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RESEARCH REPORT

Sequence features, expression profiles and biochemical characteristics of a sigma class glutathione S-transferase gene (*Ai*GSTσ) from bay scallop *Argopecten irradians*

M Wang[1, 3], B Wang[1], M Liu[1], K Jiang[1], L Wang[1, 2]

[1]Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[2]Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China
[3]Research Platform for Marine Molecular Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China

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Abstract

Glutathione S-transferases (GSTs) are a class of enzymes that facilitate the detoxification of xenobiotics and also play important roles in innate immunity. In the present study, a novel sigma class GST gene (designated as *Ai*GSTσ) was cloned from the bay scallop *Argopecten irradians* via rapid amplification of cDNA ends (RACE) technique. The complete cDNA sequence of *Ai*GSTσ consisted of a 5' untranslated regions (UTR) of 48 bp, a 3' UTR of 113 bp with a poly A tail and an open reading frame (ORF) of 618 bp. The ORF encoded a polypeptide of 205 amino acid residues with a calculated molecular mass of approximately 23.11 kDa and a theoretical isoelectric point of 5.354. The deduced amino acid sequence of *Ai*GSTσ contained a GST_N domain and a GST_C domain, and exhibited high similarity with other reported sigma class GSTs. In the phylogenetic tree, *Ai*GSTσ was located in the sigma class GSTs sub-branch. The *Ai*GSTσ mRNA transcripts were constitutively expressed in the tissues of hemocytes, muscle, mantle, gill, hepatopancreas and gonad, with the highest expression level in hemocytes, and the mRNA expression levels of *Ai*GSTσ were significantly up-regulated in hemocytes after various pathogen associated molecular patterns (PAMPs) stimulation. The purified recombinant *Ai*GSTσ protein exhibited catalytic activity against the common substrate 1-chloro-2, 4-dinitrobenzene (CDNB) with low thermal stability and narrow optimum pH spectrum. All these results indicated that *Ai*GSTσ was a fragile but efficient antioxidant enzyme and was potentially involved in the innate immune responses of scallop.

Key Words: *Argopecten irradians*; glutathione S-transferase; innate immunity

Introduction

The innate immunity is almost the solo defence mechanism for invertebrates that protects hosts against microbial invaders (Song *et al*., 2015). In the innate immune defence mechanism, hemocytes can phagocytize and kill the microbial pathogens (Lu *et al*., 2013; Chen *et al*., 2014; Wang *et al*., 2014). When the host is attacked by microbial invaders, phagocytosis is activated with high oxygen consumption named the respiratory burst and followed by mass reactive oxygen intermediates (ROI) and reactive oxygen species (ROS) production (Shao *et al*., 2017). Therefore, organisms employ the antioxidant system to maintain ROI and ROS at the normal physiological levels (Zhang *et al*., 2017a, b). As an essential kind of antioxidant enzymes, glutathione S-transferases (GSTs, EC 2.5.1.18) are a superfamily of multifunctional phase II enzymes primarily catalyzing reduced glutathione to both endogenous and exogenous electrophiles (Sheehan *et al*., 2001). GSTs have been identified from the cytosol, mitochondria and microsomes of all the prokaryotic and eukaryotic organisms that have been studied (Raza *et al*., 2002). Generally, based on their primary and tertiary structures, substrate and inhibitor specificity, and immunological cross reactivity, GSTs could be grouped into at least fifteen classes, which were termed as alpha (α), beta (β), delta (δ), epsilon (ε), kappa (κ), lambda (λ), mu (μ), omega (ω), phi (φ), pi (π), sigma (σ), tau (τ), theta (θ), zeta (ζ) and rho (ρ) (Wilce and Parker, 1994).

Corresponding author:
Lei Wang
Key Laboratory of Experimental Marine Biology
Institute of Oceanology
Chinese Academy of Sciences
Qingdao 266071, China
E-mail: wanglei@qdio.ac.cn

Table 1 Primers used in the present study

Primer	Sequence (5`-3`)	Brief information
adaptor primer	GGCCACGCGTCGACTAGTAC	Anchor primer for 3` RACE
adaptor primer-oligo (dT)	GGCCACGCGTCGACTAGTACT_{17}VN	Olido (dT) for cDNA synthetize
*Ai*actin-qRT-F	CAAACAGCAGCCTCCTCGTCAT	Internal control for real-time PCR
*Ai*actin-qRT-R	CTGGGCACCTGAACCTTTCGTT	Internal control for real-time PCR
*Ai*GSTσ-CDS-F	ATGCCTTCCTACAAACTTATCTAC	Gene specific primer for CDS
*Ai*GSTσ-CDS-R	TTAGATCACGCTCTCGGGACGCGA	Gene specific primer for CDS
*Ai*GSTσ-qRT-F	CTGATCCGTCTCGCTTTCGCT	Gene specific primer for real-time PCR
*Ai*GSTσ-qRT-R	GCTGTTTCCCGTCCACTTCCA	Gene specific primer for real-time PCR
*Ai*GSTσ-RACE-F1	CCCAAATTTGCCGAAATC	Gene specific primer for RACE
*Ai*GSTσ-RACE-F2	AGTTGAACCCAGATTGTTTGAAGG	Gene specific primer for RACE
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	Vector primer for sequencing
RV-M	GAGCGGATAACAATTTCACACAGG	Vector primer for sequencing
T7	ACATCCACTTTGCCTTTCTC	Vector primer for sequencing
T7-ter	TGCTAGTTATTGCTCAGCGG	Vector primer for sequencing

Among all the GSTs classes, sigma class GST (GSTσ) comprises one of the largest GST subfamilies identified from invertebrates to vertebrates, which was believed to evolve from ancestral GST genes and exhibit high levels of enzymatic activity toward the common substrate 1-chloro-2, 4-dinitrobenzene (CDNB) (Flanagan and Smythe, 2011). Recently, several sigma class GSTs were identified and investigated in marine invertebrates (Boutet *et al*., 2004; Lee *et al*., 2007; Wan *et al*., 2008; Ren *et al*., 2009; Li *et al*., 2012; Umasuthan *et al*., 2012; Yang *et al*., 2012; Zhang *et al*., 2012a, b; Wang *et al*., 2013a; Li *et al*., 2015). Among these sigma class GSTs, the *ab*GSTsigma gene from abalone *Haliotis diversicolor* was significantly expressed in the hemocytes, gill, mantle and digestive gland of bacteria-challenged abalone (Ren *et al*., 2009). Bacterial challenge could significantly up-regulate the mRNA expression of both *Vp*GST-1 and *Vp*GST-2 from Manila clam *Ruditapes* (*Venerupis*) *philippinarum* (Li *et al*., 2012). The mRNA expression level of *Sg*GST-S1 in hemocytes was significantly up-regulated after razor clam *Solen grandis* was stimulated by peptidoglycan (PGN) or β-1, 3-glucan (glucan) (Yang *et al*., 2012). While after bacterial challenge, the mRNA expression levels of sigma class GSTs in hemocytes were all significantly higher than those of the control group in mussels *Mytilus galloprovincialis* (Wang *et al*., 2013a). All these research achievements revealed that sigma class GSTs from marine invertebrates were functional diversity and might not only serve as an antioxidant enzyme involving in the detoxification but also play important roles in the modulation of innate immune responses.

Bay scallop *Argopecten irradians* was introduced from USA in 1982 and has become one of the most important aquaculture species in China, due to its high economic value, fast growth rate and adaptation ability to different regions for aquaculture (Li *et al*., 2007). And *A. irradians* was also considered as an attractive model to study immunology because of its relatively simple innate immune system and its propensity to undergo various manipulations, which allows researchers to study the effects of both biological and non-biological factors on the innate immune responses (Matozzo, 2016). By now, several antioxidant enzyme genes have been identified and characterized in *A. irradians*, such as metallothionein (MT) (Wang *et al*., 2009), peroxiredoxin (PRX) (Li *et al*., 2011) and superoxide dismutase (SOD) (Bao *et al*., 2008, 2009a, b, 2010), however, no information about GST genes was available in bay scallop till now. To bridge this gap, the main objectives of the present study were (1) to clone the full-length cDNA of sigma class GST from *A. irradians* (designated as *Ai*GSTσ), (2) to investigate the tissue distribution of *Ai*GSTσ mRNA transcripts and their temporal expression after different pathogen associated molecular patterns (PAMPs) stimulation, and (3) to validate the activities of recombinant *Ai*GSTσ protein under different treatments.

Materials and Methods

Scallops, immune stimulation and sample collection

The bay scallops used in the present study were obtained from a local farm in Qingdao, China, and all the experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The study protocol and all the experimental design were conducted with approval from Experimental Animal Ethics Committee of Institute of Oceanology, Chinese Academy of Sciences. Approximately 200 scallops with an average 50 mm in shell length were employed for the PAMPs stimulation treatment. The scallops were randomly divided into 6 groups and each group contained about 30 - 40 individuals. The scallops were received an injection of 50 μL phosphate buffered saline (PBS, 0.14 mol L^{-1} sodium chloride, 3 mmol L^{-1} potassium chloride, 8 mmol L^{-1} disodium hydrogen phosphate dodecahydrate, 1.5 mmol L^{-1}

Table 2 Information of GST proteins used in phylogenetic analysis

Class	Species	Accession Number
omega	*Chlamys farreri*	ADF32018
	Craassostrea gigas	XP_011429380
	Danio rerio	NP_001002621
	Haliotis discus discus	ABO26600
	Haliotis madaka	ALU63761
	Perna viridis	AGN03944
sigma	*Argopecten irradians*	ANG56313
	Chlamys farreri	ACF25904
	Chlamys farreri	ADF32019
	Hyriopsis cumingii	AGU68336
	Pinctada fucata	JAS04242
	Ruditapes philippinarum	AEW46325
rho	*Chlamys farreri*	ACF25903
	Cyprinus carpio	BAS29983
	Ruditapes philippinarum	AEW46331
	Sebastes schlegelii	ANW83217
	Siniperca chuatsi	ACI32418
	Solea senegalensis	BAG12568
zeta	*Chlamys farreri*	ADD82544
	Cyprinus carpio	BAS29981
	Oplegnathus fasciatus	ADY80028
	Xenopus laevis	XP_018084636
microsomal	*Chlamys farreri*	ADF45336
	Gallus gallus	NP_001129022
	Microtus ochrogaster	XP_005364596
	Osmerus mordax	ACO10098
	Sinonovacula constricta	ALC77324
	Xenopus tropicalis	NP_001011245

potassium phosphate monobasic, pH 7.4), lipopolysaccharides from *Escherichia coli* 0111:B4 (LPS, L2630, Sigma-Aldrich, USA, 0.5 mg mL^{-1} in PBS), PGN from *Staphylococcus aureus* (77140, Sigma-Aldrich, USA, 0.5 mg mL^{-1} in PBS), glucan from baker's yeast *Saccharomyces cerevisiae* (G5011, Sigma-Aldrich, USA, 0.5 mg mL^{-1} in PBS) or polyinosinic-polycytidylic acid (poly IC, P1530, Sigma-Aldrich, USA, 0.5 mg mL^{-1} in PBS), respectively. The injected scallops were returned to seawater tanks immediately and five individuals were randomly sampled from each stimulated and unstimulated group at 3, 6, 12, 24 and 48 h post injection. The hemolymphs were collected and centrifuged at 800 *g*, 4 °C for 10 min to harvest the hemocytes for RNA preparation. Hemocytes, muscle, mantle, gill, hepatopancreas and gonad from five untreated scallops were collected to determine mRNA transcripts of *Ai*GSTσ.

RNA isolation and cDNA synthesis

Total RNA was isolated from the hemocytes of scallops with RNAiso plus reagent (9108, Takara, Japan). The first-strand synthesis was carried out using the DNase Ⅰ (RQ1, M6101, Promega, USA) treated raw RNA as template and adaptor primer-oligo (dT) as primer (Table 1). The reaction were performed at 42 °C for 1 h, terminated by heating at 95 °C for 5 min, and then stored at -80 °C till use.

EST analysis and cloning of full-length Ai*GSTσ cDNA*

An EST (Ai_F00346) from bay scallop cDNA library in National Center for Biotechnology Information (NCBI) homologous to previously identified sigma class GST genes was selected for further cloning the cDNA of *Ai*GSTσ. Two gene-specific primers, *Ai*GSTσ-RACE-F1/F2 (Table

1), were designed to clone the 3' sequence of *Ai*GSTσ cDNA by rapid amplification of cDNA ends (RACE) technique. And the coding sequence (CDS) of *Ai*GSTσ was amplified and confirmed using another two gene-specific primers, *Ai*GSTσ-CDS-F/R. All PCR amplification was performed in an A300 Fast Thermal Cycler (LongGene, China), and the PCR products were purified using Monarch DNA Gel Extraction Kit (T1020S, NEB, USA) and cloned into the pMD18-T simple vector (D103A, Takara, Japan). After being transformed into the competent cells *Escherichia coli* strain DH5α (CB101, Tiangen, China), the positive recombinants were identified via anti-ampicillin selection and verified by PCR screening using M13-47 and RV-M primers (Table 1). Three of the positive clones were sequenced using a PRISM 3730XL automated sequencer (Thermo Fisher Scientific, USA).

Bioinformatical analysis of cDNA and protein sequences

The protein sequences information for homologous and phylogenetic analysis was listed in Table 2. The search for protein sequence similarity was conducted with blastp 2.6.0. The deduced protein sequences were analyzed by the EditSeq module in Lasergene program suite 14.0.0.88. The function domains were predicted using Simple Modular Architecture Research Tool (SMART) 7.0. Multiple sequence alignments were performed with Clustal Omega 1.2.4 and visualized by multiple alignment show module in Sequence Manipulation Suite 2.0. A Neighbor-Joining (NJ) phylogenic tree was constructed with MEGA 7.0.26. To derive confidence value for the phylogeny analysis, bootstrap trials were replicated 1,000 times.

Expression patterns analysis via quantitative real-time PCR

The mRNA transcripts of *Ai*GSTσ in different tissues or their temporal expression patterns in hemocytes of scallops stimulated with various PAMPs were investigated by quantitative real-time PCR (qRT-PCR). All qRT-PCR reactions were performed with the SYBR premix ExTaq (Tli RNaseH plus) (RR420, Takara, Japan) using 100 ng cDNA template in a LineGene K FQD-48A (A4) Fluorescence Quantitative PCR Detection System (Bioer, China). All the primers using in qRT-PCR were listed in Table 1. The mRNA expression levels of *Ai*GSTσ were normalized to those of β-actin for each sample. The relative mRNA expression levels of *Ai*GSTσ were generated using comparative C_T method ($2^{-\Delta\Delta Ct}$ method) (Schmittgen and Livak, 2008). All the data were subjected to one-way analysis of variance (ANOVA) followed by a multiple comparison using CoStat 6.400, and the *p* values less than 0.05 were considered statistically significant.

Recombinant and purification of Ai*GSTσ in* E. coli

The CDS of *Ai*GSTσ was amplified using two gene-specific primers, *Ai*GSTσ-CDS-F/R (Table 1), and ligated to the expression vector p*EASY*-Blunt E1 (CE111, Transgen, China). The recombinant plasmid, p*EASY*-Blunt E1/*Ai*GSTσ, was isolated by Monarch Plasmid Miniprep Kit (T1010S, NEB, USA) and then transformed into *E. coli* strain BL21 (DE3) (CD601, Transgen, China). The positive transformants, *E. coli* BL21 (DE3)/p*EASY*-Blunt E1/*Ai*GSTσ, were incubated in ArtMedia Protein Expression auto-inducing medium (CP101, Transgen, China) containing 100 mg L^{-1} ampicillin (GG101, Transgen, China) at 28 °C with shaking at 220 rpm for 24 h. The recombinant protein (designated as r*Ai*GSTσ) was purified using a His-tag Protein Purification Kit (P2226, Beyotime, China) under natural condition. The resultant protein was separated by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Protein Stains H (C510041, Sangon, China).

*Analysis of enzymatic activity of r*Ai*GSTσ*

The specific activities of r*Ai*GSTσ were measured as described in previous reports (Habig *et al*., 1974 Wan *et al*., 2008; Umasuthan *et al*., 2012). Briefly, the reaction was carried out in a 1 mL mixture containing 100 mM PBS, 10 mM GSH (S0073, Beyotime, China), and an appropriate amount of r*Ai*GSTσ. The enzyme mixture was incubated at 25 °C for 5 min before the reaction was initiated by adding 1 mM CDNB (703318, Cayman Chemical, USA) and absorbance was monitored for 5 min at 340 nm while the reaction was maintained at 25 °C. The changes in absorbance per minute were converted into amounts of substrate conjugated per min per mg enzyme by using the molar extinction coefficient for CDNB ε_{340} = 9.6 mM^{-1} cm^{-1}. To characterize the r*Ai*GSTσ, enzymatic activity was evaluated at different temperature and pH. To determine the optimal temperature, protein samples were treated at 10 °C intervals between 10 °C and 90 °C for 1 h. To investigate the optimal pH, protein samples were treated between pH 3.5 and 10.5 at 1.0 pH intervals using different buffers for 1 h. Acetate, phosphate and glycine-NaOH buffers were used to obtain the pH ranges of 3.5-5.5, 6.5-7.5 and 8.5-10.5, respectively, according to previously reports (Wang *et al*., 2013b, 2015).

Results

Sequence features of Ai*GSTσ*

A sigma class GST gene, *Ai*GSTσ, was identified from the bay scallop EST database, and its full-length cDNA sequence was obtained via RACE technique and deposited into GenBank under the accession number KU301768. The full-length cDNA sequence of *Ai*GSTσ comprised 779 bp, containing a 5' untranslated regions (UTR) of 48 bp, a 3' UTR of 113 bp with a poly A tail and an open reading frame (ORF) of 618 bp. The ORF encoded a polypeptide of 205 amino acid residues with a calculated molecular mass of approximately 23.11 kDa and a theoretical isoelectric point of 5.354. No signal peptide was revealed in the deduced amino acid sequence of *Ai*GSTσ by SignalP program. A GST_N domain (from Y^4 to R^{73}) and a GST_C domain (from I^{92} to N^{190}) were found in the deduced amino acid sequence of *Ai*GSTσ (Fig. 1).

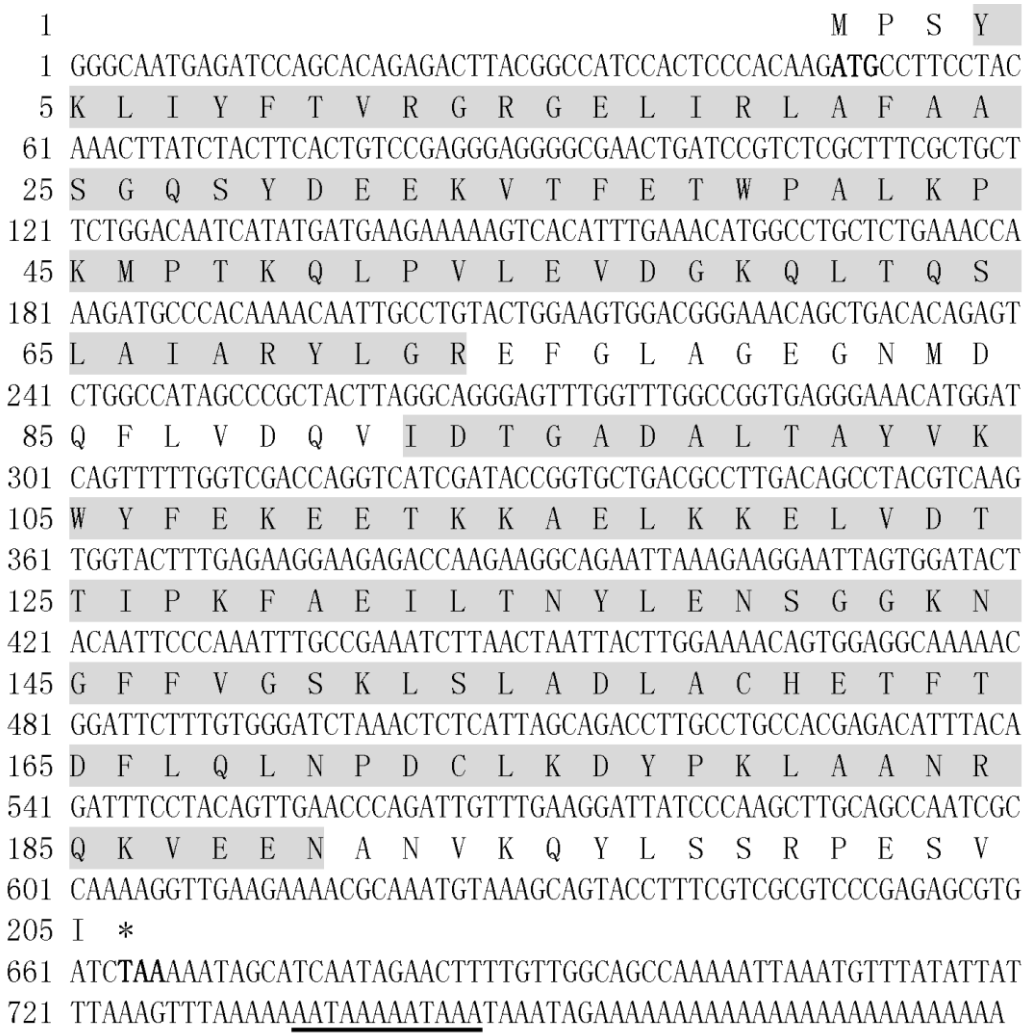

Fig. 1 Nucleotide and deduced amino acid sequences of *Ai*GSTσ. The nucleotides and amino acids were numbered along the left margin. The function domain was in shade. The asterisks indicated the stop codon. Two single typical polyadenylation signal sequences (AATAAA AATAAA) was underlined.

Phylogenetic ananlysis of Ai*GSTσ*

The deduced protein sequence of *Ai*GSTσ exhibited high similarity with other previously identified sigma class GSTs, such as 78 % identity with that of sigma class GST 2 from *Chlamys farreri* (ADF32019). The NJ phylogenetic tree based on protein sequences from multiple GST genes was positioned separately into five main branches, and *Ai*GSTσ were clustered with sigma class GST 2 from *C. farreri* and located in the sigma class GSTs sub-branch (Fig. 2).

Tissue distribution of Ai*GSTσ mRNA transcripts*

The qRT-PCR technique was employed to detect the distribution of *Ai*GSTσ mRNA transcripts in different tissues with β-actin gene as internal control (Fig. 3). The highest mRNA expression level of *Ai*GSTσ was found in hemocytes, which was 21.30-fold ($p < 0.05$) of that in muscle, while that in hepatopancreas was 13.28-fold ($p < 0.05$) of that in muscle.

Expression profiles of Ai*GSTσ mRNA transcripts*

The temporal mRNA expression profiles of *Ai*GSTσ in hemocytes after various PAMPs stimulation were also examined via qRT-PCR (Fig. 4A-D). The mRNA transcripts of *Ai*GSTσ all increased for the first time at 3-6 h and reached the peak at 12 h post different PAMPs stimulation. The mRNA transcripts of *Ai*GSTσ significantly increased at 3 h after LPS stimulation (2.79-fold compared with the origin level, $p < 0.05$, Fig. 4A), with the highest level observed at 12 h (17.74-fold, $p < 0.05$, Fig. 4A). The mRNA expression level of *Ai*GSTσ was up-regulated at 6 h post PGN stimulation (3.13-fold, $p < 0.05$, Fig. 4B) and then up-regulated to the highest level at 12 h (6.82-fold, $p < 0.05$, Fig. 4B), and finally down-regulated to the normal level at 48 h. In the glucan stimulation group, after a significant increase at 3 h post stimulation (3.18-fold, $p < 0.05$, Fig. 4C), the mRNA transcripts of *Ai*GSTσ increased to the peak at 12 h (12.15-fold, $p < 0.05$, Fig. 4C), and finally decreased to the original level at 48 h.

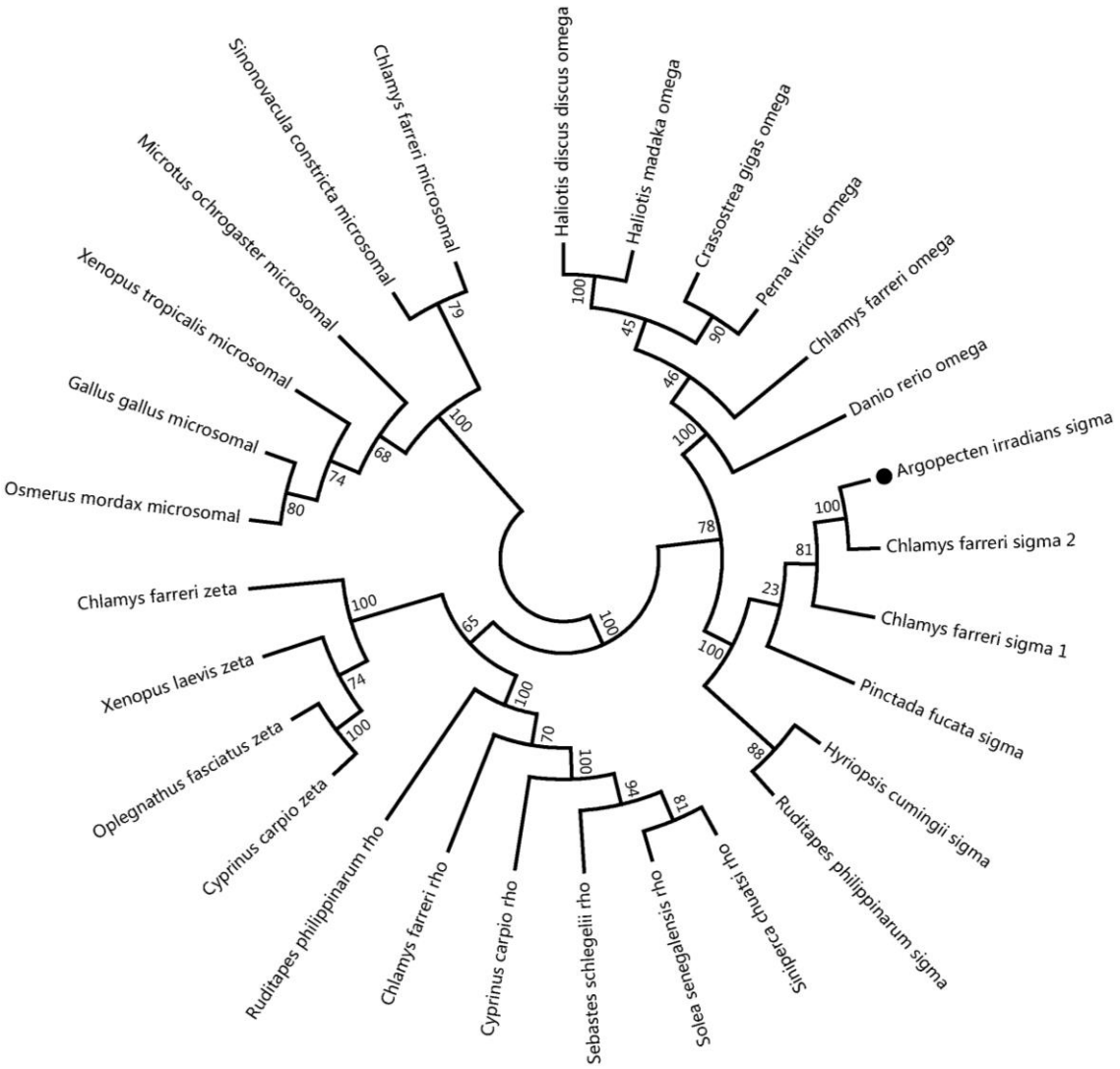

Fig. 2 Consensus neighbor-joining phylogenetic tree based on the amino acid sequences of GSTs from different organisms. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analyzed. All positions containing gaps and missing data were eliminated. The numbers at the forks indicated the bootstrap value. The sequence information has been listed in Table 2.

The mRNA transcripts of *Ai*GSTσ significantly increased at 6 h post poly IC stimulation (6.71-fold, $p < 0.05$, Fig. 4D), reached the peak at 12 h (9.07-fold, $p < 0.05$, Fig. 4D), and then decreased to the normal level at 24 h. In the normal group, no significant change of *Ai*GSTσ mRNA expression level was observed during the whole experiment, while after PBS injection, a slight but significant increase was observed at 6 h (2.93-fold, $p < 0.05$, Fig. 4A-D).

Purification of recombinant Ai*GSTσ protein*

To investigate the potential activities of *Ai*GSTσ, the recombinant plasmid p*EASY*- Blunt E1/*Ai*GSTσ was transformed into *E. coli* strain BL21 (DE3). After auto-induction, the whole-cell lysate was separated by SDS-PAGE, and a distinct band of r*Ai*GSTσ was revealed (Fig. 5).

Biochemical characteristics of recombinant Ai*GSTσ protein*

According to the method previously described, the activity of r*Ai*GSTσ was measured for five times, and r*Ai*GSTσ exhibited detectable activity towards CDNB, which was 3.28 ± 0.03 μmol min^{-1} mg^{-1}. To investigate the stability of *Ai*GSTσ, the enzymatic activities of r*Ai*GSTσ were measured at different pH and temperature. For the optimal pH assay, r*Ai*GSTσ could maintain more than 50 % of its activity at a pH range from 7.5 to 9.5, but lost more than 60 % of its activity when the pH was lower than 6.5 or at 10.5 (Fig. 6A). While when the temperature increased from 10 °C to 20 °C, r*Ai*GSTσ exhibited stable enzymatic activities, but lost more than 60 % of its enzymatic activity over 30 °C and almost devitalized at 50 °C (Fig. 6B).

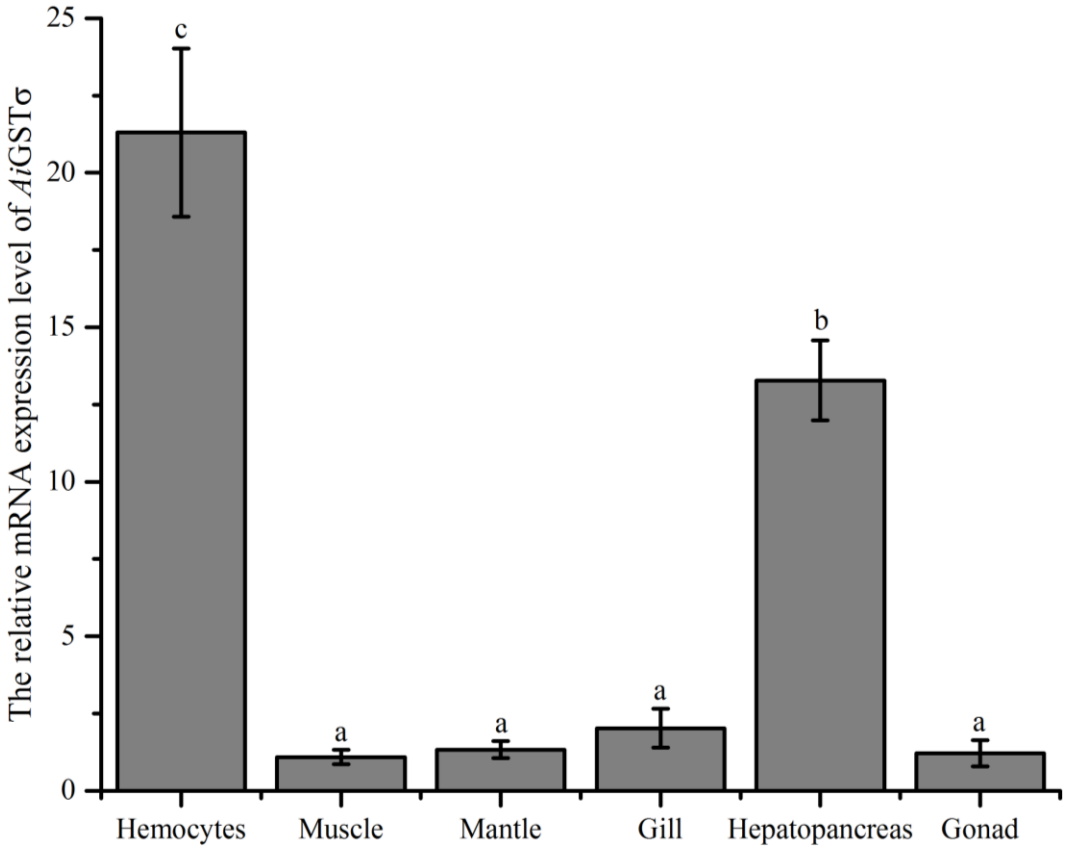

Fig. 3 Tissue distribution of *Ai*GSTσ mRNA transcripts detected by qRT-PCR. The β-actin gene was used as an internal control to calibrate the cDNA template for each sample. The mRNA expression level of *Ai*GSTσ in hemocytes, muscle, mantle, gill, hepatopancreas and gonad of five adult scallops was normalized to that of muscle. Vertical bars represented mean ± SD (n = 5), and bars with different characters indicated significantly different ($p < 0.05$).

Discussion

Sigma class GSTs are a large sub-family of GSTs (Flanagan and Smythe, 2011), and accumulating research achievements revealed that sigma class GSTs from marine invertebrates were functional diversity and might not only serve as an antioxidant enzyme involving in the detoxification but also play important roles in the modulation of innate immune responses (Boutet *et al*., 2004; Lee *et al*., 2007; Wan *et al*., 2008; Ren *et al*., 2009; Li *et al*., 2012; Yang *et al*., 2012; Umasuthan *et al*., 2012; Zhang *et al*., 2012a, b; Wang *et al*., 2013a; Li *et al*., 2015). In the present study, the full-length cDNA sequence of *Ai*GSTσ was obtained from bay scallop *A. irradians*. The deduced polypeptide of *Ai*GSTσ consisted of 205 amino acids, and its calculated molecular weight was 23.11 kDa, which was very close to GSTs of vertebrate and invertebrate. The amino acid sequence of *Ai*GSTσ shared as high as 78 % identity with the previously identified sigma class GST 2 from *C. farreri*. In the phylogenetic tree, *Ai*GSTσ was located in the sigma class GSTs sub-branch. Its sequence characteristics, high similarity with other known sigma class GSTs and the phylogenetic relationship collectively suggested that *Ai*GSTσ is a novel member of invertebrate sigma class GST family and may have similar function with sigma class GSTs from other marine invertebrates.

Sigma class GST acts as the principal scavenger of xenobiotics (Flanagan and Smythe, 2011), and it was reported to be ubiquitously distributed in multiple tissues in marine invertebrates (Boutet *et al*., 2004; Lee *et al*., 2007; Wan *et al*., 2008; Ren *et al*., 2009; Li *et al*., 2012; Yang *et al*., 2012; Umasuthan *et al*., 2012; Zhang *et al*., 2012a, b; Wang *et al*., 2013a; Li *et al*., 2015). In the present study, the tissue distribution of *Ai*GSTσ mRNA transcripts was detected by qRT-PCR to investigate its possible function, and the ubiquity of *Ai*GSTσ transcripts indicated that it could be involved in many important physiological processes of scallops. Similar to the observation in sigma class GSTs from *M. galloprovincialis*, *S. grandis* and *V. philippinarum* (Yang *et al*., 2012; Zhang *et al*., 2012a; Wang *et al*., 2013a), the highest mRNA expression level of *Ai*GSTσ was observed in hemocytes, followed by hepatopancreas. The variable tissue distribution of *Ai*GSTσ mRNA transcripts was speculated to be related with tissue dependent oxidative load. The hemocytes have been considered to play pivotal roles

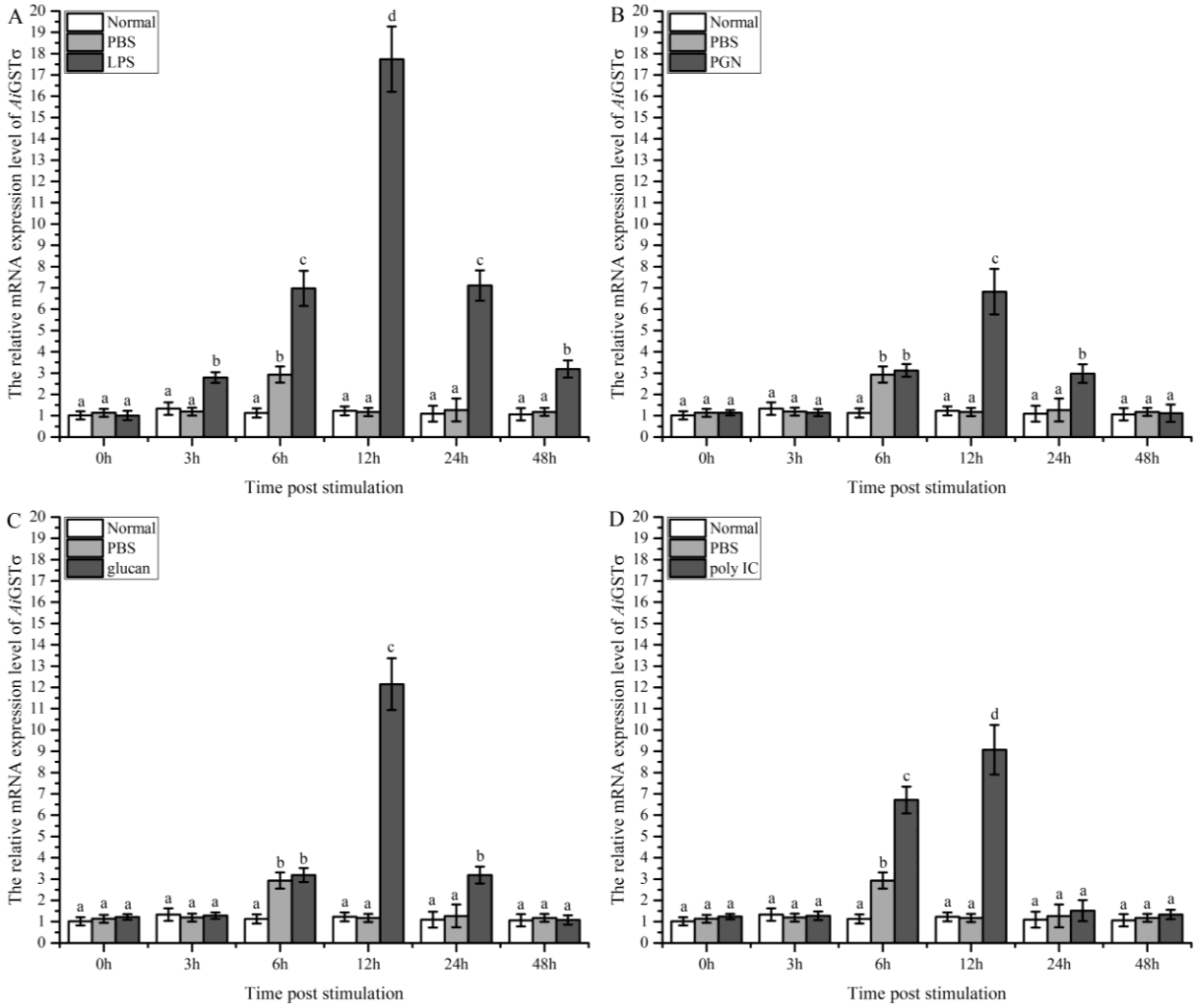

Fig. 4 Temporal mRNA expression profiles of *Ai*GSTσ detected by qRT-PCR in hemocytes at 3, 6, 12, 24 and 48 h post different PAMPs stimulation (**A**: LPS, **B**: PGN, **C**: glucan, **D**: poly IC). The β-actin gene was used as an internal control to calibrate the cDNA template for each sample. Each values was shown as mean ± SD (n = 5), and bars with different characters indicated significantly different ($p < 0.05$).

in the innate immune response in invertebrates mainly via phagocytosis, which was usually companied with oxidative stress, while the hepatopancreas is regarded as the main organ where multiple oxidative reactions and antioxidant defenses occur with high metabolic activity (Song *et al.*, 2015). Additionally, hemocytes and hepatopancreas were also considered as the main immune related organs in scallops (Song *et al.*, 2015), the high mRNA expression level of *Ai*GSTσ in these two organs indicated that it could be involved in the innate immunity of scallop.

It has been reported that sigma class GSTs could rapidly respond to various foreign particles or invading microbes in mRNA levels. For examples, a sigma class GST gene from *H. diversicolor* could be significantly induced in the hemocytes, gill, mantle and digestive gland of bacteria-challenged abalone (Ren *et al.*, 2009). Bacterial challenge could significantly induce the mRNA expression of two sigma class GSTs from *V. philippinarum* (Li *et al.*, 2012). The mRNA expression of a sigma class GST in hemocytes was significantly up-regulated after razor clam was stimulated by PGN or glucan (Yang *et al.*, 2012). While after bacterial challenge, the mRNA expression levels of sigma class GSTs in hemocytes were all significantly up-regulated in *M. galloprovincialis* (Wang *et al.*, 2013a). In the present study, the mRNA transcripts of *Ai*GSTσ could be significantly induced by the stimulation of four typical PAMPs, confirming the hypothesis that it could be involved in the innate immune response of scallops. Additionally, a slight but significant increase of *Ai*GSTσ mRNA transcripts was also observed at 6 h after PBS injection, indicating *Ai*GSTσ might be also involved in the responses to injury in scallop.

To further investigate the potential role of *Ai*GSTσ in bay scallop, the catalytic activity of its recombinant protein was determined *in vitro* using

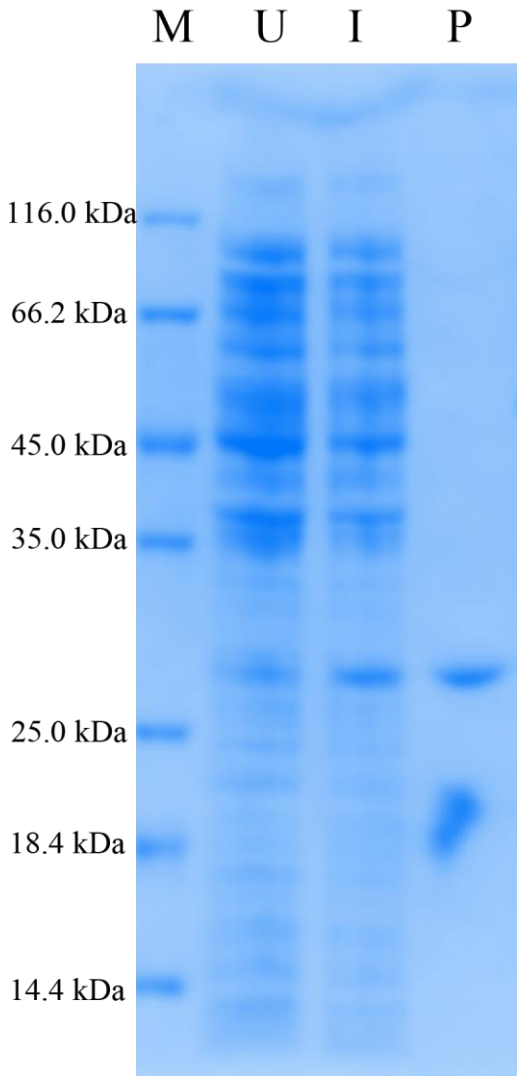

Fig. 5 SDS-PAGE analysis of the r*Ai*GSTσ protein in *E. coli* strain BL21 (DE3). Line M was the unstained protein marker (26610 Thermo Fisher Scientific, USA). Line U was the supernatant of non-induced bacteria lysate. Line I was the supernatant of auto-induced bacteria lysate. Line P was the purified recombinant protein.

CDNB as substrate. In a previous research, the recombinant *Hd*GSTS1 and *Hd*GSTS2 proteins in *H. discus discus* exhibited catalytic activities of 0.17 ± 0.01μmol min^{-1} mg^{-1} and 1.06 ± 0.02 μmol min^{-1} mg^{-1}, respectively, with relatively broad optimum pH spectrum and temperature range (Wan *et al.*, 2008). While r*Rp*GSTσ from *R. philippinarum* demonstrated a high catalytic ability toward CDNB of 4.64 ± 0.17 $μmol^{-1}$ min^{-1} mg^{-1}, but xhibited narrow optimal pH spectrum and temperature range (Umasuthan *et al.*, 2012). Similarly, in the present study, *Ai*GSTσ exhibited a high enzymatic activity of 3.28 ± 0.03 μmol min^{-1} mg^{-1}, but lost more than 60% of its activity when the pH was lower than 6.5 or when the temperature was over 30 °C. It has been reported that both sea surface temperature rise and ocean acidification affect survival and reproduction of marine organisms negatively, including scallop (Zhang *et al.*, 2014; Lagos *et al.*, 2016). So, the lower active stability of r*Ai*GSTσ, especially susceptible to low pH or high temperature, might provide valuable insights into a possible mechanism of large scale mortalities of cultured bay scallops in summer.

In conclusion, the full-length cDNA encoding a sigma class GST was identified from bay scallop *A. irradians*. It was constitutively expressed in all the tested tissues, including hemocytes, muscle, mantle, gill, hepatopancreas and gonad, and the mRNA expression levels of *Ai*GSTσ were all up-regulated in hemocytes after various PAMPs stimulaton. The purified r*Ai*GSTσ protein exhibited relatively high catalytic activity against CDNB with low thermal stability and narrow optimum spectrum of pH. All these results indicated that it was a fragile but efficient antioxidant enzyme and was potentially involved in the innate immune responses of scallop. This study would enrich the understanding of the scallop innate immunity.

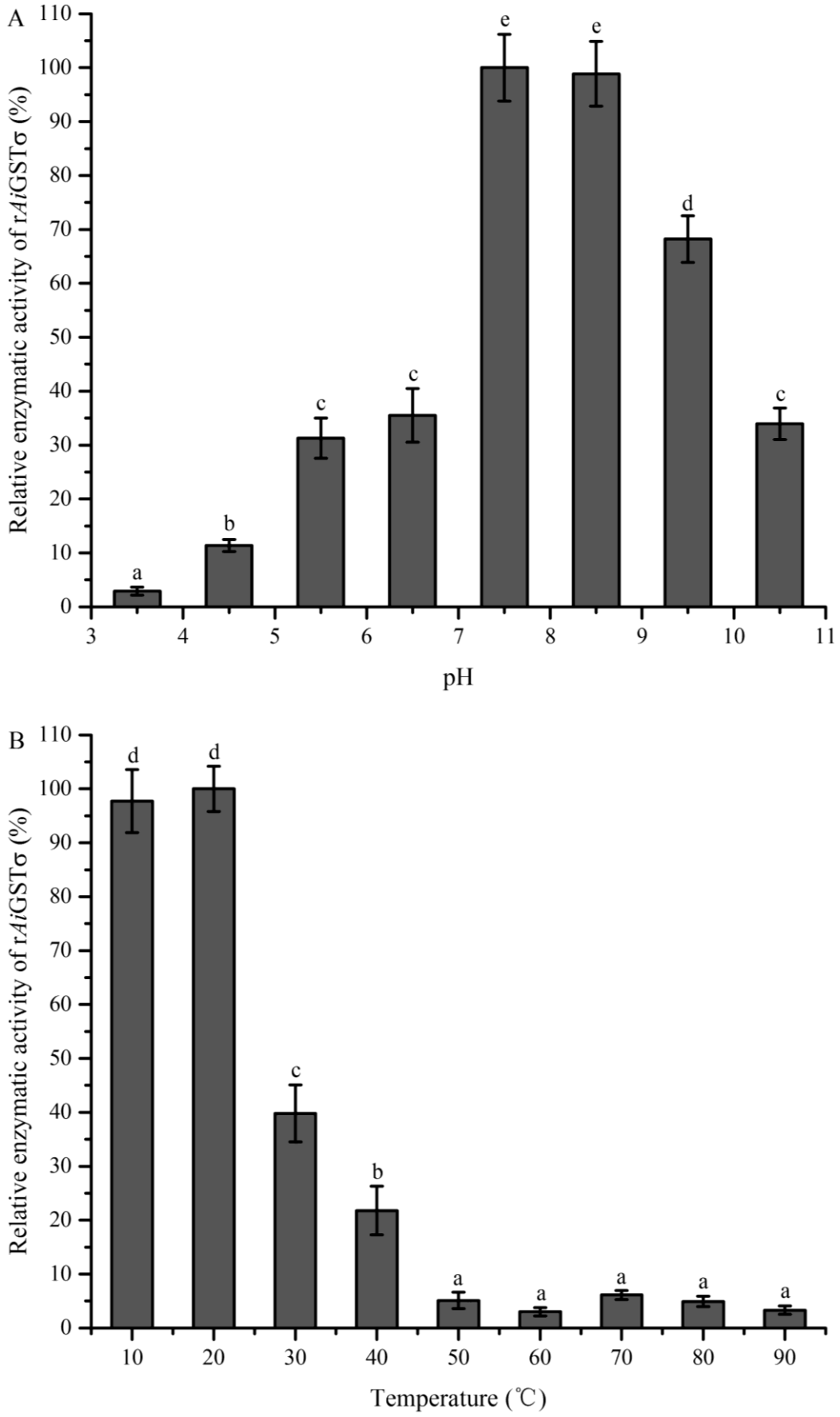

Fig. 6 The enzymatic activities of r*Ai*GSTσ under different treatment (**A**: pH, **B**: temperature). Each values was shown as mean ± SD (n = 5), and bars with different characters were significantly different ($p < 0.05$).

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