Peptide fraction identification by SE-HPLC and LC-MS/MS analysis of the body mucus from Portugal coastal fish *Halobatrachus didactylus*

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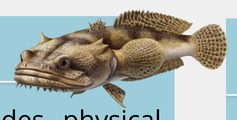
Introduction



The mucus covers the fish's body, working as a protective barrier. Besides physical protection, mucus provides molecules that protect the fish from damaging pathogens [1,2]. Has been reported that antimicrobial peptides are secreted in the mucus, which play an essential role in defense against microbial pathogens since these belong to the innate immune system [2,3].

Objectives

The present study aimed to identify and characterize new peptides with bioactive potential in mucus samples by chromatography analysis.

Methodology

Sampling

For this study, we captured two adult *Halobatrachus didactylus* individuals from the wild in Sesimbra. Then, we collected mucus by scraping the dorsal-lateral body of the fish with a sponge.

Methods for chromatography analysis

SE-HPLC

SEC Fractionation

LC-MS/MS

PEAKS

Proteomic Software

Results

1. SE-HPLC analysis of mucus samples

Figure 1 shows mucus sample chromatographic profiles of two wild fish *H. didactylus* from Sesimbra (**A**- HDSES20210414_04B and **B**- HDSES20210414_05B). At the retention time of 11 minutes, it was observed an intense peak that appeared in the both samples. This peak could represent a peptide with a molecular weight of ca. 775 Da.

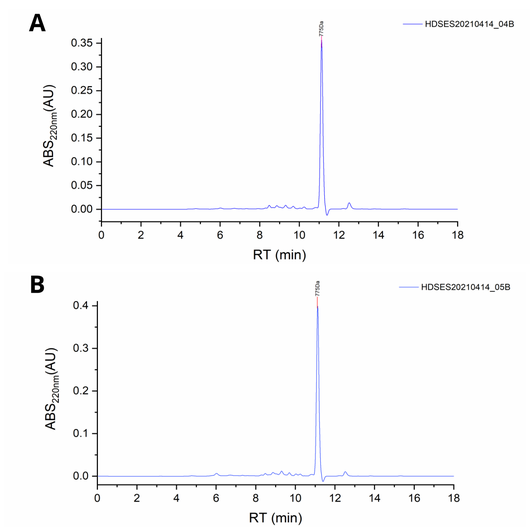


Figure 1. SE-HPLC chromatograms of the *H. didactylus* individuals. **(A)** HDSES20210414_04B and **(B)** HDSES20210414_05B.

2. Fractionation by SEC of pooled sample

A pool of HDSES20210414_04B and HDSES20210414_05B was fractionated by size exclusion chromatography (SEC) to separate the most intense peak (**Figure 2**). The collected fraction was subsequently analyzed by LC-MS/MS to identify the peptides.

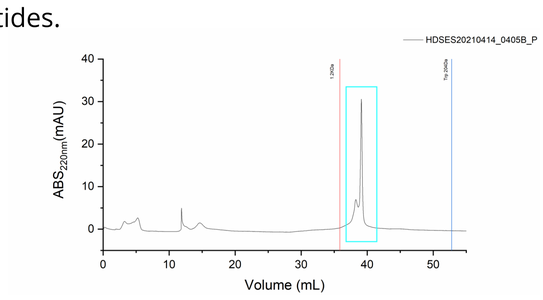


Figure 2. SEC chromatogram of the *H. didactylus* pooled sample HDSES20210414_04B05B _P.

3. Identification of peptide fraction by LC-MS/MS

Due to the lack of information in the proteomic databases for *Halobatrachus didactylus*, reliable results in peptide identification of the sample were not achieved. Therefore, it was decided to apply de novo sequencing using one of the most reliable software, PEAKS Studio©. The number of MS scans was 25686, with a number of MS/MS scans of 3329. In the analysis process, the number of De novo peptides after the score filter (De novo score ≥80) was 37 (**Figure 3**). The analysis was satisfactory in relation to the high degree of confidence of the sequencing.

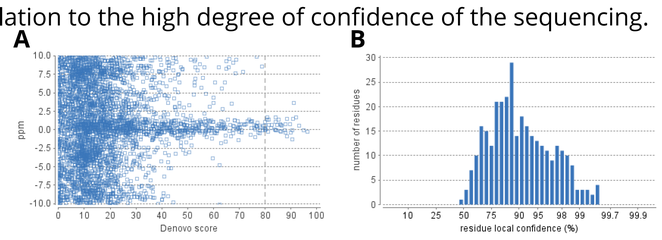
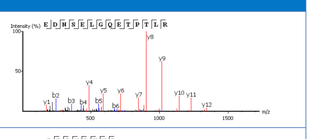
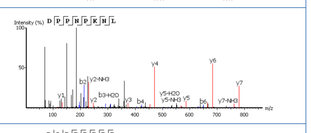
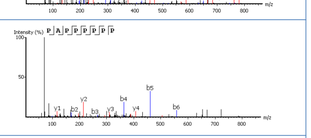
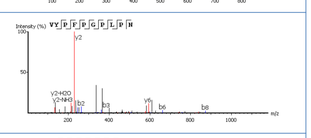
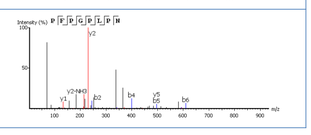


Figure 3. Denovo Statistics representation. **(A)** Scatterplot of peptide Denovo score versus precursor mass error. **(B)** Distribution of residue local confidence in the filtered result.

Five identified peptides were selected according to their bioactivities predicted *in silico* (**Table 1**).

Table 1. Mass spectrums of the identified peptides.

Code	MW (Da)	Sequence	Mass spectrum
HdKTLR	1588.4	EDNSELGQETPTLR	
HdKNL	893.8	DPPNPKNL	
HdPPP	768.8	PAPPPPPP	
HdVLPN	1099.6	VYPFPGPLPN	
HdLPN	837.5	PFPGPLPN	

4. SE-HPLC analysis and validation of identified peptides

We sent the selected peptides to be synthesized. To validate the size of each peptide, we analyzed them by SE-HPLC under standard conditions, with a mobile phase of 0.15 M NaH2PO4 pH 7.0. Under these conditions, the HdKTLR and HdKNL peptides showed larger sizes, between 3 and 4 times their size. Assuming a pH-related aggregation, we changed the mobile phase using 0.5% trifluoroacetic acid (TFA), resulting in a more approximated molecular size for HdKTLR (**Figure 4**). In the case of HdKNL, the size was about twice as large, so the conditions for analysis still need to be improved. On the other hand, the peptides HdVLPN and HdPN did not show aggregation confirming the expected molecular weight (**Figure 5**).

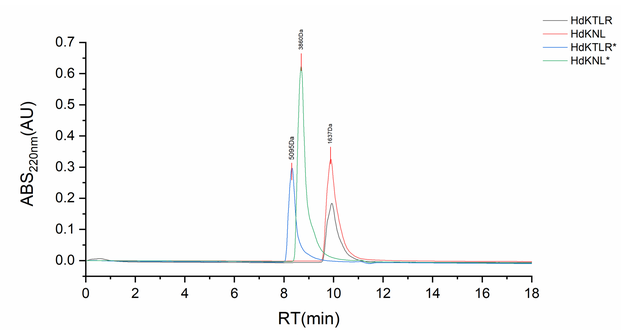


Figure 4. SE-HPLC chromatogram of the aggregated (HdKTLR* and HdKNL*) and non-aggregated (HdKTLR and HdKNL) peptides.

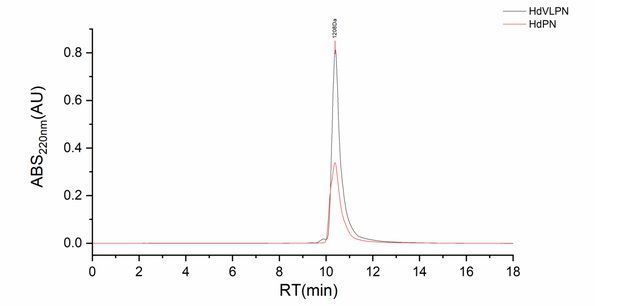


Figure 5. SE-HPLC chromatogram of the peptides HdVLPN and HdPN.

Conclusions

Overall, this chromatographic approach enabled the identification of promising peptides whose bioactivities will be evaluated *in vitro* in future work. Concerning the analysis of peptides by SE-HPLC, the standard conditions may not be adequate, so it should be considered to perform the analysis under different conditions for the samples, in order we can avoid aggregation and confirm the expected molecular weight.

Acknowledgements

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