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*Cg*AATase with specific expression pattern can be used as a potential surface marker for oyster granulocytes

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ABSTRACT

Granulocytes are known as the main immunocompetent hemocytes that play important roles in the immune defense of oyster *Crassostrea gigas*. In the present study, an alcohol acyltransferase (designed as *Cg*AATase) with specific expression pattern was identified from oyster *C. gigas*, and it could be employed as a potential marker for the isolation of oyster granulocytes. The open reading frame (ORF) of *Cg*AATase was of 1431 bp, encoding a peptide of 476 amino acids with a typically conserved AATase domain. The mRNA transcripts of *Cg*AATase were highest expressed in hemocytes, lower expressed in hepatopancreas, mantle, gonad, gill, ganglion, adductor muscle, and labial palp. The mRNA expression level of *Cg*AATase in hemocytes was significantly up-regulated at 3–12 h and reached the highest level (27.40-fold compared to control group, $p < 0.05$) at 6 h after *Vibrio splendidus* stimulation. The total hemocytes were sorted as granulocytes, semi-granulocytes and agranulocytes by Percoll® density gradient centrifugation. *Cg*AATase transcripts were dominantly observed in granulocytes, which was 8.26-fold ($p < 0.05$) and 2.80-fold ($p < 0.05$) of that in agranulocytes and semi-granulocytes, respectively. The monoclonal antibody against *Cg*AATase was produced and employed for the isolation of granulocytes with the immunomagnetic bead. *Cg*AATase protein was mainly detected on the cytomembrane of granulocytes. About 85.7 ± 4.60% of the granulocytes were positive for *Cg*AATase and they could be successfully separated by flow cytometry with immunomagnetic bead coated with anti-*Cg*AATase monoclonal antibody, and 97.7 ± 1.01% of the rest hemocytes (agranulocytes and semi-granulocytes) were negative for *Cg*AATase. The isolated primary granulocytes could maintain cell activity for more than one week *in vitro* culture that exhibited numerous filopodia. These results collectively suggested that *Cg*AATase was a potential marker of oyster granulocytes, and the granulocytes could be effectively isolated from total circulating hemocytes by immunomagnetic bead coated with the anti-*Cg*AATase monoclonal antibody.

1. Introduction

Granulocytes are a class of leukocytes characterized by the presence of lobulated nuclei and secretory granules filled with a variety of enzymes that involved in the attack and digestion of invading pathogens [1,2]. They are arisen from both the classical lymphoid and myeloid branches, and released as terminally differentiated cells (eosinophilic, neutrophilic and basophilic polymorphonuclear cells) into the blood stream [3–5]. In vertebrates, three different types of granulocytes, eosinophils, neutrophils and basophils, have been described and isolated from different sites and states with histochemical staining properities [6] and the cluster of differentiation (CD) antigens [7]. For example, granulocytes were identified as $CD45^+$ cells [8,9], and neutrophils, eosinophils and basophils which specially expressed cell surface antigens CD15 and CD66b [10], CD69 and CD11b [11], and CD63 and CD203c [12], respectively. The capability of granulocytes, phagocytosis of neutrophils, cytotoxic effector functions of eosinophils, as well as chemotaxis of basophils, has been considered historically as an effective way to defense the extracellular microorganisms [13,14].

The invertebrate granulocytes are highly conserved with relatively simple forms [15], and are mainly characterized and identified by their physical and chemical characteristics with light and electron microscopy combined with gradient centrifugation as well as magnetic activated cell sorting (MACS) [16–18]. So far, granulocytes have been

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morphologically, structurally, functionally characterized in various invertebrates, such as insects and decapods of arthropod taxa [16,18,19], bivalve and gastropod of mollusc [20–22]. Generally, granulocytes with a small nucleo-cytoplasmic (N:C) ratio are abundant of cytoplasmic granules containing a mixture of hydrolytic enzymes. There are many reports on the morphology classification of hemocytes in bivalves. It is accepted that bivalve hemocytes are divided into granulocytes and agranulocytes based on size and granularity, and the granulocytes are sub-classified into acidophilic and basophilic granulocytes based on hematoxylin-eosin staining [23–26]. Despite these general categories, there is currently no unified nomenclature and method to describe and identify bivalve granulocytes [27].

The primary potential functions of granulocytes have been evidenced in several invertebrates with significant heterogeneity. For example, granulocytes were required for phagocytosis and encapsulation of foreign targets in insects [28,29]. It was also reported that granulocytes were the main immune cells performing phagocytosis in molluscs, such as octopus, mussels, scallops, and oysters [30–32]. In Chinese mitten crab, the hemocytes containing granules displayed limited phagocytic ability, only with approximately 5.0% of granulocytes and 6.3% of semi-granulocytes displaying positive phagocytic ability against the invading polystyrene beads *in vivo* [33].

Monoclonal antibody technique combing with immunomagnetic bead has been applied to isolate cells that express specific antigens. For example, *Xenopus* natural killer (NK) cells were identified by novel anti-NK monoclonal antibodies (1F8, 4D4 and 1G5) [34]. A monoclonal antibody against porcine hematopoietic stem cells was generated to identify porcine CD34 on a subset of porcine bone marrow (BM) stem/progenitor cells [35]. Recently, monoclonal antibody technique has also been used in identifying hemocyte subpopulations in invertebrates. The monoclonal antibody specific for all hemocytes or granulocytes were reported in shrimp *Penaeus monodon* [36], *Fenneropenaeus chinensis* [37,38] and *Chlamys farreri* [22], respectively. However, these monoclonal antibodies are not strictly specific for single species and certain type hemocytes. For example, monoclonal antibodies against granulocytes in *C. farreri* showed cross-reactivity with hemocytes of other five bivalve species [22]. Therefore, the specific and membranous localized granulocyte antigens would be ideal markers for the isolation and function clarified of granulocytes.

The Pacific oyster *C. gigas* is one of the most commercially and ecologically important bivalves worldwide [39]. In our previous study, three hemocyte subtypes (granulocytes, semi-granulocytes and agranulocytes) in oyster *C. gigas* were described according to morphology, ultrastructure and staining properties, and they could be isolated by flow cytometry and Percoll® density gradient centrifugation. Granulocytes were identified functionally as the main phagocytic and encapsulating population with prominent activity of lysosome, superoxide anion, and nitric oxide [17]. Single cell transcriptome sequencing of granulocytes revealed that *Cg*AATase was highly and specially expressed in granulocytes (Unpublished data). In the present study, the cDNA and monoclonal antibody were prepared of *Cg*AATase from the oyster *C. gigas* with the main objectives (1) to examine the expression pattern of *Cg*AATase mRNA in Pacific oyster *C. gigas*, (2) to determine the sub-cellular localization of *Cg*AATase protein in oyster granulocytes, (3) to isolate the granulocytes by immunomagnetic bead coated with the anti-*Cg*AATase monoclonal antibody, and hopefully to find an effective and specific marker and establish a potential isolation method of granulocytes for future study on developmental and differentiation.

2. Materials and methods

2.1. Oysters, hemocytes and tissues collection

Adult Pacific oysters, with an average length of 13.0 cm, were collected from a commercial farm in Dalian, Liaoning Province, and acclimated in filtered and aerated seawater at 18–20 °C for a week before subsequent experiments.

After an adaptation period, hemolymph was extracted from the hematococoel using a sterile syringe with pre-cooled modified Alsever's solution (MAS, glucose 20.8 g/L, sodium citrate 8.0 g/L, ethylenediamine tetraacetic acid 3.36 g/L, sodium chloride 22.5 g/L, pH 7.5) at the ratio of 1:1. After centrifugation at 800 × *g*, 4 °C for 10 min, the hemocyte pellets were resuspended in modified L-15 medium (M-L15, supplemented with 0.54 g/L KCl, 0.6 g/L $CaCl_2$, 1 g/L $MgSO_4$, 3.9 g/L $MgCl_2$, 20.2 g/L NaCl) for the later cell sorting and immunofluorescence staining experiments. Meanwhile, the tissues of hepatopancreas, mantle, gonad, gill, hemocytes, ganglion, adductor muscle and labial palp were collected from six adult oysters for mRNA and protein extraction.

Three subtypes of hemocytes (granulocytes, semi-granulocytes and agranulocytes) were separated by the 55%/30% percoll® density gradient. The hemocyte sub-populations were immersed in TRIzol™ Reagent (Thermo Fisher Scientific, USA) for RNA isolation according to previous reports [17].

Twenty oysters were randomly divided into experimental and control groups, and they received an injection of 100 μL live *V. splendidus* suspended in PBS (NaCl 137 mmol/L, KCl 2.7 mmol/L, Na_2HPO_4 4.3 mmol/L, KH_2PO_4 1.4 mmol/L, pH 7.4) at 2×10^8 CFU/mL and an injection of 100 μL PBS, respectively. The hemocytes were randomly sampled at 0, 3, 6, 9, 12, 24, 48, 72 and 96 h after injection.

2.2. cDNA synthesis and gene cloning of full-length cDNA

Total mRNA was extracted from samples using TRIzol™ Reagent according to the manufacture's protocol. The quality and quantity of extracted mRNA were evaluated by Nanodrop 2000 and electrophoresis on 1% agarose gel, respectively. The first strand of cDNA was synthesized using total mRNA (treated with DNase I) as template and oligo dT-adaptor as primer according to the protocol of manufacturer (TaKaRa, Japan). The synthesis reaction was performed at 42 °C for 1 h, and terminated by heating at 95 °C for 5 min. The cDNA template was diluted to 1:30 for subsequent experiments and stored at −80 °C.

Specific primers P1 and P2 (Table 1) were designed based on the predicated *Cg*AATase (GenBank accession number: XM_020065799) and used to clone the open reading frame (ORF) of *Cg*AATase from *C. gigas*. The PCR products were purified and confirmed through sequencing after transformed into competent cells of *Escherichia coli* Trans5α (TransGen Biotech, China). The sequences were analyzed with the Simple Modular Architecture Research Tool SMART 7.0 (http://smart.embl-heidelberg.de/).

Table 1
Primers used in this paper.

Primers	sequence (5′-3′)
Clone primers	
P1(Forward)	ATGGTTGATCCGGGACATTT
P2(Reverse)	TTACAAATTACTATAATTCTGTA
Recombination primers	
P3(Forward)	GGGTTTCATATGGTTGATCCGGGACATTT
P4(Reverse)	CCGCTCGAGCAAATTACTATAATTCTGTA
RT primers	
P5(Forward)	CAACGACTGTCTCAAGATGCGG
P6(Reverse)	ACAAACCATCGCCTCCGTCA
P7 (EF-RTF)	AGTCACCAAGGCTGCACAGAAAG
P8 (EF-RTR)	TCCGACGTATTTCTTTGCGATGT
Sequencing primer	
M13-47	CGCCAGGGTTTTCCCAGTCACGAC
RV-M	GAGCGGATAACAATTTCACACAGG
T7-T	TGCTAGTTATTGCTCAGCGG
T7-P	TAATACGACTCACTATAGGG

2.3. Real-time PCR analysis of CgAATase mRNA expression

Quantitative real-time PCR (qRT-PCR) was carried out with SYBR Green Master Mix (TaKaRa, Japan) using a Light Cycler 7500 Real-Time PCR System (Applied Biosystems®, USA). Primers P5 and P6 (Table 1) were used for the qRT-PCR analysis, and the dissociation curve analysis of amplification products about 233bp was performed at the end of each PCR to confirm the specificity. The relative expression levels of *Cg*AATase in different tissues and three subtypes of hemocytes were normalized to the expression of *Cg*EF (Elongation Factor)-1α, and analyzed by the $2^{-\Delta\Delta CT}$ method according to previous description [40].

2.4. Recombinant expression and purification of CgAATase protein

PCR was performed using primers P3 and P4 with *Nde*I and *Xho*I sites (Table 1), which were designed according to the ORF sequence of *Cg*AATase. The amplified product with *Nde*I and *Xho*I sites was inserted into the *NdeI*/*Xho*I sites of expression vector pET-22b (Novagen, Germany) using T4 DNA ligase. The recombinant plasmid (pET-22b-*Cg*AATase) was transformed into *E. coli* Transetta (DE3) (TransGen Biotech, China). After induced by isopropy-b-D-thiogalactoside (IPTG), r*Cg*AATase was purified by Ni^{2+} chelating Sepharose column (Sangon Biotech, China), pooled by elution with 400 mmol/L imidazole under denatured condition (8 mmol/L urea). r*Cg*AATase was refolded against gradient urea dialysis buffer (50 mmol/L NaCl, 50 mmol/L Tris-HCl, 10% glycerol, 0.2 mmol/L oxide glutathione, 2 mmol/L reduced glutathione, a gradient urea concentration of 6, 5, 4, 3, 2, 1, and 0 M, pH 8.0, each gradient at 4 °C for 12 h). After dialyzed three times in PBS buffer at 4 °C for 12 h, the resultant protein was detected by reducing 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and visualized with coomassie bright blue R250. The concentration of purified r*Cg*AATase was quantified by BCA method [41]. The obtained protein was stored at −80 °C before use.

2.5. Monoclonal antibody preparation and Western blotting analysis

The preparation of monoclonal antibody was conducted according to previous report [42] with minor modification. One hundred microliter of r*Cg*AATase (1 mg/mL) was emulsified with 100 μL complete and incomplete Freund's adjuvant (Sigma, USA) to immunize BALB/C female mice of six weeks as previous description [43]. Three days after the fourth boost, the splenocytes were collected from immunized BALB/C female mice, and fused with SP2/0 myeloma cells at a ratio of 4:1 using pre-warmed polyethylene glycol (PEG 2000) (Roche, Switzerland). The hybridoma cells were resuspended with RPMI 1640 medium (GE, USA) containing HAT (hypoxantin, aminopterin, thymidin) (Sigma, USA) and 20% Fetal Bovine Serum (Gibco, USA), and then distributed in 96-wells microplate at a density of 100 μL/well [44]. Three weeks later, the hybridoma cells became macroscopically visible, and the culture supernatants were screened for positive reactivity by ELISA. The cells with positive reacting supernatant were selected and cloned by limiting dilution for three times.

2.6. Immunomagnetic bead separation

To isolate high purity and live granulocytes, immunomagnetic beads (Sangon Biotech, China) were balanced with 3 mL magnetic activated cell sorting (MACS) buffer (M-L15 medium containing 2 mM EDTA and 0.5% BSA) according to the manufacturer's instructions. They were incubated with anti-*Cg*AATase monoclonal antibody at room temperature for 1 h. Meanwhile, the hemocytes were blocked with MACS buffer at room temperature for 1 h. The anti-*Cg*AATase antibody-coated beads were then incubated with the hemocytes at room temperature for 1 h. After three times rinse with MACS buffer and magnetic adsorption, the magnetically labeled and unlabeled hemocytes were collected, resuspended in M-L15 medium, and analyzed by FCM, respectively.

2.7. Immunofluorescence assay of C. gigas granulocytes

Sub-cellular localization of *Cg*AATase on isolated granulocytes was determined by immunohistochemistry. The granulocytes, harvested from the labeled magnetical hemocytes as mentioned above, were deposited on the clean slides in the wet chamber, and incubated for 3 h to adhere the surface of glass slides. The supernatant was dislodged, and 4% PFA (Sangon Biotech, China) was used to fix the granulocytes at room temperature for 1 h. After three times of washing with PBST, the slides were blocked with 200 μL of 3% BSA in PBST at 37 °C for 1 h. The supernatant was removed, and the slides were incubated with 200 μL monoclonal antibody of anti-*Cg*AATase (diluted 1:1000 in blocking buffer). After washing three times with PBST, the slides were incubated with Alexa Fluer 488-labeled goat-anti-rat antibody (diluted 1:2000 in locking buffer, Sigma, USA) as the second antibody at 37 °C for 1 h. After three times washing with PBST, DAPI (diluted 1:1000 in PBS, Solarbio life sciences, China) and Dil (diluted 1:1000 in PBS, Beyotime technology, China) were added on the surface of slides to stain the nucleus and membrane, respectively. After the final three times of wash, the slides were mounted with buffered glycerin (50%) for observation by Fluorescence microscope (ZEISS, Germany).

2.8. In vitro cultivation of C. gigas granulocytes

Primary culture of the isolated granulocytes was cultivated in 12-wells microplate with 2 mL M-L15 medium (supplemented with 1% gentamycin and 1% streptomycin) at 28 °C. The medium was replaced every fourth day by replacing 3/4 of the spent medium with an equal volume of fresh and pre-warmed medium [45].

2.9. Statistical analysis

All data were subjected to analyze and graph by ANOVA and Student's t-test. Differences were considered as significant at $p < 0.05$. All data were represented as mean ± standard error ($N \geq 3$).

3. Results

3.1. The characters of CgAATase gene

The complete ORF of *Cg*AATase gene was of 1431 bp, encoding a peptide of 476 amino acids with a predicted molecular weight of 54.5 kDa (Fig. 1). There was an AATase domain from 25 to 389 amino acids in protein sequence of *Cg*AATase (Fig. 2).

3.2. Recombinant protein of CgAATase and the specificity of its monoclonal antibody

The recombinant protein of *Cg*AATase (r*Cg*AATase) containing AATase domain was expressed and purified by the His-tag Protein Fusion and Purification System. A distinct band with molecular weight of 56 kDa was revealed by SDS-PAGE, which was consistent with the predicted molecular mass of *Cg*AATase (54.5 kDa) together with a "LEHHHHHH-" tag (about 1.6 kDa) (Fig. 3A).

According to the hybridoma technology, the positive hybridoma cells were selected after the fusion, and cloned by limiting dilution for the first time. The single hybridoma cell cluster that secreted monoclonal antibody reacting specifically with granulocytes was obtained, and then the stable and single hybridoma cell cluster was cloned by limiting dilution for two times (Table 2). The specificity of monoclonal antibody was investigated by Western blotting assay, and there was a clear reaction band about 54.5 kDa (Fig. 3B).

```
1     ATGGTTGATCCGGGACATTTTCATTTTCAATACAACGACTGTCTCAAGATGCGGTATGAA
1      M  V  D  P  G  H  F  H  F  Q  Y  N  D  C  L  K  M  R  Y  E
61    AGAAAACTGGGACCTCTAGAAACCATGTACCATGTTTATTACATTCGTGGTGTAGACATC
21     R  K  L  G  P  L  E  T  M  Y  H  V  Y  Y  I  R  G  V  D  I
121   TATGCCCAAATGGCTACCCTGATGTGCGAAAAATTTGTCACCAAAGCGGAAGTGACGGAG
41     Y  A  Q  M  A  T  L  M  C  E  K  F  V  T  K  A  E  V  T  E
181   GCGATGGTTTGTTTGTTAAAAAGACACCCAATGTTGAGGATGACAATCAAAGAAACAGAC
61     A  M  V  C  L  L  K  R  H  P  M  L  R  M  T  I  K  E  T  D
241   AAGGAATACCCCGAGTTTAAATTCGTCGAAATGAACCCTGTCAAACTTGACATTCAAGTT
81     K  E  Y  P  E  F  K  F  V  E  M  N  P  V  K  L  D  I  Q  V
301   TCTTCTTCTTGTGACAAAGAAACTCTATTACACGACGAATCGTGCAAATCCTTTGCTGTA
101    S  S  S  C  D  K  E  T  L  L  H  D  E  S  C  K  S  F  A  V
361   AAAGACGGTCCGTTATGGAGACTGACGATCGTGAGAAATACGAATGGAGAACCAATTGGA
121    K  D  G  P  L  W  R  L  T  I  V  R  N  T  N  G  E  P  I  G
421   CCAAGAGCAAACGGCCACGAGTTTTCTTTCGTGTTCTGTTTTCATCACAGTTTAGCTGAT
141    P  R  A  N  G  H  E  F  S  F  V  F  C  F  H  H  S  L  A  D
481   GGAATTTACCTTCGGACTCTCTTTGCCGATTTCATTGAATTCTTGGACATGGTTCAAAGA
161    G  I  Y  L  R  T  L  F  A  D  F  I  E  F  L  D  M  V  Q  R
541   GAGACGATAGATGCGAGTTCTGTGAAAGAAATCGAGATTCTTCCCGCCATTGAGTACCTC
181    E  T  I  D  A  S  S  V  K  E  I  E  I  L  P  A  I  E  Y  L
601   CTTCCCTTATTAAACCCCAATCGCAGGGTGGTCTCTTTGGATTGTAACAGCGGAGATCCT
201    L  P  L  L  N  P  N  R  R  V  V  S  L  D  C  N  S  G  D  P
661   TCAAAATTCATCGATGCTCTTCCAGCGTATGAGGCACACTTCTATGATGAAATCGAACAG
221    S  K  F  I  D  A  L  P  A  Y  E  A  H  F  Y  D  E  I  E  Q
721   TTACGGAACCAAAAACAATCGACAAAGTCCATTCGATCCCATTTACAGTCAACGAAGTCT
241    L  R  N  Q  K  Q  S  T  K  S  I  R  S  H  L  Q  S  T  K  S
781   AAGAAATTCCTACTGCAATGCAAGCAAAACAGTGTGACAGTTACGGGTGCCTGCATTGCG
261    K  K  F  L  L  Q  C  K  Q  N  S  V  T  V  T  G  A  C  I  A
841   GCGTCTTGTATTGCGTTTGGACAGTTGATCAAATCTTCAATAGCAGACGATGTTAAAGTC
281    A  S  C  I  A  F  G  Q  L  I  K  S  S  I  A  D  D  V  K  V
901   TTAATGATTCCAGTGGAGATTATGGTAAATATGAGGCGTTATACGCAGCAAAACAGCTTG
301    L  M  I  P  V  E  I  M  V  N  M  R  R  Y  T  Q  Q  N  S  L
961   TATCAAGCCTACCCAGGGGTCGCTGCCGTACACCTTCCGTTAACAGTCAAACTTCCGCTC
321    Y  Q  A  Y  P  G  V  A  A  V  H  L  P  L  T  V  K  L  P  L
1021  GTTGAGAACCCGCGGGAACACTTTTGGTACCTTGCAAAACAGTGCACTGACGATATTAAT
341    V  E  N  P  R  E  H  F  W  Y  L  A  K  Q  C  T  D  D  I  N
1081  AACAAGATCATATCCGGTTATCCCTTGGAATATATGTCCACCGAGGTAGCCCAAGAAATT
361    N  K  I  I  S  G  Y  P  L  E  Y  M  S  T  E  V  A  Q  E  I
1141  CACTCCACGCCCAACACGGGAAAATCTCCCTACGTCCTGTGCATAACCAATATGTCGACA
381    H  S  T  P  N  T  G  K  S  P  Y  V  L  C  I  T  N  M  S  T
1201  GTTGATGGCATCGTGAAGCCAAACCAGAGGGCGCGGTTCCAGTTGAAGGAGTTTCCCGCC
401    V  D  G  I  V  K  P  N  Q  R  A  R  F  Q  L  K  E  F  P  A
1261  ATGACGCAAATCGGAATCGACGACATGCCGATTTTCTATGTCGGGATTTTATCCATGGCG
421    M  T  Q  I  G  I  D  D  M  P  I  F  Y  V  G  I  L  S  M  A
1321  CAGGAACTTCATTTGGATATCGGACACTGTCAACGATATACGTCACAATCTACGGCGGCC
441    Q  E  L  H  L  D  I  G  H  C  Q  R  Y  T  S  Q  S  T  A  A
1361  AAATTCTCGTGGAATGTAATGTCCATGTTACAGAATTATAGTAATTTGTAA
461    K  F  S  W  N  V  M  S  M  L  Q  N  Y  S  N  L  -
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Fig. 1. The Nucleotide and deduced amino acid sequence of *Cg*AATase.
The nucleotides and amino acids are numbered along the left margin. The dash (−) indicates the stop codon. The AATase domain is shadowed.

3.3. The expression pattern of *CgAATase*

qRT-PCR was performed to analyze the mRNA expression of *Cg*AATase. The mRNA transcripts of *Cg*AATase were expressed in all the eight tested tissues including hepatopancreas, mantle, gonad, gill, hemocytes, ganglion, adductor muscle, and labial palp. The significant highest expression of *Cg*AATase mRNA was detected in hemocytes, which was about 37.31-fold ($p < 0.05$) higher than that in hepatopancreas. There were no significant differences among the other seven tested tissues (Fig. 4A).

The three subtypes of hemocytes were separated by Percoll® gradient with optimized concentration and volume. Three distinct layers were separated after centrifugation. The granulocytes, semi-granulocytes and agranulocytes accounted for 32.27 ± 2.61%, 40.01 ± 1.92% and 27.63 ± 1.60% of the total hemocytes, respectively [17]. The mRNA expressions of *Cg*AATase in three subtypes of hemocytes were further examined by qRT-PCR. The highest expression level of *Cg*AATase mRNA was detected in granulocytes, which was about 8.26-fold ($p < 0.05$) and 2.80-fold ($p < 0.05$) of that in agranulocytes and semi-granulocytes, respectively (Fig. 4B).

3.4. The temporal mRNA expression of *CgAATase* post *V. splendidus* stimulation

The mRNA expressions of *Cg*AATase in hemocytes were detected at 0, 3, 6, 12, 24, 48, 72 and 96 h after *V. splendidus* stimulation. The expression level of *Cg*AATase was gradually up-regulated from 3 h post *V. splendidus* stimulation, and ascended to the peak level at 6 h, which was 27.40-fold ($p < 0.05$) higher than that in control group. Then, it was gradually decreased, and still significantly higher than that in control group (12 h: 20.96-fold, $p < 0.05$; 24 h: 11.29-fold, $p < 0.05$; 48 h: 7.25-fold, $p < 0.05$; 72 h: 9.20-fold, $p < 0.05$). At 96 h after *V. splendidus* stimulation, the expression level of *Cg*AATase recovered to the normal level with no significant difference compared with that in the control group (0.81-fold, $p > 0.05$) (Fig. 5).

3.5. The isolation and characterization of granulocytes via anti-*CgAATase* monoclonal antibody

Two subpopulations of *C. gigas* hemocytes, P1 and P2 (P1: agranulocytes and semi-granulocytes; P2: granulocytes), were separated by the surface marker of granulocytes, and they were scattered in separated plots according to the granule intensity_MC_Ch06 and Normalized Frequency. Agranulocytes and semi-granulocytes accounted for a high percentage (88.1 ± 3.94%) of the total hemocytes, while granulocytes made up a low percentage of 11.2 ± 3.63% (Fig. 6). After incubated with anti-*Cg*AATase antibody-coated bead, the magnetically labeled and unlabeled hemocytes were separated by immunomagnetic bead with magnetic adsorption. The P2 magnetically labeled hemocytes displayed the same morphologic characters as the granulocytes with high granule intensity_ MC_Ch06 and the biggest cell size approximately 12–15 μm in diameter, numerous large granules in cytoplasm, and a relatively small N:C ratio under the flow cytometry. The membrane of labeled hemocytes was also observed coating with many immunomagnetic beads (Fig. 7A), and the *Cg*AATase positive hemocytes accounted for 85.7 ± 4.60% of the total hemocytes (Fig. 7B). The P1 unlabeled hemocytes were consistent with agranulocytes and semi-granulocytes in a low granule intensity_ MC_Ch06, and characterized with smaller cell size (5–12 μm) and fewer granules, no immunomagnetic bead on the membrane (Fig. 7C), and the purity rate was up to 97.7 ± 1.01% (Fig. 7D).

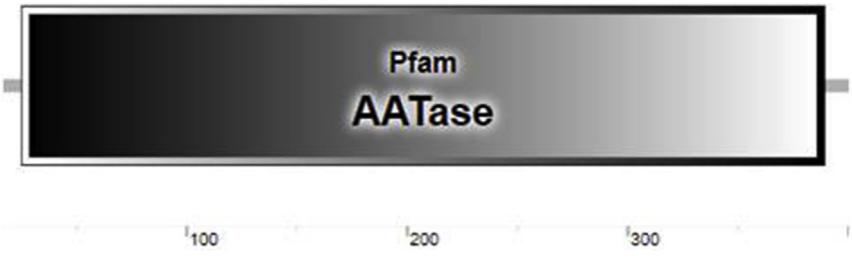


Fig. 2. The protein domain of *Cg*AATase predicted by SMART (http://smart.embl.de/).

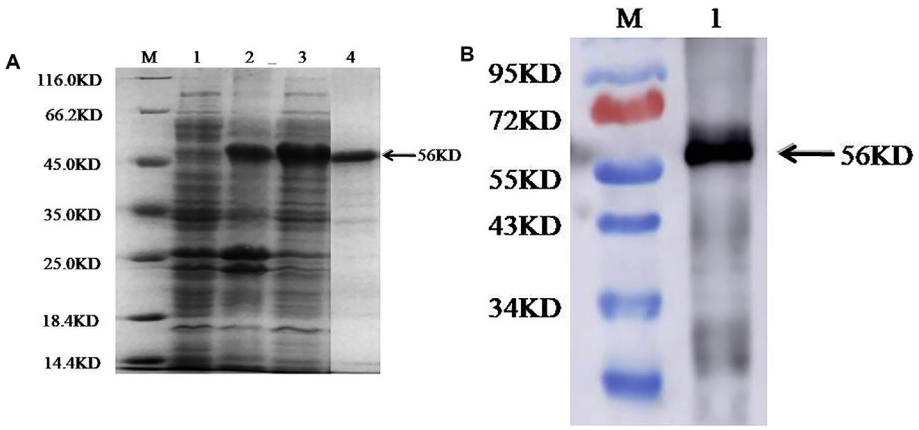


Fig. 3. The recombination protein of *Cg*AATase and the specificity of its monoclonal antibody.
A: SDS-PAGE analysis of r*Cg*AATase. Lane M: Standard protein molecular weight marker; Lane 1: Negative control (without IPTG induction); Lane 2: The supernatant of induced r*Cg*AATase; Lane 3: The sedimentation of induced r*Cg*AATase; Lane 4: The purified r*Cg*AATase;
B: Western bolt of r*Cg*AATase protein with *Cg*AATase monoclonal antibody. Lane M: Standard protein molecular weight marker; Lane 1: r*Cg*AATase protein.

Table 2
Positive values of monoclonal antibodies.

Hybridoma cell	First generation	Second generation	Third generation
1.04	1.53	0.96	1.09

Negative value (PBS) = 0.09.

3.6. The sub-cellular localization of CgAATase in C. gigas granulocytes

The sub-cellular localization of *Cg*AATase on granulocytes was determined by immunohistochemistry assay. After incubation of granulocytes with anti-*Cg*AATase monoclonal antibody, the positive signals of FITC labeled antibody were detected in green fluorescence, which were mainly located on the membrane of granulocytes. Dil and DAPI were employed to stain membrane and nucleus of granulocytes in red and blue fluorescence, respectively (Fig. 8).

3.7. In vitro cultivation of C. gigas granulocytes

The isolated granulocytes were cultured in M-L15 medium, and their morphological features were examined everyday with light microscope. After one week cultivation, granulocytes still maintained good cell viability. Highly viable and a dynamic development of filopodia relating to adherence were observed under light microscope

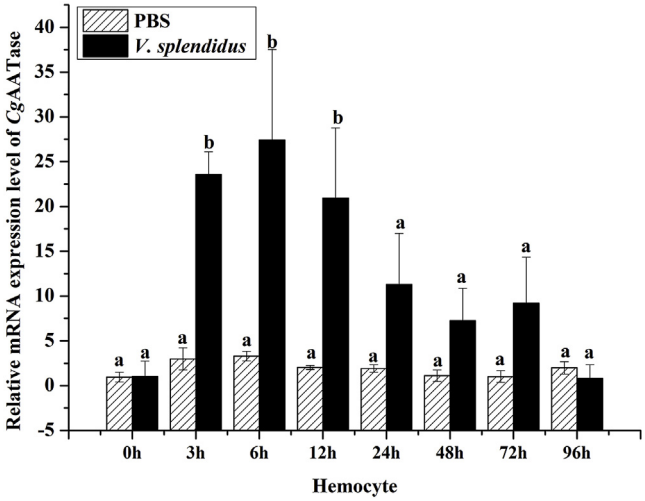


Fig. 5. The mRNA expression pattern of *Cg*AATase after *V. splendidus* stimulation.
The oyster Elongation Factor (EF, GenBank accession NM_001305313) gene was used as an internal control to calibrate the cDNA template for all the samples. Each value was shown as mean ± S.D. (Vertical bars represent the mean ± S.D.) (N ≥ 3).

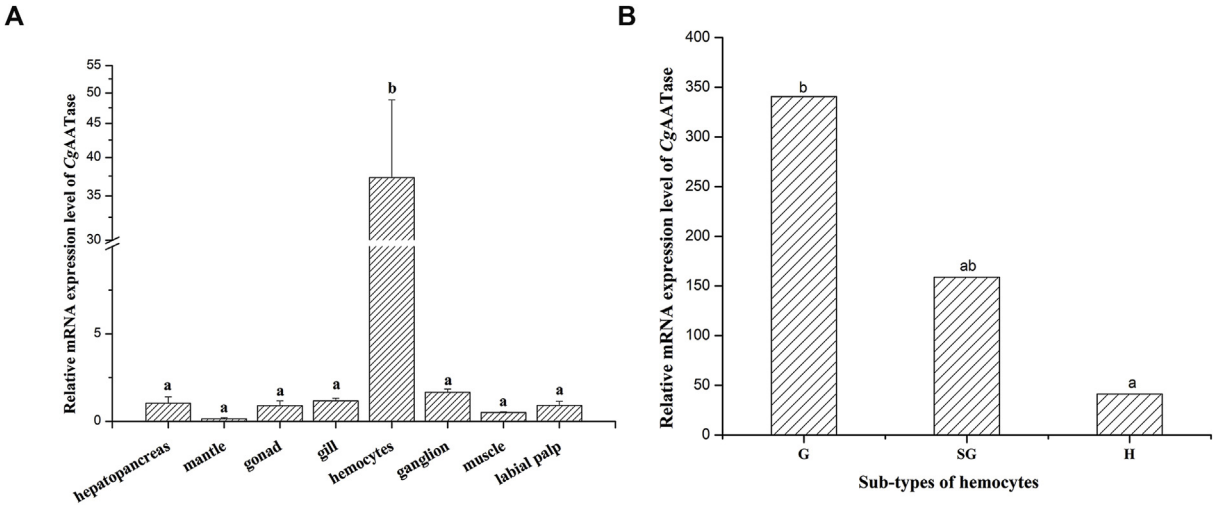
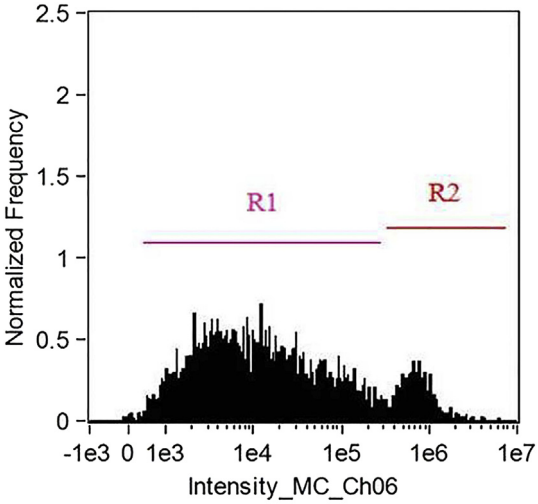


Fig. 4. Expression of *Cg*AATase mRNA in various tissues and three sub-types of oyster hemocytes.
A: *Cg*AATase mRNA expression levels (relative to Elongation Factor) in different tissues hepatopancreas, mantle, gonad, gill, hemocytes, ganglion, adductor muscle and labial palp were normalized to that of hepatopancreas. Vertical bars represent the mean ± S.D. (N ≥ 3) for each tissue. The different letters (a, b) indicated significant differences ($p < 0.05$, ANOVA). Vertical bars represent the mean ± S.D. (N ≥ 3)B: *Cg*AATase mRNA expression levels in the three subtypes of hemocytes.



Intensity_MC_Ch06

Population	Count	%Gated
All	5000	100
R1	4405	88.1 ± 3.94
R2	560	11.2 ± 3.63

Fig. 6. The total hemocytes analyzed by Flow cytometry.
R1: semi-granulocytes and agranulocytes; R2: granulocytes.

(Fig. 9). Dead cells loosed adherence were observed floating, and they were pipetted off during replacement of medium.

4. Discussion

Granulocytes have been morphologically characterized in Pacific oyster *C. gigas*, and considered as the main immunocompetent hemocytes. However, the specific immunological function, ontogenesis as well as differentiation of granulocytes are remained unclear, in part due to the limitation of specific markers. In the present study, *Cg*AATase was identified as a potential surface marker for granulocytes in Pacific oyster *C. gigas*, which could be used for cell sorting of high purity granulocytes and further molecular cytological studies.

The AATases are bisubstrate enzymes to catalyze the transfer of acyl chains from an acyl-coenzyme A (CoA) donor to an acceptor alcohol [46,47]. A number of AATases and orthologs containing AATase domain have been identified in yeasts [48], plants [49] and bacteria species [50], and they are confirmed to be involved in metabolic engineering of ester biosynthesis and enzyme engineering [51]. However, the relevant report about AATases is still very limited in animal kingdom. In the present study, an AATase (*Cg*AATase) with a typical AATase domain was first identified in *C. gigas*, and its potential function was further investigated.

The biological functions of AATase are of membrane dependent, and it play roles in ester biosynthesis and metabolic engineering [52,53]. A series of investigations have now defined the contribution of AATase to ester production in *Saccharomyces cerevisiae* at the cellular level [46]. The esters are central part of metabolism and play critical roles in immune system [54]. The esters have been identified to regulate the immune cell activation and differentiation in the Macrophages and T lymphocytes [55,56]. The function and phenotype of immune cell are related of various esters. For example, the pathways promoting ester synthesis and accumulation tend to drive a pro-inflammatory phenotype, while pathways enhancing ester efflux push immune cells toward an anti-inflammatory phenotype [55]. In the present study, *Cg*AATase was found to be highly expressed in hemocytes compared to that in other tissues, and its mRNA expression level in hemocytes was significantly up-regulated after *V. splendidus* stimulation, indicating its involvement in immune response.

Immune defense is an energy consuming process, and the organism has to reallocate energy metabolism to meet the demands of immune response, in return, the systemic metabolic switch is regulated by adenosine released from immune cells [57]. AATase has been reported that regulated the energy during the immune response by mediating ester biosynthesis and hydrolysis. The energy metabolic pathways are distinctive in different types of immune hemocytes or the same type immune hemocytes under different conditions [58,59]. Interestingly, *Cg*AATase was found to be mainly expressed in granulocytes, and granulocytes had been demonstrated as the most immune-active hemocytes in oyster [17]. Immunofluorescence assay corroboratively revealed that *Cg*AATase was located at the cell membrane of granulocytes. Immune cells require ester during proliferation to maintain and remodel cell membrane during the immune response. It was assumed that *Cg*AATase might play a certain role through the regulation of energy metabolism pathway in the immune system. In addition, *Cg*AATase is also necessary for production of esters which comprise the cellular membrane derived signaling mediators. *Cg*AATase provides a potential molecular marker *in vivo* to study the metabolic changes and their regulation during immune response. Specific cell markers have been proved to be effective in clarifying the function of hemocytes. In vertebrates, CDs are usually used as the specific molecular markers of different type granulocytes, such as CD11b, CD16 and CD66b for neutrophils, CD69 and CD11b for eosinophils, and CD63 and CD203c for basophils. In the silkworm *Bombyx mori*, BmintegrinαPS3, α subunit of integrin, was specifically expressed in larval granulocytes, and demonstrated to be a specific marker for granulocytes [39]. Taken together, *Cg*AATase could be used as specific marker for the isolation of oyster granulocytes, which would allow the identification to be based on molecular feature rather than morphological criteria. It also provides a useful tool to separate sub-populations of hemocytes and go into their origin, development, and more immunological roles [60].

Magnetic bead with the ability to bind a variety of antibodies has been applied to positive selection of cells. For example, approximately 98% of rat sinusoidal endothelial cells (SEC) could be isolated by virtue of a monoclonal antibody (SE-1) that recognized a membranous antigen expressed in SECs [61]. Similarly, the granulocytes were successfully separated using immunomagnetic bead coated with anti-*Cg*AATase monoclonal antibody, and exhibited active cell viability after cultivated *in vitro*. Cultivation of cells *in vitro* has established numerous cell lines of insect species (silkworm and mosquito) as diverse as plants and humans, and provides a helpful tool in attain a deeper understanding of developmental biology, biochemistry, and functional genomics [45]. However, due to the sensitivity and reactivity of mollusc hemocytes, it is difficult to perform functional studies because the coagulation reaction and uncontrolled cell activation regularly occur when the mixed hemocyte samples are investigated *in vitro*. Only one mollusk cell line has been established [62,63], and the primary cell culture from a variety of molluscan species have to be used for the studies of complex physiological processes. *Cg*AATase could provide a cell surface biomarker to enrich or purify the granulocytes from oyster.

In conclusion, a potential cell surface marker *Cg*AATase for granulocytes was identified for the first time in oyster *C. gigas*. *Cg*AATase was membrane associated and special highly expressed in granulocytes. The mRNA expression of *Cg*AATase was significantly increased after *V. Splendidus* stimulation, suggesting that *Cg*AATase might play significant role in the immune response. With the aid of anti-*Cg*AATase monoclonal antibody, oyster granulocytes could be separated effectively with highly purity, which provide a useful tool for the biological analysis of granulocytes.

Acknowledgements

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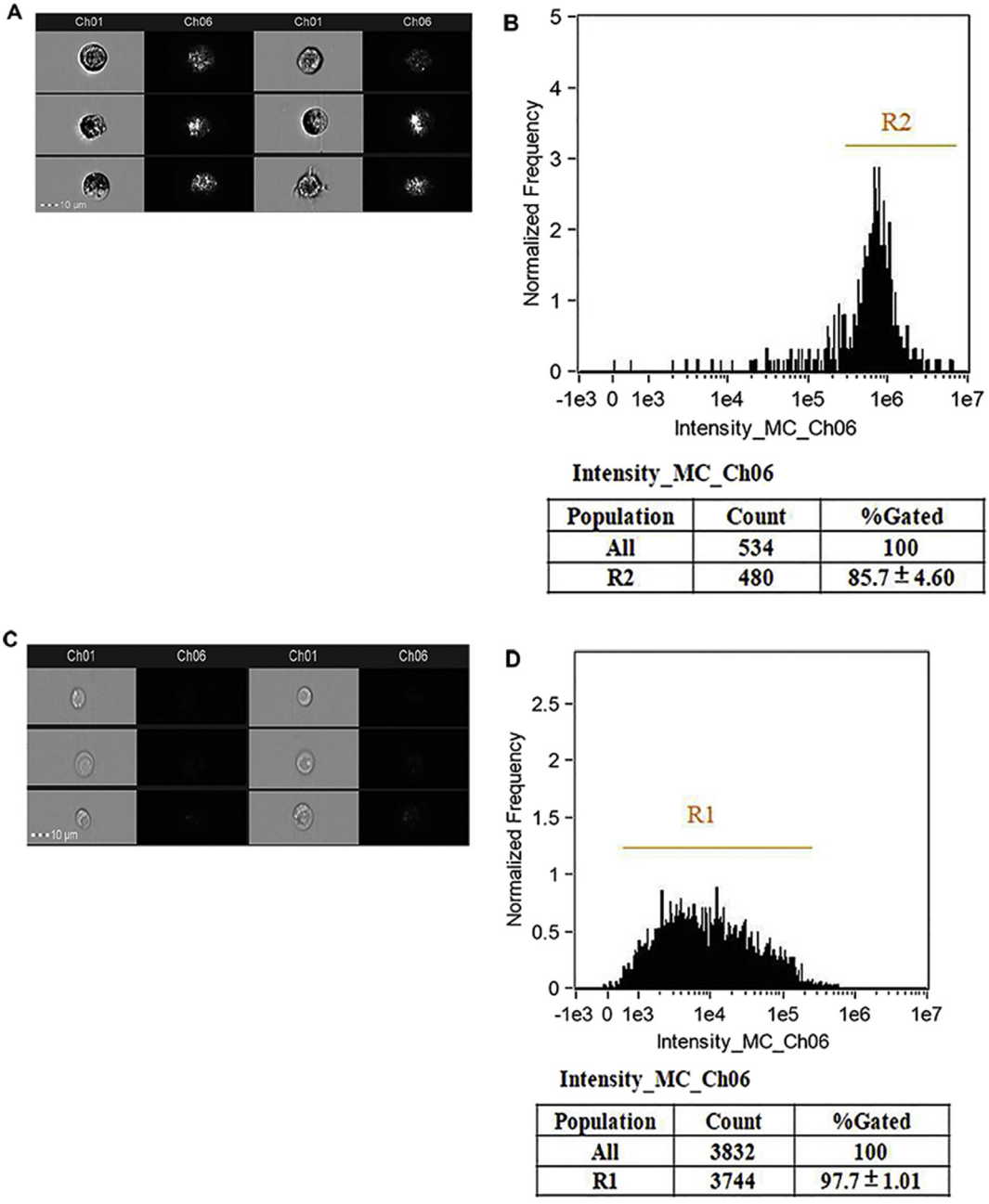

Intensity_MC_Ch06

Population	Count	%Gated
All	534	100
R2	480	85.7 ± 4.60

Intensity_MC_Ch06

Population	Count	%Gated
All	3832	100
R1	3744	97.7 ± 1.01

Fig. 7. The characterization of the labeled and unlabeled magnetical hemocytes separated with immunomagnetic bead by Flow cytometry analysis
A: The labeled granulocytes with the immunomagnetic bead on their membrane; B: The purity of labeled granulocytes; C: The unlabeled semi-granulocytes and agranulocytes; D: The purity of semi-granulocytes and agranulocytes.

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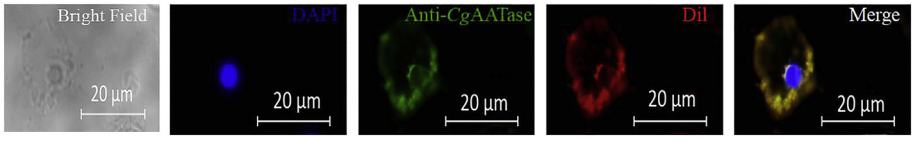

Fig. 8. Subcellular localization of *Cg*AATase in *C. gigas* granulocytes.
r*Cg*AATase was found to be located on the cell membrane of *C. gigas* granulocytes in green conjugated to Alexa-fluor 488. The contour of cells was shown in bright field. The nucleuses were stained with DAPI in blue, and the membrane was stained with DiI in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

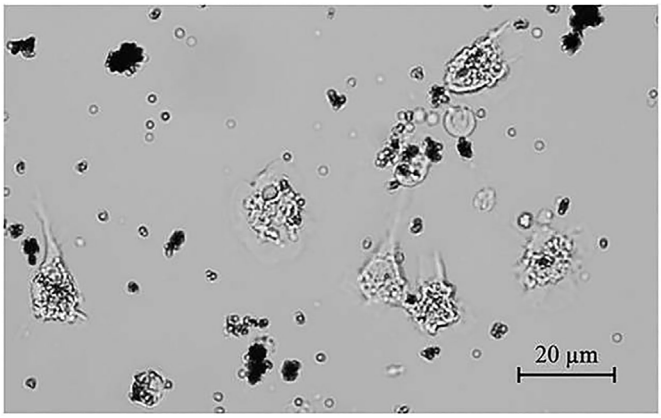

Fig. 9. Cultured granulocytes of oyster *C. gigas in vitro*.

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