Interspecies variation in the hepatic biotransformation of zearalenone: Evidence for bio-inactivation of mycoestrogen zearalenone in sturgeon fish

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Abstract

Zearalenone (ZEA) as mycoestrogen is found in human foods and animal feeds. Its estrogenic potency depends on its biotransformation fate. The hepatic biotransformation of ZEA in two species of sturgeon fish (*Acipenser persicus* and *Huso huso*) was investigated. ZEA was incubated with the hepatic microsomal and post-mitochondrial sub-fractions in the presence of NADPH and the metabolites were determined by means of HPLC. Moreover, the rate of glucuronidation for ZEA and its metabolites were estimated in the presence of uridine diphosphateglucuronic acid. β-zearalenol (β-ZOL) was found to be the major metabolite of ZEA by both sub-fractions. Enzymatic kinetics studies revealed that the maximum velocity (V_{max}) in microsomal and post-mitochondrial fractions for β-ZOL production was found 5- and 7-folds in *Huso huso* and 8- and 12-folds in *A. persicus* higher than that for α-ZOL production, respectively. The *H. huso* hepatic post-mitochondrial fraction mainly glucurinated ZEA while in *A. persicus*, the metabolites and in particular β-ZOL were glucuronidated. Data suggest that the hepatic biotransformation of ZEA in studied sturgeons resulted in detoxification of ZEA as the main metabolite tends to be β-ZOL with weaker estrogenic property. Moreover, clear differences in glucuronidation profile are indicating interspecies variety in hepatic biotransformation of ZEA.

Keywords: *Acipenser persicus*; *Huso huso*; Hepatic biotransformation; Glucuronidation; Subcellular fractions; Zearalenone.

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Introduction

Sturgeons as one of the most valuable aquatic resources are specifically found in large rivers, lakes, coastal waters and inner seas of the northern hemisphere. In addition of being a major source of income and employment, sturgeons' meat and caviar as unfertilized sturgeon roe are among the most valuable foods for human. Like other fish species, successful sturgeon farming also depends on the proper management and high quality water and feed. Several factors influence the feed quality including having complete nutrients and being free of contaminations. Mycotoxins are among the main feed contaminants, the substances which are produced by various fungi. Previous studies have shown that plant ingredients including vegetable oil, soybean meal, corn and wheat gluten make up the main parts of fish diet (Nizza and Piccolo, 2009). At the same time it has been reported that mycoestrogen zearalenone had been found at the highest level in the corn samples used for fish diet preparation. Recently ZEA was reported as the more prevalent mycotoxin with an average level of 67.9 μg kg^{-1} fish feed (Pietsch *et al*., 2013).

ZEA, 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone, is produced by *Fusarium* species including *F. culmorum* and *F. graminearum* (Hestbjerg *et al.,* 2002). These molds contaminate crops such as maize, barley and wheat (Yamashita *et al.*, 1995; Jimenez and Mateo, 1997). The concentration of ZEA in feed materials can vary from a few micrograms up to 276 mg/kg (Vrabcheva *et al*., 1996). Since ZEA binds to estrogen receptors (ERs) after ingestion and acts as an estrogenic compound, thus it is referred as a mycoestrogen (Malekinejad *et al*., 2005). Animals exposed to ZEA or ZEA-contaminated feed, show symptoms of hyperestrogenism. In this regards, previous data indicated that pigs are the most sensitive species to ZEA (Decasto *et al*., 1995; Yang *et al.*, 1995). The estrogenisity of ZEA in rainbow trout has also been previously demonstrated by using the relative binding affinity method (Knudsen and Pottinger, 1999).

Previous investigations on effects of ZEA in fish have focused mainly on the reproduction of zebrafish (Johns *et al*., 2009). It is well documented that the estrogenic potency of ZEA could largely be affected by hepatic biotransformation and the conversion of ZEA into α-zearalenol or β-zearalenol. We also have shown that there are species differences in hepatic biotransformation of ZEA in terms of different metabolite production and the rate of glucuronidation (Malekinejad *et al.*, 2006). Sturgeons have a relatively long life span and depending on the species their sexual maturation starts at the age of six years onward, thus there are possibilities of consuming the ZEA contaminated diet. Although there is no direct report about the ZEA-contaminated sturgeon diet, ZEA

contamination has been previously reported in soils, drainage water, rivers and lakes of the United States and several other countries, ranged up to 220 ng/L (Maragos, 2012). In our previous study, we investigated the hepatic biotransformation of ZEA by subcellular fractions of rainbow trout and demonstrated that the hepatic metabolism of ZEA resulted in detoxification and lessening the estrogenic potency of ZEA by converting it to weaker estrogenic metabolite of β-zearalenol (Malekinejad *et al*., 2012). As there is currently a lack of knowledge about the hepatic biotransformation of ZEA in sturgeons, hence in this study the hepatic biotransformation of ZEA including both phases I and II reactions by sub-cellular fractions of *H. huso* and *A. persicus* were investigated.

Materials and methods

Reagents

The test compounds ZEA, α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), uridine diphosphate glucuronic acid (UDPGA), and nicotinamide dinucleotide phosphate (NADPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of reagent grade.

Animals

Sixty sturgeons fish (average wt. 200 ± 5 g) including *Huso huso* (n = 30) and *A. persicus* (n= 30) were obtained from the Artemia and Aquatic Animals Research Institute (Urmia, Iran) and stocked in separate concrete ponds containing groundwater freshwater. The fish were cultured in a flow-through system with a flow rate of 50 L/min. Dissolved oxygen was maintained above 8 mg/L using constant aeration and fish were exposed to a natural photoperiod of approximately 12:12 L:D. The water temperature was 13.5 ± 1.0˚C, and the pH was maintained between 7.3-7.5. The fish were fed on commercially standard and mycotoxin free formulated sturgeon diet (Esfahan Mokammel CO., Iran) for a period of three months.

Fish tissue collection and preparation of the liver subcellular fractions

The fish were kept in accordance with the guidelines of the Local Ethical Committee roles applying to principles of Laboratory Animal Care, NIH publication No. 86-23, revised in 1985 (NIH, 1985). For tissue collection, eight randomly collected male individuals (average wt. 420 ± 20 g) were anesthetized by immersion in eugenol solution (20 mg/L) and immediately dissected to remove the liver. The collected liver samples were rinsed three times with chilled normal saline to get rid of the extra blood.

The sub-cellular fractions of the liver were prepared as described previously (Malekinejad *et al*., 2005). Briefly, immediately after anesthesia, the liver specimens were collected and cut into small pieces. Two volumes of KCl (1.15%) and EDTA (0.1mM) solution were added to the tissue samples and

the mixtures were homogenized in a Potter-Elvehjem apparatus with a Teflon pestle (Krackeler Scientific Inc. Albany, NY, USA). The homogenates were centrifuged (Beckman Coulter Inc. USA) at 9,000 *g* for 30 min at 4 °C and an aliquot of the supernatant was collected as the post-mitochondrial fraction. The remaining sample was centrifuged at 100, 000 *g* for 90 min at 4 °C in order to obtain the microsomal fraction. After centrifugation, the supernatant was discarded and the pellet was carefully dissolved in the same volume of phosphate buffer (0.05 M; pH 7.4) with glycerol (20%), and then was homogenized with an ultra-turrax homogenizer (T 25, IKA, Germany). The individual fractions extracted from the fish samples were pooled and stored at –70°C until use. Protein concentrations of the microsomal and the post-mitochondrial fractions were determined according to Lowry method (Lowry *et al*., 1951).

Incubation of ZEA with hepatic sub-cellular fractions (Phase I reactions)

To determine the biotransformation metabolites, various concentrations of ZEA (10, 25, 50, 100, 250, 500 μM, dissolved in methanol) were added to the reaction mixtures, containing either pooled liver microsomes (0.505 mg protein) or the post-mitochondrial fraction of the liver (2.008 mg protein) and supplemented with NADPH (0.5 mM) in a final volume of 250 μl phosphate buffer (50 mM, pH 7.4). The samples were incubated in a shaking water bath (Memmert, Germany) at 20 °C for 30 min. The reaction was stopped by transferring the samples to an ice-cold environment followed by extraction with chloroform (1.25 mL). One milliliter of the organic phase was collected and evaporated to dryness under a gentle stream of N_2. The residue was re-dissolved in the mobile phase and was subsequently analyzed by high performance liquid chromatography (Malekinejad *et al*., 2005).

Determination of the extent of glucuronidation

To evaluate the rate of glucuronidation, the post-mitochondrial fraction was dialyzed overnight using cellulose membranes (width 25 mm, diameter 16 mm; retaining proteins with a MW>12 KDa and hence all enzymes) with a dialysis buffer containing 1.15% (w/v) KCl and 0.1 mM EDTA, which was refreshed twice. Subsequently, increasing concentrations of ZEA were added to the dialyzed fraction (2.008 mg protein) dissolved in a phosphate buffer (50 mM, pH 7.4) and supplemented with 0.5 mM NADPH (pre-dissolved in phosphate buffer) in the absence or presence of 10 mM UDPGA, and incubated in shaking water bath for 30 min at 20°C. The reaction was stopped by placing the samples on ice and adding 1.25 ml ice-cold chloroform. After vigorous mixing and centrifugation at 3000 g for 5 minutes at 4°C, one ml of the organic phase was collected and evaporated to

dryness under a stream of nitrogen. The residue was subsequently analyzed by HPLC. The rate of glucuronidation (%) was calculated using the following equation:

% of glucuronidation = 100 – (100 × the amount of produced metabolites in the presence of UDPGA / the amount of produced metabolite in the absence of UDPGA)

Determination and confirmation of ZEA metabolites using HPLC method

ZEA and its metabolites were determined using high-performance liquid chromatography (HPLC), according to previously described method (Malekinejad *et al*., 2005). Shortly, the chromatographic system consisted of an auto sampler (Autosampler Triathlon type 900, Germany) and dual pumps (Wellchrom HPLC pump, K-1001, KNAUER Germany). Twenty microlitres of the extracted sample was injected into a LUNA 5μ C18 (150×4.60 mm, Phenomenex) column. The mobile phase consisted of a mixture of methanol- water (70:30, v/v) eluted at a flow-rate of 0.8 mL/min. ZEA and its metabolites were detected by means of a fluorescence detector (RF-10AXL KNAUER, Germany), set at an excitation and emission wavelength of 236 and 418 nm, respectively. ZEA and its metabolites were quantified by measuring peak areas and comparing them to the relevant calibration curves. To obtain calibration curve for each single compound, we subjected a concentration range from zero to 100 μM (r^2= 0.9992 for ZEA, r^2= 0.9975 for α-ZOL and r^2=0.9985 for β-ZOL).

Statistical analysis

All results are presented as means ± SD. For kinetic studies the Michaelis-Menton constant (K_m) and the maximum velocity (V_{max}) were obtained. Differences between the amounts of the individual products were analyzed with a two-way ANOVA followed by a Bonferroni test, using Graph Pad Prism 4.00, Graph Pad Software. p<0.05 was considered significant.

Results

ZEA was bio-inactivated by both sturgeons' hepatic biotransformation

Incubation of ZEA with the hepatic subcellular fractions of sturgeons, followed by chloroform extraction and HPLC analyses, revealed that although both known metabolites of ZEA including α- and β-ZOL are produced by either studied fractions, however β-ZOL found to be the dominant metabolite. Fig. 1 shows both standard and sample chromatograms with identical retention time for individual compounds. The β-ZOL production by *Huso huso* hepatic microsomes was found 3 fold higher than that of *A. persicus*. We did not find any significant (p>0.05) differences between the level of produced metabolites by two post-mitochondrial fractions of the two different species of studied sturgeons.

Comparison of the biotransformation capacity of the studied fractions showed that the microsomal fraction produced significantly (p<0.05) more quantities of both metabolites than those produced by post-mitochondrial fraction in both examined fishes (Fig. 2).

Enzymatic kinetics studies revealed that the maximum velocity (V_{max}) in microsomal and post-mitochondrial fractions of *H. huso* for β-ZOL production was found approximately 5- and 7-fold higher than that for α-ZOL production, respectively. The Km value for the α-ZOL production by hepatic microsomal fraction of *H. huso* was 1.7-fold higher than that for the β-ZOL production, while it was found in contrary in the post-mitochondrial fraction as the Km value for β-ZOL production was approximately 3-fold higher than that for the α-ZOL production (Table 1). At the same time, the V_{max} values for β-ZOL production by the hepatic microsomal and post-mitochondrial subfractions of *A. persicus* was estimated 8- and 12-folds higher than that for α-ZOL production. The highest Km value was found for β-ZOL production by the post-mitochondrial fraction in *A. persicus*.

Glucuronidation of ZEA and its hydroxylated metabolites

The rate of glucuronidation of ZEA and its hydroxylated metabolites by the post-mitochondrial fraction of sturgeons was determined. The first finding of the conjugation study revealed that there is a clear difference between two sturgeons in terms of glucuronidation profile as *H. huso* hepatic post-mitochondrial fraction mainly glucurinated ZEA while in *A. persicus*, the metabolites and in particular β-ZOL were glucuronidated. We found that *Huso huso* conjugated α-ZOL significantly (p<0.05) more than β-ZOL, while *A. persicus* post-mitochondrial sub-fraction mainly glucuronidated β-ZOL. Our results showed a reverse level of glucuronidation as the high concentration of ZEA, the low percentage of glucuronidation, indicating a saturable condition of glucuronidase, which is responsible for glucuronidation of ZEA and its reduced metabolites (Table 2).

Discussion

The hepatic biotransformation of ZEA in two types of sturgeon was investigated. The results showed that, although there are some similarities in the production of β-ZOL as the dominant metabolite by hepatic sub-fractions of both either sturgeons, but there are also substantial differences in the glucuronidation of ZEA and its metabolites by post-mitochondrial fractions.

Table 1: Kinetics of ZEA biotransformation by the sturgeon's hepatic subcellular fractions.

Metabolite	Microsomes	Post-mitochondrial
Huso huso		
(α-ZOL)		
V_{max} (pMol/min/mg)	19.5 ± 2.5	2.9 ± 0.09*
K_m (µMol/L)	91.2 ± 34.6	16.9 ± 2.5*
r^2	0.68	0.88
β-ZOL		
V_{max} (pMol/min/mg)	98.8 ± 6.5	20.8 ± 1.04*
K_m (µMol/L)	52.8 ± 11.9	55.25 ± 9.4
r^2	0.85	0.91
A. persicus		
(α-ZOL)		
V_{max} (pMol/min/mg)	3.2 ± 0.08	1.7 ± 0.06*
K_m (µMol/L)	13.01 ± 1.7	28.5 ± 4.4*
r^2	0.90	0.89
β-ZOL		
V_{max} (pMol/min/mg)	25.4 ± 1.2	21.3 ± 1.02*
K_m (µMol/L)	17.3 ± 3.8	72.3 ± 10.9
r^2	0.76	0.95

Table 2 : Percentage of glucuronidation of ZEA and its metabolites by the hepatic post-itochondrial fraction of rainbow trout.

ZEA (µM)	ZEA	α-ZOL	β-ZOL
Huso huso			
10	71.7±9.1	33.1± 2.8	16.3 ± 1.2
25	64.9±8.0	29.0 ± 0.5	9.6 ± 1.8
50	45.7 ± 13.9	27.5 ± 1.3	6.4 ± 0.3
125	40.2 ± 2.7	26.1 ± 2.2	5.5 ± 3.3
250	29.5 ± 12.4	11.9 ± 1.1	4.4 ± 0.6
500	19.7± 4.6	4.7 ± 2.3	3.2 ± 1.1
A. persicus			
10	23.6 ± 1.1	100	100
25	24.0 ± 2.3	100	100
50	16.7 ± 2.9	31.2 ± 0.7	100
125	13.2 ± 1.3	28.7 ± 0.4	44.0 ± 0.3
250	7.3 ± 0.5	23.3 ± 3.1	24.5 ± 0.5
500	5.7 ± 0.6	19.2 ± 0.6	12.3 ± 0.9

Very first finding of the current study was that the hepatic biotransformation of ZEA resulted in detoxification as β-ZOL with rather weaker estrogenic activity was found as the main metabolite in both sturgeons. There are numerous studies indicating that β-ZOL in comparison to α-ZOL and the original compound (ZEA) is a weaker mycoestrogen (Le Guevel *et al*., 2001; Malekinejad *et al.,* 2005; Filannino *et al*., 2011).

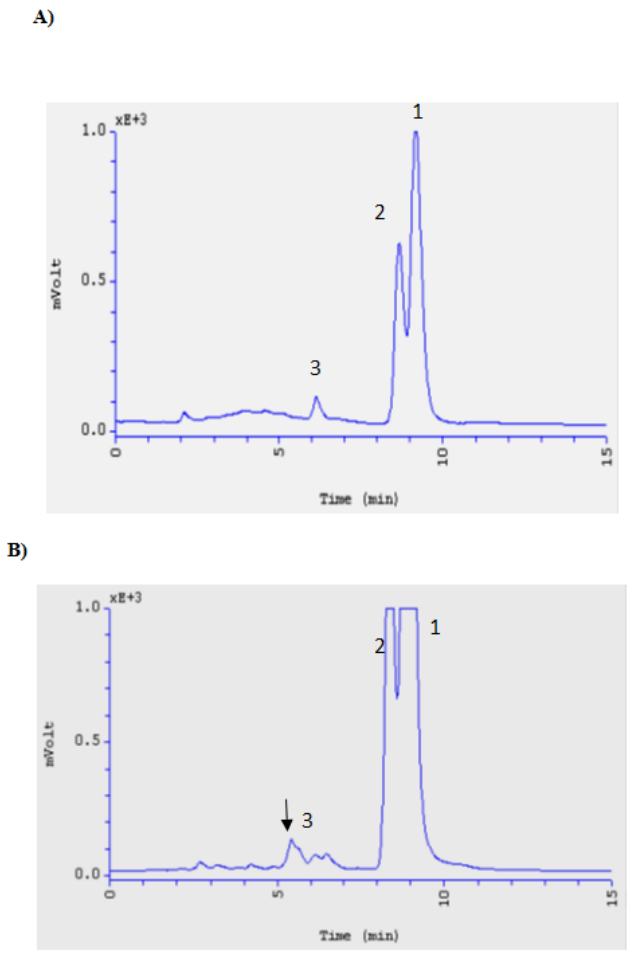

Figure 1: Standard (A) and sample chromate grams (B) with identical retention time for individual compounds; 1: Zearalenone, 2: α-Zearalenol and 3: β-Zearalenol.

Cosnefroy and co-workers recently demonstrated the same order of estrogenic potency for ZEA and its reduced metabolites by using a zebrafish specific *in vitro* stable reporter gene assay (Cosnefroy *et al*., 2012). Comparing two examined sturgeons indicates that there are clear differences between the amounts of produced metabolites by the hepatic sub-fractions of two sturgeon species, suggesting interspecies variety in the ZEA hepatic biotransformation. Although there is no direct report about the ZEA biotransformation in sturgeons, however our previous studies in mammalians showed considerable interspecies differences between various ruminants including cattle and sheep (Malekinejad *et al.*, 2006).

Enzymatic analyses of this study clarified that V_{max} for production of both metabolites in microsomal fraction was higher than that in post-mitochondrial fraction in both tested sturgeons, indicating dense localization of enzymes in microsomal fraction.

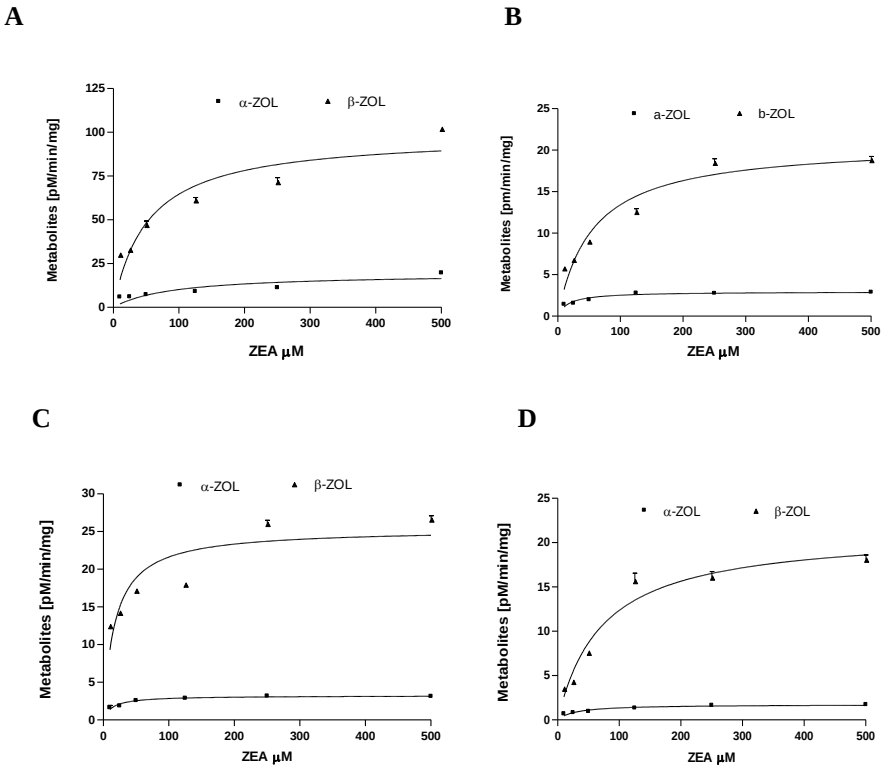

Figure 2: Biotransformation of ZEA by A) huso huso hepatic microsomes, B) huso huso hepatic post-mitochondrial fraction, C) A. persicus hepatic microsomes and D) A. persicus post-mitochondrial fraction.

This finding is in a good accordance with previous works (Malekinejad *et al*., 2005; Malekinejad *et al*., 2006; malekinejad *et al*., 2012). Our data suggest a higher efficiency (V_{max}/K_m ratio) of the microsomal enzymes than post-mitochondrial enzymes in examined sturgeon's liver and this finding is not consistent with our previous finding in the rainbow trout (Malekinejad *et al*., 2012) suggesting various affinity of enzymes toward the ZEA between different species of fish. Additionally, under the assumption that 3β-HSD converts ZEA into β-ZOL, the obtained enzyme kinetics indicate a higher efficiency of 3β-HSD than 3α-HSD in examined sturgeon's liver. Our data also indirectly confirm the expression and localization of both isoenzymes albeit with differences in the liver of examined sturgeons.

We also investigated the glucuronidation rate of ZEA and its reduced metabolites by the hepatic post-mitochondrial fraction of sturgeons. It is known that the rate of glucuronidation mainly depends on the capacity of UDPGTs and availability of UDPGA (Belanger *et al.*, 1990; Tukey and Strassburg, 2000). The estimated percentage of the conjugation rate for all three compounds indicates that there are profound differences between two examined sturgeons in terms of glucuronidation profile of ZEA and its metabolites. The main finding in this regard is indicating that the *H. huso*

hepatic post-mitochondrial fraction mainly glucuronidated ZEA, while ZEA reduced metabolites were largely glucuronidated by *A. persicus*, indicating another difference between two sturgeons in the biotransformation of ZEA. There is molecular evidence indicating the presence of multiple UGT genes in zebra fish, suggesting the fact that the different glucuronidation profile of ZEA and its metabolites in our study may be related to the presence of distinct UDP-glucuronosyltransferases in two examined sturgeons (George and Taylor, 2002). Nevertheless, clarifying the presence and function of UGT enzyme(s) in sturgeons is required.

The data described in the present study is the first report showing that ZEA is converted mainly into β−ZOL which could account for detoxification method. Moreover, different glucuronidation profile of ZEA and its metabolites in two examined sturgeons confirmed the interspecies variety of the ZEA hepatic biotransformation.

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