SUPPLEMENTARY MATERIAL

Supplementary methods

Identification of parvalbumins by mass spectrometry

For the sample preparation for liquid chromatography-tandem mass spectrometry (LC-MS/MS), gel bands of interest stained by Coomassie Brilliant Blue were excised from 1D or 2D gels and washed with ammonium bicarbonate buffer and acetonitrile to remove the staining. Proteins were reduced with 10 mM dithiothreitol and alkylated with 55 mM iodoacetamide, followed by overnight digestion with proteomics-grade trypsin (Sigma-Aldrich, Taufkirchen, Germany) with a 1:30 enzyme-substrate ratio, and finally cleaned up using C18 ZipTips (Sigma-Aldrich, Taufkirchen, Germany). The resulting peptides were used for LC-MS/MS. The peptides were chromatographically separated using the nLC system RSLC UltiMate 3000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using A) 0.1% formic acid in water, and B), 0.1% formic acid in acetonitrile as the mobile phase. Samples were chromatographically resolved using a 1–20 min 5–70% – 95% B gradient at a flow rate of 250 nL/min for 23 min. Peptides were analyzed using Orbitrap Exploris 240 (Thermo Fisher Scientific Inc., Bremen, Germany) in the data-dependent mode with the 20 most intense precursors subjected to fragmentation by collision-induced dissociation. Peptides were identified using the PEAKS X Pro platform (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada). Precursor mass tolerance was set to 10 ppm, fragment mass tolerance to 0.05 Da and, digestion mode to semi-specific with maximum missed cleavages 2. Protein filters were as follows: protein −10 lgP ≥ 20, proteins unique peptides ≥ 1, and “A” Score for confident PTMs identification of at least 20. Peptide filters were as follows: false discovery rate for peptide-spectrum matches < 0.5%; therefore, the resulting false discovery rate of the peptide sequence was lower than 1%. MS/MS spectra were searched using the PEAKS DB algorithms or the Proteome Discoverer Software (Thermo Fisher Scientific). The fish protein database was created for each species using entries under the specific taxonomy IDs (see supplementary Table S2) from NCBI Genbank and UniProtKB databases (accessed in December 2023 for Wels catfish, tench and eel parvalbumins and October 2021 for the other parvalbumins).