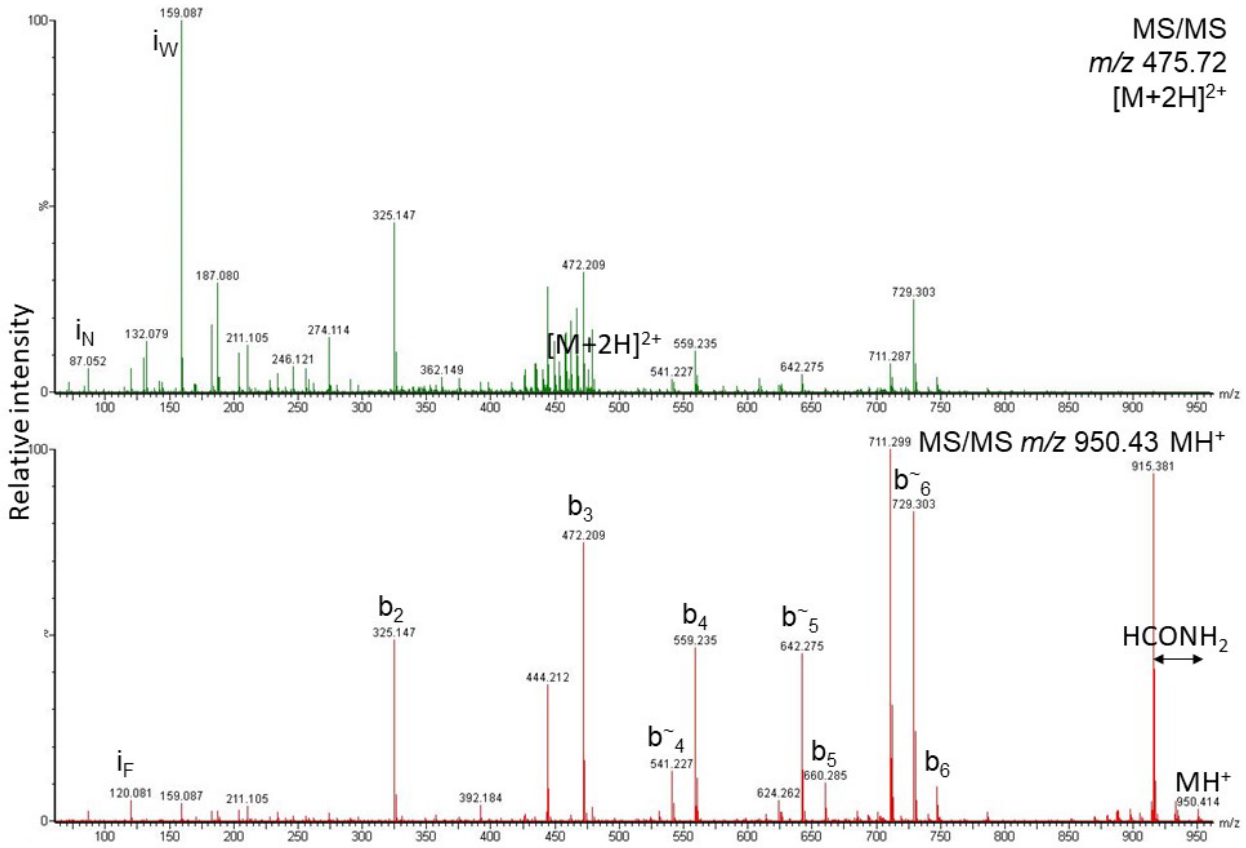

Figure 3: MS/MS spectra for the singly and the doubly-charged ion (top trace) of Dappu-RPCH showing the b-ion series with multiple water losses and a dominant immonium ion for tryptophan.

The equivalent of 1 pmol synthetic Dappu-RPCH (derived from spiking 10 pmol into hemolymph, followed by peptide purification) was easily detected using the trace for the singly-charged ion (Fig. 4, 5). At this concentration, the trace for the doubly-charged ion did not show good quality spectral information, but it improved with higher injection volumes (4 µl). Conclusively, the use of the MS/MS of the singly-charged ion is sufficient for target analysis. Spiking of lower Dappu-RPCH amounts into hemolymph did, however, not provide successful results, possibly due to residual proteolytic activity and losses incurred during extraction. Unfortunately, neither in pure hemolymph (i.e. not spiked with synthetic peptide), nor in the whole animal extracts was a signal detected for endogenous Dappu-RPCH with this methodology, which can confidently assign peptides at the level of 20-50 fmol.

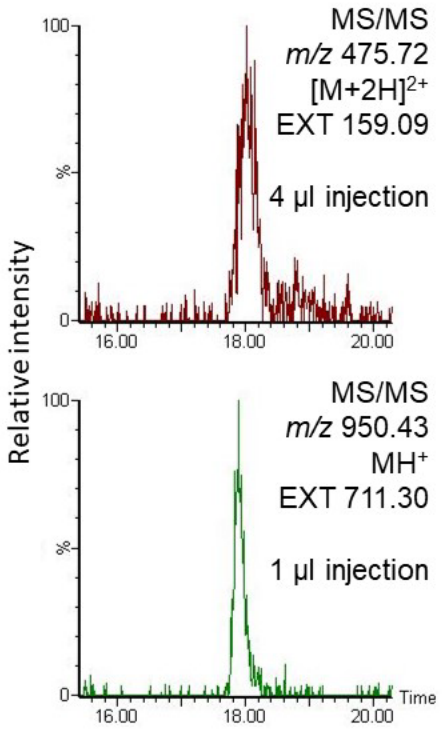

Figure 4: Recovery experiment: 10 pmol Dappu-RPCH spiked into hemolymph. LC-chromatogram for extracted fragment ion masses of the singly-charged ion for 1/10 (bottom trace) and of the doubly-charged ion for 4/10 of the recovered material.

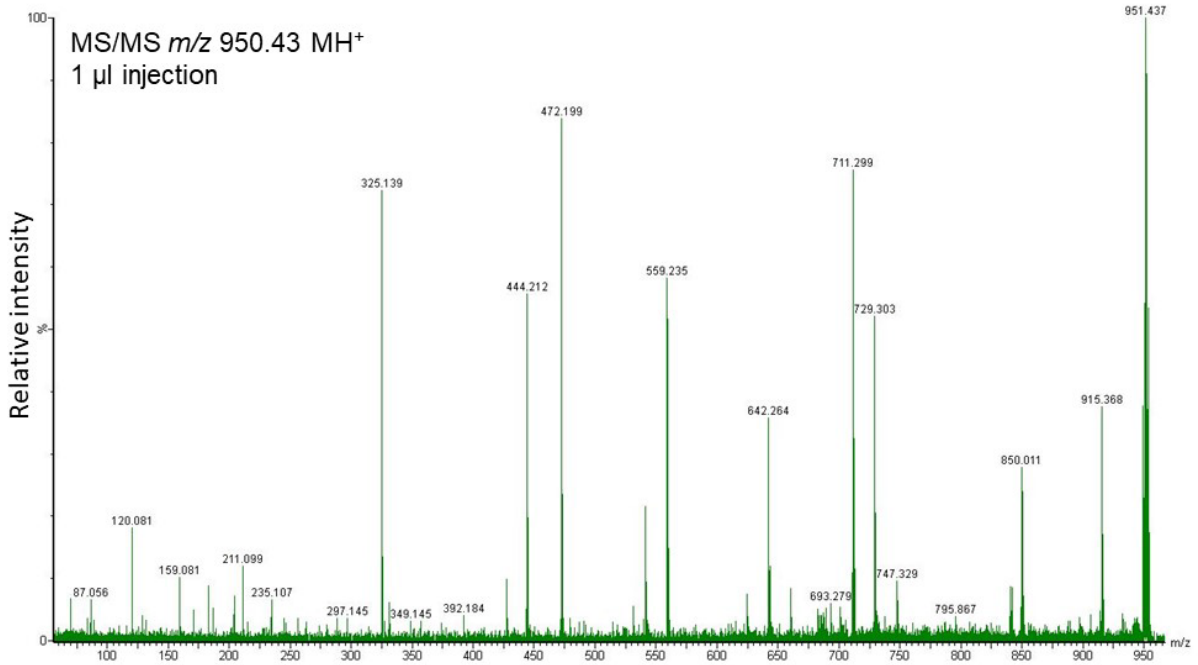

Figure 5: Recovery experiment: 10 pmol Dappu-RPCH spiked into hemolymph. MS/MS of singly-charged ion of 1/10 of the recovered material. Note that in the parent ion peak the second isotope carries the label.

Conclusion

A method for MS-based target analysis of Dappu-RPCH with low fmol sensitivity was developed. When the synthetic reference peptide was, however, mixed with hemolymph from *D. magna* and subsequently recovered, considerable losses were encountered. High fmol to pmol equivalents of spiked peptide were needed to obtain a reasonable MS signal. To counteract this reduced peptide recovery, an increased hemolymph volume (obtained via a greater number of animals) was used for the detection of endogenous Dappu-RPCH in *D. magna* to no avail. The use of total animal extracts proved even more difficult due to the increase in background. A separate preparation method including an additional purification step, e.g. solid phase extraction, would be required in future experiments for that purpose. So far, we have learned that, if Dappu-RPCH is, indeed, present in its predicted form in *Daphnia* hemolymph, then it is likely available at low concentration – not unexpected in a GPCR signaling system with signal transduction amplification after binding of the primary messenger (see [9]). Possibly, proteolysis or other processes in the animals may also cause modifications of the peptide so that it eludes detection by this target method.

Acknowledgment

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