Top-Down, High-Throughput of Thermo-Stable Allergens Using Complementary MS/MS Fragmentation Strategies

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Overview

Purpose: Development of a simple and fast strategy based on ultra high mass resolution that allows simultaneously the identification of food allergens and the authentication of fish species. This technique could be widely expanded and applied to determine fish species and as a tool to enforce the guidelines from the U.S. Food and Drug Administration with respect to fish labeling.

Methods: We used a two-step workflow. First, the fraction of thermo-stable proteins from the tissue was purified, and second, direct infusion using an Orbitrap™ Fusion

Results: We have developed an analytical strategy that allows to identify the presence of fish allergens and it also provides fish traceability in minutes using a high throughput LC-MS platform and top-down proteomics. These methods are the fastest in the allergen and food identification arena.

Introduction

LC/MS/MS provides an accurate and sensitive way to test for allergens in food products. However, in the case of fish allergens, it is not only important to identify the allergen, but also determine the species that belongs to a given sample in order to asses traceability. Here, we present a protein based method that identifies common commercially available fish species in minutes using a simple protein extraction protocol couple to multiplex top-down proteomics using an Orbitrap Fusion Tribrid mass spectrometer modified with an UVPD source. The high mass accuracy and resolution, and the different fragmentation modes allow the classification of proteins with high protein sequence homology, but species dependent amino acid substitutions.

Methods

Sample Preparation

Hake was employed in this study. These were purchased at local fish markets in Vigo spain. ~1g of fresh tissue was homogenized in ~1mL of water, centrifuged and supernatants were heated at 70ºC for 5 minutes. The samples were then centrifuged at 10,000g. Supernatant with the purified parvalbumin fraction were desalted using a MSPac™ DS-10 desalting cartridge . Proteins were eluted in 80% ACN 0.1% FA and direct infused into the mass spectrometer.

Mass Spectrometry

All data was acquired on a Thermo Scientific Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA). A 193-nm ArF excimer laser (Coherent ExciStar XS) was coupled to the mass spectrometer to allow for UVPD fragmentation. For direct infusion, desalted samples were direct infused in 80% ACN, 0.1% FA. A 500 µL Hamilton syringe was used for direct infusion, and the flow rate was set to 2 µL/min. Instrument control software Tune V 1.1 with EThcD, ETciD and UVPD as built-in methods was used. However, some of the data was processed using embedded Lua directly to the Orbitrap Fusion instruments. All data was acquired in the Orbitrap mass analyzer at a resolution of 120,000 (full width at half-maximum, fwhm) in intact protein mode (3 mTorr ion-routing multipole (IRM) pressure). Twenty scans, each consisting of 200 microscans were averaged. The most abundant proteoforms were subjected to fragmentation. Spectra for all fragmentation methods were acquired using a mass range of 150−2000 m/z. Precursor ion isolation was performed with the mass selecting quadrupole, and the isolation window was set to 1.5 m/z. In source fragmentation was used at 50V to avoid protein clustering in the gas phase. The precursor automatic gain control (AGC) target value was 5e5, maximum injection time 200 ms. For HCD, normalized collision energy (NCE) was set to 8, 12, 15, 17, 20, and 25%. ETD ion/ion reaction times (anion AGC 2e5, 200 ms maximum injection time), supplemental collisional activation (SA) in ETciD, and NCE in EThcD varied as indicated.

Data Analysis

Deconvoluted peaks were copied into the clipboard and paste into ProSight Lite v12. Intact protein spectra were deconvoluted with XTRACT with a signal-to-noise ratio (S/N) threshold of 10, a fit factor of 44%, mass range from 400 to 1600, and a remainder threshold of 25% (Thermo Scientific). Deconvoluted peaks were copy into the clipboard and paste into ProSight lite V12 (Proteomics Center of Excellence, Evanston, IL) for protein characterization

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Workflow

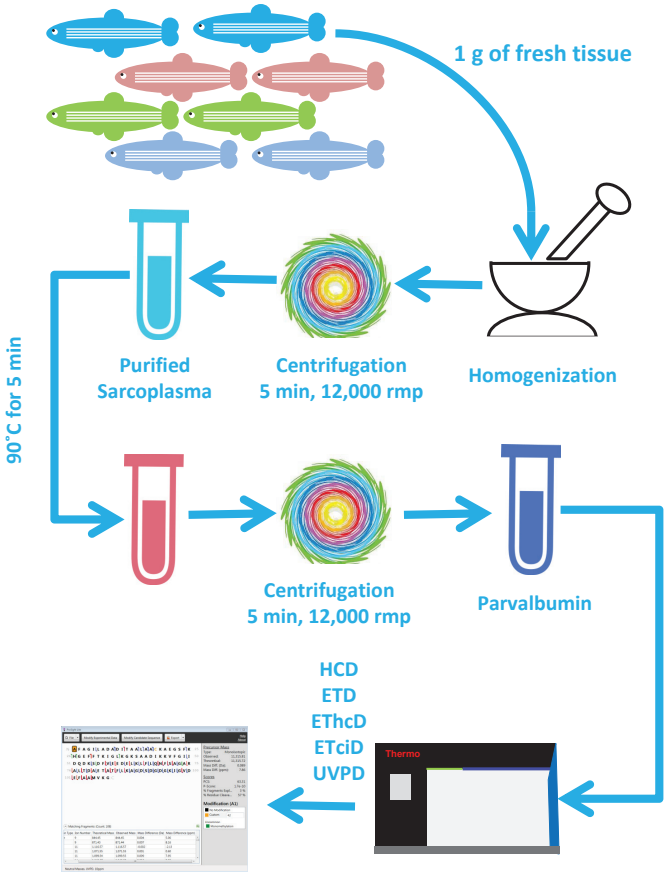

FIGURE 1. General overview of the analytical workflow. Reference muscle samples are processed. The thermo-stable proteins, beta-parvalbumins are purified and analyzed by top-down mass spectrometry

Results

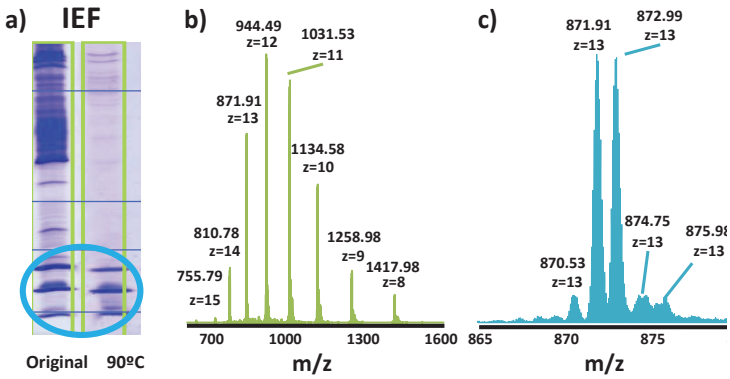

FIGURE 2. a) IEF gel showing the effect of the thermal treatment in the purficiati of parvalbumins. b) Orbitrap Fusion MS data for purified parvalbumins from Merluccius merluccius. c) Zoom in on the m/z region 860 to 880 is shown, displaying ions originating from the different parvalbumins from their +13 precursor.

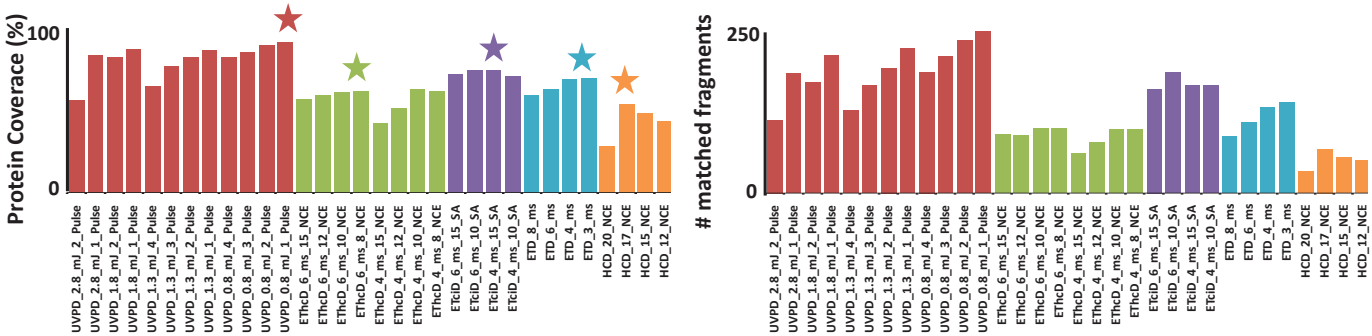

FIGURE 3. Summary of the achieved sequence coverage for Parvalbumin beta-1 (Merluccius merluccius) based on the fragmentation of the +13 precursor ion (m/z 871.9) resulting from the different fragmentation methods and the experimental parameters that were evaluated.

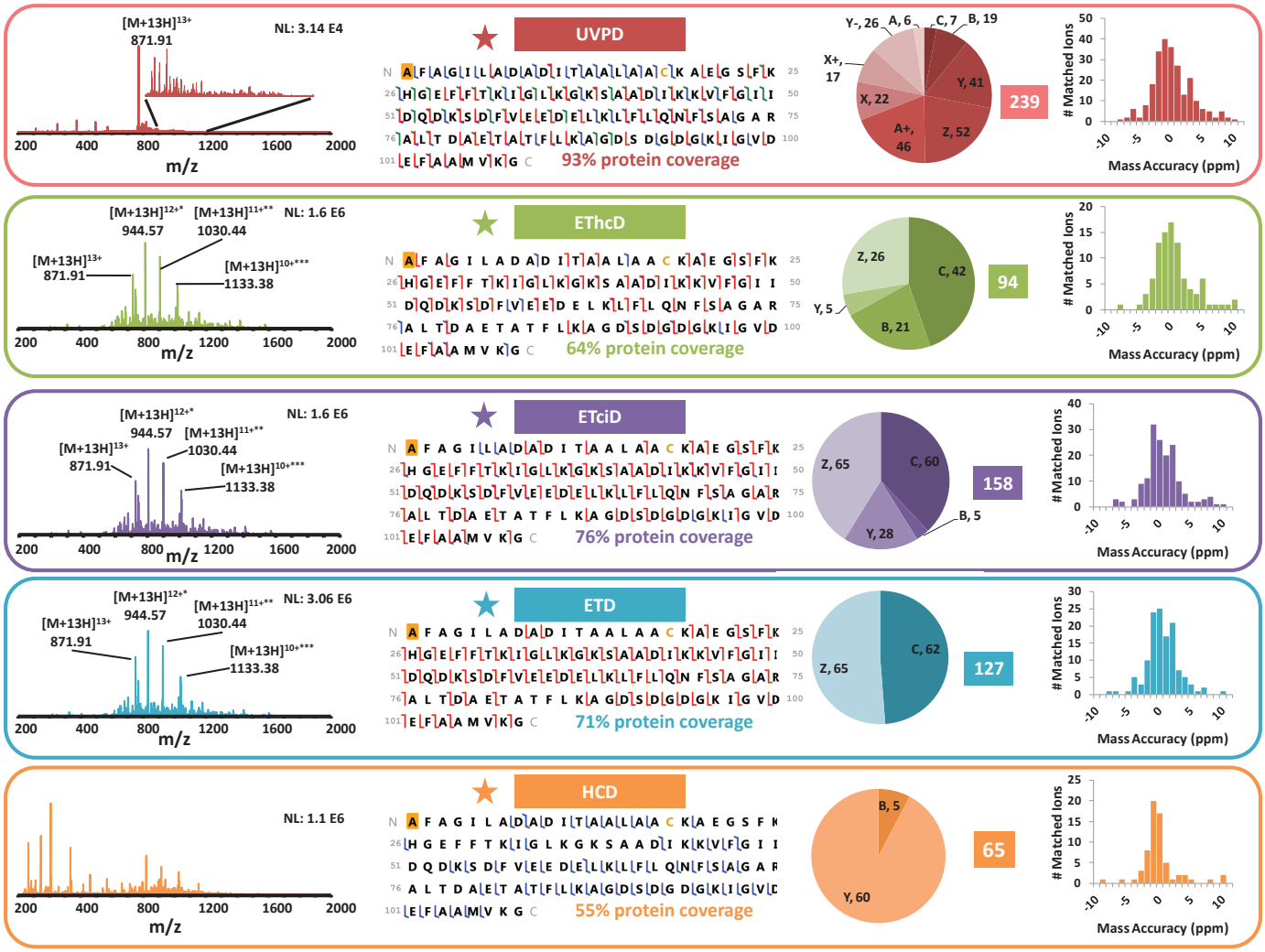

FIGURE 4. Summary of the best results obtained for each fragmentation methods (star). Including Orbitra Fusion MSMS data, sequence coverage, total number of assigned fragment ions and histogram of the mass error for each of the matched ions.

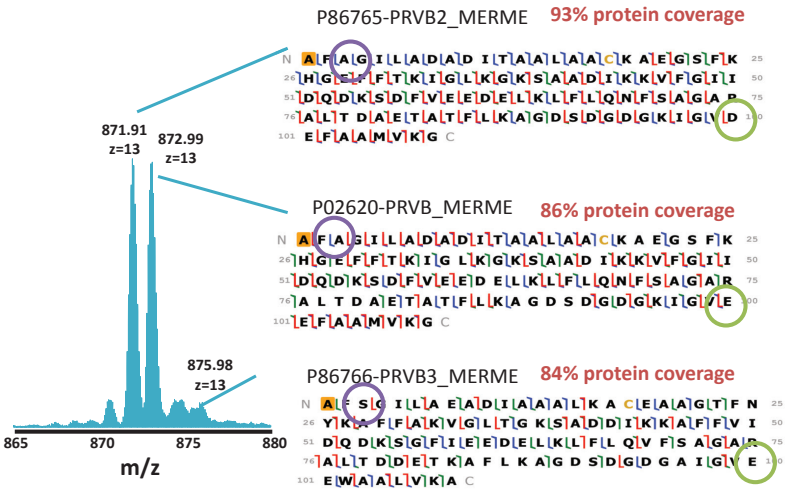

FIGURE 5. Full characterization of the three different main isoforms for the European hake. The high ion transmission at isolation widths down to 1 amu not only allows for high sensitivity that allows the identification and characterization of the three main isoforms, but also allows for high sensitivity, specifically in the case of PRVB3 which is the less abundant of the three of them. Circles in the amino acid sequence indicate the different substitutions.

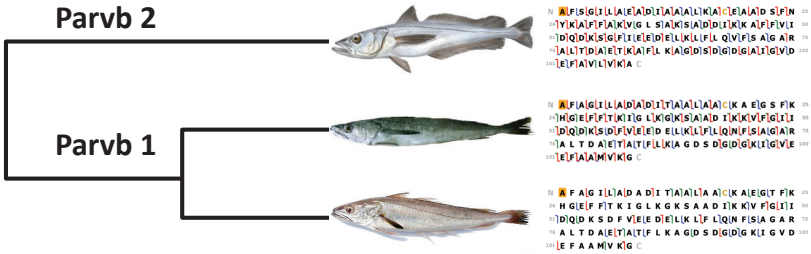

FIGURE 5. Characterization and Identification of 3 different hake species. The combination of the two parvalbumin isoforms allows for the complete differentiation of the three close related fish species. Isoform 1 allows for the unique identification of *M. paradoxus*, while the isoform 2 alows for the discrimination between *M. gayi* and *M. merluccius*.

Conclusion

An easy and robust method for fish speciation has been developed utilizing the high speed, high resolution and fragmentation capabilities of the Orbitrap Fusion Mass Spectrometry. Using parvalbumin proteoforms as a signature for the species identification reveals the following benefits:

- We show that the sequence coverage achieved using UVPD dramatically outperforms the other fragmentation techniques
- Minimal sample preparation
- High sensitivity and throughput
- Bypass extensive *de novo* sequencing due to the high homology among the amino acid sequences from the different species.

References

1. Carrera M, Cañas B, Gallardo JM *J Proteomics. 2013 ;78:211-20*
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3. *Brunner A.M et al. Analytical Chemistry 2015 87 (8), 4152-4158*