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Method for detection and quantitation of fathead minnow vitellogenin (Vtg) by liquid chromatography and matrix-assisted laser desorption/ionization mass spectrometry

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Abstract

Vitellogenin (Vtg) is a well-recognized biomarker of estrogen exposure in many species, particularly fish. This large protein shares a high degree of sequence homology across a large number of species. Quantitative measurement is currently done using antibody-based assays. These assays frequently require purification of Vtg and antibody production from each species because there is poor cross reactivity between antibodies for different fish. Therefore, complementary methods of measuring Vtg are desirable. Mass spectrometric (MS) analysis coupled to database searching offers the promise of a general method for protein identification. In this study, we used the well-characterized Vtg from rainbow trout (*Oncorhynchus mykiss*) to evaluate the analytical parameters for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis of the intact protein. The low sensitivity observed for the intact protein demonstrated that a proteolytic digestion would be necessary for MALDI-MS identification of Vtg. An analytical scale high performance liquid chromatography (HPLC) separation combined with MALDI-MS was used to measure and confirm the identity of Vtg from the plasma of an important species for regulatory agencies, fathead minnow (*Pimephales promelas*). The small volume requirement of this method (<10 μL) was found to be compatible with the plasma volume obtained from a few minnows. Importantly, the HPLC–MALDI-MS measurement of fathead minnow Vtg abundance after exposure to estradiol was similar to previously obtained enzyme linked immunosorbant assays (ELISA) data.

Keywords: Vitellogenin; Ethynyl estradiol; HPLC; MALDI-MS; Peptide mass fingerprinting

1. Introduction

Vitellogenin (Vtg) is a large (190 kDa), dimeric, highly modified egg yolk precursor protein belonging to a large family of lipoproteins (Roubal et al.,

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1997). Vtg is synthesized in the liver of fish and other oviparous vertebrates before being transported to the ovaries and the developing oocytes where it will provide nutrition for developing embryos (Bon et al., 1997). There is a conserved overall structure to the rainbow trout (*Oncorhynchus mykiss*) Vtg molecule with an N-terminal section, lipovitellin I (1100 amino acids) heavily phosphorylated serine rich region (phosvitin) and C-terminal lipovitellin II similar to other vertebrate Vtg (Mouchel et al., 1996). Two potential glycosylation sites have also been identified; one within phosvitin and the other within the lipovittellin II portions of the molecule. The conserved function and sequence similarity of the lipovitellin I and II sequences have led to their use in comparative studies between higher chordates (Lim et al., 2001). In fish, a wealth of lipovitellin I sequences (>50) are present in Genbank that share significant homology with blast scores >200.

Synthesis of Vtg is under estrogen regulation, which can also be stimulated by synthetic estrogens (Schultz et al., 2001; Rose et al., 2002). Because Vtg can be induced in large quantities in either sex after exposure to estrogen or estrogen mimics, it has been used as a biomarker for xenobiotic exposure to estrogenic compounds in many species (Brion et al., 2000; Roubal et al., 1997). To be effectively utilized as a biomarker, assays are required to accurately measure the concentration of Vtg in plasma. The principal methods in use are enzyme linked immunosorbant assays (ELISA) or other types of immunoassays utilizing specific antibodies (Parks et al., 1999; Bon et al., 1997). This approach has proven to be a reliable and sensitive way of measuring Vtg levels. Although a high degree of sequence conservation for Vtg is displayed across taxa, there is still considerable variability in the immunological determinants in different species (Brion et al., 2000). Typically, best results are obtained with ELISA assays that use monoclonal antibodies produced towards Vtg from a specific fish species (Nilsen et al., 2004). The latter process can be time consuming to develop and is currently only available for a few fish species. Thus, it is more common to use polyclonal antibodies from species specific or closely related fishes resulting in a wide variety of published Vtg ELISA assay methods.

The measurement of Vtg is now becoming routinely incorporated into laboratory studies of endocrine disruptors, which increases the need for a complimentary method to ELISA that does not require the availability of antibodies. Mass spectrometric (MS) analysis of proteins and other large biomolecules has allowed for a general approach to be utilized for identifying proteins (Mann et al., 2001). One type of ionization technique for MS that allows analysis of large biomolecules without fragmentation is matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (Gross and Strupat, 1998). By producing ions from very large, intact molecules, a mass measurement can be obtained that is far more accurate than polyacrylamide gel electrophoresis (PAGE) can provide. However, the ionization efficiency decreases with increasing molecular weight, which can limit the sensitivity and size of intact proteins for analysis. Furthermore, the appropriate choice of matrix and solvent conditions is important and post-translational modifications such as glycosylation can dramatically reduce the ability to measure large proteins (Kim et al., 2001). A recent study has shown that both the intact and protease-digested forms of rainbow trout Vtg could be analyzed by MALDI-MS (Banoub et al., 2003). Structural information on Atlantic salmon (*Salmo salar*) and rainbow trout Vtg has also been gained by generating peptides from specific cleavage and subsequent tandem mass spectrometry analysis of the peptides generated (Banoub et al., 2003, 2004).

In order to identify a protein from a database of sequences, information beyond the mass measurement alone is required. This is due to both the presence of post-translational modifications that affect the measured mass (but are not represented in the protein databases) and the inadequacy of a mass measurement alone to uniquely identify a protein. The technique of peptide mapping using MALDI-MS is the method of choice for identifying proteins separated by PAGE (Egelhofer et al., 2002). This technique relies on the mass measurement of peptides produced by proteolytic digestion and comparison with predicted peptide masses from each protein in the database (Bienvenut et al., 1999). Algorithms are then used to compare and determine a probability score to match the experimental data with candidates in the database. The mathematical tools for identifying proteins using this approach are available publicly through an easily navigable web interface (Zhang and Chait, 2000). Protein identification using the peptide mapping approach is limited to a small (mixtures of four or fewer) number of proteins,