In vitro reduction of zearalenone to β-zearalenol by rainbow trout (*Oncorhynchus mykiss*) hepatic microsomal and post-mitochondrial subfractions

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Summary

Mycoestrogen zearalenone (ZEA) is found in human foods and animal feeds. Its estrogenic potency mainly depends on its biotransformation fate. The hepatic biotransformation of ZEA in rainbow trout was investigated in this study. Various concentrations of ZEA were separately 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 reduced metabolites were estimated in the presence of uridine diphosphate glucuronic acid. β-zearalenol (β-ZOL) was found to be the major metabolite of ZEA by both sub-cellular fractions. The enzymatic kinetics analyses indicated that the α-ZOL and β-ZOL production by microsomal fraction were 8- and 2-fold higher than those by post-mitochondrial fraction, respectively. High percentages of ZEA and its metabolites are conjugated with glucuronic acid at the lower concentrations. Data suggest that the hepatic biotransformation of ZEA in rainbow trout resulted in its detoxification as the main metabolite tends to be β-ZOL with weak estrogenic property. Moreover, at certain concentrations, the produced metabolites are entirely conjugated with glucuronic acid, which may consequently cause a prolonged duration of action due to entero-hepatic cycle.

Key words: Zearalenone, Hepatic biotransformation, Subcellular fractions, Glucuronidation, Rainbow trout

Introduction

Fish nutritionists use plant ingredients along with other sources of proteins for fish diet formulating. According to the United Nations Food and Agriculture Organization (FAO) reports and similar reports by individual scientific groups, it has become clear that plant ingredients including vegetable oil, soybean meal, corn gluten and wheat percentages in whole fish diet has been significantly elevated (Nizza and Piccolo, 2009). As there are increasing interests for using the vegetable sources in fish diet, it would be essential to consider the safety of diet including being free from mycotoxins, too. The occurrence of various mycotoxins including zearalenone in fish meal and different plant materials such as maize, wheat, rice, and soybean has been reported (Weidenbörner, 2007).

ZEA, 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone, is produced as a secondary metabolite 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; Jiménez 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). ZEA is a stable compound and is not degraded by routine food and feed processing procedures. Since ZEA binds to estrogen receptors (ERs) after ingestion and acts as an estrogenic compound, it is referred as a mycoestrogen (Malekinejad *et al*., 2005). Animals exposed to ZEA or ZEA-contaminated feed, show symptoms of hyperestrogenism. In this regard, previous data indicated that pigs are the most sensitive species (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). ZEA has also been shown to cause a significant increase in mRNA level of cytochrome P450 1A (so called xenobiotic effects) in liver of juvenile rainbow trout (Woźny *et al*., 2008).

There are remarkable differences between various species in hepatic and extra-hepatic biotransformation of ZEA explaining the most logical reason for species sensitivity/resistance to ZEA (Malekinejad *et al*., 2006a). In previous works, we demonstrated 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 showed that there are species differences in hepatic biotransformation of ZEA in terms of different metabolite production and the rate of glucuronidation (Malekinejad *et al*., 2006a). As there is currently a lack of knowledge about the hepatic biotransformation of ZEA in rainbow trout, in this study the hepatic biotransformation of ZEA including both phase I and II by sub-cellular fractions of rainbow trout were investigated.

Materials and Methods

Reagents

The test compounds zearalenone (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 rainbow trout fish (average wt. 200 ± 5 g) were obtained from a local trout farm and transported to the Artemia and Aquatic Animals Research Institute, Urmia, Iran, in oxygenated tank. They were then stocked in a concrete pond containing underground 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 pH = 7.3-7.5. The fish were fed on commercially standard and mycotoxin free formulated trout diet (Milad Mahabad Co., West Azerbaijan, Iran) throughout the culture period of 6 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 rainbow trout 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 (0.1 mM) 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 analysed 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 min and 4°C, one ml of the organic phase was collected and evaporated to dryness under a stream of nitrogen. The residue was subsequently analysed 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 the previously described method (Malekinejad *et al*., 2005). In short, the chromatographic system consisted of an auto sampler (Autosampler Triathlon type 900, Germany) and dual pumps (Wellchrom HPLC pump, K-1001, KNAVER Germany). Twenty microlitres of the extracted sample was injected into a LUNA 5 µ 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 analysed 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

β-ZOL is produced as the major phase I metabolite of ZEA

Incubation of a wide ranging con-

centration of ZEA with the hepatic subcellular fractions of rainbow trout, followed by chloroform extraction and ultimately HPLC analyses, revealed that both known metabolites of ZEA including α- and β-ZOL are produced by both studied fractions. Figures 1A and B show both standard and sample chromatograms with identical retention time for individual compounds. The obtained data indicate that at all given concentrations of ZEA the β-ZOL production was the statistically ($P<0.05$) dominant metabolite in both subcellular fractions (Figs. 2A and B).

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.

Analyses of the obtained data from

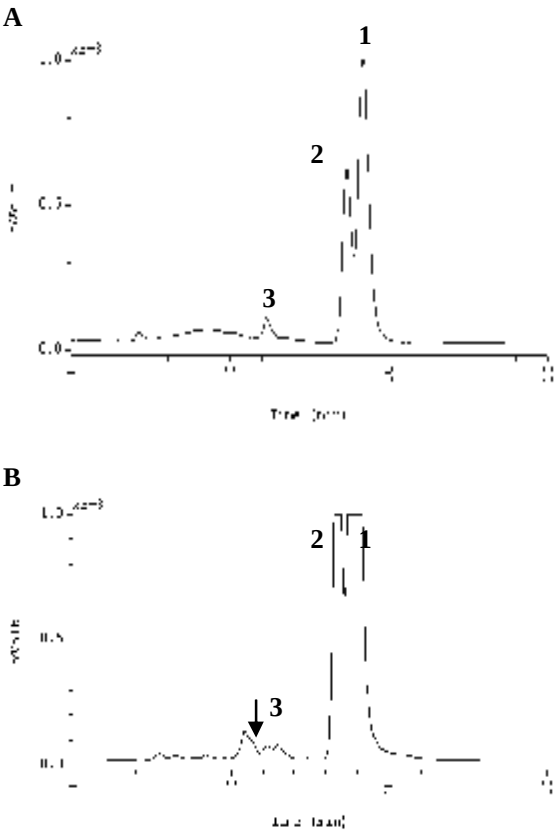

Fig. 1: HPLC chromatogram of ZEA and its metabolites. A) the chromatogram representing a mixture of standard ZEA (1), α- and β-ZOL (2 and 3) and B) the chromatogram shows the peaks (1, 2 and 3) after incubation of ZEA with the rainbow trout hepatic microsomes for 30 min

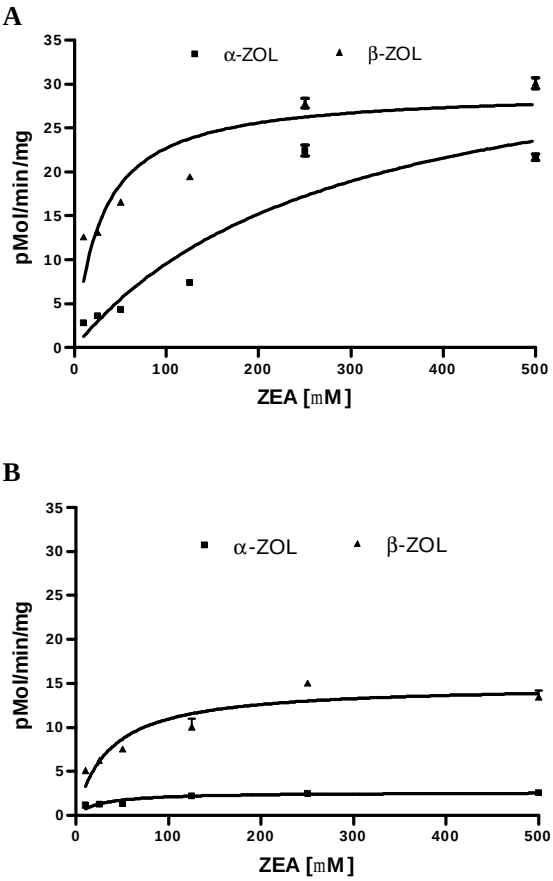

Fig. 2: Biotransformation of ZEA by A) hepatic microsomes and B) hepatic post-mitochondrial fractions of the rainbow trout; Data represent means ± SD from three independent experiments

enzymatic kinetics point of view revealed that the maximum velocity (V_{max}) in microsomal fraction was found approximately 8-fold and 2-fold higher than those in post-mitochondrial fraction for α- and β-ZOL, respectively. The K_m value for the α-ZOL production by microsomal fraction was found approximately 10-fold higher than that for post mitochondrial fraction, however, no significant differences were found for K_m values for the β-ZOL production by two subcellular fractions (Table 1).

Glucuronidation of ZEA and its hydroxylated metabolites

The second part of this study was devoted to determine the rates of glucuronidation of ZEA and its hydroxylated metabolites by the rainbow trout post-mitochondrial fraction. Our results with the

various studied concentrations of ZEA showed a reverse level of glucuronidation as the high concentration of ZEA, the low percentage of glucuronidation, representing a saturable condition of glucuronidase, which is responsible for glucuronidation of ZEA and its reduced metabolites. The highest rate of glucuronidation was found for α-ZOL at concentrations lower than 125 μM, followed by ZEA and β-ZOL. Interestingly, all of the produced α-ZOL was conjugated with glucuronic acid at concentrations lower than 125 μM (Table 2).

Discussion

The purpose of this study was to investigate the hepatic biotransformation of ZEA in rainbow trout. Results showed that sub-cellular fractions of the rainbow trout's liver converted ZEA into mainly β-ZOL and to a lesser extent into α-ZOL. This study also demonstrated that ZEA and its reduced metabolites are conjugated with glucuronic acid.

Although there is no direct report from trout diet and ZEA contamination at high levels, there are reports that the concentration of ZEA can vary from a few micrograms up to 276 mg/kg in animal feed materials, and between 0.001 and 175 mg/kg in cereal grains intended for human consumption, depending on plant variety, geographic region and climatic conditions in the production year (Vrabcheva *et al*., 1996; Binder *et al*., 2007).

It has been reported that the ZEA biotransformation is similar to steroids metabolism which is catalyzed by hydroxy steroid dehydrogenases (HSD) (Olsen and Kiessling, 1983; Malekinejad *et al*., 2005). During phase I metabolism on ZEA that is catalyzed by 3α- or 3β-HSD, the keto group at C-6 is reduced and α-ZOL and/or β-ZOL are produced. There are two known isoforms of 3-HSD which produce 3α- and 3β-hydroxysteroids. 3α-HSD acts as oxidoreductase and is physiologically expressed in the liver, brain, prostate, lung, kidney, and the gonads. Additionally, 3α-HSD is able to metabolize many other non-steroidal substances, as well (Penning *et al*., 1996; Pirog and Collins, 1999). The expression of 3β-HSD in the equine placenta, corpus luteum, ovarian follicles and rat sciatic nerve has been reported (Chavatte *et al*., 1995; Boerboom and Sirois, 2001; Coirini *et al*., 2003). Moreover, human 3β-HSD is expressed mainly in the prostate, placenta, mammary glands and endometrium, which functions as dehydrogenases/isomerases (Thomas *et al*.,

Table 1: Kinetics of ZEA biotransformation by the rainbow trout hepatic subcellular fractions

Metabolites/subcellular fractions	Microsomes	Post-mitocondrial
α-ZOL		
V_{max} (pMol/min/mg)	21.8 ± 7.1	2.6 ± 0.1*
K_m (μMol/L)	287.5 ± 49.8	26.1 ± 5.1*
r^2	0.881	0.847
β-ZOL		
V_{max} (pMol/min/mg)	32.8 ± 1.6	14.8 ± 0.8*
K_m (μMol/L)	28.9 ± 6.3	35.5 ± 7.5*
r^2	0.801	0.849

Stars indicate a significant difference (P<0.05) between two tested subfactions

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

ZEA concentration (μM)	ZEA	α-ZOL	β-ZOL
10	85.88 ± 6.1	100	100
25	83.9 ± 0.8	100	100
50	75.8 ± 5.8	100	31.9 ± 8.8
125	39.6 ± 10.2	53.6 ± 3.0	26.3 ± 3.8
250	33.8 ± 4.2	50.2 ± 1.1	18.3 ± 1.8
500	23.1 ± 6.3	37.2 ± 3.5	9.1 ± 1.4

2002). We have already reported that these two isomers are expressed in extra-hepatic organs such as porcine cumulus oocyte complexes (COCs) and granulose cells (Malekinejad *et al*., 2006b). We also demonstrated that both 3α- and 3β-HSD catalyze the conversion ZEA into hydroxylated metabolites (Malekinejad *et al*., 2006c). In this respect, the expression of 3β-HSD in the ovarian follicles of rainbow trout has been reported (Sakai *et al*., 1993). Immunohistochemical studies have also shown the expression of 3β-HSD in interstitial leydig cells of immature and mature testes of rainbow trout (Kobayashi *et al*., 1998). Although there is little data about the expression of HSDs in the liver of rainbow trout, our results in this study indicate that most likely both 3α-and 3β-HSD are expressed in the liver converting ZEA into its metabolites.

Enzymatic analyses of this study clarified that V_{max} for production of both metabolites in the microsomal fraction is higher than that in post-mitochondrial fraction, indicating dense localization of enzymes in microsomal fraction. This finding is in good accordance with previous works (Malekinejad *et al*., 2005; Malekinejad *et al*., 2006a). Our data suggest a higher efficiency (V_{max}/K_m ratio) of the post-mitochondrial enzymes than microsomal enzymes in trout liver. Additionally, under the assumption that 3β-HSD converts ZEA into β-ZOL, the obtained enzyme kinetics indicate a much higher efficiency of 3β-HSD than 3α-HSD in trout liver. High K_m values for α-ZOL production indicate a low affinity of trout hepatic 3α-HSD for parent substrate (ZEA). A higher and lower affinity of 3β-HSD for pregenolone in blacktip shark than human and rat, respectively, have been reported (Nunez and Trant, 1998).

Our previous studies, in agreement with others, revealed that high sensitivity of pigs to ZEA is due to the bioactivation of ZEA into α-ZOL with highly potent estrogenecity. It was the same for chickens as a resistant animal against ZEA due to the production of β-ZOL as the dominant and a weak estrogenic metabolite (Malekinejad *et al*., 2006a). Regardless of other possible factors that could influence the sensitivity of the rainbow trout against ZEA, our metabolic studies indicate that this specific species might be categorized as resistant animal to ZEA as the hepatic main metabolite of ZEA was found to be β-ZOL. It should be noted that the estrogenic effect of ZEA has been reported in trout as ZEA showed estrogenic potency in displacing specifically bound [^{3}H]estradiol from estrogen receptors of the rainbow trout's liver and brain (Knudsen and Pottinger, 1999). There are other reports that ZEA increases mRNA level of both ERα and CYP1A via the AhR signaling pathway in rainbow trout's liver (Woźny *et al*., 2008).

The second part of this study was devoted to investigate the glucuronidation rate for ZEA and its reduced metabolites by the rainbow trout's liver. It is known that the rate of glucuronidation mainly depends on the capacity of UDPGTs and availability of UDPGA (Bélanger *et al*., 1990; Tukey and Strassburg, 2000). The computed percentage of the conjugation rate for all three compounds indicates that, albeit with differences there are limitations for the glucuronidation, as at low concentrations either entire molecules or a majority of them are conjugated, while at higher concentrations the percentage of conjugation was declined. Our findings about the rate of glucuronidation of two produced metabolites of ZEA by the rainbow trout's liver is consistent with previous reported data where it was shown that the rate of β-ZOL glucuronidation in the rat liver is higher than that of α-ZOL. As the entire β-ZOL at the lower concentrations is conjugated with glucuronic acid, it may cause the prolongation of the presence of β-ZOL in the body due to entreo-hepatic cycle. The profile of glucuronidation by the rainbow trout's liver resembles that in rat and the rate of β-ZOL glucuronidation is significantly higher than that for α-ZOL (Malekinejad *et al*., 2006a).

The data described in the present study is the first report showing that ZEA is converted mainly into β-ZOL which could account for the detoxification method. Moreover, the original compound, along with the dominant reduced conjugated metabolites may cause a prolonged duration of action of both mentioned chemicals as

these could undergo entero-hepatic cycle.

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