Short communication

Response of selenium-dependent glutathione peroxidase in the freshwater bivalve *Anodonta woodiana* exposed to 2,4-dichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol

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ABSTRACT

2,4-dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6-TCP), and pentachlorophenol (PCP) pose a health risk to aquatic organism and humans, and are recognized as persistent priority pollutants. Selenium dependent glutathione peroxidase (Se-GPx) belongs to the family of selenoprotein, which acts mainly as an antioxidant role in the cellular defense system. In the current study, a Se-GPx full length cDNA was cloned from *Anodonta woodiana* and named as AwSeGPx. It had a characteristic codon at 165TGA167 that corresponds to selenocysteine(Sec) amino acid as U44. The full length cDNA consists of 870 bp, an open reading frame (ORF) of 585 bp encoded a polypeptide of 195 amino in which conserved domain (68LGFPCNQF75) and a glutathione peroxidase-1 GPx active site (32GKVLVENVASLUGTT47) were observed. Additionally, the eukaryotic selenocysteine insertion sequence (SECIS) was conserved in the 3'UTR. The AwSeGPx amino acid sequence exhibited a high similarity with that of other Se-GPx. Real-time PCR analysis revealed that AwSeGPx mRNA had a widely distribution, but the highest level was observed in hepatopancreas. AwSeGPx mRNA expression was significantly up-regulated in hepatopancreas, gill and hemocytes after 2,4-DCP, 2,4,6-TCP and PCP exposure. Under similar environment, clams *A. woodiana* showed a more sensitive to PCP than that of 2,4-DCP and 2,4,6-TCP. These results indicate that AwSeGPx plays a protective role in eliminating oxidative stress derived from 2,4-DCP, 2,4,6-TCP and PCP treatment.

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1. Introduction

Products chlorophenols (CPs) are ubiquitous globally in surface waters, groundwater, wastewater, sludge and drinking waters because they are widely used in agriculture and industry as pesticides, wood preservatives, and personal care formulations [1,2]. Furthermore, CPs are the most direct precursors of dioxin and polyaromatic compounds in incinerator gas emissions [3]. CPs have received worldwide concern due to their toxicity to aquatic life, persistence, and potential to bioaccumulation [4,5]. Among CPs,

2,4-dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6-TCP), and pentachlorophenol (PCP) are ubiquitous in significant quantities, which pose a health risk to aquatic organism and humans and are recognized as persistent priority pollutants in the United States, Europe and China [6,7]. At present, 2,4-DCP, 2,4,6-TCP and PCP exist widely in over 600 sites in the 7 major watersheds and 3 drainage areas of China [8]. They have been shown to induce reproductive toxicity, endocrine activity and oxidative stress in aquatic organism [9]. Previous studies indicate that principal mechanisms of action of PCP-induced toxicity are related to the uncoupling of oxidative phosphorylation in mitochondria and the generation of reactive oxygen species (ROS) [9,10].

In healthy organism, total ROS level stay a relatively low level and ROS will be rapidly eliminated by an array of anti-oxidant enzymes to maintain a balance between ROS level and anti-oxidant enzyme activity [11,12]. Moderate oxidative stress in a shorter

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time may function as an important player to induce different anti-oxidant enzyme expressions to deal with different ROS [13,14]. However, higher ROS concentrations exceed the scavenging capacity of anti-oxidant enzymes could bring about the risk of DNA damage, lipid peroxidation and protein carbonylation [15,16]. Orchestration of anti-oxidant enzymes is a key hallmark of organism homeostasis. Among of these enzymes, glutathione peroxidases (GPx) are one major component of the antioxidant system, which catalyze the reduction of hydrogen peroxide, two isoforms had been identified: selenium-dependent GPx (Se-GPx) and selenium independent GPx. SeGPx catalyzes the reduction of organic and inorganic peroxides like hydrogen peroxide (H_2O_2) while selenium-independent GPx reduces only organic peroxide [17].

Now, highlight of Se-GPx enzyme protecting organisms from oxidative damage gained widely attention. In mammals, four major members of Se-GPx (GPx-1 to GPx-4) have been identified, and show specificities for the hydroperoxide substrates. GPx-1 and GPx-2 reduce rapidly hydrogenperoxide or fatty acid hydroperoxides, GPx-4 can react with phospholipid hydroperoxides [18,19]. It has demonstrated that enhancement of Se-GPx activities contribute to attenuate oxidative injury in mammals and some fish species [20]. In mussel *Mytilus galloprovincialis*, inhibition of Se-GPx activity by Hg could promote a shift in the balance between oxidants and antioxidants in favor of oxidants, resulted in the enhancement of Hg induced oxidative and genotoxic effects [21]. In *Venerupis philippinarum*, administration of heavy metals, such as Cd and Cu, can results in a significant induction of VpSe-GPx expression [22].

Freshwater clams, *Anodonta woodiana* are widely distributed in the world and functions as a main criteria required for a bio-indicator organism. Earlier studies have revealed the ability of *A. woodiana* to accumulate trace elements and pesticides, as well as its potential to detect genotoxicity [23]. In addition, these mussels have important ecosystem functions such as particle filtration and processing, nutrient release, and sediment mixing [23,24]. Considering here, their decline of population can profoundly affect aquatic ecosystem. Take into consideration of great interest to investigate the effect of 2,4-DCP, 2,4,6-TCP and PCP on *A. woodiana*, in the current study, one complete sequence of Se-GPx has been cloned and named AwSeGPx, and temporal expressions derived from 2,4-DCP, 2,4,6-TCP and PCA exposure were determined by quantitative real-time PCR.

2. Materials and methods

2.1. Ethics statement

All handling methods of clams were conducted in accordance with the guidelines on the care and use of animals for scientific purposes set up by the Institutional Animal Care and Use Committee of Nanyang Medicine College, Nanyang, China.

2.2. Materials

Approximately 1-year-old of clams *A. woodiana* (shell length, 6.5 ± 0.5 cm) were obtained from the Baihe River of Nanyang, Henan Province, China. Prior to experiment, animals were maintained in a recirculation system containing filtered freshwater at 24 °C for 2 weeks in laboratory. 2,4-DCP, 2,4,6-TCP and PCP were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO) and stock solutions were prepared by dissolution in dimethylsulfoxide (DMSO). The experiment was conducted in rectangular plastic boxes (40 cm × 25 cm; 10 cm height) containing 10 L artificial pond water and the concentration of DMSO in the water was not excess 0.01%. Clams *A. Woodiana* were randomly grouped into CPs treated

groups in which animals were respectively administrated with 5 test concentrations of 2,4-DCP, 2,4,6-TCP and PCP, and control group with similar volume DMSO. Concentration ranges for viability studies were as follows: 60 µg/L, 120 µg/L, 240 µg/L, 480 µg/L, 960 µg/L for 2,4-DCP, 50 µg/L, 100 µg/L, 200 µg/L, 400 µg/L, 800 µg/L for 2,4,6-TCP, and 20 µg/L, 40 µg/L, 80 µg/L, 160 µg/L, 320 µg/L for PCP. Each group had triplicates per 10 clams and those were not fed during the study. Survival in each box was assessed at 48 h and median lethal concentration (LC50) value was calculated using the log-probit method.

In order to determine the tissue distribution of AwSeGPx, several of tissues including foot, gill, hepatopancreas, adductor muscle, heart and mantle were sampled prior to the treatment. According to above mentioned way of animal treatment, 440 clams were selected to determine the expressions of AwSeGPx. Hepatopancreas, gill and hemocytes were pooled at 0, 6, 12, 24 and 48 h, then immediately frozen in liquid nitrogen and stored at −80 °C until total RNA extraction.

2.3. Total RNA isolation and reverse transcription

Total RNA was extracted using TRIzol (Invitrogen Life Technologies, USA) according to the manufacturer's protocol. Quality of RNA was monitored by 1.2% agarose gel electrophoresis and those with complete rRNA bands were selected to produce cDNA. First-strand cDNA was synthesized using M-MLV First-Strand cDNA synthesis Kit (Takara, China) according to the manufacturer's instructions and used as the template for PCR reaction.

2.4. Cloning of AwSeGPx cDNA

GPx fragment was amplified using two degenerate primers G1 and G2 (Table 1), which were designed according to conserved domains of SeGPx of other species including bivalve, gastropod, insect, crustacean and vertebrate. The PCR products were subcloned into the pMDT-19 (Takara, China), sequenced from both directions (Invitrogen Life Technologies, China) and identified SeGPx partial cDNA sequence. Highly stringent primers (Table 1) designed from the partial cDNA sequences were used to characterize the 5' and 3' regions of the AwSeGPx cDNA by rapid amplification of cDNA ends (RACE) approaches (Takara, China) according to the manufacturer's protocol. 5' Raceouter primer and GPx5-1 (Table 1) were used for the first-round PCR of AwSeGPx 5' RACE, 3' Raceouter

Table 1

Description of the primes used in this study. G1 and G2 were degenerate primers and used to isolate partial cDNA of SeGPx. 5' Race Outerprimer, 5' Race Innerprimer, GPx5-1 and GPx5-2 were used to characterize the 5' RACE of the AwSeGPx in the nest PCR, 3' Race Outerprimer, 3' Race Innerprimer, GPx3-1 and GPx3-1 for 3' RACE. GPx-F and GPx-R as well as β-F and β-R were selected to isolate AwSeGPx and β-actin in real-time PCR, respectively.

Primer	Sequence (5'–3')
G1	TTGTAGCCNGTCTNCCAGG
G2	GTGTCGNGTGAGANGTAATAGAA
5'Race Innerprimer	CATGGCTACATGCTGACAGCCTA
5'Race Outerprimer	CGCGGATCCACAGCCTACTGATGATCAGTCGATG
GPx5-1	TCCTTCTCATCGTGGTGCA
GPx5-2	ACCACGCCAAACTTGCCCT
3'Race Outerprimer	TACCGTCGTTCCACTAGTGATT
3'Race Innerprimer	CGCGGATCCTCTCCACTAGTGATTCTACTATAGG
GPx3-1	CCTGAAATCTCCAGCATATG
GPx3-2	ATCCTGCGACCCAGACACTG
GPx-F	CGTTGAAGTATGTGCGACCTGG
GPx-R	GCCATCAGGAGCAATCAGGAAC
β-F	CATCCCTTGCTCTCCAACATATG
β-R	CTGGAAGGTAGAGAGAGAAGCCAAG

primer and GPx3-1 (Table 1) for 3' RACE. Subsequently, 5' nested PCR was performed by the 5' first-round PCR product used as template, 5' Raceinner primer included in the kit and GPx5-2, 3' nested PCR using 3' first-round PCR products, 3' Raceinner primer and GPx3-2 (Table 1). The 5' RACE and 3' RACE nest PCR products were cloned and five clones were sequenced using the method described above.

2.5. Sequence and phylogenetic analysis

AwSeGPx sequence was analyzed and compared using the BLAST program with a GenBank database search (www.ncbi.nlm.nih.gov/blast). The signal peptide was predicted by signal program (<http://www.cbs.dtu.dk/services/SignalP>). Prediction of protein domain was carried out with the Simple Modular Architecture Research Tool (<http://smart.embl-heidelberg.de/>). The SECIS element and stem loop structure was predicted and generated by a SECISearch 2.19 program (<http://genome.unl.edu/SECISearch.html>). Multiple sequence alignments of AwSeGPx gene were performed using the DANMEN analysis program. Prediction of AwSeGPx3-D structure was fulfilled by Swiss-model (<http://swissmodel.expasy.org/>). Phylogenetic trees constructed from the alignment were generated by the Neighbor-joining method using MEGA5.0 software. Reliability of trees obtained was assessed by bootstrapping, using 1000 bootstrap replications.

2.6. Quantification of AwSeGPx expression by real-time PCR

To determine the mRNA levels of AwSeGPx derived from hepatopancreas and other tissues, real-time quantitative PCR was undertaken following the manufacture instruction of SYBR Premix Ex Taq™ (TaKaRa, China). Firstly, AwSeGPx primers as well as β -actin primers (Table 1) were designed based on isolated sequences of *A. Woodiana*, respectively, used to isolated target genes in common PCR instrument, and only one band was detected in the PCR production by agarose gel electrophoresis. PCR products were sequenced and identified as the partial sequence of target genes. Subsequently, real-time PCR was fulfilled using an ABI 7500 Real-Time Detection System (Applied Biosystems, USA). Based on constructed standard curve, expression levels of AwSeGPx were calculated by $2^{-\Delta\Delta CT}$, and the data obtained were subjected to the statistical analysis followed by an unpaired sample *t*-test. Significant difference was accepted at $P < 0.05$.

3. Results

3.1. Molecular characterization of AwSeGPx cDNA

A 870 bp nucleotide sequence represented the cDNA sequence of AwSeGPx was cloned and deposited in GenBank under accession number KU821031. The full-length cDNA of AwSeGPx had a 5'-untranslated region (UTR) of 36 bp, a 3'UTR of 69 bp, and an open

GAAAAATAGTTACAGCGGACCTAGCGACAGCAACTC	36
ATGGCAGTACGAAGTCCCGTGA AACTACAAATTTCTTCGAATTTACTGCCAAACTTTA	96
M A V R S P V K L Q N F F E F T A K T L	20
AACGCCAAGAAGCCAACCTTAGTAAATTCAAAGGAAAGTAATTCTAGTAGAGAATGTA	156
N G Q E A N L S K F K G K V I L V E N V	40
GCCTCTCTGAGGAACAACGGTCCGGGATTTACCCAGATGAACGAGCTTGCTCCAAA	216
A S L U G T T V R D F T Q M N E L A S K	60
TCTGAAGGTAAGCTTGTATCCTGGGATTCCTGTGTAACAGTTTGGTCATCAGGAAAT	276
S E G K L V I L G F P C N Q F G H Q E N	80
GGCAATCAGGAAGAAATTTGAATTCGTTGAAGTATGTGCGACCTGGTAATGGTTTGAA	336
G N H E E I L N S L K Y V R P G N G F E	100
CCCGCTTTTCCAATTATGGAGAAATGTGACGTCATGGTAGCAACGCTCACCCTATTC	396
P A F P I M E K C D V N G S N A H P L F	120
CAGTTTCTCAGGGAAAACTTCCGTTGCCAAGTGACGATCCAGTATCACTGATGACTCAC	456
Q F L R E K L P L P S D D P V S L M T H	140
ACCAAGAATATCATTTGGGAACCAAGTCACAAGAAATGATATTCCTGGAACCTTGAAAAG	516
T K N I I W E P V T R N D I S W N F E K	160
TTCTGATTGCTCCTGATGGCATGCCGTATAAAGTTACAGCAGACAATTTCAAACAATT	576
F L I A P D G M P Y K R Y S R Q F Q T I	180
AATATTCAGAATGATATTAAGGACTTATTCAAAAGTTTGGTATGTAAGTATCGCATACA	636
N I Q N D I K G L I Q K F G M *	195
GTAATTCACGTCTGATTTAAATTTAAATAGCTTGCTGTAATAATACATCTAATCGACTG	696
CAGATGATCAGCGACAATGTACCACTCTTGAGTCTATGATTGTAAACCCCTAAACAGCA	756
<i>GTCTGCCTGGGTTTGCTTGACGCTTTCTCAAGGATGGAATATTGTCCAGATGTCTCAG</i>	816
GATAATGCAATATGGTACGTAAATAAGACAAAAGTATACCGTAAAAA	870

Fig. 1. Nucleotide and deduced amino acid sequences of AwSeGPx. The start and stop codons are indicated with bold. Putative polyadenylation signal "AATAAA" is showed with wavy line. The selenocysteine (U44) are marked with box. The GPx signature motif 2 (68LGFPCNQF75) and active site motif (156WNFEKF161) are highlighted with shadow. The arginine residues (R94, R151) involved in binding glutathione are marked with underline. Catalytically important residues of Gln (Q74) and Trp (W146) are bold in a box. The predicted SECIS element in the 3'UTR is marked with italic.

reading frame (ORF) of 585 bp encoded a polypeptide of 195 amino acids with an estimated molecular mass of 22.04 kDa and theoretical isoelectric point of 8.70 (Fig. 1). Termination signal (AATAAA) was located at the positions 838–843 in the 3' UTR. A codon (165TGA167) encoded Sec amino acid as U was observed in predicted amino acids sequence of AwSeGPx, which is the main characteristic feature of the Se-GPx family. Result of SignalP 3.0 program showed AwSeGPx had no signal peptide in predicted amino acid sequence. In the N-terminal region, AwSeGPx had a conserve domain of GPx consisted of 8 amino acid (68LGFPNCQF75) and a glutathione peroxide-1 GPx active site (32GKVLVENVASLUGTT47) (Fig. 1). In addition, an extra active site motif (156WNFEKF161), two amino acids residues Gln(Q74) and two Trp residues (W156) responded for fixation of Se as well as two arginine residues (R94, R151) for directing donor glutathione substrate towards the catalytic center in GPx were also found in AwSeGPx (Fig. 2). Furthermore, the predicted secondary structure of AwSeGPx protein contained eight (alpha)-helices and seven (beta)-sheets (Fig. 3A) that was very similar to that of Se-GPx secondary structure of other species, especially to SeGPx of *Homo sapiens*. The alignments of AwSeGPx 3-D structure showed a high-degree similarity with that of Se-GPx (Fig. 3B).

3.2. SECIS element comparison and stem-loop structure of AwSeGPx

The nucleotide sequence of AwSeGPx was analyzed by SECISearch 2.19 for occurrence of stem-loop structure resembling SECIS

element. Results showed that a 101-bp SECIS element appeared in the AwSeGPx 3'UTR, which formed a stem loop secondary structure in the mRNA. Predicted secondary structure of the AwSeGPx SECIS element were composed of two helices separated by an internal loop, a SECIS core structure, a quartet located at the base of helix 2, and an apical loop (Fig. 4). The quartet, the main functional site of the stem loop structure, was formed by four non-Watson Crick interacting base pairs. The SECIS elements were predicted in AwSeGPx including of UAUGAU in the 5' stem, AA in the internal loop, and the UGA in the 3' stem (Fig. 4).

3.3. Evolutionary relationship of AwSeGPx

BLAST analysis revealed that AwSeGPx matched closely with other GPx V family members. The overall deduced amino acid sequence of AwGRX exhibited 85.64% identity to *Hyriopsis cumingii*, 64.80% to *Danio rerio* and 30.26% to *Halotia discus discus*. To establish the molecular evolutionary relationship of AwSeGPx, a phylogenetic trees were generated by the MEGA 5.0 neighbor joining method using different Se-GPx orthologs selected from vertebrate and invertebrate species respectively (Fig. 5). AwSeGPx was closest to that of mollusk clusters, then vertebrate, last insect and crustacean (Fig. 5). In evidenced sequences of mollusk, the most relationship of evolution with AwSeGPx was the calm of fresh water, then shellfish and snail of sea. The phylogenetic analysis of AwSeGPx reflects early divergence and independent evolution from other ASeGPxs.

Anodonta woodnata	---MAVRSPVKLQNFEEFTAKTLNGQEANLSKF-----KG	32
Danio rerio	-----MAGTMKKFYDLASAKLLSGDLLNFSSL-----KG	28
Octopus vulgaris	-----MTTIAKSFFELSAKTLKGEHIDFSRF-----MG	28
Rattus norvegicus	MSAARLS-AVAQSTVYAFSARPLAGGEPVSLGSL-----RG	36
Homo sapiens	MCAARLAAAAAQSVYAFSARPLAGGEPVSLGSL-----RG	36
Halotia discus discus	MAAATSWTAGLATLVTALCAARAAQKVTCQVPAKTTNTFYDFKINDVFDEKLIDFSDFRN	60
Anodonta woodnata	KVILVENVASLUGTTVRDFTQMNELASKSEGL-LVILGFPCNQFGHQEN-GNHEEILNSL	90
Danio rerio	KVVL IENVASLUGTTVRDYTQMNELHSRYADQGLVVLGAPCQFGHQEN-CKNEEILQSL	87
Octopus vulgaris	KVILVENVASLUGTTTRDYMKNLVSQFADK-LVVLGFPCNQFGHQEN-GNGEEILLSL	86
Rattus norvegicus	KVLL IENVASLUGTTTRDYTEMNDLQKRLGPRGLVVLGFPCNQFGHQEN-GKNEEILNSL	95
Homo sapiens	KVLL IENVASLUGTTVRDYTQMNELQRRGLGPRGLVVLGFPCNQFGHQEN-AKNEEILNSL	95
Halotia discus discus	KVVLIVNVATYUGHTHQYNGLNALMTDYAGQGDFLVLGLPCNQFLKQEPGANGTEIMNGV	120
Anodonta woodnata	KYVRPGNGFEPAPFIMEKCDVNGSNAHPLFQFLREKLPLPSDDPVSLMTHTKNI IWEPT	150
Danio rerio	KYVRPGNGFEPKFQILEKLEVNGENAHPLFAFLKEKLPQPSDDPVSLMGDPKFIIWSPVC	147
Octopus vulgaris	QSVRPGNGFEPNFTIMEKVEVNGENTHAFVQFLKNHLPYPSDDSTFSMKNPSSINWTPVA	146
Rattus norvegicus	KYVRPGGGFEPNFTLFEKCEVNGEKAHPLFTFLRNALPAPSDDPALMTDPKYIIWSPVC	155
Homo sapiens	KYVRPGGGFEPNFMFLFEKCEVNGAGAHPLFAFLREALPAPSDDPALMTDPKLITWSPVC	155
Halotia discus discus	KYVRPGLT--PLFNLTKIDVNGEHQHPYLRFLKSYCKRVESVFRPSN---LLFYEPKE	174
Anodonta woodnata	RNDISWNFEKFLIAPDGMPYKRYSRQFTINIQNDIKGLIKFGM---	195
Danio rerio	RNDISWNFEKFLIGPDGEPFKYRRTITIDIDADIKELLKRTK----	191
Octopus vulgaris	RNDISWNFEKFLIAPDGKPLRYSKSFQTIETIQDKISLIEKFS----	190
Rattus norvegicus	RNDISWNFEKFLVPGDGVPRYRRTITIDIEPDIEALLSKQSPNP-	202
Homo sapiens	RNDVWNFEKFLVPGDGVPLRRYRRTITIDIEPDIEALLSQGLSCA-	202
Halotia discus discus	IGDVVWNFEKFLVAGDHVKFRYSLEVQPIDVRPDIEALLGRHVPVVG	222

Fig. 2. Multiple alignment of AwSeGPx with other Se-GPx. The Se-GPx conserved domain (68LGFPNCQF75) and active site motif (156WNFEKF161) are marked with shadow, respectively. The selenocysteine (U44) are bold in a box. Catalytically important residues Gln (Q74) and Trp (W146) are indicated with bold. The arginine residues (R94, R151) involved in binding glutathione are highlighted in a line.

Fig. 3. Predicted secondary and 3D structures of AwSeGPx deduced amino acids. A: The secondary structure of AwSeGPx. B: The 3D structure of AwSeGPx.

Fig. 4. Predicted secondary structure in SECIS of AwSeGPx and disk abalone *H. discus discus*. A: Schematic representations of stem-loop structures of form 1 and 2, B: *A. woodiana*, C: *H. discus discus*. Conserved nucleotides in the SECIS core (Quartet) and apical loop are shown in bold.

3.4. Tissue distribution of AwSeGPx

Results of real time PCR showed that expressions of AwSeGPx had widely distribution including foot, gill, hepatopancreas, adductor muscle and mantle (Fig. 6). The mRNA levels of AwSeGPx showed a higher level in the hepatopancreas and heart, a moderate level in mantle, foot and gill, but a lower level in adductor muscle (Fig. 6).

3.5. Acute toxicity

Dead calms in each box was determined at 48 h and LC50 value were calculated using the log-probit method, data indicated the LC50 of 2,4-DCP, 2,4,6-TCP and PCP was 0.246, 0.194 and 0.139 mg/L33 for *A. woodiana*, respectively (Table 2).

3.6. Temporal expressions of AwSeGPx in hepatopancreas exposed to 2,4-DCP, 2,4,6-TCP, and PCP

Temporal expressions of AwSeGPx mRNA in hepatopancreas were significantly affected by 2,4-DCP, 2,4,6-TCP, and PCP challenge using RT-qPCR with β -actin as internal control (Fig. 7). In all the 2,4-DCP treated groups (60, 120, 240, 480 and 960 μ g/L), mRNA levels of AwSeGPx increased more than 1.79 times ($P < 0.05$) from 6 h to 48 h compared with that of control group. In all the 2,4,6-TCP treated groups (50, 100, 200, 400 and 800 μ g/L) at 6, 12, 24 and 48 h, except of 50 μ g/L of 2,4,6-TCP at 6 h, AwSeGPx mRNA levels in others exhibited a significant up-regulation in contrasted with that of control group, which increased more than 1.01 times (Fig. 7).

In the PCP treated groups (20, 40, 80, 160 and 320 μ g/L), at 6, 12 and 24 h post exposure, expressions of AwSeGPx were significantly

Fig. 5. Phylogenetic relation of AwSeGPx from other organisms. *Anodonta woodnata* is marked with bold. *Hyriopsis cumingii* (accession number AEG21072.1), *Mytilus galloprovincialis* (accession number ADY38576.1), *Sepiella maindroni* (accession number AEK48346.1), *Octopus vulgaris* (accession number AGZ63440.1), *Lethenteron camtschaticum* (accession number AIU44099.1), *Danio rerio* (accession number NP_001007282.2), *Rattus norvegicus* (accession number NP_110453.3), *Homo sapiens* (accession number AAA67540.2), *Metapenaeus ensis* (accession number ACB42237.1), *Haliotis discus discus* (accession number AJP06025.1), *Daphnia magna* (accession number JAM75890.1), *Danaus plexippus* (accession number EHJ74560.1), *Aedes aegypti* (accession number AF394234_1), *Bombyx mori* (accession number NP_001040104.1), *Eriocheir sinensis* (accession number ACV41935.1), *Aplysia californica* (accession number NP_001191508.1).

Fig. 6. Real-time PCR analysis of AwSeGPx transcript from different tissues. $n = 3$ replicates.

Table 2
48 h LC50 values for *A. Woodnata* exposed to 2,4-DCP, 2,4,6-TCP and PCP.

PCs	LC50 (mg/L)	95% Confidence intervals	
		Lower	Upper
2,4DCP	0.246	0.1905	0.3185
2,4,6TCP	0.194	0.1520	0.2487
PCP	0.139	0.1003	0.2180

induced compared with that of control group (Fig. 7). At 48 h, the significant up-regulations were still observed in 20, 40, 80 $\mu\text{g/L}$ PCP treated groups. But, in 160 and 320 $\mu\text{g/L}$ PCP treated groups, the expression of AwSeGPx declined and returned to normal level at

48 h (Fig. 7).

3.7. Temporal expressions of AwSeGPx in gill and hemocytes exposed to 2,4-DCP, 2,4,6-TCP, and PCP

In all the 2,4-DCP, 2,4,6-TCP and PCP treated groups, expressions of AwSeGPx mRNA in gill were significantly induced from 6 h to 48 h. Meanwhile, at 48 h of post exposure, the significant up-regulation of AwSeGPx showed a decline trend that was observed in the 2,4,6-TCP treated groups (200, 400 and 800 $\mu\text{g/L}$) and PCP treated groups (160 and 320 $\mu\text{g/L}$) (Fig. 8) in contrasted with that of previous sampled time.

In the hemocytes, expressions of AwSeGPx mRNA were obviously effected by 2,4-DCP, 2,4,6-TCP, and PCP treatment. A dose- and time-dependant up-regulation of AwSeGPx expression was observed in 2,4-DCP treated groups (60, 120 and 240 $\mu\text{g/L}$), 2,4,6-TCP treated groups (50, 100 and 200 $\mu\text{g/L}$) and PCP treated groups (20, 40 and 80 $\mu\text{g/L}$). However, expressions of AwSeGPx showed a biphasic profile characterized by up-regulation trend in the early stage and down-regulation trend in the latter stage at 48 h in higher concentration groups, such as 2,4-DCP treated groups (480 and 960 $\mu\text{g/L}$), 2,4,6-TCP treated groups (400 and 800 $\mu\text{g/L}$) and PCP treated groups (160 and 320 $\mu\text{g/L}$) (Fig. 9).

4. Discussion

The coding sequence of AwSeGPx cDNA contained a nonsense TGA, suggesting AwSeGPx is a member of Se-GPx family. This codon is firstly indicated to encode a selenocysteine for in mouse, subsequently in human and rat [25,26]. Now, the codon is widely observed in Se-GPx of mollusk, such as bivalve *Dreissena polymorpha*, scallop *Mizuhopecten yessoensis*, clam *V. philippinarum*, and disk abalone *H. discus discus* [21,22,27]. So, the selenocysteine is identified as the catalytic site in this enzyme from invertebrate to vertebrate.

Fig. 7. Temporal expressions of AwSeGPx in hepatopancreas after 2,4-DCP, 2,4,6-TCP and PCP challenge as measured by quantitative real-time PCR. A: Effect of 2,4-DCP on AwSeGPx expression in hepatopancreas. B: Effect of 2,4,6-TCP on AwSeGPx expression in hepatopancreas. C: Effect of PCP on AwSeGPx expression in hepatopancreas. Bars represented means $\pm$ SE; $n = 3$ /each group/each time point. * $P < 0.05$, # $P < 0.01$ vs control group at the same time.

Two conserved motifs of Se-GPx family were also found in the deduced amino acids sequence of AwSeGPx, one was the Se-GPx signature 2 at 67LGFPNCNQH75, and another was the active site at 156WNFEKF161. In the two domains, Gln(Q) and Trp(W) are considered as important residues for GPx catalytic activity, and involved in the fixation of selenium [28]. In addition, results multiple alignment showed two arginine residues of AwSeGPx at position 94 and 151 have a high homology with that of other Se-GPx in which these arginine residues are contribute to the electrostatic architecture that directs the donor substrate (glutathione) towards the catalytic center in such a way that its sulfhydryl group must react with the selenium [28]. Therefore, to shed light of functions of these conserved residues is an interesting subject to understand the AwSeGPx role.

The SECIS element is a major characteristic feature of the mRNA sequence of Se-GPx, which directs the SeC into the UGA opal codon during the translation process [29]. Length of SECIS elements of AwSeGPx had 101 bp which are composed of two helices separated by an internal loop I, a SECIS core structure, a quartet located at the base of helix 2, an apical loop, and an internal loop II (stem-loop). According to stem-loop secondary structure, the location of a

conserved stretch of two or three adenosines (A), and the size of the apical loop, SECIS elements are grouped into two main forms, form 1 and form 2. Inform 1, the AA stretch is located at the apical loop, whereas inform 2, this stretch is shifted to an internal loop II region [30]. Therefore, SECIS element of AwSeGPx belongs to the member of form 2 SECIS elements with respect with AA located of internal loop II. However, additional experiments are required to determine the real function of the stem-loop structure of the SECIS element in AwSeGPx.

AwSeGPx showed a widely distribution in gill, mantle, abductor muscle, foot, heart and hepatopancreas. Ubiquitous distribution suggests that AwSeGPx is an important enzyme for the tissues to keep the balance of redox. The variation of AwSeGPx mRNA levels in different tissues is associated with the balance of various processes acting on the production of ROS generated in different environment. In selected tissues, the highest level was observed in hepatopancreas that is related to the roles of hepatopancreas as the main metabolic organ and the main defense tissue against oxidative stress [31].

There are relatively fewer published reports providing sensitivity of freshwater bivalve responded to organic compounds. In the

Fig. 8. Temporal expressions of AwSeGPx in gill after 2,4-DCP, 2,4,6-TCP and PCP challenge as measured by quantitative real-time PCR. A: Effect of 2,4-DCP on AwSeGPx expression in gill. B: Effect of 2,4,6-TCP on AwSeGPx expression in gill. C: Effect of PCP on AwSeGPx expression in gill. Bars represented means $\pm$ SE; $n = 3$ /each group/each time point. $P < 0.05$, $^{*}P < 0.01$ vs control group at the same time.

present study, 2,4-D, 2,4,6-TCP, and PCP were the three organic chemicals that elicited bivalve *A. woodiana* responses, LC50 results showed clams are more sensitive to PCP exposures than other ones. In the crustacean *Daphnia magna*, results of EC/LC50 show PCP is more potently toxic than that of 2,4-DCP and 2,4,6-TCP [1]. In addition, the recommended acute exposure water quality criteria are 286.2 μg 2,4-DCP/L, 341.5 μg 2,4,6-TCP/L and 11.4 μg PCP/L at pH 7.8, and chronic ones are 16.3 μg 2,4-DCP/L, 54.6 μg 2,4,6-TCP/L and 3.9 μg PCP/L [1]. Therefore, implying TCP likely exhibits more severe destroy for water organisms than that of 2,4-DCP and 2,4,6-TCP under similar environment. As to the reason, that is likely with the transformation of PCP in the tissue. It has indicated that oxidative dechlorination what PCP is catalyzed by cytochrome P450 enzymes to form tetrachlorohydroquinone (TCHQ) can result in oxidative stress on cells and tissues [32]. Meanwhile, TCHQ is considered as a more toxic metabolic product of PCP, it can induce DNA strand breakage and deplete glutathione content [33,34]. In order to explore of mechanism of PCP action in the bivalve, great efforts are required in the future.

In hepatopancreas, gill and hemocytes, administration of different concentrations of 2,4-DCP, 2,4,6-TCP as well as PCP could result in a significant up-regulation of AwSeGPx expressions in contrast with that of control group, suggest enhancement of AwSeGPx is contribute to eliminate ROS stress derived from of 2,4-

DCP, 2,4,6-TCP and PCP challenges. As to aerobic organisms, production of ROS is a common physiological phenomenon. In healthy organism, total ROS levels are tightly controlled by an array of anti-oxidant enzymes to maintain a balance between ROS level and anti-oxidant enzyme activity [11,12]. Clams *A. woodiana* are filter-feeding freshwater bivalves [20,23]. PCs, as important pollutant in water, are easier to pass through the cell membrane and accumulate in body that causes an obvious increase of ROS production [9,10]. On the other hand, anti-oxidant enzyme expressions are induced to evaluate the scavenging capacity of ROS. Se-GPx enzyme catalyzes the reduction of hydrogen peroxide into water, or the reduction of organic peroxides to their corresponding stable alcohols by oxidizing the reduced glutathione into its oxidized form. In *V. philippinarum*, VpSe-GPx expressions are significantly up-regulated to against oxidative stress caused by heavy metals [22]. In disk abalone *H. discus discus*, enhancement of selenium dependent glutathione peroxidase may be involved in a protective role against H_2O_2 oxidative stress [27]. Therefore, these studies point out that the inductions of AwSeGPx expression contribute to prevent cells from injury of ROS.

Notably, in hepatopancreas and hemocytes, the AwSeGPx expression in lower concentration of 2,4-DCP, 2,4,6-TCP and PCP treated groups showed a dose- and time-dependent manner. However, in higher concentration of this three PCs treated ones

Fig. 9. Temporal expressions of AwSeGPx in hemocytes after 2,4-DCP, 2,4,6-TCP and PCP challenge as measured by quantitative real-time PCR. A: Effect of 2,4-DCP on AwSeGPx expression in hemocytes. B: Effect of 2,4,6-TCP on AwSeGPx expression in hemocytes. C: Effect of PCP on AwSeGPx expression in hemocytes. Bars represented means $\pm$ SE; $n = 3$ /each group/each time point. * $P < 0.05$, # $P < 0.01$ vs control group at the same time.

AwSeGPx expression showed a biphasic profile characterized by up-regulation trend in the early stage and down-regulation trend in the latter stage, such as 480 and 960 $\mu\text{g/L}$ of 2,4-DCP treated groups, 400 and 800 $\mu\text{g/L}$ of 2,4,6-TCP treated groups, 80, 160 and 320 $\mu\text{g/L}$ of PCP treated groups, this pattern is associated with scavenging ability of animals faced persistent production of ROS. With elongation of 2,4-DCP, 2,4,6-TCP and PCP treated time, ROS should increasingly be produced and accumulated in the cells. On another hand, animals are enhancing anti-oxidant enzyme activities to eliminate ROS [35]. Once levels of abruptly increased ROS is exceed the eliminating ability of cells that cause redox imbalance possibly via a shift between oxidants and antioxidants in favor of oxidants, and result in chronic inflammation, apoptosis of immune cells, etc [36,37]. In the condition, if animals cannot produce new cells to compensate for death ones, number of living cells should be gradually decreased. Following, expressions of AwSeGPx are also decline alone treated time. Thus, a biphasic phenomenon was detected in higher concentration groups of three PCs. Similar results are also reported in other studies. For instance, in the goldfish, weak oxidative stress activates antioxidant defense mechanisms, strong oxidative stress generated when Cd accumulates in tissues results in a decrease in metabolic activity and reduced antioxidant enzyme mRNA expression [38,39].

At 6 h, administration of different concentrations of 2,4-DCP, 2,4,6-TCP and PCP could result in a significant up-regulation of

AwSeGPx expression in the gill, but this scenario of significant up-regulation was only occurred in higher concentration-treated groups in hepatopancreas and hemocytes. It is suggesting that the gills reflect early-phase responses as external organs in direct contact with contaminants, whereas the hepatopancreas and hemocytes reflect late-phase responses, as internal tissues. Similar results are also reported in the previous studies [26,40,41]. In addition, expressions of AwSeGPx showed different characterizations in groups treated with 2,4-DCP, 2,4,6-TCP and PCP at similar tissue as well as one compound of these PCs at different tissues. As to the phenomena, one interpretation is likely associated with the different physiological functions of these tissues. The hepatopancreas of mollusca showed double properties combined liver with pancreas, is involved in digestion, neutralizes large amounts of toxicants that are ingested by the organism and the major site of toxicants accumulation [42,43]. In contrast, the gills are located in the ventilated mantle cavity and direct interact with the outer environment and their epithelium is very thin [26,40]. Thus, the gills are also frequent target organs of environmental pollutants [44]. Hemocytes of mussels are the important line of defense against pathogenic infection as well as reducing ROS, through phagocytosis and stimulation of respiratory burst and widely used as models in environmental toxicology [21,26]. It is well known that heavy metals' toxicity could be a result of oxidative stress that is associated with respiratory burst processes in the induction of

metal-mediated oxidative and genotoxic events in hemocytes of mussels [26,45]. Considering here, respiratory burst plays a potential role in eliminating toxic effects induced by heavy and PCs. Another interpretation is that, based on mentioned above, various toxic metabolic products generated from 2,4-DCP, 2,4,6-TCP and PCP show different toxic effects in *A. woodiana*. Therefore, many complex elements are likely involved into regulation of AwSeGPx expression in the hepatopancreas, gill and hemocytes.

In conclusion, we clone a Se-GPx cDNA from *A. woodiana* and its full-length cDNA contains highly conserved motifs and residues important for antioxidant defense that similar to other Se-GPx family proteins. Correlating the tissue specific mRNA expression and temporal expressions, suggests up-regulation of AwSeGPx are contribute to eliminate the oxidative stress derived from 2,4-DCP, 2,4,6-TCP and PCP exposure. It worth noting that PCP is likely to be more detrimental for aquatic organisms than 2,4-DCP and 2,4,6-TCP under a similar environment. In order to prevent or mitigate extensive negative impacts on aquatic organisms in freshwater and conserve aquatic organism biodiversity, it would thus be of critical importance for government to take measures controlling diffusion of PCs.

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