Functional Characterization of Alpha-Class Glutathione S-Transferases from the Turkey (Meleagris Gallopavo)

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Key Words: glutathione S-transferases; aflatoxin B1; aflatoxin B1-8,9-epoxide; turkeys.

Six Alpha-class glutathione S-transferase (GST) subunits were cloned from domestic turkey livers, which are one of the most susceptible animals known to the carcinogenic mycotoxin aflatoxin B1. In most animals, GST dysfunction is a risk factor for susceptibility toward AFB1, and we have shown that turkeys lack GSTs with affinity toward the carcinogenic intermediate \( \text{exo-aflatoxin B}_{1}-8,9\text{-epoxide} \) (AFBO). Conversely, mice are resistant to AFB1 carcinogenesis, due to high constitutive expression of mGSTA3 that has high affinity toward AFBO. When expressed in \( \text{Escherichia coli} \), all six tGSTA subunits possessed conjugating activities toward substrates 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (ECA), and cumene hydroperoxide (CHP) with tGSTA1.2 appearing most active. Interestingly, IGST1A.1, which lacks one of the four Alpha-class signature motifs, possessed enzymatic activities toward all substrates. All had comparable activities toward AFBO conjugation, an activity absent in turkey liver cytosols. \( \text{E. coli} \)-expressed mGSTA3 conjugated AFBO with more than 3-fold greater activity than that of tGSTAs and had higher activity toward GST prototype substrates. Mouse hepatic cytosols had approximately 900-fold higher catalytic activity toward AFBO compared with those from turkey. There was no apparent amino acid profile in tGSTAs that might correspond to specificity toward AFBO, although tGSTA1.2, which had slightly higher AFBO-trapping ability, shared Tyr\(^{168} \) with mGSTA3, a residue postulated to be critical for AFBO trapping activity in mammalian systems. The observation that recombinant tGSTAs detoxify AFBO, whereas their hepatic forms do not, implies that the hepatic forms of these enzymes are silenced by one or more regulatory mechanisms.

Glutathione S-transferases (GSTs: EC2.5.1.18) are a family of dimeric enzymes that among several other functions, catalyze the detoxification of reactive electrophilic compounds, many of which are toxic and carcinogenic intermediates, via conjugation with the endogenous tripeptide glutathione (GSH) (Hayes et al., 2005). Cytosolic GSTs exist as dimeric subunits of 23–30 kDa with an average length of 199–244 amino acids (Hayes and Pulford, 1995; Mannervik and Danielson, 1988). Each subunit consists of a thioredoxin-like domain containing the G-site for GSH-binding and an \( \alpha \)-domain containing the H-site for binding the hydrophobic electrophile (Wang et al., 2009). Because of their importance in disease resistance, cancer susceptibility, and responsiveness to drug therapy, mammalian GSTs have been intensively studied. GSTs are primarily cytosolic enzymes, but microsomal forms also exist (Kelner et al., 1996). While most GSTs are widely expressed in many tissues, some forms show tissue specificity (Fedulova et al., 2010; Thomson et al., 2004). Alpha- and Mu-class GSTs appear to be the most diverse, typically containing four or more distinct genes in mammals (Morel et al., 2002). Alpha-class GSTs play an important role as antioxidant enzymes modulating stress-induced signaling pathways (Yang et al., 2001). Recently, new classes of nonmammalian GSTs have been identified, sequenced, and heterologously expressed (Cha et al., 2002; Konishi et al., 2005; Lee et al., 2007; Shahein et al., 2008; Sheehan et al., 2001).

Glutathione S-transferases play a central role in detoxifying the hepatotoxic and hepatocarcinogenic mycotoxin aflatoxin B1 (AFB1), a common contaminant of corn and peanut-based foods and feeds (Eaton and Bammler, 1999; Hayes and Strange, 1995; Newberne and Butler, 1969). The resulting AFB-GSH adduct is a detoxification product that is safely eliminated (Kim and Lee, 2007; Salinas and Wong, 1999). Upon ingestion, AFB1 is activated by hepatic cytochrome P450s (P450) to form the reactive electrophilic metabolite \( \text{exo-aflatoxin B}_{1}-8,9\text{-epoxide} \) (AFBO), which is responsible for the toxic and carcinogenic properties of this mycotoxin (Klein et al., 2000). In the domestic turkey, AFB1 is bioactivated principally by hepatic microsomal cytochromes P450 1A5 and P450 3A37, which have been cloned, mapped, and functionally characterized (Rawal et al., 2009; Yip and Coulombe, 2006).
Numerous studies have demonstrated that individual and species susceptibility to AFB1-induced toxicity and hepatocarcinogenicity is strongly associated with GST-mediated detoxification of AFB1 (Monroe and Eaton, 1988; Ramsdell and Eaton, 1990). Mice are resistant to the acute and chronic toxic effects of AFB1, apparently because of the extremely high affinity toward AFB1 exhibited by their hepatic Alpha-class A3 subunit (mGSTA3, previously designated Yc and Ya3) (Buetler et al., 1992; Hayes et al., 1992). Conversely, Alpha-class GSTs from rats, which are relatively susceptible, have little affinity toward AFB1; however, its affinity can be enhanced more than 200-fold when combination of six critical mGSTA3 amino acid residues (Ile103, Tyr108, His111, Phe207, Asp208, and Lys17) are substituted into the rat GSTA3-3 (previously Yc) sequences (Van Ness et al., 1998). Furthermore, mGSTA3 confers protection against AFB1 when transfected into hamster V79 cells (Fields et al., 1999). Very recently, the centrality of mGSTA3 in AFB1 resistance was affirmed by the demonstration that GSTA3 knockout mice are susceptible species known to AFB1 (Klein et al., 1995; Pier et al., 1980). We have previously shown that like rats, turkeys efficiently bioactivate AFB1, catalyzed by hepatic P450 1A5 (Yip and Coulombe, 2006) and P450 3A37 (Rawal et al., 2009). Bradford Protein Assay Kit was used for quantification of protein (Bio-Rad, Philadelphia, PA). E. coli BL21 (DE3) and chemically competent cell DH5α (Invitrogen, Carlsbad, CA) were the host strain for expression and subcloning of recombinant GSTAs. For cloning and amplification, zero blunt PCRII vector (Invitrogen) pET21a vector (Novagen, Madison, WI), and plu/high Ultra High-Fidelity DNA Polymerase (Strategene, La Jolla, CA) were used. 

Multiple alignments of amino acid sequences and secondary structures. Full-length cDNAs of six Alpha-class GSTs (tGSTA1.1, tGSTA1.3, tGSTA2, tGSTA3, tGSTA5, and tGSTA4) were cloned from domestic turkey liver (Kim et al., 2010). The similarities of all putative amino acids among GSTAs and Alpha-class GST subunits of other species (chicken, human, mouse, and rat) were compared using ClustalW 2.0.12 (http://www.ebi.ac.uk/Tools/clustalw2/). All putative amino acid sequences of tGSTAs were aligned. Their predictive secondary structures were compared with Alpha-class GSTs of other species (turkey, tGSTA1.1-tGSTA4; chicken, cGSTA1-cGSTACL3; human, hGSTA1-hGSTA5; mouse, mGSTA3; and rat, rGSTA5-5) using PSSPRED Protein Structure Prediction Server software program (http://bioinf.cs.ucl.ac.uk/psspred/).

GenBank accession numbers cited in this article are as follows: tGSTA1.1 (M. gallopavo, ACU44693), tGSTA1.2 (M. gallopavo, ACU44694), tGSTA1.3 (M. gallopavo, ACU44695), tGSTA2 (M. gallopavo, ACU44696), tGSTA3 (M. gallopavo, ACU44697), tGSTA4 (M. gallopavo, ACU44698), tGSTA3 (G. gallus, Q08392), tGSTA2 (G. gallus, Q08393), tGSTA3 (G. gallus, NP_990149), tGSTA3 (G. gallus, Q08392), tGSTA2 (G. gallus, Q08393), tGSTA3 (G. gallus, NP_990149), tGSTA3 (G. gallus, P26697), hGSTA1 (Homo sapiens, P08263), hGSTA2 (H. sapiens, P09210), hGSTA3 (H. sapiens, Q16772), hGSTA4 (H. sapiens, CA02451), hGSTA5 (H. sapiens, Q7RTV2), mGSTA3 (Mus musculus, NP_034886), and rGSTA5-5 (Rattus rattus, P46418).

Plasmid construction and expression in E. coli. Full-length cDNA of each tGSTA was subcloned and transformed into E. coli. PCR was applied to introduce restriction enzyme sites flanking the cDNA of tGSTA for fusion with C-terminal 6× His-tag in pET21a. The gene-specific primers with two restriction sites, NdeI at 5’-end (BanHI at 5’-end for tGSTA4) and XhoI at 3’-end, were designed to amplify the open reading frame (ORF) fused C-terminal 6× His-tag to generate recombinant tGSTAs (Table 1). In addition to the six tGSTA cDNA clones, NdeI/XhoI sites were introduced into mGSTA3 (gift from Dr Dave Eaton, University of Washington) to make a recombinant 6× His-tag-fused protein as a positive control. The PCR profile was 2 min at 94°C, 30 s at 94°C, 30 s at 60-63°C (25 cycles), and 1 min and 40 s at 72°C, followed by a final extension for 8 min at 72°C. The gel-purified gene PCR fragments were ligated with T4 DNA ligase and then cloned into pET21a with NdeI/XhoI (BanHI/XhoI) for tGSTA4. Clones were confirmed by colony PCR and sequence analysis with Lasergene SeqManII software (DNA STAR Inc., Madison, WI).

The recombinant 6× His-tagged constructs were transformed into E. coli strain BL21 (DE3). Colonies were inoculated to 30 ml of LB containing ampicillin (100 mg/ml) and grown overnight at 37°C. The next day, this starter culture was inoculated (1:100 dilution) into 500 ml of LB containing ampicillin (100 mg/ml) and incubated at 37°C until OD600 reached 0.6-0.65 in a shaking incubator (250 rpm). The expression of 6× His-tag-fused GSTA proteins was induced with
0.4mM isopropyl-β-D-thiogalactopyranoside (IPTG). E. coli cells were harvested after 3 h induction and pelleted by centrifugation (5000 g) for 20 min at 4°C.

**Purification of recombinant GSTA proteins.** Affinity purification of recombinant 6× His-tag-fused proteins was performed using Ni-NTA agarose resins and with polypropylene column. Harvested cell pellets were thawed on ice for 20 min and resuspended in cold lysis buffer (2.5mM NaH₂PO₄, 300mM NaCl, and 10mM imidazole, pH 8.0) and incubated for 30 min after adding lysozyme (1 mg/ml), DNase, DNase I (50 μg/ml), 1,2-dichloro-4-nitrobenzene (DCNB) and ethanolic acid (ECA) (Habig et al., 1974; Habig and Jakoby, 1981), and cumene hydroperoxide (CHP) (Konishi et al., 2005; Lawrence and Burk, 1976). Conditions of all assays were optimized in a final 1ml volume containing 100mM potassium phosphate buffer at room temperature (25°C) using a spectrophotometer (Thermo Scientific, Madison, WI). (1) 1mM CDNB, 1mM GSH, A₆₅₀ nm (Extinction coefficient: 9.6/mM/cm), and buffer pH 3.65; (2) 1mM DCNB, 1mM GSH, and buffer pH 6.0; (3) 1mM CDNB, 1mM GSH, and buffer pH 7.5 (3) 0.2mM ECA, 0.25mM GSH, A₆₅₀ nm (5/mM/cm), and buffer pH 6.5, and (4) glutathione peroxidase activity with CHP as substrate was determined with 1.2mM CHP, 2mM GSH, 1U Glutathione reductase, 0.2mM NADPH, A₆₃₀ nm (6.22/mM/cm), and buffer pH 7.0.

**SDS-PAGE and immunoblots.** Purified recombinant GSTA proteins were visualized by Coomassie Brilliant Blue staining on a 15% Tris-HCl Ready Gel. The SDS-PAGE gel was blotted onto PVDF membrane and blocked with 2% nonfat milk powder in tris-buffered saline and Tween 20 (TBST) buffer (20mM Tris-HCl, pH 7.6, 137mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. After washing, the membrane was incubated with anti-His probe overnight (1:200) overnight at 4°C. The next day, the membrane was incubated with bovine anti-mouse IgG HRP-conjugated secondary antibody (1:8000) for 1 h at room temperature and washed five times with TBST buffer for 60 min. Images were visualized by chemiluminescence using the Luminol reagent system and a NucleoVision E20 Imaging Workstation (Nucleotech, Hayward, CA).

**Preparation of turkey and mouse hepatic cytosols.** The activities of turkey and mouse GSTAs were compared with that from their respective hepatic cytosols. Turkey and mouse liver cytosols were prepared as described by Klein et al. (2000). All steps were done at 4°C. Frozen liver was homogenized using a Polytron (Brinkman, Westbury, NY) in 2 vol of cold lysis buffer (50mM Tris, 1mM EDTA, 0.25M sucrose, 150mM KCl, 20mM BHT, and 200mM PMSF buffer, pH 7.4). The homogenate was centrifuged at 10,000 g for 10 min. The subsequent was centrifuged at 100,000 g for 10 min and then was further centrifuged at 150,000 g for 1 h. The final supernatant (cytosols) was collected and stored at −80°C.

**Prototype GST activities.** Specific enzyme activities of E. coli-expressed GSTA proteins (six tGSTAs and mGSTA3) and two hepatic cytosolic GSTA proteins (turkey and mouse) were assayed for their conjugation activities toward prototype substrates 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB) and ethanolic acid (ECA) (Habig et al., 1974; Habig and Jakoby, 1981), and cumene hydroperoxide (CHP) (Konishi et al., 2005; Lawrence and Burk, 1976). Conditions of all assays were optimized in a final 1ml volume containing 100mM potassium phosphate buffer at room temperature (25°C) using a spectrophotometer (Thermo Scientific, Madison, WI). (1) 1mM CDNB, 1mM GSH, A₆₅₀ nm (Extinction coefficient: 9.6/mM/cm), and buffer pH 3.65; (2) 1mM DCNB, 1mM GSH, and buffer pH 6.0; (3) 1mM CDNB, 1mM GSH, and buffer pH 7.5 (3) 0.2mM ECA, 0.25mM GSH, A₆₅₀ nm (5/mM/cm), and buffer pH 6.5, and (4) glutathione peroxidase activity with CHP as substrate was determined with 1.2mM CHP, 2mM GSH, 1U Glutathione reductase, 0.2mM NADPH, A₆₃₀ nm (6.22/mM/cm), and buffer pH 7.0.
and the amount of metabolite injected, using an authentic exo-AFB1-GSH standard.

**Statistical analysis.** Enzyme activities of recombinant GSTAs (tgGSTA1–A4 and mGSTA3) were compared and analyzed by ANOVA and **post hoc** LSD, which were used for mean separation. Enzyme activities of two hepatic cytosol GST groups (turkey and mouse) were analyzed by Student t-test. Data were analyzed using MYSTAT software (version 12, Systat Software, Inc., Chicago, IL). Significance was set at p < 0.05.

## RESULTS

### Secondary Structure of Alpha-Class GSTs

From the full-length cDNAs of tGSTAs, the turkey Alpha-class genes were found to consist of a cluster of six tGSTA subunits (tgGSTA1.1, 663bp; tgGSTA1.2, 666bp; tgGSTA1.3, 666bp; tgGSTA2, 669bp; tgGSTA3, 672bp; and tgGSTA4, 690bp). These were isolated, sequenced, and mapped using 5'- and 3’-RACE (Kim *et al.*, 2010). The ORF of each gene, which was predicted to be in the range of 25–26 kDa, was composed of the following: tgGSTA1.1 encoding a protein of 220 residues with a predicted molecular mass of 25 kDa (GenBank accession no. ACU44693); tgGSTA1.2 encoding 221 residues with a predicted mass of 25.3 kDa (ACU44694); tgGSTA1.3 of 221 residues with a predicted mass of 25.3 kDa (ACU44695); tgGSTA2 with 222 residues with a predicted mass of 25.5 kDa (ACU44696); tgGSTA3 encoding 223 residues with a predicted mass of 25.5 kDa (ACU44697); and tgGSTA4 of 229 residues with predicted molecular mass 26.3 kDa (ACU44698). Used here as a positive control, mgSTA3 encoded a protein of 221 residues with predicted molecular mass of 25.4 kDa (NP_034486). With the exception of A1.1, A1.2, and A1.3, which had an average amino acid similarity of 83%, amino acid similarities among other tGSTAs were in the range of 62–71%. Amino acid sequences between mgSTA3 and rGSTA5-5 are 90% similar, and all tGSTAs have 63% amino acid similarity with mgSTA3 and 63% with rGSTA5-5 (Table 2).

In order to compare the amino acid residues and secondary structure of GSTA proteins among species, we performed multiple alignments with tgGSTA1–A4 with those of Alpha-class GSTs from chicken, (cGSTA1–cGSTACL3), human (hGSA1–hGSTA5), mouse (mGSTA3), and rat (rGSTA5-5).

Figure 1 shows that all GSTAs contain a typical GST structure, consisting of two distinct conserved domains and four Alpha-class signature motifs. Two domains consist of the thioredoxin-like N-terminal domain (residues 3–83aa) and the helical C-terminal domain (residues 85–209aa). All Alpha-class GSTs possess ten α-helices and five β-strands; N-terminal domain contains α1–α3/β1–4 and C-terminal contains α4–α10/β5. These alignments showed that Alpha-class GST proteins possess four Alpha-class signature motifs, except that tgGSTA1.1 lacked one motif (PVxEKVLxKxGxxxL; residues 134–148) in the C-terminal domain (Kim *et al.*, 2010). However, the lack of this motif did not appear to have any discernible effect on enzyme activity toward CDNB, DCNB, ECA, CHP, and AFBO (Table 3).

### Expression and Identification of Recombinant GSTA Proteins

Seven recombinant 6× His-tagged clones (tgGSTA1.1, tgGSTA1.2, tgGSTA1.3, tgGSTA2, tgSTA3, tgSTA4, and mgSTA3) were harbored in pET21α and expressed in *E. coli* BL21 (DE3). Bacterial cells were harvested at different time points to determine optimal protein expression, which occurred at 3 h after induction with 0.4mM IPTG at 37°C. The molecular mass of 6× His-tagged recombinant tGSTA and mgSTA3 proteins was predicted: tGSTA1.1 (26.3 kDa); tGSTA1.2 (26.4 kDa); tGSTA1.3 (26.5 kDa); tGSTA2 (26.7 kDa); tGSTA3 (26.7 kDa); tGSTA4 (28.8 kDa); and mgSTA3 (26.6kDa). Figure 2 shows the electrophoretic separation of affinity purified recombinant tGSTAs and mgSTA3 proteins. Immunoblots revealed bands of approximate mass of 28 kDa in all 6× His-tag fused recombinant GSTA proteins (Fig. 3).

### *E. coli*-Expressed GSTAs-Mediated Conjugating Activities on Substrate Specificities

Activities of the six cDNA-expressed recombinant GSTAs (six tGSTAs and mgSTA3) as well as their hepatic cytosolic forms toward conjugation of CDNB, DCNB, ECA, and CHP, measured under standard conditions, were then determined and compared. All recombinant tGSTA proteins possessed detectable activities toward these prototype substrates (Table 3). tgSTA1.2 and A3 had the highest activities toward CDNB, ECA, and CHP, whereas tgSTA1.1 possessed the highest activity toward DCNB. Interestingly, in spite of lacking the

### TABLE 2

Percent Amino Acid Sequence Similarities among Turkey Alpha-Class GSTs, Mouse mGSTA3, and Rat rGSTA5-5

<table>
<thead>
<tr>
<th>tGSTA1.1</th>
<th>tGSTA1.2</th>
<th>tGSTA1.3</th>
<th>tGSTA2</th>
<th>tGSTA3</th>
<th>tGSTA4</th>
<th>mgSTA3</th>
<th>rGSTA5-5</th>
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<tr>
<td>80</td>
<td>80</td>
<td>80</td>
<td>70</td>
<td>65</td>
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signature motif PVxEKVLKxHGxxxL in residues 134–148, tGSTA1.1 showed measurable catalytic activity toward all GST substrates. Recombinant mouse GSTA3, which was included as a positive control, had activities similar to those in a previous study (Hayes et al., 1992). In every case, activities catalyzed by recombinant mGSTA3 toward prototype substrates were significantly greater than that from tGSTAs ($p < 0.001$).

As was the case for the expressed tGSTAs, turkey hepatic cytosol possessed activities toward all GST prototype substrates. However, these activities were significantly lower than that observed for mouse cytosol except that for ECA. The greatest activity difference was observed for mouse cytosolic DCNB, which was 60-fold greater than that of turkey. Mouse cytosolic activities toward these substrates were similar to those previously observed from the Swiss Webster mouse with the exception for CHP, in which the activity in the present study was approximately 10-fold greater than that reported by Borroz et al. (1991). It is possible that this difference is due to the fact that the liver cytosol used in our study was from BHA-induced mice. BHA is a known inducer of GSTA activity in mammals (Buetler et al., 1992).

AFBO-Conjugating Activities of Recombinant GSTAs and Cytosolic GSTs

When GST activity specific to trapping AFBO was measured, all recombinant tGSTAs possessed similar activity toward conjugation of this in situ-generated reactive intermediate (Table 3). Interestingly, all tGSTAs possessed similar activities, which were not statistically different from one another. As was seen with activity against prototype GST substrates, it is noteworthy that tGSTA1.1, which lacks at least one motif in the ORF, also possessed AFBO-conjugating activity. Recombinant mGSTA3 possessed significantly higher AFBO-conjugating activity than any of the GSTAs expressed from turkey. Unlike the expressed tGSTAs, turkey liver cytosol completely lacked AFBO conjugation activity, an observation we have repeatedly made.
As expected, liver cytosol from BHA-induced mice had substantial AFBO-conjugating capability and was in the same order of magnitude previously observed for Swiss Webster mice (Borroz et al., 1991).

**DISCUSSION**

Species susceptibility to AFB1 has been described as a balance between P450-mediated AFBO production, and the efficiency with which this reactive intermediate is detoxified through GST conjugation (Eaton and Gallagher, 1994). Mice, which efficiently bioactivate AFB1 to produce AFBO through high-affinity P450s, are remarkably AFB1 resistant owing to the expression of the A3 subunit (mGSTA3), which has a high catalytic activity toward AFBO. Current knowledge thus indicates that efficiency of GST conjugation is a principal “rate-limiting” determinant for AFB1 action in individuals and species (Ilic et al., 2009). Investigations from our laboratory have demonstrated that turkeys possess two P450 enzymes with high AFB1 epoxidation activity: P450 1A5 (Rawal et al., 2009; Reed et al., 2007; Yip and Coulombe, 2006) and 3A37 (Rawal et al., 2009, 2010b). In combination with this, turkey liver is also deficient in AFBO-conjugating GSTs (Guarisco et al., 2008; Klein et al., 2000, 2003). The extreme sensitivity of turkeys toward AFB1 was graphically demonstrated as an etiological agent of Turkey 3 Disease, which caused

### TABLE 3

Comparison of Recombinant Alpha-Class GSTs and Hepatic Cytosolic GSTs Catalytic Activities toward CDNB, DCNB, ECA, CHP, and AFBO

<table>
<thead>
<tr>
<th>Specific enzyme activity (nmol/min/mg protein)</th>
<th>CDNB</th>
<th>DCNB</th>
<th>ECA</th>
<th>CHP</th>
<th>AFBO</th>
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<tr>
<td><strong>Recombinant GSTAs</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>mGSTA3</td>
<td>17 327.00 ± 148.4a</td>
<td>70.59 ± 0.00a</td>
<td>3614.00 ± 6.54a</td>
<td>30 505.40 ± 220.13a</td>
<td>71.19 ± 1.47a</td>
</tr>
<tr>
<td>tGSTA1.1</td>
<td>1674.61 ± 48.15b</td>
<td>16.35 ± 0.80b</td>
<td>44.31 ± 2.00cd</td>
<td>451.83 ± 12.41b</td>
<td>19.54 ± 1.42b</td>
</tr>
<tr>
<td>tGSTA1.2</td>
<td>7720.94 ± 75.81b</td>
<td>10.85 ± 0.09b</td>
<td>164.51 ± 6.18b</td>
<td>1076.77 ± 11.45b</td>
<td>22.49 ± 1.97b</td>
</tr>
<tr>
<td>tGSTA1.3</td>
<td>1063.48 ± 25.39b</td>
<td>1.46 ± 0.15b</td>
<td>50.58 ± 0.24e</td>
<td>166.97 ± 68.47de</td>
<td>21.98 ± 2.26b</td>
</tr>
<tr>
<td>tGSTA2</td>
<td>3045.23 ± 16.92d</td>
<td>7.55 ± 0.59d</td>
<td>34.49 ± 0.00d</td>
<td>314.06 ± 7.29de</td>
<td>21.71 ± 0.54b</td>
</tr>
<tr>
<td>tGSTA3</td>
<td>4254.39 ± 37.32c</td>
<td>1.25 ± 0.00c</td>
<td>174.32 ± 1.54b</td>
<td>850.25 ± 59.46b</td>
<td>18.04 ± 2.67b</td>
</tr>
<tr>
<td>tGSTA4</td>
<td>302.57 ± 44.57f</td>
<td>0.71 ± 0.28f</td>
<td>21.38 ± 0.98e</td>
<td>895.6 ± 43.68a</td>
<td>20.06 ± 0.33b</td>
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<td><strong>Hepatic cytosolic GSTs</strong></td>
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<td></td>
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<tr>
<td>Mouse</td>
<td>2888.7 ± 43.98a</td>
<td>64.55 ± 1.10a</td>
<td>61.10 ± 0.12b</td>
<td>805.56 ± 43.68a</td>
<td>889.61 ± 80.38**</td>
</tr>
<tr>
<td>Turkey</td>
<td>1027.2 ± 17.81b</td>
<td>1.51 ± 0.54b</td>
<td>89.58 ± 0.80b</td>
<td>180.87 ± 0.97b</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Note. Different superscripts are significantly different (p < 0.05). (A) Recombinant GSTs were analyzed by ANOVA and post hoc LSD for mean separation. (B) Hepatic cytosol GSTs were analyzed by Student t-test (p < 0.05). CDNB, 1-Chloro-2,4-dinitrobenzene; GSH (1mM); DCNB, 1,2-Dichloro-4-nitrobenzene; GSH (5mM); ECA, Ethacrynic acid; GSH (2.5mM); CHP, Cumene Hydroperoxide; GSH (2mM); AFBO, AFB1-8,9-epoxide; GSH (5mM); n.d., not detected; *Specific enzyme activity (pmol/min per mg protein); Mean ± SE of triplicate determination.

(Coulombe et al., 2005; Guarisco et al., 2008; Klein et al., 2000, 2002, 2003). As expected, liver cytosol from BHA-induced mice had substantial AFBO-conjugating capability and was in the same order of magnitude previously observed for Swiss Webster mice (Borroz et al., 1991).
widespread deaths of turkeys and other poultry in the 1960’s, traced to contaminated feed (Blount, 1961). We recently cloned and mapped six Alpha-class GST genes (tGSTAs) from turkey liver (Kim et al., 2010), and it was therefore of interest to explore their functional characteristics, with particular emphasis on their ability to conjugate AFBO.

In the present study, six Alpha-class GST subunits were cloned and expressed in E. coli. molecular weights of expressed tGSTA subunits were consistent with that expected for Alpha-class GSTs (Prova, 2006; Hayes et al., 2005; Kim et al., 2010). Since all six tGSTA subunits contained two highly conserved GST domains (the GSH-binding site and the hydrophobic substrate-binding site), Alpha-class signature motifs and specific conserved residues (Fig. 1), all tGSTA enzymes shared functional similarities on prototypical substrates though amino acid similarity among tGSTAs were in the range 62–71% with the exception of A1.1, A1.2, and A1.3, which had an average amino acid similarity of 83% (Tables 2 and 3). As was observed in turkey hepatic cytosol, expressed tGSTAs all possessed catalytic activities toward the GST substrates CDNB, DCNB, ECA, and CHP, though that toward CDBN and CHP appeared to be the greatest. With some exceptions, tGSTA1.2 and tGSTA3 appeared to possess the greatest activities toward these prototypical GST substrates. On the other hand, tGSTA1.1, tGSTA1.2, and tGSTA1.3 share 83% sequence similarity and have similar AFBO trapping activities. Comparing mGSTA3 with average 63% amino acid similarity, mGSTA3 showed highly significant AFBO activity than tGSTA subunits. In addition, with respect to prototype enzyme activities (CDNB, DCNB, ECA, and CHP), mGSTA3 showed significantly higher substrate conjugating activities than all tGSTAs. Turkey hepatic cytosol and tGSTAs possessed the least activity toward DCNB, which is known as a Mu-class GST substrate (Arakawa et al., 2010; Buettler and Eaton, 1992; Vanhaecke et al., 2000). Unlike the hepatic forms of turkey GSTs, which show no detectable AFBO-trapping ability (Coulombe et al., 2005; Guarisco et al., 2008; Klein et al., 2000, 2002, 2003), all heterologously expressed tGSTAs possessed measurable and comparable AFBO-conjugating activity. We observed that tGSTA1.1 which lacks one of the four Alpha-class signature motifs (residue 134–148; PVxEKVLxHGxxL) possessed enzymatic activities toward all substrates including AFBO. Therefore, it is possible that one missing motif in these Alpha-class GSTs may not have a substantial affect on the catalytic action of these enzymes but that a smaller number of residues may confer the majority of activity toward AFBO and other substrates.

In the present study, there was no apparent correlation between individual amino acid residues and AFBO-conjugation activity among the tGSTAs. Although tGSTA1.2, with slightly higher AFBO activity, shares Tyr108 with mGSTA3, it did not exhibit significantly different AFBO-conjugating activity than other tGSTAs (Fig. 1 and Table 3). Site-directed mutagenesis with molecular modeling may be useful to elucidate the relative contributions of specific amino acid residues to the catalytic function of tGSTAs.

Several studies have demonstrated that single amino acid site change in GSTs can influence catalytic properties, substrate specificity, and stereoselectivity (Bammler et al., 1995; Wang et al., 2000; Zimmia et al., 1994). For example, Met104 in mGSTA4-4 determined the selectivity and specificity toward 4-hydroxynonenal (Nanduri et al., 1996). A single amino acid change from Arg to Pro (residue 11) significantly altered substrate specificity in Pi-class GSTs in mice (Bammler et al., 1995). In chicken cGSTA1-1, two residues, Lys15 and Ser208, appeared to be responsible for high specificity and selectivity toward ethacrylic acid (ECA) (Liu et al., 1997). McDonagh et al. (1999) demonstrated that substitutions of Tyr108 and Asp208 with other amino acids reduced activity toward AFBO by altering the orientation in the binding site of three different GSTs (human GSTA1-1, rat GSTA3-3, and rat GSTA5-5). Site-directed mutagenesis studies determined substrate specificity among Alpha-class GSTs by individual amino acid residues (Liu et al., 1997; McDonagh et al., 1999; Van Ness et al., 1998). The substitution of six amino acid residues (Ile104, Tyr108, His111, Phe207, Asp208, and Lys217) from mGSTA3 into rGSTA3 increased the AFBO-trapping ability of this enzyme 200-fold (Van Ness et al., 1998). In our study, all tGSTs showed AFBO activity without these six amino acid residues, although tGSTA1.2 possesses only Tyr108. Therefore, the combination of several critical amino acids may be responsible for conjugation activity toward AFBO.

While the amino acid sequence almost certainly explains the difference in AFBO conjugation between mGSTA3 and tGSTAs, that recombinant tGSTAs possess this activity while hepatic forms do not is unexplained. Though unlikely, it may be that cytosolic GSTAs in turkey cytosol are labile and do not survive the preparation process. More plausible reasons for this discrepancy include a variety of genetic and epigenetic regulatory mechanisms. Mechanisms of GSTA silencing in turkey liver may include downregulation or reduced activation of nuclear factor erythroid-related factor 2 (Nrf2), a key transcription factor regulator, which upon activation is released from the cytosolic repressor Kelch-like ECH-associated protein-1 (Keap1), and subsequent localization at the antioxidant response element (ARE) (Chen and Kunsch, 2004; Yu et al., 2010). Knockout of Nrf2 in mice substantially reduced the expression of ARE-mediated detoxifying and antioxidant enzymes and rendered these mice highly susceptible to carcinogens and/or oxidative damage (Yu et al., 2010). Loss of the Nrf2 transcription factor in both wild-type and nrf2-null mice was associated with a reduction in basal expression of mGSTA3 (Jowsey et al., 2003). AFBO metabolizing mGSTA3 is transcriptionally regulated through AREs and Nrf2 transcription factor, which confer protection against xenobiotics (Chanas et al., 2002; Jowsey et al., 2003). In addition, posttranscriptional modifications (e.g., methylation, glucosylation,
and phosphorylation) of tGSTAs might repress the enzyme activities or GST-specific inhibitors might affect GST-conjugating activity toward AFBO.

The presence of potential mutations in 5′-regulatory elements, splice variations, and epigenetic changes are currently being investigated for their role in silencing hepatic forms of tGSTAs.

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