A glutamic acid decarboxylase (CgGAD) highly expressed in hemocytes of Pacific oyster *Crassostrea gigas*

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Abstract:

Glutamic acid decarboxylase (GAD), a rate-limiting enzyme to catalyze the reaction converting the excitatory neurotransmitter glutamate to inhibitory neurotransmitter γ -aminobutyric acid (GABA), not only functions in nervous system, but also plays important roles in immunomodulation in vertebrates. However, GAD has rarely been reported in invertebrates, and never in molluscs. In the present study, one GAD homologue (designed as CgGAD) was identified from Pacific oyster

Crassostrea gigas. The full length cDNA of CgGAD was 1689 bp encoding a polypeptide of 562 amino acids containing a conserved pyridoxal-dependent decarboxylase domain. CgGAD mRNA and protein could be detected in ganglion and hemocytes of oysters, and their abundance in hemocytes was unexpectedly much higher than those in ganglion. More importantly, CgGAD was mostly located in those granulocytes without phagocytic capacity in oysters, and could dynamically respond to LPS stimulation. Further, after being transfected into HEK293 cells, CgGAD could promote the production of GABA. Collectively, these findings suggested that CgGAD, as a GABA synthase and molecular marker of GABAergic system, was mainly distributed in hemocytes and ganglion and involved in neuroendocrine-immune regulation network in oysters, which also provided a novel insight to the co-evolution between nervous system and immune system.

Key words: *Crassostrea gigas*; GABA synthase; Immune response; Hemocyte specific

1. Introduction

The GABAergic system is a ubiquitous inhibitory neurotransmitter system which cooperates with excitatory glutamatergic system to maintain a balance in the central nervous system (CNS) of vertebrates (Murphy et al., 2005; Shen et al., 2014). This system exists not only in nervous system, but also in immune cells like monocytes and macrophages to exert a profound effect on the immune function (Dionisio et al., 2011). In vertebrates, GABAergic system is related to suppression of

immune-mediated pro-inflammatory reactions (Reyes-García et al., 2009), modification of cell proliferation and migration of the dendritic cells (Fuks et al., 2012; Jin et al., 2013). In addition, GABAergic system also participates in immune-related diseases, such as psoriasis, experimental autoimmune encephalomyelitis, rheumatoid arthritis and Alzheimer disease (Limon et al., 2012; Nigam et al., 2010). GABAergic system is also detected in nervous system of some invertebrates, including *Caenorhabditis elegans* (McIntire et al., 1993), *Haliotins asinine* (Soonklang et al., 2013), *Eledone cirrhosa* (Cornwell et al., 1993), and it plays effective roles in the regulation of larval swimming (Katow et al., 2013), settlement and metamorphosis during development of sea urchin and bivalve mollusc (García-Lavandeira et al., 2005). However, the information about the distribution and immunomodulatory function of GABAergic system is still very limited in invertebrates.

GABAergic system is composed of four primary parts: i) GABA synthase: glutamic acid decarboxylase (GAD); ii) GABA catabolism enzyme: GABA transaminase (GABA-T); iii) GABA transporters (GAT); iv) GABA receptors (Dionisio et al., 2011). Among them, GAD is the key rate-limiting synthase of GABA and the starter of GABAergic system, which is always used as a molecular marker of GABAergic neurons. It catalyzes the reaction from excitatory neurotransmitter glutamate to inhibitory neurotransmitter γ -aminobutyric acid (GABA) with the cofactor pyridoxal 5'-phosphate (PLP) (Taherzadeh et al., 2015; Jin et al., 1999). Two GAD isoforms,

GAD65 and GAD67, have been identified in vertebrates. Although both isoforms of GAD can synthesize GABA, they are encoded by different independently regulated genes, and have distinct biochemical properties and intracellular distributions (Erlander et al., 1991). GAD65 appears to be targeted to membranes and nerve endings, and preferentially synthesizes GABA for vesicular release. Whereas GAD67 is widely distributed in cells, and preferentially synthesizes cytoplasmic GABA (Soghomonian et al., 1998). So far, only one GAD isoform has been identified in the primitive invertebrates. For example, one GAD gene locus was identified in *Caenorhabditis elegans* and it was necessary for synaptic transmission in GABAergic nervous system (McIntire et al., 1993; Jin et al., 1999). In *Dugesia japonica*, only one GAD gene was reported and required for GABA biosynthesis and photosensitivity (Nishimura et al., 2008). Therefore, it has been postulated that the two GAD isoforms from phylogenetically distant organisms evolved from a common ancestor (Dunathan et al., 1974).

Accumulating evidences have demonstrated that GAD not only functions in the nervous system, but also participates in immunomodulation through synthesizing GABA in vertebrates. It has been reported that GAD can be detected in dendritic cells and macrophages, and its expression can be induced by LPS stimulation (Bhat et al., 2010). In invertebrates, GAD is always regarded as a useful molecular marker for GABAergic neurons (Nishimura et al., 2008), while its role in immunomodulation is still far from well understood. Recently, it was proposed that immune and

neuroendocrine system evolved from a common origin (Ottaviani et al., 2007), and crayfish adult-born neurons were found to be derived from hemocytes, indicating that neuronal precursors may be derived from cells in the innate immune system (Benton et al., 2014). Therefore, the information of the distribution and immune function of GAD in invertebrates may help us to further understand the possible co-evolution of immune system and nervous system.

The Pacific oyster *Crassostrea gigas* is one of most important marine mollusc, which contributes greatly to the aquaculture industry worldwide. With the convenience of oyster genome information, investigations of the distribution and immune function of GABAergic system in oyster *C. gigas* will further understand the neuro-endocrine-immune modulation and the relationships between the nervous system and immune system. The purposes of this study were to (1) identify the homologue of GABA synthase GAD in oyster *C. gigas*, (2) survey the enzyme function and distribution of CgGAD in different tissues and hemocytes, (3) investigate the response of CgGAD after LPS stimulation.

2. Materials and methods

2.1 Oysters and LPS stimulation

Adult oysters *C. gigas* with an average shell length of 12.0-15.0 cm were collected from a local farm in Qingdao, Shandong Province, China, and maintained in the aerated seawater at 15-18°C for a week before experiments.

For LPS stimulation experiment, a narrowed notch was sawed in the closed side of the oyster shell adjacent to the adductor muscle, and the oysters were acclimated for another one week for experiments. The oysters were randomly divided into 2 groups and each group contained 80 individuals. The oysters in control group received an intramuscular injection of 100 μ L phosphate buffered saline (0.27 g L⁻¹ KH₂PO₄; 3.58 g L⁻¹ Na₂HPO₄·12H₂O; 8.00 g L⁻¹ NaCl; 0.20 g L⁻¹ KCl; pH 7.4), while the oysters in stimulation group received an injection of 100 μ L LPS from *Escherichia coli* 0111:B4 (Sigma Aldrich, 0.5 g L⁻¹ in PBS). After stimulation, the oysters were returned to filtered seawater immediately, and 12 individuals were randomly sampled at 0, 3, 6, 12, 24 and 48 h. The hemolymph ' was aseptically collected from sinus of four different individuals with 10-mL syringe (1.6×30-gauge needle), pooled together and then centrifuged at 800 g, 4°C for 10 min to harvest the hemocytes for RNA preparation. At the same time, different tissues, including ganglion, gills, hepatopancreas, mantle, gonad and adductor muscle, were sampled and kept in Trizol reagent at -80°C.

2.2 RNA isolation and cDNA synthesis

Total RNA were extracted from hemocytes and different tissues using Trizol reagent according to the protocol (Invitrogen, USA). The first strand cDNA synthesis was carried out based on Promega M-MLV RT Usage information using the DNase I (Promega)-treated total RNA as template and oligo (dT)-adaptor as primer P1 (Table 1). The reaction was performed at 42°C for 1 h and terminated by heating at 95°C for

5 min. The cDNA mix was diluted to 1:40 and stored at -80°C for subsequent gene cloning and SYBR Green fluorescent quantitative real-time PCR.

2.3 Cloning the full-length cDNA of *CgGAD* and sequence analysis

A pair of specific primers P2 and P3 (Table 1) were designed according to the nucleotide sequence of *CgGAD* to clone the coding sequence. The PCR product was gel-purified, cloned into the pMD19-T simple vector (TaKaRa), and sequenced with primers P4 and P5 (Table 1). The sequence was verified and subjected to cluster analysis. The homology searches of the cDNA sequence and amino acid sequences of *CgGAD* were conducted with BLAST algorithm at the National Center for Biotechnology Information (<http://www.ncbi.nlm.gov/blast>). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (<http://www.expasy.org>). The protein domains were predicted with the simple modular architecture research tool (SMART) version 4.0 (<http://www.smart.emblheidelberg.de/>). The ClustalW Multiple Alignment program (<http://www.ebi.ac.uk/clustalw/>) was used to create the multiple sequence alignment. An unrooted phylogenetic tree of various GADs were estimated in Mega 6.06 by maximum-likelihood (ML) based on the JTT matrix-based model. The reliability of the branching was tested using bootstrap of 1000 (Tamura et al., 2013).

2.4 Recombination, purification, antibody preparation of CgGAD and western blotting analysis

The cDNA fragment encoding CgGAD was amplified using ExTaq polymerase and primers P6 and P7 (Table 1). The PCR products were gel purified and cloned into pET-30a expression vector with His tag. The recombinant plasmid (pET-30a-CgGAD) was transformed into *E. coli transetta* (DE3) (TransGen). Positive transformants were incubated in LB medium at 37°C with shaking at 220 rpm, and IPTG was added into the medium at a final concentration of 0.24 g L⁻¹ when the culture mediums reached OD₆₀₀ of 0.4-0.6. The recombinant protein of CgGAD (designed as rCgGAD) was purified by Ni affinity chromatography, and extensively dialyzed out of urea to renature the protein. The renatured protein of rCgGAD was used to immunize to 6-week old mice to acquire polyclonal antibody as previous description (Cheng et al., 2006).

After SDS-PAGE, samples (purified rCgGAD) were electrophoretically transferred onto nitrocellulose membrane. The membrane was blocked by 5% skim milk powder solution (in TBS-T) for 2 h, and then incubated with the antiserum solution (1:1000, v/v) at 4°C overnight. After thoroughly washing with TBS-T (2.40 g L⁻¹ Tris-HCl; pH 8.0; 8.80 g L⁻¹ NaCl and 0.05% Tween 20), the membrane was incubated with 1:2000 (v/v) diluted horse radish peroxidase (HRP)-conjugated anti-mouse IgG (ABclonal, USA) for 1 h. After thoroughly washing with TBS-T, the membrane was finally incubated with ECL detection reagents (Thermo scientific, USA) and then exposed to

films. Mouse pre-immune serum was used as negative control.

2.5 Cell transfection and quantification of GABA concentration in HEK293 cell

The recombinant plasmid used for transfection assay was constructed as the previous report with modifications (Xin et al., 2015). The open reading fragment (ORF) of CgGAD was amplified with primers P12 and P13 (Table 1), and sub-cloned into mcherry-N1 vector (Clontech, Japan) to construct the plasmid of GAD-mcherry-N1. The null mcherry-N1 vector was used as negative control group and the untreated HEK293 cell as blank group.

HEK293 cells were plated in 12-well plates and cultured with Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 12% fetal bovine serum (FBS, Invitrogen) at 37°C under 5% CO₂. When the cells coverage reached 50-80%, transfections were carried out with Lipofectamine 2000 (Life Technologies) at a final concentration of 10 mg L⁻¹ plasmid following the manufacturer's recommendations. After incubation for 12 h, the substrate (Glutamate) and cofactor (PLP) of the GAD enzyme were added into HEK293 cell supernatant and incubated at 37°C for another 12 h. The cells were collected and lysed with 100 µL lysis buffer (Beyotime Biotechnology, China) to determine the concentration of GABA. All assays were carried out in three replicates.

The concentration of GABA in HEK293 cells was quantified by GABA ELISA Kit (Mlbio Shanghai Enzyme-linked Biotechnology, China) based on a double antibody sandwich method. According to manufacturer's instruction, the HEK293 cell

lysate (40 μ L) and 50 μ L HRP-labeled antibody of GABA were added to a 96 micro-well plate which was coated with purified anti-GABA antibody. After tetramethylbenzidine (TMB) chromogenic reaction under the catalysis of HRP at 37°C for 10 min, the fluorescence intensity of the mixture in micro-well plate was measured at 450 nm (Biotek, USA). The concentration of GABA was determined from standard curves generated according to the kit protocol.

2.6 Real-time PCR analysis of CgGAD mRNA expression

The expression level of CgGAD mRNA was measured by SYBR Green fluorescent quantitative real-time RT-PCR in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). One pair of specific primers P8 and P9 (Table 1) for CgGAD were used to amplify a fragment of 249 bp, and the *C. gigas* elongation factor (CgEF) fragment amplified with primers P10 and P11 (Table 1) was used as the endogenous control. The program of real-time PCR was applied and designed as follow: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Dissociation curve analysis of the amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The $2^{-\Delta\Delta CT}$ method was used to analyze the expression level of genes, and all data were given in terms of relative mRNA expression of mean $\pm$ S.E. (N = 4).

2.7 Immunohistochemical analysis of CgGAD protein in different tissues

Immunohistochemical assay was performed according to the previous description

with little modification (Yang et al., 2015). Tissue samples, including gills, adductor muscle, hepatopancreas and ganglion, were fixed in Bouin's fluid at room temperature for 24 h, faded in 70% ethanol 2 h for 4 times, and dehydrated in 80%, 95% and 100% ethanol sequentially. Then the samples were embedded in paraffin wax, sectioned (5 μ m) and mounted on slides. The slides were deparaffinated in xylene, rehydrated in diluted ethanol series from 95% up to distilled water. After antigens were refolded in sodium citrate-hydrochloric acid buffer, the slides were blocked with 3% BSA in PBS at 37°C for 30 min, incubated with antiserum of CgGAD (diluted 1:1000 in 3% BSA) at 37°C for 1 h, washed in PBS containing 0.05% Tween-20 (PBS-T), and then incubated with Alexa Fluor 488-labeled goat-anti-mouse secondary antibody (ABclonal, diluted 1:1000 in 3% BSA with Evans blue dye) at 37°C for 50 min. Finally, the slides were washed with PBS-T, covered with cover slides with glycerin, and observed under fluorescence microscopy (Olympus). Mouse pre-immune serum was used as negative control.

2.8 Immunofluorescence analysis of CgGAD protein in oyster hemocytes by confocal microscopy and flow cytometer

For immunofluorescence analysis by confocal microscopy, hemolymph was collected from sinus from six oysters with 10-mL syringe (1.6×30-gauge needle) and seeded on cell culture dish directly. Four hours later, the supernatant was discarded and hemocytes were fixed with 4% paraformaldehyde (PFA) for 15 min. After washing with TBS-T, hemocytes were permeabilized with 0.5% Triton-100 for 5 min,

blocked with 3% BSA in PBS at room temperature for 30 min, and incubated with the antiserum of CgGAD (diluted 1:1000 in 3% BSA) at room temperature for 1 h. Following washing with TBS-T, hemocytes were incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (ABclonal, diluted 1:1000 in 3% BSA) for 1 h. Finally, hemocytes were washed three times with TBS-T before incubation with DAPI (Beyotime life, diluted 1:10000 in PBS) for 5 min, and observed under a laser confocal scanning microscopy (Carl Zeiss LSM 710, Germany).

For immunofluorescence analysis by flow cytometer, the hemolymph was aseptically collected from sinus from six oysters with 10-mL syringe (1.6×30-gauge needle) containing equal volume of pre-chilled anticoagulant (6.06 g L⁻¹ Tris-HCl; 2% glucose, 2% NaCl; 5.84 g L⁻¹ EDTA; pH 7.4), then centrifuged at 800 g, 4°C for 10 min to harvest the hemocytes. Then the hemocytes were fixed, permeabilized, blocked and incubated respectively with the antiserum of CgGAD and Alexa Fluor 488-conjugated goat anti-mouse secondary antibody as aforementioned description. Finally, the hemocytes were re-suspended with modified L15 medium (with additional saline 20.2 g L⁻¹ NaCl, 0.54 g L⁻¹ KCl, 0.6 g L⁻¹ CaCl₂, 1 g L⁻¹ MgSO₄, 3.9 g L⁻¹ MgCl₂) and detected by flow cytometer (Becton, USA).

2.9 Phagocytosis assay

To detect the distribution of CgGAD in the phagocytes, *in vitro* phagocytosis assay was performed according to the previous report with slight modification (Wang et al.,

2015). Briefly, hemocytes were harvested from oysters as mentioned in the assay of immunofluorescence analysis by flow cytometer, and then adjusted to 10^6 cell mL^{-1} in modified L15 medium. Five microliters of fluorescent orange latex beads (Sigma L9529, 1.0×10^9 mL^{-1}) was added to 500 μL of hemocytes suspension, and incubated at room temperature for 1 h with continual rotation. After centrifuged at 4°C , 800 g for 10 min, the hemocytes were collected and fixed in 4% PFA for 15 min, and then permeabilized with 0.5% Triton-100 for 5 min before being treated with 3% BSA in PBS at room temperature for 30 min. Then the antiserum of CgGAD (diluted 1:1000 in 3% BSA) was added to the suspension, and Alexa Fluor 488-conjugated goat anti-mouse IgG (ABclonal) (diluted 1:1000 in 3% BSA) was employed as the secondary antibody. Finally, the hemocytes were detected by flow cytometer (Becton, USA).

2.10 Statistical analysis

All data were presented as mean $\pm$ S.D., and analyzed by Statistical Package for Social Sciences (SPSS) 16.0. The results were graphed by Origin 8.1 (OriginLab, Northampton, MA, USA). The differences between treatments for each assay were tested by two-tailed Student t test, and differences were considered statistically significant at $p < 0.05$ and extremely significant at $p < 0.01$.

3. Results

3.1 The molecular characters of *CgGAD*

According to the genome annotation of Pacific oyster *C. gigas*, three candidate genes of GAD (Genebank Accession NO. JH821014.1, JH819059.1 and XM_011420388.1) were identified from NCBI. The protein encoded by the first gene (JH821014.1) lacked two conserved active site residues (Leu158, Asn179) of GAD. The remaining two candidates were found to be located on the same gene locus, and only one gene product of 1689 bp (XM_011420388.1) was amplified from cDNA library of Pacific oyster. Its open reading frame (ORF) encoded a polypeptide of 562 amino acids with a predicted molecular mass of 63.8 KDa and a theoretical isoelectric point of 8.05. SMART program analysis revealed that *CgGAD* contained a pyridoxal-dependent decarboxylase conserved domain (E-value $\leq 1.1\text{e-}104$) from amino acid 197 to 472.

The deduced amino acid sequence of *CgGAD* shared high similarity with other reported GADs: 49.10% similarity with *Mus musculus* (NP_032103), 47.74 % similarity with *Danio rerio* (NP_919400), 45.33% similarity with *Drosophila melanogaster* (NP_523914), and 42.26% similarity with *Caenorhabditis elegans* (AAD19958). Three conserved active site residues (Leu158, Asn179, Gly422) and a 'NPHK' pyridoxal binding site from amino acid 368 to 371 were identified in *CgGAD* by multiple alignment (Xu et al., 2015) (Fig.1).

3.2 The phylogenetic relationship of CgGAD with other GADs

A phylogenetic tree of CgGAD with GADs from other species was constructed using Maximum Likelihood (ML) method, and all the members were distinctly separated into two groups of invertebrate and vertebrate. The CgGAD was closely clustered with GAD from *Dugesia japonica* (BAG48266), and then gathered with GADs from *D. melanogaster* (NP_523914), *C. elegans* (AAD19958), *Ciona intestinalis* (NP_001027785), *Toxocara canis* (KHN81955) and *Rhipicephalus microplus* (AFX68719) to form the invertebrate branch (Fig.2). The other GADs from *Homo sapiens* (AAA62368), *D. rerio* (NP_919400), *Sus scrofa* (NP_999059), *Columba livia* (NP_001302440), *Pan troglodytes* (NP_001029285), *Larimichthys crocea* (KKF28290), *Pteropus alecto* (ELK04690), *Xenopus laevis* (NP_001079270) and *Rattus norvegicus* (NP_058703) were clustered into the vertebrate branch (Fig.2).

3.3 The function of CgGAD to promote the synthesis of GABA in HEK293 cells

To determine the function of CgGAD in the synthesis of GABA, the recombinant CgGAD gene was successfully transfected into HEK293 cells. Under fluorescent microscope, strong red fluorescence could be detected in the recombinant plasmid CgGAD-mcherry-N1 and the null mcherry-N1 vector transfected HEK293 cells (Fig. 3A). There was no significant change of GABA concentration between blank group ($0.88 \pm 0.14 \text{ mg L}^{-1}$) and negative group ($0.77 \pm 0.20 \text{ mg L}^{-1}$), whereas the concentration of GABA in GAD-mcherry-N1 group ($1.82 \pm 0.37 \text{ mg L}^{-1}$) was

significantly higher ($p < 0.05$) than that in other two groups (Fig. 3B).

3.4 Distribution of CgGAD mRNA in different oyster tissues

Quantitative real-time PCR was employed to detect the expression level of CgGAD mRNA in different oyster tissues, including hepatopancreas, mantle, gonad, adductor muscle, gills, hemocytes and ganglion. The highest expression level of CgGAD mRNA was detected in hemocytes, which was 39.79 ± 3.49 fold ($p < 0.01$) higher than that in gills (Fig. 4). The expression level of CgGAD mRNA was also high in ganglion, which was 17.42 ± 2.68 fold ($p < 0.01$) higher than that in gills (Fig. 4). There was no significant difference of CgGAD mRNA transcripts among the rest tissues, including hepatopancreas, mantle, gonad and adductor muscle (Fig. 4).

3.5 Recombinant protein and polyclonal antibody of CgGAD

The recombinant plasmid (pET-30a-CgGAD) was transformed into *E. coli transetta* (DE3) as described above and the whole cell lysate of positive clone was analyzed by SDS-PAGE after IPTG induction. A distinct band with a molecular mass of nearly 66 KDa was revealed as predicted (Fig. 5). The purified rCgGAD protein was used to obtain polyclonal antibody. The antibody specificity was tested by western blotting and a clear band was revealed (Fig. 5). Meanwhile, as negative group, no clear band was observed with mouse pre-immune serum (data not shown).

3.6 Distribution of CgGAD protein in oyster hemocytes and other tissues

CgGAD in hemocytes and other tissues was detected by immunohistochemistry assay, and the green positive signal was dominantly located in the tissue of ganglion (Fig. 6A) under fluorescence microscope, while it was barely detected in other examined tissues including gills, hepatopancreas, adductor muscle and negative control (Fig. 6A). As to the hemocytes, CgGAD was only detectable in the cytoplasm of hemocytes which were much bigger in size, more granular and looked like granulocytes (Fig. 6B). The population of CgGAD positive hemocytes was further detected by flow cytometer.

3.7 Character of CgGAD positive hemocyte population in oyster

In order to further verify the character of CgGAD positive population, the localization of CgGAD in hemocytes was analyzed by flow cytometer. The FSC and SSC parameters were used to indicate cell size and granularity, respectively. The CgGAD positive population reached about 16.8% of the whole hemocytes in oysters (Fig. 7A). The hemocytes with CgGAD positive signal, marked with green (FITC-conjugated), had higher SSC and FSC compared with those of CgGAD negative population (Fig. 7A).

According to the previous reports in which the hemocytes with the high SSC and FSC were phagocytes (Donaghy et al., 2009; Goedken et al., 2004; Pech et al., 1996), the localization of CgGAD in phagocytes was further detected. The hemocytes with a

relative higher *CgGAD* expression level were marked green, and the phagocytes with fluorescent orange latex-beads inside were marked red (Fig. 7B). The percentage of *CgGAD* positive population (*CgGAD*⁺) and phagocytes (beads⁺) was about 16.8% and 27.7%, respectively. While the double positive (*CgGAD*⁺/ beads⁺) hemocytes, marked with blue, composed just about 4.6% of the whole hemocytes and 27.4% of *CgGAD*⁺ hemocytes (Fig. 7B). In the whole experiment, no GAD positive signal was detected in negative control group.

3.8 Temporal expression of *CgGAD* mRNA in oyster hemocytes after LPS stimulation

The expression level of *CgGAD* mRNA in hemocytes of oysters was detected by quantitative real-time PCR at 0, 3, 6, 12, 24 and 48 h after LPS stimulation. The expression level of *CgGAD* decreased significantly ($p < 0.05$) at 3, 6 and 12 h after LPS stimulation, compared with that in PBS group (Fig. 8). However, a significant ($p < 0.01$) up-regulation of *CgGAD* mRNA transcripts was observed at 48 h after LPS stimulation, which was about 3.35-fold that in PBS group (Fig. 8). During the whole experiment, there was no significant difference of *CgGAD* mRNA expression between the blank and control group.

4. Discussion

The CNS and the immune system play important roles in the process of homeostasis, adaptation and immune defense in both vertebrate and invertebrate

animals (Demas et al., 2011; Mašek et al., 2003). The CNS can regulate the function of immune system through secreting neurotransmitters. However, some neurotransmitters, such as NO and biogenic amines have also been reported in the immunocytes of some protostome species without complex nervous system (Ottaviani et al., 1996; Jiang et al., 2013). Therefore, some scientists have proposed that there is a common evolutionary origin for the immune and neuroendocrine systems (Ottaviani et al., 2007). In addition, recent study in crayfish showed that adult-born neurons can derive from hemocytes, which further support the possible common evolutionary origin of the nervous and immune systems (Benton et al., 2014).

The GABAergic system is a ubiquitous inhibitory neurotransmitter system in the CNS of vertebrates, which also plays important role in immunomodulation (Jin et al., 2013). Glutamic acid decarboxylase (GAD) is one of the key components of GABAergic system, which is always used as a molecular marker of GABAergic system. In vertebrates, GAD is required for GABA signaling in CNS, but it also exists in immune cells and responds to stimulation (Bhat et al., 2010). However, the information about GABAergic system and the function of GAD in immunity is still very limited in invertebrates. The investigation of GAD in mollusk may pave the way to know its role in immunomodulation and further understand the co-evolution of neuroendocrine system and immune system.

In the present study, only one gene encoding GAD (designated *CgGAD*) was identified from the genome of Pacific oyster *C. gigas*. The deduced amino acid

sequence of CgGAD shared high similarity (42.2%-49.3%) with those of other reported GADs. The lysine (K³⁷¹) in conserved pyridoxal binding site 'NPHK' which can form a Schiff's base with the PLP aldehyde group (Sukhareva et al., 2002) was also found in CgGAD. The high amino acid similarity together with its conserved functional domain suggested that CgGAD was a homologue of GAD. In the phylogenetic tree, CgGAD was closely clustered with GADs from primitive invertebrates, such as the Platyhelminthes *Dugesia japonica* and the Nematoda, and then gathered together with GADs from Arthropoda and Urochordata to form an invertebrate branch, while the remaining GADs from amphibian and mammal formed the vertebrate branch. Interestingly, there are two GAD isoforms in *Drosophila* and *Strongylocentrotus purpuratus*, but only one GAD isoform has been found in molluscs and nematodes (Jackson et al., 1990; Soghomonian et al., 1998; Katow et al., 2013; Erlander et al., 1991; Nishimura et al., 2008; Jin et al., 1999). It was suspected that the duplication and divergence of GAD isoforms during the phylogenetic evolution probably occurred after molluscs.

Currently, the functional study of GAD is mainly focused on the producing of GABA or as a molecular marker of GABAergic neurons (Nishimura et al., 2008). GAD is the only biosynthetic enzyme of GABA which is the principal inhibitory neurotransmitter in CNS (Ben-Ari, 2002). In the present study, the enzyme function and the distribution of CgGAD were examined to illustrate its potential physiological roles. It was proved that CgGAD could promote the production of GABA in

transfected HEK293 cell. In addition, as a marker of GABAergic neurons, the mRNA transcripts of CgGAD in ganglion were indeed much higher than that in the tissues of hepatopancreas, mantle, gonad, adductor muscles and gills. Unexpectedly, the highest expression level of CgGAD was detected in hemocytes, which was 2.00-fold higher than that in ganglion. Meanwhile, the positive immunohistochemical signal of CgGAD could be mostly detected in hemocytes and ganglion at protein level. These results collectively suggested that hemocytes rather than ganglion were the main place to produce GABA in oyster, and CgGAD might implicate in the immune response and play important role in the neuroendocrine-immune regulatory network of oyster.

In invertebrates, hemocytes have been considered as an important defensive tool of innate immunity, which could produce abundant immune related molecules to eliminate the invading pathogens (Allam et al., 2001; Canesi et al., 2002). Recently, the accumulating evidences demonstrated that neurotransmitter could be released by hemocytes to modulate the immune response (Guo et al., 2013; Jiang et al., 2013; Zhou et al., 2011). GABA, as one of the neurotransmitters, has been proved to exist in oyster and plays the inhibitory role in the immunomodulation (Li et al., 2016). In the present study, CgGAD, as the GABA synthase, could also respond to the immune stimulation. After the oysters were stimulated with LPS, the expression of CgGAD mRNA in hemocytes decreased significantly at 3-12 h ($p < 0.05$), but increased significantly at 48 h ($p < 0.05$), which was similar to the change of GABA concentration in oysters hemolymph supernatant (Li et al., 2016) or mice

macrophages activated by LPS (Tannahill et al., 2013). These results implied that CgGAD could be induced in response to LPS stimulation and involved in the inhibitory immunomodulation of the immune response in oysters.

As a molecular marker of GABAergic neural system, CgGAD was found to be mainly expressed in hemocytes of oyster *C. gigas*, suggesting that these cells function in both immune and neuroendocrine system. Due to the heterogeneity of hemocytes in oyster, the specific identity of CgGAD⁺ hemocytes was not determined. In oyster, the hemocytes have been classified in at least two cell types: granulocytes, hyalinocytes or agranulocytes (Aladaileh et al., 2007; Donaghy et al., 2009; Xue et al., 2001). In the present study, CgGAD⁺ hemocytes were characterized by high granularity, which was confirmed by confocal microscopy and flow cytometer. It was reported that granulocytes in oyster were involved in phagocytosis (Donaghy et al., 2009; Goedken et al., 2004; Sun et al., 2006). Similarly, the professional phagocytes are deemed as fundamental players in immune and neuroendocrine crosstalk such as macrophages in vertebrates (Malagoli et al., 2015). However, most CgGAD⁺ hemocytes in oyster were proved to be the granulocytes without phagocytic capacity. It was reported in crayfish that adult-born neurons could be generated from circulating hemocytes with fine cytoplasmic granules which are more likely the semi-granular hemocytes (Benton et al., 2014). Thus, the granulocytes or semi-granulocytes in oyster seem more likely to be the progenitor cells of immune and neuron cells in the early evolution of animals. Moreover, it has been proposed that the immune-neuroendocrine role of immunocytes

predates the split of protostomian and deuterostomian superphyla (Malagoli et al., 2015). Collectively, the study of *CgGAD* can represent valuable evidence for the possible common evolutionary origin of immune system and nervous system.

In conclusion, one GABA synthase gene *CgGAD* was identified from Pacific oyster *C. gigas*, which could promote the synthesis of neurotransmitter GABA. As a GABA synthase and molecular marker of GABAergic system, *CgGAD* was mainly distributed in ganglion and the granulocytes without phagocytic capacity, and might be involved in neuroendocrine-immune regulation network in oyster, which also provided a novel insight to the co-evolution between nervous system and immune system.

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Table 1 Primers used in this study

Primer name	Sequence (5'-3')
<i>Clone primers</i>	
P1 (oligo dT-adaptor)	GGCCACGCGTCGACTAGTACT
P2 (forward)	ATGGAAGATCTACCAAGAAGAAAAG
P3 (reverse)	TCAACAAAAGATGTCTTTTGACAA
<i>Sequence primers</i>	
P4 (M13-47)	CGCCAGGGTTTTCCCAGTCACGAC
P5 (RV-M)	GAGCGGATAACAATTCACACAGG
<i>RT primers</i>	
P6 (CgGAD-RTF)	GCTATGTGCGGATTACCTCTACCAG
P7 (CgGAD-RTR)	GATTCGCTAAGTCTTGGGTTGGATA
P8 (EF-RTF)	AGTCACCAAGGCTGCACAGAAAG
P9 (EF-RTR)	TCCGACGTATTTCTTTGCGATGT
<i>Recombination primers</i>	
P10 (forward)	CGGGGTACCATGGAAGATCTACCAAGAAGAAAAG
P11 (reverse)	CCGCTCGAGACAAAAGATGTCTTTTGACAA
<i>Cell transfection primers</i>	
P12 (forward)	GCGCTACCGGACTCAGATCTCGAGATGGAAGATC

	TACCAAGAAGAAAAG
	TGGATCCCGGGCCCGCGGTACCGTACAAAAGATG
P13 (reverse)	TCTTTTGACAA

Figure legends

Fig.1. Multiple sequence alignment analysis of putative domains of CgGAD with other GADs deposited in GenBank, including GADs from *Caenorhabditis elegans* (AAD19958), *Mus musculus* (NP_032103), *Drosophila melanogaster* (NP_523914), *Ciona intestinalis* (NP_001027785), *Homo sapiens* (AAA62368), *Danio rerio* (NP_919400), *Chilo suppressalis* (AKL78865). The identical amino acid residues are shaded in black and the similar amino acid are shaded in grey. The conserved active site residues are marked with blue balls. The functional residues are indicated by red filled triangles and the pyridoxal binding site 'NPHK' is marked with a red wireframe.

Fig.2. Maximum likelihood (ML) phylogenetic tree based on the amino acid sequences of different GADs. The scale bar represents the conversion of branch length to genetic distance between clades. The GADs used for phylogenetic analysis include: *Dugesia japonica* (BAG48266), *Drosophila melanogaster* (NP_523914), *Caenorhabditis elegans* (AAD19958), *Ciona intestinalis* (NP_001027785), *Toxocara canis* (KHN81955), *Rhipicephalus microplus* (AFX68719), *Homo sapiens* (AAA62368), *Danio rerio* (NP_919400), *Sus scrofa* (NP_999059), *Columba livia* (NP_001302440), *Pan troglodytes* (NP_001029285), *Larimichthys crocea*

(KKF28290), *Pteropus alecto* (ELK04690), *Xenopus laevis* (NP_001079270) and *Rattus norvegicus* (NP_058703).

Fig.3. Concentration of GABA in HEK293 cells transfected with CgGAD. (A) Microscopic examination of HEK293 cells transfected with CgGAD. a,b: HEK293 cells transfected with null mcherry-N1 vector; c,d: HEK293 cells transfected with CgGAD-mcherry-N1; CgGAD fused mcherry tag and mcherry tag were successfully expressed in transfected HEK293 cells (red). (B) Concentration of GABA in HEK293 cells. Untreated and null vector transfections were used as control. Vertical bars represented means $\pm$ S.D (N = 4), **: $p < 0.01$.

Fig.4. Expression of CgGAD mRNA in different tissues of oyster detected by real-time PCR. Data were represented as the ratio of the CgGAD mRNA level to that of gills and normalized to that of elongation factor (CgEF). Vertical bars represent the mean $\pm$ S.D. (N = 4). **: $p < 0.01$.

Fig.5. SDS-PAGE and western-blot analysis of rCgGAD. Lane 1: protein molecular standard; lane 2: negative control for rCgGAD (without induction); lane 3: induced rCgGAD; lane 4: purified rCgGAD; lane 5: western blotting based on the sample of line 3 to assay the specificity of the polyclonal antibody against rCgGAD.

Fig.6. Distribution of *CgGAD* in different tissues and hemocytes of oyster. A: Immunohistochemical analysis of the distribution of *CgGAD* in hepatopancreas, adductor muscle, gills and ganglion by fluorescence microscope. The red background was stained by Evan Blue dye which labeled the cell membrane. Negative means that the tissues were incubated with the pre-immune serum instead of recombinant *CgGAD*. The positive signal was shown by white arrow. B: Cellular distribution of *CgGAD* in oyster hemocytes detected by confocal microscope. The nuclei of hemocytes were stained with DAPI (blue). Positive signal of *CgGAD* was green.

Fig.7. Expression of *CgGAD* in oyster hemocytes as detected by flow cytometer. FSC and SSC parameters were used to indicate cell size and granularity. Alexa Fluor 488-conjugated goat anti-mouse antibody was used to mark *CgGAD* positive hemocytes (*CgGAD*⁺), which were labeled green. The orange latex-beads were used to mark phagocytes (beads⁺), which were labeled red. The *CgGAD* positive hemocytes with phagocytic ability (*CgGAD*⁺/beads⁺ hemocytes) were labeled blue. A: The percentage of *CgGAD* positive hemocytes in the whole oyster hemocytes, which was 16.8%. B: The percentage of *CgGAD* positive hemocytes, phagocytes and *CgGAD*⁺/beads⁺ hemocytes in the whole oyster hemocytes, which were 16.8, 27.7 and 4.6%, respectively.

Fig.8. Temporal expression of *CgGAD* transcripts in oyster hemocytes after LPS

stimulation measured by Real-time PCR. Data were represented as the ratio of the CgGAD mRNA level to that of 0 h and normalized to that of CgEF. Vertical bars represent the mean $\pm$ S.D. (N = 4). *: $p < 0.05$; **: $p < 0.01$.

Homo sapiens MASSTPSSSATSSNAGADPNTTNLRP--TTYDTWCGVAHGCTRKLGKICGFLQRTNSLEEKSRVSAFKERQS-SKNLLSCENSDDRARFRRTETDFSN 97
Mus musculus MASSTP-SPATSSNAGADPNTTNLRP--TTYDTWCGVAHGCTRKLGKICGFLQRTNSLEEKSRVSAFRERQS-SKNLLSCENSDDGARFRRTETDFSN 96
Danio rerio MASSAP----SSSAGDMDPNTANLRQPATTSDAWYGVAGHGCTRKLGKICGFLQKNSLDEKSRMVGSFKES---AKNQMSCDNNE---RFTRDETDFSN 90
Drosophila melanogaster -----MSKLTLDGFFSYCKQIAE-HIQLGERMAALSQGQTPKLTSAVGN 16
Chilo suppressalis -----MQQSAWRFRSS-SVGLIKVEPNN--DDYSMNNGDFEN 43
Ciona intestinalis -----MSSAAADESDAVLEN 15
Caenorhabditis elegans -----MEDLPRRKENKKLYNASTKTLQIDPQVRRPVENVSSKIQGSLLKGEASENRETRKRNQRNQEWGKFEG 68
Crassostrea gigas

Homo sapiens LFARDLLPAKN-GEEQTVQFLLEVVDFLLNLYVRKTFDRSTKVLDFFHHPHQLLEGMEG--FNLELSDHPESLEQILVDCRDTLKYGVRTGHPFRFNQLSTG 194
Mus musculus LFAQDLLPAKN-GEEQTAQFLLEVVDFLLNLYVRKTFDRSTKVLDFFHHPHQLLEGMEG--FNLELSDHPESLEQILVDCRDTLKYGVRTGHPFRFNQLSTG 193
Danio rerio LFARDLLPAKN-GEEPTIQFLLEVVDFLLNLYVRKTFDRSTKVLDFFHHPHQLLEGMEG--FNLELSDHPESLEQILVDCRDTLKYGVRTGHPFRFNQLSSG 187
Drosophila melanogaster LTAYDLMPTTVTAGPETREFLLKVIDVLDVFKATNDRNEKVLDFHHPEDMKRLDLDD---VP-DRALPLQLLIEDCATTLKYGVRTGHPFRFNQLSSG 111
Chilo suppressalis LYYDSILPYREDANTQTREFLARVVDVLLDEVQVNDNRNEKILEFRMPPEEMMKMLDLQ---LH-DEPLPLRLLEDCKITLKNQVKTGHPFRFNQLSSG 138
Ciona intestinalis SSSADLLPFGK-SDEKTQLFLKEIFEILLKYISKSFDRCKKILDFHHPHQLLEGIEG--FSLNNGEAESELEQILVDCRDTLKYGVRTGHPFRFNQLSSG 132
Caenorhabditis elegans LIAKEILPQTG-NWEGTEEFLLNRIVQVLLKYIKDQNDRDQKILEFHHHPDKMQMLMDLS---IP-EKPESLLKLKSCEDVLRGVRTGHPFRFNQLSSG 109
Crassostrea gigas VYYKDLQIDSK---VQKDFMLEMARLLNTYMTDQCQGVKVVDFHHPHQLQEMMGD---CLTVDAVPRDLHQVLSDCKETLKCYCKTGHPFRFNQLSSG 161

Homo sapiens LDIIGLAGEWLT-STANTNMFTYEIAFVFVLMQITLKKMREIVGWSKDGDFGIFSPGGATSNMYSIMAARYKYFPEVKTKGMAAVP-KLVLFTESESHY 292
Mus musculus LDIIGLAGEWLT-STANTNMFTYEIAFVFVLMQITLKKMREIVGWSKDGDFGIFSPGGATSNMYSIMAARYKYFPEVKTKGMAAVP-KLVLFTESESHY 291
Danio rerio LDIIGLAGEWLT-STANTNMFTYEIAFVFVLMQITLKKMREIVGWSKDGDFGIFSPGGATSNMYSIMAARYKYFPEVKTKGMAAVP-KLVLFTESESHY 285
Drosophila melanogaster LDLISMAGEWLT-ATANTNMFTYEIAFVFILMENVVLTLMREIIGWS--GGDSILAPGGISNLYAFLAARHKKMFNYKEHGSVGLPGTLVMTLSDQCHY 208
Chilo suppressalis LDIISLAGEWLT-AAANTNMFTYEIAFVFILMENVVLEKMRSMIGWK--SGDSILAPGGISNLYAFLAARHKKMFNYKEHGSVGLPGTLVMTLSDQCHY 235
Ciona intestinalis LDIVSLADWVT-STANTNMFTYEIAFVFILMEDVVIKRMKIKIGWENIDG--IFSPGGISNLYAFLAARHKKMFNYKEHGSVGLPGTLVMTLSDQCHY 228
Caenorhabditis elegans LDLVSMAGEWLT-ATANTNMFTYEIAFVFILMEKSVMARMWEAVGWDPEKADGIFAPGGATSNMYSIMAARYKYFPEVKTKGMAAVP-KLVLFTESESHY 207
Crassostrea gigas LDPVGVAGEWLT-AAVNTNMFTYEIAFVFVLMQITLKKMREIVGWSKDGDFGIFSPGGATSNMYSIMAARYKYFPEVKTKGMAAVP-KLVLFTESESHY 258

Homo sapiens SIKKAGAAAGGFTDNVILIKCNERGKIIPADLEAKILEAKQKGVYVFNATAGTITVYGAEDFIQEIADICEKYNLWHDVDAWGGGGLLMSRKHRH-KLN 391
Mus musculus SIKKAGAAAGGFTDNVILIKCNERGKIIPADLEAKILEAKQKGVYVFNATAGTITVYGAEDFIQEIADICEKYNLWHDVDAWGGGGLLMSRKHRH-KLS 390
Danio rerio SIKKAGAAAGGFTDNVILIKCNERGKIIPADLEAKILEAKQKGVYVFNATAGTITVYGAEDFIQEIADICEKYNLWHDVDAWGGGGLLMSRKHRH-KLS 384
Drosophila melanogaster SIKSCAAVCGGLGTDCIVVPSDEHGKMITSELERLILERKAKGDIIPFVFNATAGTITVYGAEDFIQEIADICEKYNLWHDVDAWGGGGLLMSRKHRH-KLS 308
Chilo suppressalis SVKSCASVCGGLGTDCIVVPSDEHGKMITSELERLILERKAKGDIIPFVFNATAGTITVYGAEDFIQEIADICEKYNLWHDVDAWGGGGLLMSRKHRH-KLS 335
Ciona intestinalis SNKRPAAILGGLNNDICIEVDGERGKMPEDLEKILQSKLDGKVPFVFNATAGTITVYGAEDFIQEIADICEKYNLWHDVDAWGGGGLLMSRKHRH-LVA 327
Caenorhabditis elegans SIKSASAVLGTADYCFNIPTDKNGKMIPEALEAKIIECKKEGLTGFACCTAGSTTVYGAEDFIQEIADICEKYNLWHDVDAWGGGGLLMSRKHRH-KLA 306
Crassostrea gigas SIKKAAAILGGLMNNVCCIPCDERGKMKVDVLEIREIQRTLSGGVPLMVNATCGTITVLAAYDVFQKIDICEKYNLWHDVDAWGGGGLLMSRKHRH-KLA 357

Homo sapiens GIERANSVTWNPHKMMGVLLQCSAILVREKGIILQGCNQMCAGYLEQBDKQYDVS YDTGDKAIQCGRHVDIFKFWLMWKAAGTGVGFENQINKCLELAEYLY 491
Mus musculus GIERANSVTWNPHKMMGVLLQCSAILVREKGIILQGCNQMCAGYLEQBDKQYDVS YDTGDKAIQCGRHVDIFKFWLMWKAAGTGVGFENQINKCLELAEYLY 490
Danio rerio GIERANSVTWNPHKMMGVLLQCSAILVREKGIILQGCNQMCAGYLEQBDKQYDVS YDTGDKAIQCGRHVDIFKFWLMWKAAGTGVGFENQINKCLELAEYLY 484
Drosophila melanogaster GVERADSVTWNPHKMLMGALQCSITHFEDGLLISCNQMSAEYLEMTDKQYDIS YDTGDKAIQCGRHVDIFKFWLMWKAAGTGVGFENQINKCLELAEYLY 408
Chilo suppressalis GIERADSVTWNPHKMLMGALQCSITHFEDGLLISCNQMSAEYLEMTDKQYDIS YDTGDKAIQCGRHVDIFKFWLMWKAAGTGVGFENQINKCLELAEYLY 435
Ciona intestinalis GIERADSVTWNPHKMLMGALQCSITHFEDGLLISCNQMSAEYLEMTDKQYDIS YDTGDKAIQCGRHVDIFKFWLMWKAAGTGVGFENQINKCLELAEYLY 427
Caenorhabditis elegans GIERANSVTWNPHKMLMGALQCSITHFEDGLLISCNQMSAEYLEMTDKQYDIS YDTGDKAIQCGRHVDIFKFWLMWKAAGTGVGFENQINKCLELAEYLY 406
Crassostrea gigas GVERADSVTWNPHKMMGVLLQCSAILVREKGIILQGCNQMCAGYLEQBDKQYDVS YDTGDKAIQCGRHVDIFKFWLMWKAAGTGVGFENQINKCLELAEYLY 457

Homo sapiens AKIKNREEFEMVFNAGEPEHTSVCFWYIPQSLRGVPS-PORREKLHVAPKIKALMMESGITMVGYPQGDKANFFRMVISNPAATQSDIDFLIEEIERL 590
Mus musculus AKIKNREEFEMVFNAGEPEHTSVCFWYIPQSLRGVPS-PORREKLHVAPKIKALMMESGITMVGYPQGDKANFFRMVISNPAATQSDIDFLIEEIERL 589
Danio rerio NKIKNREGYEMVFEGQPHNTNVCFWYIPQSLRGVPS-PORREKLHVAPKIKALMMESGITMVGYPQGDKANFFRMVISNPAATQSDIDFLIEEIERL 583
Drosophila melanogaster KRIREQSDRFHLIL-EPECVNVSWFWYIPQSLRGVPS-PORREKLHVAPKIKALMMESGITMVGYPQGDKANFFRMVISNPAATQSDIDFLIEEIERL 506
Chilo suppressalis RRIREQSDRFHLIL-EPELVNVSWFWYIPQSLRGVPS-PORREKLHVAPKIKALMMESGITMVGYPQGDKANFFRMVISNPAATQSDIDFLIEEIERL 533
Ciona intestinalis RKIKKTPGFQILVFQ-EPEYSNVCFWYIPQSLRGVPS-PORREKLHVAPKIKALMMESGITMVGYPQGDKANFFRMVISNPAATQSDIDFLIEEIERL 525
Caenorhabditis elegans RRIKETEGFELIIE-NPEFLNICFWYIPQSLRGVPS-PORREKLHVAPKIKALMMESGITMVGYPQGDKANFFRMVISNPAATQSDIDFLIEEIERL 503
Crassostrea gigas EKVKQRDNFTLVN-EVEGPSVSWFWYIPQSLRGVPS-PORREKLHVAPKIKALMMESGITMVGYPQGDKANFFRMVISNPAATQSDIDFLIEEIERL 556

A

B

KDa

1

2

3

4

5

116.0

66.2

45.0

35.0

25.0

18.4

14.4