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Mass spectrometry based detection of common vitellogenin peptides across fish species for assessing exposure to estrogenic compounds in aquatic environments

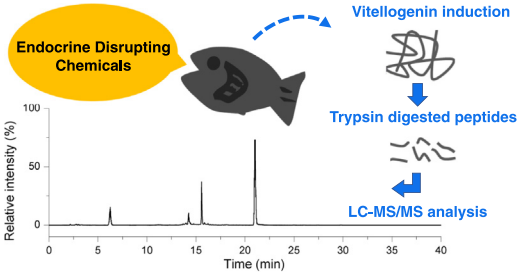
Ping He [a], Eryn K. Matich [a], Lance T. Yonkos [b], Alan E. Friedman [a], G. Ekin Atilla-Gokcumen [a], Diana S. Aga [a,*]

[a] Department of Chemistry, University at Buffalo, The State University of New York, Buffalo, New York 14260, United States
[b] Department of Environmental Science and Technology, College of Agriculture and Natural Resources, University of Maryland, College Park, Maryland 20742, United States

HIGHLIGHTS

- A mass spectrometry based method to identify common peptides in VTG from multiple fish species was developed.
- Functional validation was performed by using female fish, estrogen-exposed male fish, and unexposed male fish.
- LC-MS/MS proved useful in determining VTG biomarkers of endocrine disruption in fish.

GRAPHICAL ABSTRACT



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ABSTRACT

The identification of myriad of chemicals in the environment that mimic hormones and affect the endocrine functions of exposed organism is a daunting analytical challenge for environmental scientists and engineers. Many of these endocrine disrupting chemicals (EDCs) are present at very low concentrations in the aquatic systems, but yet affect the metabolic, developmental, and reproductive functions in exposed fish and wildlife. Vitellogenin (VTG) protein is a widely used biomarker in fish for assessing exposure to EDCs, and is commonly measured using species-specific immunochemical techniques. In this study, we developed a liquid chromatography tandem mass spectrometry (LC-MS/MS) method that can measure common peptides from digested VTG in multiple fish species. In the initial experiments using high resolution mass spectrometry, two peptides (ALHPELR and FIELIQLLR) were identified as common fragments in the digested VTG protein isolated from three different fish species (*Pimephales promelas*, *Micropterus salmoides*, and *Fundulus heteroclitus*). Then, a quantitative analysis using LC-MS/MS under selected reaction monitoring mode was developed for the detection of these two peptides in trypsin-digested plasma from female fish (positive control), estrogen-exposed male fish (test sample), and unexposed male fish (negative control) using two of the same species used for identifying the common peptides (*P. promelas*, and *M. salmoides*) and one new species (*Ameiurus nebulosus*) that was not included during the selection of peptides. Results from this study demonstrate the potential of LC-MS/MS as an effective cross-species method to detect VTG in fish, which can be an alternative analytical technique for assessing endocrine disruption in multiple fish species.

1. Introduction

Endocrine disrupting chemicals (EDCs), which include natural and synthetic hormones, pesticides, and many classes of compounds used

* Corresponding author.
E-mail address: dianaaga@buffalo.edu (D.S. Aga).

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in consumer products and industrial applications (Diamanti-Kandarakis et al., 2009) are constantly introduced in the aquatic environment from sewage treatment plant effluents (Kime, 1998; Sumpter and Jobling, 1995; Laganà et al., 2004), agricultural and livestock run-off (Goksoyr, 2006), and industrial emissions (Sidhu et al., 2005). A wide range of synthetic chemicals and their degradation products that have been identified to have potential estrogenic effects include brominated flame retardants (Pérez-Fuentetaja et al., 2015), alkylphenolic chemicals (Sumpter and Jobling, 1995; Barber et al., 2015; Hansen et al., 1998; Thorpe et al., 2001), organochlorine pesticides (Sumpter and Jobling, 1995; Hansen et al., 1998; Thorpe et al., 2001), and synthetic estrogens (Sumpter and Jobling, 1995; Gutendorf and Westendorf, 2001). Exposure to these chemicals even at low concentrations has been associated with negative physiological responses in fish, which have been used as sentinels for assessing environmental pollution (Kime, 1998; Hutchinson et al., 2006; Purdom et al., 1994; Polzonetti-Magni et al., 2004).

Identification and quantitation of estrogenic compounds at ng/L concentrations in the aquatic systems have been performed using mass spectrometry-based methods (Tso et al., 2011; Farré et al., 2007; Rodriguez-Mozaz et al., 2004a; Rodriguez-Mozaz et al., 2004b). In addition, *in vitro* bioassays have been successfully used to screen for estrogenic activities in source and treated drinking water in the United States (Conley et al., 2017; Alvarez et al., 2013). However, because of the ever increasing number of chemical pollutants being released into the aquatic systems, it has become analytically challenging to identify unknown EDCs and define their endocrine disrupting effects in fish and wildlife living in the affected environment (Matozzo et al., 2008). Laboratory investigations have shown that a mixture of different estrogenic compounds could act synergistically and be more potent than the additive effects of each of the individual chemicals when tested separately (Sumpter and Jobling, 1995; Thorpe et al., 2001).

Therefore, it is important to develop an analytical approach to assess the effects of EDCs at environmentally relevant concentrations and provide a tool that will allow scientists and regulators to better assess the overall effects of mixtures of chemicals in contaminated environments. One of the most effective ways to evaluate endocrine disruption is to measure a biomarker that is induced in response to EDC exposure (Sumpter and Jobling, 1995; Hansen et al., 1998). Vitellogenin (VTG), a liver-derived precursor protein of egg yolk that is detected in meaningful concentrations only in sexually mature female fish (Polzonetti-Magni et al., 2004), is the most widely used indicator of estrogenic exposure using male fish (Sumpter and Jobling, 1995; Hansen et al., 1998; Hutchinson et al., 2006). Numerous *in vivo* and *in vitro* studies have shown that production of plasma VTG can be induced within the liver of male fish upon exposure to estrogen agonists (Kime, 1998; Sumpter and Jobling, 1995; Del Giudice et al., 2012; Pait and Nelson, 2002; Hong et al., 2009). Thus, elevated plasma VTG in male fish is considered a sensitive biomarker of exposure to EDCs (Sumpter and Jobling, 1995; Hong et al., 2009; Yang et al., 2015).

Enzyme-linked immunosorbent assay (ELISA) is the most widely used analytical method for the quantification of VTG in fish serum (Wheeler et al., 2005). However, a major limitation of ELISA is the need to develop specific antibodies for each fish species, which is both time-consuming and costly. Commercial ELISA kits designed for a certain fish species are rarely applicable for another fish species due to poor cross-reactivity between antibodies for different fish species (Simon et al., 2010; Mourot and Le Bail, 1995). A mass spectrometry-based method for the detection of specific peptides from digested VTG protein has been demonstrated as a promising alternative to ELISA (Simon et al., 2010; Wunschel et al., 2005). The sequences of peptides could be confirmed by matching tandem mass spectrometry (MS/MS) data with theoretical fragmentation ions from a database. Peptide identification work is more challenging for species where VTG amino acid sequence is not available. In such cases, *de novo* sequencing of individual peptides could be performed (Cohen et al., 2006; Cohen et al., 2005). Mass spectrometric quantification is based on identification of particular peptides from VTG protein following enzymatic digestion with synthetic isotope-labeled peptides as standards (Simon et al., 2010). To date, mass spectrometry-based methods have been successfully established for VTG quantification in fathead minnow (*Pimephales promaelas*) (Wunschel et al., 2005), zebrafish (*Danio rerio*) (Yang et al., 2015), Greenland halibut (*Reinhardtius hippoglossoides*) (Cohen et al., 2009), Atlantic cod (*Gadus morhua*) (Cohen et al., 2005), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Cohen et al., 2006).

The objectives of the present study are to identify common VTG peptides from different fish species, and to demonstrate the potential of liquid chromatography-tandem mass spectrometry (LC-MS/MS) as an effective method for the detection of VTG from multiple fish species. We hypothesize that because large proteins with the same function may share a high degree of sequence homology across species (Dayhoff et al., 1983), VTG protein from many species will share common peptide sequences. The ultimate goal is to develop an LC-MS/MS based quantification method that targets a specific set of VTG peptides observed across various fish species. To test this hypothesis, first, common peptides resulting from trypsin enzymatic hydrolysis of purified VTG standards from three different fish species (fathead minnow, *P. promelas*; largemouth bass, *Micropterus salmoides*; and killifish, *Fundulus heteroclitus*) were identified using a LC equipped with a high-resolution quadrupole time-of-flight tandem mass spectrometer (Q-TOF/MS/MS) system. Second, method validation was performed by targeted analysis of common peptides from fish plasma using LC-MS/MS with a triple quadrupole mass spectrometer for low-level detection of peptides from trypsin-digested VTG. Finally, functional validation of the identified common peptides was performed by determining their occurrence in trypsin-digested plasma samples from female fish (positive control), estrogen-exposed male fish (test sample), and unexposed male fish (negative control).

2. Experimental section

2.1. Chemicals and materials

Acetonitrile (Optima™ LC-MS grade), ammonium bicarbonate, sodium dodecyl sulfate, and formic acid, were purchased from Fisher Scientific (Pittsburgh, PA). Iodoacetamide (IAM) was from Alfa Aesar (Tewksbury, MA). Tris and dithiothreitol (DTT) were from VWR (Radnor, PA). Bromophenol blue was from Acros Organics (Morris Plains, NJ). Glycerol was from EMD Millipore (Billerica, MA). Trypsin (protein sequencing grade lyophilized powder) and synthetic internal standard peptide Angiotensin II (≥93%, powder, amino acid sequence DRVYIHPF) were obtained from Sigma-Aldrich (St. Louis, MO). We choose synthetic peptide Angiotensin II as internal standard, because of its stability and solubility in water, and because the amino acid sequence of Angiotensin II does not occur in any of the fish species included in this study, as has been checked in BLAST (Basic Local Alignment Search Tool) program. Synthesized peptides ALHPELR and FIELIQLLR were from LifeTein (Hillsborough, NJ). Coomassie blue G-250 solution was from Bio-Rad (Hercules, CA). Oasis™ PRiME hydrophilic-lipophilic balance (HLB™) solid-phase extraction cartridges were purchased from Waters (Mildford, MA). 17β-estradiol (β-E2) and 17α-ethinylestradiol (EE2) were from Sigma Aldrich (St. Louis, MO).

2.2. Identification of common peptides from three fish species

2.2.1. In-gel trypsin digestion of VTG standards

The procedure for fish collection and sources of fish samples are described in the Supporting Information (SI). Plasma was collected from each fish following procedure described previously (Yonkos et al., 2010). The VTG standards for fathead minnow, largemouth bass, and killifish were isolated and purified from the plasma of β-E2-induced