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Characterization of porcine Alpha-class glutathione transferase A1-1

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ABSTRACT

An Alpha-class glutathione transferase (GST) has been cloned from pig gonads. In addition to two conservative point mutations our nucleotide sequence presents a frame shift resulting from a missing A as compared to a previously published porcine GST A1-1 sequence. The deduced C-terminal amino-acid segment of the protein differs between the two variants. Repeated sequencing of cDNA isolated from different tissues and animals ruled out the possibility of a cloning artifact, and the deduced amino acid sequence of our clone showed higher similarity to related mammalian GST sequences. Hereafter, we refer to our cloned enzyme as GST A1-1 and to the previously published enzyme as GST A1-1*. The study of the tissue distribution of the GSTA1 mRNA revealed high expression levels in many organs, in particular adipose tissue, liver, and pituitary gland. Porcine GST A1-1 was expressed in *Escherichia coli* and its kinetic properties were determined using alternative substrates. The catalytic activity in steroid isomerization reactions was at least 10-fold lower than the corresponding values for porcine GST A2-2, whereas the activity with 1-chloro-2,4-dinitrobenzene was approximately 8-fold higher. Differences in the H-site residues of mammalian Alpha-class GSTs may explain the catalytic divergence.

Introduction

Glutathione transferases (GSTs,[1] EC 2.5.1.18) are pivotal enzymes for detoxication processes in animals and plants. In addition, functions not related to detoxication have been uncovered for GSTs [1]. Such roles include for example involvement in signal transduction [2–4], steroid biosynthesis [5], and intracellular transport (ligandin) [6–9]. The superfamily of GST enzymes therefore constitutes a multifaceted system with profound physiological impact. Seven classes of cytosolic GSTs have been found in humans: Alpha, Mu, Pi, Theta, Zeta, Omega and Sigma [10]. Human Alpha-class GSTs have been of particular interest due to their involvement in steroidogenesis, both directly as anabolic enzymes [5,11] and indirectly by detoxifying deleterious byproducts of the steroidogenic pathway [12].

The pig is used as a biochemical model due to the similarities between humans and pigs in size, physiology, organ development and disease progression [13]. The knowledge on enzymes involved in, inter alia, reproductive and detoxication systems would facilitate understanding of the mechanisms underlying these physiological processes. Our previous work [14] has established a functional analogy between pGST A2-2 and hGST A3-3 involved in steroidogenesis [5]. How other porcine Alpha-class GST enzymes may function and be related to the human enzymes has remained unknown since the enzymes have yet to be characterized at the protein level. Up to five Alpha-class GST enzymes have been detected in porcine tissues [15–19]. Several isoforms have been characterized immunologically and have been shown as highly expressed and regulated in the ovary and testis, where they are thought to detoxify genotoxic compounds [12,19,20].

A porcine mRNA, which is regulated by hormones in pig granulosa cells, has been isolated from an ovary EST library [21]. The corresponding protein is referred to as porcine GSTA 1-1 in Swiss-Prot database (entry P51781). However so far, the evidence for this protein has only been available at the transcript level. The aim of the present study was to clone and characterize porcine GST A1-1, with respect to gene location, expression profile, and catalytic properties.

Materials and methods

Isolation of total RNA

Ovary and testis specimens from the domesticated Large White pig intercrossed with European Wild Boar were kindly provided by Emmanuelle Bourneuf and Leif Andersson (Department of Medical

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[1] *Abbreviations used:* GST, glutathione transferase; hGST, human GST; PCR, polymerase chain reaction; pGST, porcine GST; pGST A1-1*, pGST A1-1 sequence published under Swiss-Prot entry P51781; pGST A1-1, pGST A1-1 sequence identified in the present study.

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Biochemistry and Microbiology, Uppsala University, Uppsala). Total RNA was isolated from the tissues using the RNeasy Mini Kit (Qiagen); the quantity and purity were assessed spectrophotometrically and the RNA integrity was verified by denaturating electrophoresis of 4 μg RNA according to the manufacturer's instructions.

cDNA synthesis

A 2 μg amount of total RNA was incubated with 1 U DNase I (Sigma) in a volume of 10 μl at 22 °C for 15 min and the DNase I was inactivated by the addition of Stop Solution (Sigma) followed by heating at 70 °C for 10 min and chilling on ice. The mixture was supplemented with anchored oligo$(dT)_{23}$ primers (Sigma) to a final concentration of 3.5 μM, heated at 70 °C for 10 min and chilled on ice. Then, 2 μl 10× AMV RT buffer (Sigma), 40 U RNase Inhibitor (Sigma), 2 μl 10 mM dNTPs solution (Sigma) and 20 U AMV RT (Sigma) were added to a final volume of 20 μl and incubated at 50 °C for 1 h. The reaction was stopped by heating at 85 °C for 5 min and mixtures were stored at −20 °C for subsequent pGSTA1* and pGSTA1 amplification by PCR.

Amplification of nucleotide sequence encoding pGSTA1

Primers for PCR amplification were 5′-TTTTTTGGATCCATATGG CGGGGAAGCCCATTCTTC, and 5′-TTTTTTAAGCTTAATCCGGAAAATA TTCTTGGCTTC based on pGSTA1* sequence or 5′-TTTTTTAAGCTTA TTTAATCCGGAAAATATTCTTGGCTTC based on pGSTA1 sequence (Thermo Electron GmbH). The reaction mixture (50 μl) included 2 μl cDNA, 1× Phusion HF buffer (Finnzymes), 0.2 mM dNTPs (Sigma), 0.5 μM of each primer, and 1 U Phusion DNA polymerase (Finnzymes). Three minutes at 98 °C were followed by 35 cycles of 98 °C for 1 min, 62 °C for 1 min, 72 °C for 1 min and, finally, by additional 10 min at 72 °C. PCR products were separated on 1.5% agarose/TBE gel and stained with ethidium bromide. A fragment of ≈700 bp was isolated from the gel using QIAquick Gel Extraction kit (Qiagen), and stored at −20 °C. The expected sizes of pGSTA1 and pGSTA1* PCR products were 696 bp and 693 bp, respectively.

Construction of the cloning plasmid pGEM-3Zf(+)-pGSTA1

The amplified pGSTA1 cDNA was digested with *Bam*HI and *Hin*dIII, yielding a 682 bp fragment that, after electrophoresis, was purified using QIAquick Gel Extraction kit (Qiagen). The purified DNA fragment was inserted into pGEM-3Zf(+) vector (Promega), also digested with *Bam*HI and *Hin*dIII, using T4 DNA ligase (Roche Applied Sciences). Electroporation-competent XL1-Blue cells (Stratagene) were transformed with the ligase mixture, and positive clones containing the pGSTA1 sequence were identified by PCR using colony lysate as a template and appropriate primers. The plasmid DNA was isolated from positive clones using Plasmid Mini kit (Qiagen), and sequenced. Sequencing was done using MegaBACE 1000 kit (GE Healthcare) at the Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences, Uppsala.

Construction of the expression plasmid pET-21α(+)-pGSTA1

pGEM-3Zf(+)-pGSTA1 was digested with *Nde*I and a 720 bp fragment, containing the pGSTA1 sequence, was purified using QIAquick Gel Extraction kit (Qiagen). This fragment was digested with *Hin*dIII yielding a fragment of 676 bp that was ligated into *Nde*I/*Hin*dIII-restricted pET-21α(+) expression vector (Novagen) using T4 DNA ligase (Roche Applied Sciences). The ligation mixture was used to transform electroporation-competent XL1-Blue cells (Stratagene), and positive clones containing pGSTA1 sequence were identified by PCR using colony lysate as a template and appropriate primers. The plasmid DNA from positive clones was isolated using Plasmid Mini kit (Qiagen), and the presence of the target pGSTA1 sequence was verified by the analysis of the size of fragments resulting from restriction of the construct with *Nde*I and *Hin*dIII.

Expression of pGST A1-1 in Escherichia coli

The pET-21α(+)-pGSTA1 plasmid was transformed into electroporation-competent Rosetta™(DE3) cells (Novagen). All cultures were grown in the presence of 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 30 °C in a shaker at 200 rpm. A starter culture of 100 ml Luria–Bertani medium was inoculated with single colonies of freshly transformed cells and incubated for 14 h. One and a half liters 2× YT medium were inoculated with starter culture as 1:50 and grown for 3.5 h until OD_{600} was 0.6. Then, isopropyl-β-D-thiogalactopyranoside (Sigma) was added to a final concentration of 1 mM and incubation continued for 6 h. The cell culture was chilled on ice and then harvested at 10,000 g. The cell pellets were frozen at −20 °C for subsequent protein purification.

Purification of pGST A1-1

The cell pellet from 1.5 l bacterial culture was resuspended in 50 ml lysis buffer [1 mg/ml lysozyme in 0.1 M Tris–HCl pH 8.0 (0 °C), 1 mM EDTA, 14 mM 2-mercaptoethanol, protease inhibitors (Complete EDTA-free, Roche Diagnostics GmbH)] and kept on ice for 2 h under stirring. After ultrasonication, the insoluble debris was sedimented by centrifugation. The supernatant was supplemented with ammonium sulfate (p.a., Merck) to 50% of saturation at 0 °C, incubated for 1 h on ice, centrifuged and the pellet was discarded. The supernatant was further supplemented with ammonium sulfate to 75% of saturation at 0 °C, incubated for 1 h on ice and centrifuged. The supernatant was discarded and the pellet was dissolved in 8 ml binding buffer [10 mM Tris–HCl pH 7.8 (22 °C), 5 mM 2-mercaptoethanol]. The remaining ammonium sulfate was removed by gel filtration on PD-10 columns (GE Healthcare) repeated twice (final sample volume was 30 ml). The protein solution was transferred to 10 ml S-hexylglutathione-Sepharose [22] (prepared from epoxy-activated Sepharose, GE Healthcare) equilibrated with binding buffer and left on ice for 8 h under stirring. After adsorption, the gel was washed batch-wise with 8 × 50 ml binding buffer supplemented with 0.2 M NaCl, packed into a column (1.0 cm internal diameter), and the bound protein was eluted with 50 mM glycine–NaOH pH 10 at 1 ml/min. Fractions of 2 ml were collected into tubes already containing 0.1 ml 2 M Tris–HCl pH 7.2 (22 °C) in order to lower the pH immediately after elution. The protein-containing fractions were pooled, concentrated from 42 ml to 10 ml by ultrafiltration on a polyethersulfone membrane with a 10 kDa molecular-mass cut-off (Pall Life Sciences), desalted on PD-10 columns into the 10 mM Tris–HCl pH 7.2 (22 °C) containing 3 mM dithiothreitol, concentrated to 42 mg/ml by ultrafiltration, aliquoted and stored at −80 °C. Enzyme purity was assessed by SDS–PAGE applying both optimal and excessive protein amounts to visualize possible impurities.

Kinetic characterization of pGST A1-1

The reaction rates with various substrates were followed spectrophotometrically (UV-2501PC Shimadzu, Shimadzu Inc) at 30 °C. 5-Androstene-3,17-dione and 5-pregnene-3,20-dione were purchased from Steraloids, Inc., and other substrates from Sigma–Aldrich. All substrates except glutathione were dissolved in methanol (spectroscopy grade, Acros Organics) and the enzyme was diluted in buffer D [ice-cold 0.1 M sodium phosphate pH 7.8, 45% w/v glycerol (AnalaR®, BDH), 0.2 mM dithiothreitol] prepared

as described in our earlier work (submitted to Analytical Biochemistry). Therefore, all final reaction mixtures contained 0.9% glycerol and 1% methanol. The molar extinction coefficients used for calculations were $\varepsilon_{248} = 16.3\ mM^{-1}\ cm^{-1}$ for 5-androstene-3,17-dione, $\varepsilon_{248} = 17.0\ mM^{-1}\ cm^{-1}$ for 5-pregnene-3,20-dione, $\varepsilon_{340} = 9.6\ mM^{-1}\ cm^{-1}$ for 1-chloro-2,4-dinitrobenzene, $\varepsilon_{340} = -6.2\ mM^{-1}\ cm^{-1}$ for cumene hydroperoxide, $\varepsilon_{225} = -19.22\ mM^{-1}\ cm^{-1}$ for trans-2-nonenal, and $\varepsilon_{274} = 8.89\ mM^{-1}\ cm^{-1}$ for phenethyl isothiocyanate.

Specific activities with 5-androstene-3,17-dione (0.1 mM) and 5-pregnene-3,20-dione (0.01 mM) in the presence of 1 mM GSH were measured in 25 mM Na_2HPO_4–HCl pH 8 using 50 nM and 200 nM enzyme, respectively. The activities with 1-chloro-2,4-dinitrobenzene (1 mM) in the presence of 1 or 5 mM GSH using 50 nM enzyme and with trans-2-nonenal (0.1 mM) in the presence of 0.5 or 1 mM GSH using 3 μM enzyme were measured in 0.1 M KH_2PO_4–KOH pH 6.5. The activity with cumene hydroperoxide (1.5 mM) in the presence of 1 or 2 mM GSH was measured in PBS pH 7.4 supplemented with 0.3 U/ml glutathione reductase (Sigma) and 0.1 mM NADPH (Sigma) using 250 nM enzyme. The activity with phenethyl isothiocyanate (0.1 mM) in the presence of 1 mM GSH and 30 nM enzyme was measured in 0.1 M NaH_2PO_4–NaOH pH 6.5.

For determination of steady-state parameters, the Michaelis–Menten equation was fit to reaction rates (Simfit 6.0.28 software, W.G. Bardsley, University of Manchester) determined with a fixed concentration of GSH and variable concentrations of the second substrate. Saturation curves were obtained using 0.001–0.2 mM 5-androstene-3,17-dione with 1 mM GSH, 0.002–0.01 mM 5-pregnene-3,20-dione with 1 mM GSH, 0.002–2.5 mM 1-chloro-2,4-dinitrobenzene with 5 mM GSH, 0.01–0.4 mM cumene hydroperoxide with 2 mM GSH, 0.015–0.2 mM trans-2-nonenal with 1 mM GSH, and 0.001–0.2 mM phenethyl isothiocyanate with 1 mM GSH. Other conditions including the amount of enzyme were as in specific activity measurements.

Study of tissue-specific expression of pGST A1-1

A PCR was performed using cDNAs from ten tissues of an individual Yorkshire male pig (Zyagen) as a template. Primers for the amplification of full-length pGSTA1 were identical to those used for cloning. For normalization of the data, a 202 bp fragment of porcine β-actin was amplified using primers 5′-TCCTTCCTGGGCATGGAA and 5′-GCGCGATGATCTTGATCTTCATC. The designed primers exclude amplification of non-spliced isoforms of β-actin as well as α-actin. The mixture of 25 μl contained 0.25 μl template cDNA, 0.2 mM dNTPs (Sigma), 0.5 μM of each primer, 0.01 U/μl Phusion DNA polymerase (Finnzymes) in 1× Phusion HF buffer. For pGSTA1 amplification, 3 min at 98 °C were followed by 34 cycles of 98 °C for 10 s, 63 °C for 30 s, 72 °C for 20 s and, finally, by an additional step at 72 °C for 5 min. For β-actin amplification, 3 min at 98 °C were followed by 25 cycles of 98 °C for 10 s, 60 °C for 30 s, 72 °C for 20 s and, finally, by additional step at 72 °C for 5 min. PCR products were separated on 2% agarose/TBE gel, stained with ethidium bromide and a photo was taken. Primer specificity was confirmed by sequencing of the PCR fragments from liver and testis as well as by negative control using pET-21α(+)-pGSTA2 as a template. For quantification, the intensity of PCR fragments was evaluated using Image J 1.33u software (NIH). Intensities of pGSTA1 PCR fragments were normalized to the intensities of β-actin bands from the corresponding tissues and to the intensity of the pGSTA1 band from liver. The resulting normalized intensity values were assigned to an ordinal scale as follows: (0; 0.1) = +, [0.1; 0.5) = ++, [0.5; 1) = +++, and [1; ∞) = ++++.

Results

Alternative sequence of pGST A1-1

Porcine GST A1-1 (Swiss-Prot: P51781) was identified as a protein similar to human GST A3-3 (Swiss-Prot: Q16772) by a BLASTP [SIB BLAST Network service] search among mammalia, performed against the UniProtKB. During cloning with primers derived from the published sequence it was found that the pGSTA1 cDNA we obtained differed from the pGSTA1 sequence published in the database (hereafter called pGSTA1*) at three positions (Fig. 1). C219 in the sequence of pGSTA1* was replaced by T in pGSTA1, which was a silent mutation. A440 in pGSTA1* was replaced with T in

```
                    15                  30                  45                  60                  75
A1    ATGGCGGGGAAGCCC  ATTCTTCACTATTTC  AATGGCCGAGGCAGA  ATGGAGTGTATCCGG  TGGCTCCTGGCTGCA
A1*   ···.............  ...............  ...............  ...............  ...............

                    90                 105                 120                 135                 150
A1    GCTGGAGTGGAGTTT  GAAGAGAAATTTATT  AAGACTCCAGAAGAC  CTGGATAAGTTAACA  AATGATGGGAGTTTG
A1*   ···............  ...............  ...............  ...............  ...............

                   165                 180                 195                 210                 225
A1    CTGTTCCAGCAAGTG  CCCATGGTTGAGATT  GACGGGATGAAGCTG  GTGCAGACCAGGGCC  ATCCTCAATTACATC
A1*   ···............  ...............  ...............  ...............  ........C......

                   240                 255                 270                 285                 300
A1    GCCACCAAGTACAAC  CTCTACGGGAAGGAC  GCCAAGGAGAGAGCC  CTGATTGATATGTAT  ACAGAAGGTGTGGCA
A1*   ···............  ...............  ...............  ...............  ...............

                   315                 330                 345                 360                 375
A1    GATTTGGGTGAAATG  ATCTTGCTGTTGCCA  CTGTGCCCACCCAAT  GAAAAAGATGCCAAG  GTGGCCTCGATCAAA
A1*   ···............  ...............  ...............  ...............  ...............

                   390                 405                 420                 435                 450
A1    GAAAAATCGACAAAC  CGTTATCTTCCTGCA  TTTGAAAAAGTGTTG  AAGAGCCATGGACAA  GACTTCCTTGTGGGC
A1*   ···............  ...............  ...............  ...............  ····A··········

                   465                 480                 495                 510                 525
A1    AACAAGCTGAGCAGG  GCTGACATCCAGCTG  GTTGAACTTCTCTAC  TACGTGGAAGAGCTG  GACCCCAGCCTGCTG
A1*   ···............  ...............  ...............  ...............  ...............

                   540                 555                 570                 585                 600
A1    GCCAACTTCCCTCTG  CTGAAGGCCCTGAAA  ACCAGAGTCAGCAAC  CTCCCCACGGTGAAG  AAGTTTCTGCAGCCT
A1*   ···............  ...............  ...............  ...............  ...............

                   615                 630                 645                 660                 672
A1    GGCAGCCAGAGGAAG  CCTCCTATGGATGCG  AAAAAA-TTAGAAGA  AGCCAAGAATATTTT  CCGGATTAAATAA
A1*   ···............  ...............  ......A........  ...............  ......···
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Fig. 1. Alignment of cDNA encoding pGST A1-1 and pGST A1-1*. Start and stop codons are underlined. Differing nucleotide bases are highlighted by gray background.

pGSTA1, resulting in a conservative replacement of tyrosine 174 by phenylalanine. Also, an A from a 7A-stretch (nucleotides 631–637) in the published sequence was absent in our clone, resulting in a 6A-stretch (nucleotides 631–636). Repeated sequencing of the cDNA isolated from different tissues and different animals ruled out the possibility of a cloning artifact. Our nucleotide sequence, with the 6A-stretch, is also recognized on the chromosome 7 of the pig genome (single Duroc sow) published at NCBI (sixth exon is mapped at position 372712–372837 in contig NW_001886576.1), while the pGST A1-1* sequence is only recognized for the first two differences (position 301227 and position 365403 or 429458 in the same contig). Whether the A637 in pGSTA1* has arisen as a porcine breed difference, an allelic variant, or as a cloning artifact in previous studies is not clear. We recloned the enzyme using primers derived from our pGSTA1 nucleotide sequence. The C-terminal amino acid residues encoded by our pGSTA1 were 213-LEEAKNIFRIK-223, instead of 213-IRRSQEYFPD-222 as in the published sequence of pGSTA1* (Fig. 2), and the deduced amino acid sequence of our clone showed higher similarities with pGSTA2 and hGSTA3.

Molecular cloning of pGST A1-1

Porcine pGSTA1 cDNA was cloned from ovary and testis, using the same 5′ primer as for the first cloning (see Alternative sequence of pGST A1-1), and a new 3′ primer including the codon corresponding to Lys223. Total RNA was isolated from the tissues and cDNA was synthesized using anchored oligo$(dT)_{23}$. PCR fragments were amplified using pGSTA1 specific primers and inserted into the pGEM-3Zf(+) cloning vector. At this step the identity of pGSTA1 cDNAs cloned from ovary and testis was verified by sequencing and the absence of gender sequence polymorphism was ascertained. The pGSTA1 cDNA was also subcloned into the pET-21α(+) vector for overexpression in *E. coli*.

Heterologous expression and purification of pGST A1-1

In order to characterize the cloned enzyme further, pGST A1-1 was expressed in the *E. coli* Rosetta™(DE3) strain and purified to homogeneity as well as other proteins in the study. The two-step purification procedure yielded 200 mg protein from 1.5 l bacterial culture. The purity of pGST A1-1 in the obtained specimen was at least 95% as estimated by the analysis of the corresponding SDS–PAGE gel.

Basic physical properties of pGST A1-1

Porcine GST A1-1 contains 223 amino acid residues including the initial methionine. The molecular mass of the pGST A1-1 subunit is 25.33 kDa (25.15 kDa excluding the initial methionine), but the protein is expected to be a dimer based on analogy to other soluble GSTs [23]. The molar absorption coefficient of 18.91 $mM^{-1}\,cm^{-1}$ and $Abs^{0.1\%}$ of 0.747 at 280 nM, assuming that both cysteine residues (Cys^{18} and Cys^{112}) are reduced, were calculated by the ProtParam tool [24] and used to determine the enzyme concentration. Isoelectric point was calculated to 9.00 using the same software.

Specific activities and steady-state parameters of pGST A1-1

Specific activities of pGST A1-1 with several substrates were measured under standard conditions (Table 1). The enzyme demonstrated the highest specific activity with 1-chloro-2,4-dinitrobenzene (135 and 164 μmol $min^{-1}\,mg^{-1}$ with 1 and 5 mM GSH, respectively). Activities with cumene hydroperoxide, phenethyl isothiocyanate, 5-androstene-3,17-dione, 5-pregnene-3,20-dione and nonenal were below 10 μmol $min^{-1}\,mg^{-1}$. Normalizing all values obtained in the presence of 1 mM GSH to the specific activity with 1-chloro-2,4-dinitrobenzene (100%), it can be said that pGST A1-1 has ⩽7% activity with the other substrates tested. Activity with nonenal (0.06%) was the lowest.

Porcine GST A1-1 obeyed Michaelis–Menten kinetics under the conditions used and steady-state parameters were determined. The k_{cat}/K_M value of 420 $mM^{-1}\,s^{-1}$ with 1-chloro-2,4-dinitrobenzene was highest of all the substrates tested (Table 1). Cumene hydroperoxide and phenethyl isothiocyanate proved to be moderately good substrates with k_{cat}/K_M of 84 and 79 $mM^{-1}\,s^{-1}$, respectively. Steroids and nonenal were the least active as substrates with catalytic efficiencies of 20, 16, and 1.1 $mM^{-1}\,s^{-1}$ for

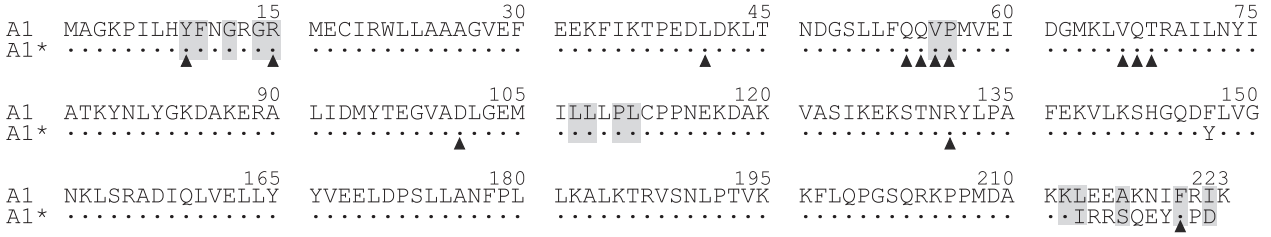


Fig. 2. Alignment of amino acid sequences of porcine GST A1-1 and A1-1*. H-site and G-site residues are highlighted by gray background and triangles, respectively. Active-site residues are inferred from the analysis of hGST A1-1, A2-2, A3-3 and A4-4 structures published in [14].

Table 1
Kinetic parameters of pGST A1-1. The parameters were determined under standard conditions specified in Materials and Methods.

Substrate	Substrate (mM)	GSH (mM)	Activity ± SD (μmol $min^{-1}\,mg^{-1}$)	k_{cat}/K_M ± SE ($mM^{-1}\,s^{-1}$)	k_{cat} ± SE (s^{-1})	K_M ± SE (μM)
1-Chloro-2,4-dinitrobenzene	1	5	164 ± 12	420 ± 15	84 ± 9	200 ± 7
		1	135 ± 3			
Cumene hydroperoxide	1.5	2	3.9 ± 0.2	84 ± 13	3.3 ± 0.2	39 ± 6
		1	5.8 ± 0.3			
Phenethyl isothiocyanate	0.1	1	9.6 ± 0.2	79 ± 11	9.5 ± 0.6	121 ± 15
5-Androstene-3,17-dione	0.1	1	3.4 ± 0.2	20 ± 2	4.8 ± 0.3	237 ± 20
5-Pregnene-3,20-dione	0.01	1	0.19 ± 0.02	16 ± 7	0.17 ± 0.04	10 ± 4
Trans-2-nonenal	0.1	1	0.075 ± 0.017	1.1 ± 0.3	0.16 ± 0.02	155 ± 33
		0.5	0.045 ± 0.004			

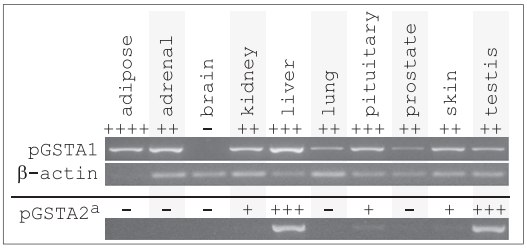


Fig. 3. Expression of pGST A1-1 in various tissues. Full-length pGSTA1 was amplified using cloning primers. Intensity of obtained PCR fragments was evaluated using ImageJ 1.33u software (NIH). For quantification, the intensities of pGSTA1 PCR fragments were sequentially normalized to the respective intensities of β-actin PCR fragments and the normalized intensity of pGSTA1 PCR fragment from liver. Final normalized intensities were assigned to an ordinal scale as follows: (0; 0.1) = +, [0.1; 0.5) = ++, [0.5; 1) = +++, and [1; ∞) = ++++. [a]From [14]. The tissue expression of pGST A2-2 was studied in parallel with that of pGST A1-1.

5-androstene-3,17-dione, 5-pregnene-3,20-dione, and nonenal, respectively.

Expression of pGST A1-1 in various tissues

Expression of pGST A1-1 in various tissues was analyzed by PCR (Fig. 3). The sequence specificity of the used primers was verified by sequencing of the entire PCR fragments obtained from liver and testis. No pGSTA1* was amplified in any case and no tissue polymorphism was detected. The highest expression levels of pGST A1-1 were found in adipose tissue, liver, and pituitary gland. The enzyme expression in adrenal gland, kidney, lung, prostate, skin and testis was also significant, but no pGSTA1 mRNA was detected in brain. In addition, expression level of pGST A1-1 in ovary appeared to be similar to that in testis, since similar amounts of pGSTA1 cDNA were obtained from ovary and testis in cloning experiment (data not shown).

Discussion

Several Alpha-class GSTs are present in porcine tissues

Mammalian species such as humans, rats, and mice appear to have five functional genes expressing Alpha-class GSTs [10]. The porcine genome assembly (NCBI build 1.1, based on Sscrofa5) has not yet been completed such that a definite comparison can be made at the gene level. Although exons encoding four or five Alpha-class GSTs (including pGSTA1/pGSTA1* and pGSTA2) can be found on chromosome 7 (contig NW_001886576.1), the corresponding genes cannot be unambiguously mapped and annotated. At the protein level, it has been reported that glutathione transferases constitute up to 6% of the total soluble protein of the porcine liver with Alpha-class GSTs accounting for the greater part of the 1-chloro-2,4-dinitrobenzene activity in this tissue [15]. One [16], three [17], four [18] or five [15] Alpha-class isoforms have been isolated from porcine liver in earlier studies. Four or five Alpha-class GSTs have also been detected in porcine ovary [19]. We have previously cloned and characterized pGST A2-2 [14] and made a corresponding characterization of pGST A1-1 in the present investigation. The mRNA of these proteins is present in liver at high concentrations, as inferred from PCR analysis (Fig. 3). Definitive identifications of the cloned GSTs with the previously reported porcine enzymes are not possible, since complete characterization of the latter proteins is lacking. Amino acid sequences of Alpha-GST peptides identified in four HPLC fractions [18] correspond to residues 47–55, 70–77 and 93–100 of pGST A1-1 and/or pGST A2-2. These sequences may also be derived from one or two additional Alpha-class enzymes identifiable in the porcine genome. The molecular masses determined by mass-spectrometry in the same study are less than monoisotopic molecular masses calculated for the pGST A1-1 and pGST A2-2 subunits (excluding the initial methionine). Comparisons of basic isoelectric points determined in [15,17] with those determined for pGST A1-1 and A2-2 in our studies (9 and 9.2 [14], respectively) do not allow unequivocal distinctions either. However, it appears that pGST A1-1 and A2-2 are represented by two of three Alpha-class GSTs with basic pI found expressed in liver [15]. Proteomic analyses and complementary studies may be required for definitive identification of the GST expression pattern in porcine tissues.

Porcine GST A1-1 expression profile is characteristic for detoxication function

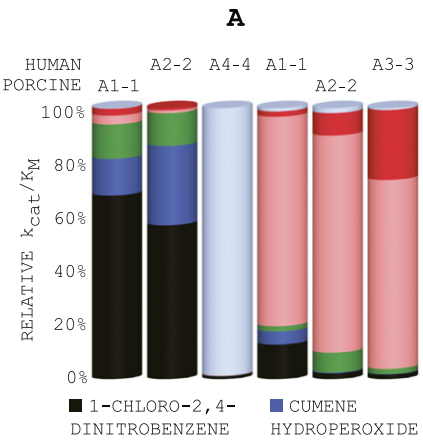
The present study revealed that pGST A1-1, originally cloned from ovary among genes expressed and regulated during ovarian folliculogenesis [21], is actually expressed in many porcine tissues. This may suggest a dissimilar cellular role of pGST A1-1 as compared to pGST A2-2, which has been characterized as an analogue of hGST A3-3 [14] involved in steroidogenesis [5]. As for other human Alpha-class GSTs, pGST A1-1 mRNA was not found in brain, which distinguishes pGST A1-1 from the ubiquitous Alpha-class enzyme hGST A4-4 mRNA [25]. In contrast, the pGST A1-1 expression profile was similar to those of human GST A1-1 and A2-2 [25], suggesting that pGST A1-1 in the pig in a similar manner is involved in general detoxication of electrophiles.

Porcine GST A1-1 and human GST A2-2 show functional similarities

Using a set of alternative substrates, the catalytic efficiency profile of pGST A1-1 was compared to those of human Alpha GSTs as well as porcine GST A2-2 (Fig. 4A). Porcine GST A1-1 appears to functionally be most similar to hGST A2-2 in terms of k_{cat}/K_M values for the particular substrates used. The main dissimilarities from other human Alpha GSTs are very low relative activities with steroids and nonenal and very high relative activity with 1-chloro-2,4-dinitrobenzene. Remarkably, the catalytic efficiencies of pGST A1-1 with 5-androstene-3,17-dione and 5-pregnene-3,20-dione are very similar, in contrast to the corresponding pairs of values that differ from one another in the cases of pGST A2-2 [14] or hGST A3-3 [11,26,27]. The main contributor to such a difference is the Michaelis constant, which is 24-fold higher for 5-androstene-3,17-dione than for 5-pregnene-3,20-dione (Table 1). 5-Pregnene-3,20-dione differs from 5-androstene-3,17-dione by an acetyl group instead of the keto group in position 17 of the steroid molecule. The bulkier part of the steroid situated far from the migrating double bond apparently facilitates binding of the substrate but at the cost of lower catalytic rate, since K_M and k_{cat} values for 5-pregnene-3,20-dione are much lower than the values for 5-androstene-3,17-dione.

Structural bases for diverging substrate selectivity of Alpha-class GSTs

Substrate selectivity profiles of characterized porcine [this study and [14]], human [11,26–39] and bovine [40] Alpha-class GSTs divide the enzymes into three groups based on prominent activities with the following substrates: (I) pGST A1-1, hGST A2-2, and bGST A1-1 with 1-chloro-2,4-dinitrobenzene; (II) pGST A2-2, hGST A1-1, and A3-3 with 5-androstene-3,17-dione; and (III) hGST A4-4 with nonenal (Fig. 4B). Out of seventeen amino acid residues forming the H-site in Alpha-class GSTs [14], ten residues were found to differ in the compared enzymes (Fig. 4B). Since the secondary and tertiary structures of these proteins (except for hGST A4-4) are very similar, we may expect the divergent



B

GROUP	ENZYME	POSITION 10 12 107 108 111 208 212 213 216 222	k_{cat}/K_M OR (SPECIFIC ACTIVITY) 1-CHLORO-2,4-DINITROBENZENE	CUMENE HYDROPEROXIDE	PHENETHYL ISOTHIOCYANATE	5-ANDROSTENE-3,17-DIONE	5-PREGNENE-3,20-DIONE	NONENAL
(I)	PORCINE A1-1	F G L L L M K L A I	420	84	79	20	16	1.1
	HUMAN A2-2	**S I** L L **F M** S L **S** F	56	29	12[a]	1	0.8[b]	–[c]
	BOVINE A1-1	F G M H L T K I A F	(6.2)	(3.3)	(4.6)	(0.7)	(0.4)	(0.6)
(II)	HUMAN A3-3	**F G** L L **L A** A L **A** F	110	9.9	120	4300	1600	40[a]
	PORCINE A2-2	F G L L L T S L A F	46	4.7	150	1630	170	36
	HUMAN A1-1	F A L L V M S L A F	92	35	14	550	14[a]	8.5
(III)	HUMAN A4-4	P G I M F P Y V V P	7.9	0.6	1[a]	0.53	0.4[b]	710
(I)	PORCINE A1-1*	F G L L L M K I S D						

Fig. 4. Catalytic properties and H-site differences of pGST A1-1 as compared to other mammalian Alpha-class GSTs. The values are from this study (pGST A1-1) and from the following publications: bGST A1-1 [40], hGST A1-1 [11,27,31–33,36,38], hGST A2-2 [29,30,33,36,37,39], hGST A3-3 [11,26–28,30], hGST A4-4 [31,33–35], and pGST A2-2 [14]. (A) Substrate selectivity profiles. Each cylinder represents one individual GST and segments of different colors relate to substrates as indicated. The length of each segment corresponds to the relative contribution of the particular k_{cat}/K_M to a total sum of the values for all tested substrates. (B) Alignment of H-site residues differing among porcine, human and bovine Alpha-class GSTs. The differing H-site residues are inferred from the analysis of hGST A1-1, A2-2, A3-3 and A4-4 structures published in [14]. The largest value of k_{cat}/K_M ($mM^{-1}\ s^{-1}$) or specific activity ($\mu mol\ min^{-1}\ mg^{-1}$) for a particular enzyme is highlighted with colored background; and the enzymes are grouped accordingly. Residues in bold govern the productive binding of steroids as identified in [30,41]. [a]The k_{cat}/K_M values for hGST A1-1 in the reaction with 5-pregnene-3,20-dione, for hGST A2-2 and hGST A4-4 in the reaction with phenethyl isothiocyanate, and for hGST A3-3 in the reaction with nonenal were estimated based on available specific activities. [b]The k_{cat}/K_M values for hGST A2-2 and hGST A4-4 in the reaction with 5-pregnene-3,20-dione were estimated based on assumption that they do not exceed 80% of those with 5-androstene-3,17-dione. [c]The k_{cat}/K_M value for hGST A2-2 in reaction with nonenal could not be estimated and is absent.

H-site residues to account for major differences in substrate preferences. The structural basis for the low steroid isomerase activity of hGST A2-2 in comparison to the Alpha-class GSTs with high steroid isomerase activity has been reported to depend on five residues (in bold, Fig. 4B) that govern the productive binding of the steroid [30,41]. Porcine GST A1-1 has only one of the unfavorable residues in the active site, Met208, whereas the other four residues are identical to those found in pGST A2-2 or hGST A3-3, which are efficient steroid isomerases. Human GST A1-1 is also characterized by Met208, but its catalytic efficiency in the 5-androstene-3,17-dione isomerization is 28-fold higher in absolute numbers than that of pGST A1-1 (Fig. 4B). This considerable difference between pGST A1-1 and hGST A1-1 is probably explained by the presence of bulky Lys212 in pGST A1-1 instead of Ser212 in hGST A1-1. Indeed, a pair of bulky residues in positions 208 and 212 seems to favor the smaller-size substrates like 1-chloro-2,4-dinitrobenzene or cumene hydroperoxide, while a pair of small residues in these positions favors the larger steroid substrates. Also, the pair Ser10 and Ile12, closely spaced in the hGST A2-2 structure (PDB entry 2WJU), occupies a similar volume as the pair Phe10 and Gly12 in other GSTs. For steroid substrates, this difference is apparently essential (compare hGST A1-1 and hGST A2-2 profiles, Fig. 4B). Smaller substrates, the binding of which is permitted by a pair of the bulky residues 208 and 212, seem to tolerate such replacement (compare pGST A1-1 and hGST A2-2 profiles, Fig. 4B).

In accordance with the reasoning above, the putative pGST A1-1* if expressed can be expected to have high activity with smaller-size substrates and low steroid isomerase activity, thereby associating with group I. It should finally be mentioned that a conservative replacement of residue 147, which does not belong to the active site, would not be expected to change the substrate selectivity profile. In contrast, it can be speculated that the C-terminal residues of pGST A1-1 Ile222-Lys223 and that of pGST A1-1* Asp222, will influence reactions with substrates bearing positive or negative charges.

Conclusions

Our study presents porcine GST A1-1 as a detoxication enzyme expressed in many tissues. Based on comparison of activity and expression profiles, we would expect this enzyme to function *in vivo* similarly to human GST A2-2, although one-to-one correspondence between GSTs in different mammalian species does not necessarily exists. Thus, porcine GST A1-1 and A2-2 would correspond to human GST A2-2 and A3-3, respectively. More Alpha-class GSTs are encoded in the porcine genome. Therefore, additional porcine Alpha-class GSTs are yet to be cloned and characterized for understanding of the physiological role of these enzymes in the pig.

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References

[1] P.D. Josephy, B. Mannervik, Glutathione transferases, in: Molecular Toxicology, Oxford University Press, Oxford, 2006, pp. 333–364.
[2] L. Romero, K. Andrews, L. Ng, K. O'Rourke, A. Maslen, G. Kirby, Biochem. J. 400 (2006) 135–141.
[3] S.G. Cho, Y.H. Lee, H.S. Park, K. Ryoo, K.W. Kang, J. Park, S.J. Eom, M.J. Kim, T.Sh. Chang, S.Y. Choi, J. Shim, Y. Kim, M.S. Dong, M.J. Lee, S.G. Kim, H. Ichijo, E.J. Choi, J. Biol. Chem. 276 (2001) 12749–12755.
[4] V. Adler, Zh. Yin, S.Y. Fuchs, M. Benezra, L. Rosario, K.D. Tew, M.R. Pincus, M. Sardana, C.J. Henderson, C.R. Wolf, R.J. Davis, Z. Ronai, EMBO J. 18 (1999) 1321–1334.
[5] F. Raffalli-Mathieu, C. Orre, M. Stridsberg, M. Hansson Edalat, B. Mannervik, Biochem. J. 414 (2008) 103–109.
[6] W.H. Habig, M.J. Pabst, G. Fleischner, Z. Gatmaitan, I.M. Arias, W.B. Jakoby, Proc. Natl Acad. Sci. USA 71 (1974) 3879–3882.
[7] G. Litwack, B. Ketterer, I.M. Arias, Nature 234 (1971) 466–467.
[8] I.M. Arias, N. Ohmi, M. Bhargava, I. Listowsky, Mol. Cell. Biochem. 29 (1980) 71–80.
[9] A.M. Caccuri, A. Aceto, F. Piemonte, C. Di Ilio, N. Rosato, G. Federici, Eur. J. Biochem. 189 (1990) 493–507.
[10] B. Mannervik, P.G. Board, J.D. Hayes, I. Listowsky, W.R. Pearson, Methods Enzymol. 401 (2005) 1–8.
[11] A.-S. Johansson, B. Mannervik, J. Biol. Chem. 276 (2001) 33061–33065.
[12] L. Benbrahim-Tallaa, F. Boussouar, C. Rey, M. Benahmed, J. Endocrinol. 175 (2002) 803–812.
[13] J.K. Lunney, Int. J. Biol. Sci. 3 (2007) 179–184.

[14] N. Fedulova, F. Raffalli-Mathieu, B. Mannervik, Biochem. J. 431 (2010) 159–167.
[15] G. Williamson, Int. J. Biochem. 21 (1989) 23–30.
[16] A. Grahnén, I. Sjöholm, Eur. J. Biochem. 80 (1977) 573–580.
[17] T. Kunze, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 116 (1997) 397–406.
[18] P. Rouimi, P. Anglade, L. Debrauwer, J. Tulliez, Biochem. J. 317 (1996) 879–884.
[19] M. Eliasson, T. Stark, J.W. DePierre, Chem. Biol. Interact. 117 (1999) 35–48.
[20] L. Benbrahim-Tallaa, E. Tabone, G. Tosser-Klopp, F. Hatey, M. Benahmed, Biol. Reprod. 66 (2002) 1734–1742.
[21] G. Tosser-Klopp, F. Benne, A. Bonnet, P. Mulsant, F. Gasser, F. Hatey, Mamm. Genome 8 (1997) 250–254.
[22] B. Mannervik, C. Guthenberg, Methods Enzymol. 77 (1981) 231–235.
[23] B. Mannervik, H. Jensson, J. Biol. Chem. 257 (1982) 9909–9912.
[24] E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvaud, M.R. Wilkins, R.D. Appel, A. Bairoch, Protein identification and analysis tools on the ExPASy server, in: J.M. Walker (Ed.), The Proteomics Protocols Handbook, Humana Press, 2005, pp. 571–607.
[25] F. Morel, C. Rauch, B. Coles, E. Le Ferrec, A. Guillouzo, Pharmacogenetics 12 (2002) 277–286.
[26] A.-S. Johansson, B. Mannervik, J. Biol. Chem. 277 (2002) 16648–16654.
[27] Y. Gu, J. Guo, A. Pal, S.S. Pan, P. Zimniak, S.V. Singh, X. Ji, Biochemistry 43 (2004) 15673–15679.
[28] N. Tetlow, M. Coggan, M.G. Casarotto, P.G. Board, Pharmacogenetics 14 (2004) 657–663.
[29] N. Tetlow, P.G. Board, Pharmacogenetics 14 (2004) 111–116.
[30] P.L. Pettersson, A.-S. Johansson, B. Mannervik, J. Biol. Chem. 277 (2002) 30019–30022.
[31] A. Gustafsson, L.O. Nilsson, B. Mannervik, J. Mol. Biol. 316 (2002) 395–406.
[32] S. Lien, A. Gustafsson, A.K. Andersson, B. Mannervik, J. Biol. Chem. 276 (2001) 35599–35605.
[33] P.L. Pettersson, B. Mannervik, J. Biol. Chem. 276 (2001) 11698–11704.
[34] P.G. Board, Biochem. J. 330 (1998) 827–831.
[35] I. Hubatsch, M. Ridderström, B. Mannervik, Biochem. J. 330 (1998) 175–179.
[36] R.H. Kolm, U.H. Danielson, Y. Zhang, P. Talalay, B. Mannervik, Biochem. J. 311 (1995) 453–459.
[37] D.J. Meyer, D.J. Crease, B. Ketterer, Biochem. J. 306 (1995) 565–569.
[38] G. Stenberg, P.G. Board, I. Carlberg, B. Mannervik, Biochem. J. 274 (1991) 549–555.
[39] W. Zhang, O. Modén, B. Mannervik, Chem. Biol. Interact. 186 (2010) 110–117.
[40] F. Raffalli-Mathieu, D. Persson, B. Mannervik, Biochim. Biophys. Acta 1770 (2007) 130–136.
[41] K. Tars, B. Olin, B. Mannervik, J. Mol. Biol. 397 (2010) 332–340.