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著者	Lin Haisheng, Ishizaki Shoichiro, Nagashima Yuji, Nagai Kiyohito, Maeyama Kaoru, Watanabe Shugo
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細菌の接種によって誘導されるアコヤガイ中の抗菌性タンパク質の探索

林　海生（海洋大・広東海洋大，中国）
石崎　松一郎，長島　裕二（海洋大）
永井　清仁　（ミキモト真珠研）
前山　薫　（御木本製薬）
渡部　終五　（北里大海洋）

本研究で対象とするアコヤガイは，日本において重要な養殖真珠の母貝として用いられる二枚貝である。腸炎ビブリオをアコヤガイ閉殻筋に直接接種したところ，鰓から得られた酸抽出物に，非接種の対照よりも強い抗菌活性を示す成分が存在することを見出した。酸抽出物はグラム陽性菌および陰性菌に抗菌活性を示し、とくにビブリオ属に強く作用した。鰓より 2 種の抗菌タンパク質 APg-1（分子量約 210 kDa）および APg-2（分子量約 30 kDa）を分離し、これらは MALDI-TOF MS 分析により，新規の抗菌タンパク質である可能性が推察された。

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Fisheries Sciences: Chemistry and Biochemistry

Exploration of the induced antibacterial proteins in pearl oyster *Pinctada fucata* by bacterial inoculation

Haisheng Lin[1,2] ·Shoichiro Ishizaki[1] ·Yuji Nagashima[1] ·Kiyohito Nagai[3] ·Kaoru Maeyama[4] ·Shugo Watabe[5]

1. Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato, Tokyo 108-8477, Japan

2. College of Food Science and Technology, Guangdong Ocean University, Haida Road 1, Mazhang, Zhanjiang 524088, China

3. Pearl Research Laboratory, Mikimoto Co., Ltd, Shima 517-0403, Japan

4. Mikimoto Pharmaceutical Co., Ltd, Ise 516-8581, Japan

5. School of Marine Biosciences, Kitasato University, Sagamihara 252-0373, Japan

Corresponding author: Dr. Shoichiro Ishizaki, Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato, Tokyo 108-8477, Japan.

TEL&FAX +81-3-5463-0614

E-mail: ishizak@kaiyodai.ac.jp

Haisheng Lin, E-mail: haishenglin@163.com

Yuji Nagashima, E-mail: yujicd@kaiyodai.ac.jp

Kiyohito Nagai, E-mail: k-nagai@mikimoto.com

Kaoru Maeyama, E-mail: maeyama@mail.mikimoto-cosme.com

Shugo Watabe, E-mail: swatabe@kitasato-u.ac.jp

Abstract

The aim of this research was to characterize immune-related antibacterial substances from pearl oyster *Pinctada fucata* induced by bacterial invasion. Bacteria inoculation was performed by injecting 0.1 ml of 1.0×10^{12} CFU/ml *Vibrio parahaemolyticus* into adductor muscle. Acidic extracts were prepared by 0.1 % trifluoroacetic acid from different tissues after 8 hours of injection and antibacterial activity against *V. parahaemolyticus* was determined via the microdilution broth method. The acidic extracts from gills of inoculated oysters (AEg) showed stronger antibacterial activity than those from non-inoculated ones. Based on this result, antibacterial proteins were purified from AEg via two-step gel filtration chromatography, followed by high-performance liquid chromatography using a TSkgel G3000 column. Protein components were analyzed by both sodium dodecyl sulfate and native polyacrylamide gel electrophoresis. As a result, two antibacterial proteins, APg-1 (with a molecular mass of approximately 210 kDa) and APg-2 (of approximately 30 kDa) were obtained from AEg. Matrix-assisted laser desorption/ionization time of flight mass spectrometry analysis and partial amino acid sequences revealed that they might be novel antibacterial proteins. These results indicate that the antibacterial proteins were potentially upregulated in the gill of pearl oysters or released therefrom to defend against the bacterial invasion.

Keywords: pearl oyster ·innate immunity · antibacterial proteins ·bacterial inoculation

Introduction

Marine organisms live in a microbe-rich environment and are under persistent threat of infection by resident pathogenic microbes. Because of their characteristic of filter feeding, bivalve mollusks are continuously and markedly exposed to potential pathogens including bacteria, viruses, fungus, and parasites [1, 2]. To prevent colonization by microbes, bivalve mollusks have developed a number of biologically active organic compounds possessing antibacterial activity, such as peptides, proteins, glycoproteins, terpenes, polypropionates, nitrogenous compounds, polypeptides, macrolides, prostaglandins, fatty acid derivatives, sterols, and other miscellaneous compounds [3]. Similarly to other invertebrates, bivalve mollusks' innate immunity relies on cellular and humoral immunities, which are composed of a comprehensive repertoire of immune cells, genes, and proteins that respond to external aggressions [4]. Upon tissue injury or infection, an acute phase response serves as the core of the immune response involving physical barriers and molecular effectors to prevent infection, clear potential pathogens, and initiate inflammatory processes, contributing to resolution and the healing process [5]. Many defense factors, including lectin (agglutinin) [6], antimicrobial peptides (AMPs) [1], peptidoglycan-recognition proteins [7], lysozymes [8], antibacterial proteins [9], and other substances (pro-phenoloxidase, protease inhibitors, lysosomal enzymes) [10], have been discovered from bivalve species including oysters, scallops, mussels, and clams [11]. Among these defense factors, antibacterial proteins have a diverse group of molecules that provide the first line of defense against invading pathogens by exerting broad-spectrum microbicidal activity [12]. Therefore, studies of antibacterial proteins and their defense mechanisms may provide valuable information leading to new antibiotic discoveries and giving new insights into disease control in aquaculture.

It is well known that adaptive immunity is not obtained by a mild infection with the unmodified pathogen in invertebrates. However, a list of humoral defense factors, such as antibacterial proteins and antimicrobial peptides, could be induced following exposure to pathogenic stimulation [13, 14]. Moreover, stimulation of defense system of the host is considered to be the most important part of biocontrol without using antibiotics for disease control, a new view advocated by Defordt et al. [15]. Therefore, antigen stimulation of the effector of the immune system might provide new insights into the study of innate immunity of ocean bivalves.

The pearl culturing industry started by Kokichi Mikimoto at the end of the 19th century in Japan is famous historically [16]. Pearl oyster *Pinctada fucata* is primarily cultured for seawater pearl production

and the pearl produced by this species is known as "Akoya pearl", which contributes enormous value to marine economic development in Japan. However, the surgery implantation process during pearl production leaves pearl oysters prone to operational injury followed by pathogen infection. Diseases caused by various pathogens during breeding and cultivation practices occur frequently, resulting in significant economic losses [17]. Since 1993, the production of pearl in Japan has been declining because of mass mortalities of cultured pearl oysters. Several reports describe infectious agents, such as viruses, bacteria, and parasites, as a cause [18]. Similar to other invertebrates, the pearl oyster has developed various innate immune components and a set of humoral and cellular immune reactions to address pathogen infection and environment stress. Once the invading pathogens gain entry into the body of the host, they encounter these complex system of innate defense mechanisms, usually at the first barrier of the gill filaments where a large number of hemocytes and glycoproteins are present [19]. In order to control disease and enhance the yields and quality of pearls, it is necessary to investigate the innate immune mechanisms in pearl oysters. Some immune-related molecules have recently been discovered in this species, including lectins (galectin and F-type lectin) [20, 21], oxidoreductase [22], and cytokines (TNF-α factor, interleukin-17, and IRF-2) [17]. However, antibacterial substances derived by stimulation using bacterial inoculation have never been described in the pearl oyster. And whether these antibacterial substances contribute to the innate immune system remains unclear.

To study the immune-related defense factors possessing antibacterial activity, pearl oysters were inoculated by injecting *Vibrio parahaemolyticus* into adductor muscle, after which antibacterial substances were extracted from the gill. Finally, two antibacterial proteins were purified and preliminarily characterized from the gill. To our best knowledge, this is the first report of finding antibacterial proteins from the pearl oyster performed by bacterial inoculation stimulation.

Materials and methods

Materials

The specimens of pearl oysters (about 8.0–9.0 cm in shell length, 2 years old), from Mie Prefecture, were acclimated in tanks containing static aerated seawater in a dark place. The water temperature was raised at a rate of 1 ℃ per day, beginning at 17 °C on the first day. When the temperature reached 23 ℃, the specimens were fed. After one week acclimation, the bacterial inoculation experiment was carried out.

121 **Bacteria inoculation experiment**

122 *V. parahaemolyticus* was cultured in trypticase soy broth (TSB, Becton Dickinson and Company, 123 Sparks, MD, USA) plus 3.0 % NaCl at 37 °C for 18 h, then the incubated enriched broth was centrifuged 124 at 4000 ×*g* for 10 min and *V. parahaemolyticus* was re-suspended in 0.85 % NaCl solution. The plate 125 count method was used for the quantitative assay of the bacterial suspension. The bacterial inoculation 126 experiment was performed by injecting 100 µl of *V. parahaemolyticus* (low dose: 1×10^4 CFU/ml, middle 127 dose: 1×10^8 CFU/ml, high dose: 1×10^{12} CFU/ml, in 0.85 % NaCl) into adductor muscle of each pearl 128 oyster specimen, with 100 µl of 0.85 % NaCl as control. All the specimens (9 individuals of each group) 129 were then put back in the tanks containing static aerated seawater. Eight hours (8 h) post-inoculation, 130 about 0.5 ml of hemolymph was collected from adductor muscle of each individual using an injection 131 syringe, and the mucus was gathered from the surface of the mantle. Different tissues including the 132 mantle, gill, digestive gland (together with gonad), and adductor muscle were split as samples from each 133 group. All these samples were stored at −85 °C for further research.

134 **Preparation of the extracts**

135 The hemolymph, mucus, and different tissues were homogenized with 3 volumes of 0.1 % 136 trifluoroacetic acid (TFA, pH 1.75) and 0.15 M NaCl-0.01 M Tris-HCl buffer (pH 6.8), respectively. 137 After centrifugation at 14,170 ×*g* for 30 min, the supernatants were harvested and then freeze-dried. The 138 lyophilized powders were dissolved with 0.01 M Tris-HCl buffer (pH 6.8) as acidic extracts and neutral 139 extracts, respectively. The protein content was measured by the Bradford method (Quick Start Bradford 140 Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA). The antibacterial activity was determined by 141 the microdilution broth method as described below. Because the acidic extract from the gill of pearl 142 oyster inoculated with 1×10^{12} CFU/ml *V. parahaemolyticus* possessed stronger antibacterial activity 143 than those from the control, it was named as AEg and used for the purification of induced antibacterial 144 proteins.

145 **Assay of antibacterial activity**

146 The antibacterial activity of extracts was examined by the microdilution broth method as reported 147 by Nagashima et al. [23]. The samples were diluted by 2, 4, 8, 16, 32, 64, 128, and 256 fold with distilled 148 water. Aliquots of 100 µl of each dilution were loaded into a 96-well microplate, and then 50 µl of *V.* 149 *parahaemolyticus* (2×10^7 CFU/ml) and 50 µl of TSB-3.0 % NaCl were added to each well. After 150 incubation at 35 °C for 24 h, the growth condition of the bacterium was recorded by observing the

turbidity of each well. Inhibitory titer (IT) was defined as the reciprocal of the highest dilution to inhibit the growth of the bacterium (incubated for 24 h). Then, 50 μl of the incubated mixture in each well was added to 100 μl of fresh TSB-3.0 % NaCl medium and incubated at 35 ℃ for an additional 48 h. The growth condition of the bacterium was again recorded by observing the turbidity. Bactericidal titer (BT) was defined as the reciprocal of the highest dilution to inhibit the growth of the bacterium (incubated for 72 h).

Radial diffusion assay was also carried out as described by Seo et al. [2] with minor modifications to evaluate the antibacterial activity. To determine the antimicrobial activity of the acid extract from the gill (AEg), a wide range of bacteria strains were used, including *V. parahaemolyticus, V. alginolyticus, V. anguillarum, Edwardsiella tarda, E. hoshinae, Shewanella putrefaciens, Escherichia coli, Lactococcus garvieae, Bacillus subtilis,* and *Micrococcus luteus*. First, 100 μl of 1×10^8 CFU/ml bacteria suspension was pipetted into sterile trypticase soy agar (TSA, Becton Dickinson and Company, Sparks, MD, USA) culture medium (*Vibrio* strains: TSA plus 3 % NaCl), vortexed and then poured into a petri dish. Then, 5 μl of sample was pipetted into a 2.0 mm diameter well and the plate was incubated at 37 ℃ for 20 h. The diameter of the clear inhibition zone was measured to the nearest 0.1 mm.

Purification of antibacterial proteins from AEg

The AEg was separated using a Sephacryl S-200 (GE Healthcare, Uppsala, Sweden) gel filtration column (2.6 × 250 cm) equilibrated with 0.15 M NaCl-0.01 M Tris-HCl buffer (pH 6.8) at a flow rate of 0.6 ml/min under monitoring of A_{280} via ultraviolet spectrometer (UV-1800, Shimadzu, Kyoto, Japan). Aliquots of 5.0 ml of each fraction were collected and the antibacterial activity was examined via the radial diffusion assay. The fraction with the strongest antibacterial activity was loaded onto a Superdex 200 column (10/300 GL, GE Healthcare, Pittsburgh, PA, USA) and eluted with 50 mM sodium phosphate buffer containing 0.15 M NaCl (pH 7.0) at a flow rate of 0.7 ml/min. Then the antibacterial proteins were purified by high-performance liquid chromatography (HPLC) using a TSKgel G3000 SWxl column (Tosoh, Tokyo, Japan). The protein components of effective fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12.5 % gel) of samples after heat-denaturation under a reducing condition and native PAGE（12.0 % gel) of samples without SDS and heating, staining with Coomassie Brilliant Blue (CBB, Kanto Chemicals, Tokyo, Japan).

Protein identification and partial amino acid sequence analysis

After analysis by SDS-PAGE, the protein bands were cut out of the gel for in-gel digestion.

Following in-gel reduction by dithiothreitol (1.5 mg/ml, 56 ℃, 30 min), alkylation by iodoacetamide (10 mg/ml, kept in a dark place for 20 min), and destaining (incubated in 50 % MeOH for 15 min × 2 times, and then in 50 % acetonitrile for 10 min × 3 times), proteins were digested by 20 ng/μl trypsin (Sequencing Grade Modified Trypsin, Promega, Madison, WI, USA) at 37 ℃ overnight. After digestion, 0.5 μl of the peptide mixture was mixed using ZipTip Pipette Tips (Millipore, Darmstadt, Germany) with 0.5 μl of α-cyano-4-hydroxycinnamic acid matrix (ABSCIEX, Foster, CA, USA) and then directly transferred to a mass spectrometer target (MTP384 target plate). Mass spectrometry (MS) was performed using a marix-assisted laser desorption/ionization time of flight/time of flight (MALDI-TOF/TOF) analyzer (4800 Plus, Applied Biosystems, Foster city, CA, USA) in the positive reflection mode. The main peptide fragments were subjected to tandem TOF-MS and the data were processed using Data Explorer. The data of the peak list from the MS spectrum was subjected to Matrix Science Mascot Search. The Mascot engine was set specifying National Center for Biotechnology Information nr (NCBI nr) and SwissProt as the databases, peptide mass fingerprint (PMF) as type of search, trypsin as enzyme, carbamidomethyl as fixed modification, monoisotopic as mass value, unrestricted as protein mass, 1.2 Da as peptide tolerance, 0.6 Da as fragment mass tolerance, and 1 as missed cleavage. In addition, de novo sequencing was performed automatically using De Novo Explorer™ software (Applied Biosystems) following the settings reported by Bringans et al. [24]. This software automatically generates candidate sequences and assigns them a score between 0 and 100. The de novo-derived candidate sequences of peptides with the highest score were rechecked based on the protease cleavage sites, and then searched in the NCBI protein database using the Basic Local Alignment Search Tool (BLAST) at http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins.

Results

Antibacterial activity of the acidic extracts

To determine which organ possesses antibacterial substances and whether it has a tissue specific response to bacterial inoculation, the effect of inoculation on antibacterial activity of acidic extracts and neutral extracts was investigated from different tissues, and the results are shown in Table 1 and Table 2. Among the extracts from the control pearl oyster, the acid extracts from the gill, mantle, adductor muscle, and digestive gland showed antibacterial activity against *V. parahaemolyticus*, and the highest antibacterial activity (IT = 128) and bactericidal activity (BT = 64) were found in the mantle extract,

211 while no inhibition was observed in the mucus and hemolymph extracts (Table 1). The neutral extracts
212 from only the gill and mantle were found to possess antibacterial activity, which showed weaker activity
213 than the acid extracts from the same tissue (Table 2). These results suggest that the 0.1 % TFA was proper
214 for the extraction of the antibacterial components from pearl oysters.

215 Moreover, only the gill extracts from inoculated oysters (low, middle, and high doses) were found
216 to have stronger antibacterial as well as bactericidal activity than those from the control (Table 1).
217 However, no significant differences were observed in the antibacterial and bactericidal activities between
218 the inoculated group and the control group extracts from the mantle, adductor muscle, and digestive
219 gland. These results indicate that most of the antibacterial substances exist in the mantle and gill in the
220 normal state, and the immune-related antibacterial substances are potentially upregulated in the gill or
221 released therefrom to defend against invasion after bacterial inoculation.

222 Therefore, to study the antibacterial properties of the gill, the bacterial sensitivity of the acid extract
223 from the gill (AEg) was determined. As shown in Table 3, the AEg displayed a certain antibacterial
224 activity against *V. parahaemolyticus, V. alginolyticus, V. anguillarum, L. garvieae,* and *B. subtilis.* This
225 result suggests that AEg possessed a broad spectrum against both Gram-positive and Gram-negative
226 bacteria, especially against the fish pathogen strains (*Vibrio* strains and *L. garvieae*). The AEg was found
227 to be more effective against three *Vibrio* strains than the Gram-positive strains *L. garvieae,* and *B. subtilis.*
228 According to our research purposes, the strain *V. parahaemolyticus* was selected for the antibacterial
229 activity assay.

230 **Fractionation of the antibacterial substances from AEg**

231 In order to clarify the antibacterial substances induced by bacterial inoculation in the gill, the AEg
232 was subjected to gel filtration chromatography using a Sephacryl S-200 column. Three fractions
233 possessing antibacterial activity were observed on the chromatogram (Fig. 1). Fractions 42-48, named
234 AEgfr1, contained several protein components with molecular weights ranging from approximately 40
235 to 200 kDa. According to the principle of gel filtration, it is strange that a thick protein band of
236 approximately 18 kDa appeared in the SDS-PAGE. This result indicates that the 18 kDa protein might
237 be derived from some proteins in AEgfr1. Fractions 86-90, named AEgfr2, showed an obvious protein
238 band of approximately 30 kDa and another one of about 10 kDa on the SDS-PAGE. However, no protein
239 band was observed in the fractions 108-112, named AEgfr3, which revealed that the antibacterial
240 substances in this fraction were not proteins.

Purification of the antibacterial proteins from AEg

As we focus on the antibacterial proteins, AEgfr1 with the strongest antibacterial activity was further purified by HPLC using a Superdex 200 column (data not shown) and a TSKgel G3000 SWxl column. Finally, an antibacterial protein with a molecular weight of about 210 kDa was separated by gel filtration HPLC, which was named APg-1 (antibacterial protein from the gill). APg-1 showed a single band by native-PAGE and a major band of about 210 kDa by SDS-PAGE, although some bands with molecular masses below 20 kDa were faintly observed on the SDS-PAGE (Fig. 2). In AEgfr2, the band of approximately 30 kDa, named APg-2, was considered to be antibacterial protein, based on the elution profile in Sephacryl S-200 column chromatography.

Characterization of the antibacterial proteins from AEg

To characterize the antibacterial proteins in the gill, the APg-1 of approximately 210 kDa and APg-2 of approximately 30 kDa in SDS-PAGE were cut out for in-gel digestion and then characterized via MALDI-TOF/TOF. The peak lists of PMFs (Fig. 3) were subjected to a Matrix Science Mascot Search using PMF mode. The search results (data not shown) showed low score (not significant) and these PMFs did not match any known proteins from the available database. In order to investigate the primary structures of these proteins, the peptide fragments were subjected to tandem MALDI-TOF/TOF analysis in positive reflection mode and the MS/MS data was processed using De Novo Explorer software to determine the partial amino acid sequences. The de novo sequencing gave a list of candidate peptide sequences (Fig. 3). (1) Partial sequences of APg-1: KVKKGMWW at *m/z* 1062.70 (score: 24.75), SSPVLGCPVR at *m/z* 1071.64 (score: 62.61), RDVRCCPR at *m/z* 1118.67 (score: 70.56), and (759.497) TMCER at *m/z* 1471.88 (score: 84.61) (Fig. 3a). (2) Partial sequences of APg-2: RWMMPAKR at *m/z* 1107.58 (score: 78.90), NCQLSLQSDKK at *m/z* 1320.64 (score: 71.84), and SSKYLLNYSVDKR at *m/z* 1572.89 (score: 82.84) (Fig. 3b). These predicted partial sequences of each protein were submitted to the NCBI protein database for BLAST search. No proteins were found to have sequence similarity to the partial peptide sequences. These results suggest that these proteins might be novel.

Discussion

In this study, we found the antibacterial proteins from the pearl oyster following bacteria inoculation. To our knowledge, this is the first report on induced antibacterial proteins from pearl oysters stimulated by bacterial inoculation.

The innate immunity of pearl oyster is composed of a comprehensive repertoire of immune cells, and a large variety of humoral defense factors constituting the first line of defense against invading microbes [25]. It has been reported that a few immune-related defense factors and inflammatory factors respond to bacterial infection in the pearl oyster *P. fucata* [22, 26]. However, implication of the defense factors including antibacterial substances in the innate immune system remains unknown. To assess the defense factors in pearl oysters responding to bacterial invasion, bacterial inoculation stimulation was carried out by injection with *V. parahaemolyticus* in this study. Then, antibacterial substances were extracted from different tissues. Unexpectedly, even acidic extracts from the gill and mantle of pearl oysters under the normal state without bacterial inoculation still showed strong antibacterial activity against *V. parahaemolyticus*, indicating that these two tissues might have a significant function in host defense against pathogens. Recent findings also showed that the pallial organs (gill and mantle) are a major portal of entry for microbes [27, 28]. The gill filaments, which provide a large surface involved in gas exchange and feeding, are highly exposed to the bacteria in seawater. Histological studies revealed that a large amount of hemocytes and effective glycoproteins are secreted from gill filaments for immune defense [19]. The mantle also has a sensory function response to unfavorable environmental conditions. An increase in hemocyte counts and soluble lysozyme activity was observed in the extrapallial fluid (between the mantle and inner shell) upon infection with bacteria [29]. In addition, immunological studies indicated that most of the immune-related genes have a high tissue-specific expression in the gill and mantle of bivalves [2, 20, 21, 30]. Therefore, it is not surprising that the gill and mantle possess strong antibacterial substances in this study. Because there are few molecules from bivalves that are able to prevent the growth of the *Vibrio* strains, it is interesting that the antibacterial substances in the gill (AEg) were found to be more effective against the marine *Vibrio* species than the other ones (Table 3). In contrast, the mantle extracts potently showed inhibition activity against *S. putrefaciens, L. garvieae,* and *M. luteus* (data not shown). Their spectra of antibacterial activity suggest that the effective substances could be different between the gill and mantle.

It is notable that the antibacterial activity of the acidic extracts from the gill significantly increased following bacterial inoculation by injection of *V. parahaemolyticus*. It has been reported that antibacterial proteins and antimicrobial peptides could be induced by bacterial infection in the innate immune system of invertebrates [13, 14]. In bivalves, defense factors such as lectin [21] and galectin [20] were highly expressed in the gill, and the expression of lectin mRNA in this tissue was dramatically up-regulated

after inoculation with bacteria. Our results suggest that the defense factors in the pearl oyster consist of various effective substances, that the antibacterial factors in the gill are potentially upregulated or released therefrom to defend against the bacterial invasion, and that the ones in the mantle exist constitutively. On the other hand, no antimicrobial activity was observed in acidic extracts of hemolymph and mucus. They seem to play a minor role in inhibition and in killing pathogens directly, although the hemocytes in hemolymph play a major role in bacterial recognition and encapsulation [29] and the bivalve mucus may act as the first host mechanical and chemical barrier encountered by microbes by particle processing [28].

It is interesting that the antibacterial substances in the gill (AEg) were more effective against three marine *Vibrio* than other strains (Table 3). There is evidence that the innate immune systems of lower organisms including insects, worms, crustaceans, and sea sponges, also possess "memory" of pathogens [31]. Recent studies on the immune system of invertebrates suggest that immunity could be enhanced by previous infections and that stronger recall responses may be mounted upon homologous pathogen challenge [32, 33]. Therefore, it is possible that exposure to *Vibrio* might result in immune "memory" and could induce antibacterial substances in pearl oyster against the attacking of bacteria.

We obtained three kinds of antibacterial fractions, AEgfr1, AEgfr2, and AEgfr3 from the acidic extract of the gill (AEg). Antibacterial substances in AEgfr1 and AEgfr2 were proteinous, but those in AEgfr3 were considered to be non-protein. Two antibacterial proteins, APg-1 and APg-2, were obtained from AEg. There is a possibility that the proteins with molecular masses below 20 kDa (18 and 12 kDa) might be subunits to combine with APg-1, because they were produced by the reducing and heating treatments for SDS-PAGE analysis. It was assumed that APg-1 might be composed of two oligomeric proteins containing with the same subunit of about 200 kDa, and another half of different subunits of about 18 and 12 kDa, respectively. The results of broad peak of APg-1 in the gel filtration chromatogram and dense bands on the PAGEs seems to be in favor of this assumption. Alternatively, it is possible that minute quantity of an unknown oligomer, which was composed of three subunits (about 200, 18, and 12 kDa), was included in APg-1. However, the structure of these antibacterial proteins still remains to be clarified. The results of PMF searches of these two proteins showed no similarity to known proteins, and BLAST search of the partial amino acid sequences revealed that no putative conserved domains were detected. Antimicrobial proteins are often lytic enzymes, nutrient-binding proteins or proteins containing sites that target specific microbial macromolecules [34]. In this research, pearl oysters were inoculated

by bacteria injection, thus the inflammation response and immune responses were potentially activated to protect the organism from the invasion of pathogens and eliminating inflammation for tissue repair. The antibacterial proteins might be defense factors against the invading bacteria by induction in the innate immune process. Further information on the expression of the proteins and their genes are needed to support these results. The cloning of full cDNA sequences of these proteins is in progress.

In conclusion, we have found two antibacterial proteins from the pearl oyster *P. fucata* induced by bacteria inoculation. Database similarity searches using PMFs and the partial amino acid sequences of these proteins revealed that they might be novel antibacterial proteins. Although these antibacterial substances have not been yet absolutely identified, the newfound antibacterial proteins might potentially upregulate in the gill or be released therefrom to defend against bacterial invasion. These promising results led us to consider a further study on the function and mechanism of these active compounds in the innate immune system of pearl oysters.

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Figure

1 **Figure captions**

2

3 **Fig. 1** Gel filtration chromatogram of the AEg using a Sephacryl S-200 column (2.6 × 100 cm). Elution
4 was performed with 0.15 M NaCl-0.01M Tris-HCl buffer (pH 6.8) at a flow rate of 0.6 ml/min and the
5 absorbance of each fraction (5.0 ml/fraction) was monitored at 280 nm. Antibacterial activity against *V.*
6 *parahaemolyticus* of each fraction was assayed via the radial diffusion method. The diameter of the
7 inhibition zone was measured including the diameter of the well (2.0 mm). Protein components were
8 analyzed by SDS-PAGE (12.5 % gel)

9

10 **Fig. 2** HPLC of APg-1 using a TSKgel G3000 SWxl column. Elution was performed with 50 mM sodium
11 phosphate buffer containing 0.15 M NaCl (pH 7.0) at a flow rate of 0.4 ml/min. The antibacterial activity
12 against *V. parahaemolyticus* of the eluate at retention times between 20.0 and 24.5 min (dashed frame)
13 was determined via the radial diffusion method. Protein components were analyzed by SDS-PAGE (12.5 %
14 gel) and native-PAGE (12.0 % gel). Molecular weight markers for gel filtration chromatography and
15 native-PAGE were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic
16 anhydrase (29 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa)

17

18 **Fig. 3** MS analysis and de novo sequencing of the peptide fragments of the antibacterial proteins. (a):
19 PMF of the tryptic peptides of APg-1, (b): PMF of APg-2. The peptide sequences marked on the MS
20 spectra were the partial amino acid sequences with the highest scores predicted by the De Novo Explorer
21 software

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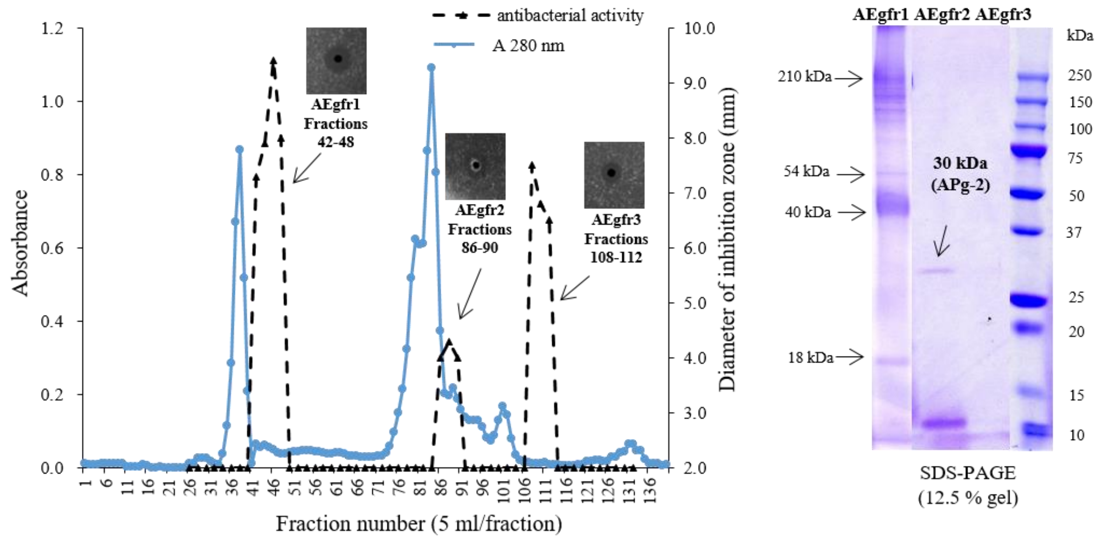

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26 Fig. 1
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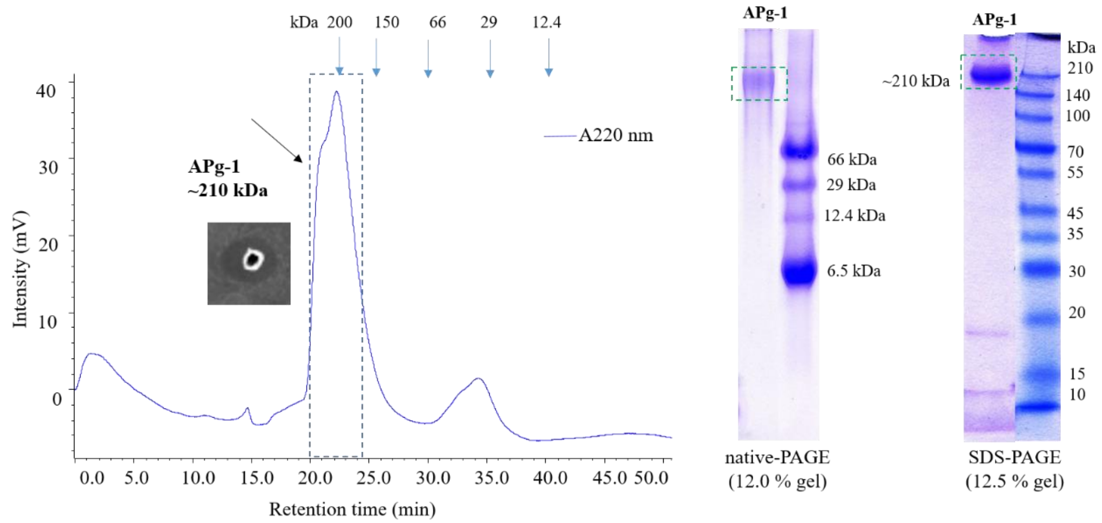

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33 Fig. 2
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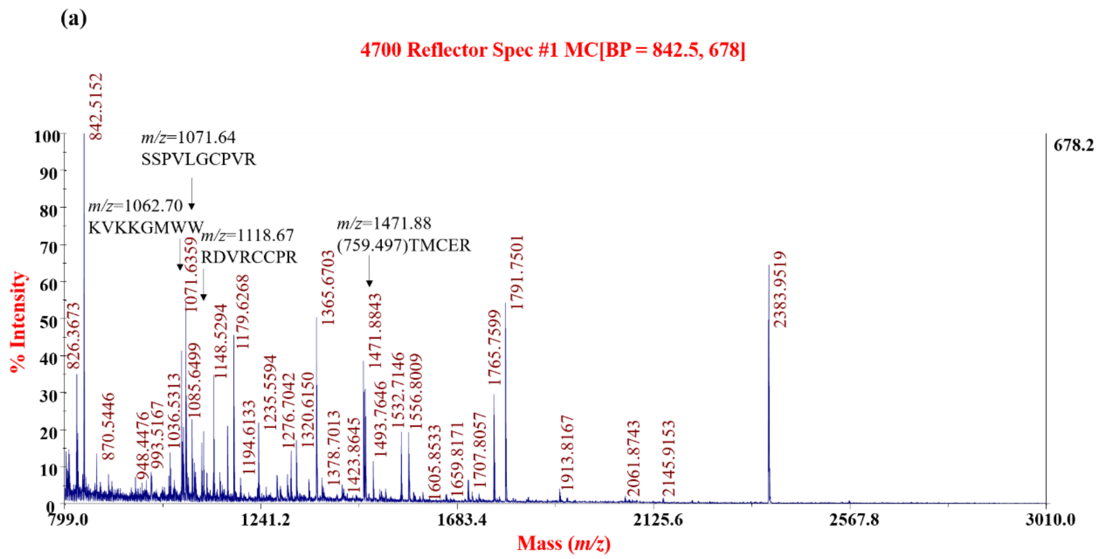
(a)



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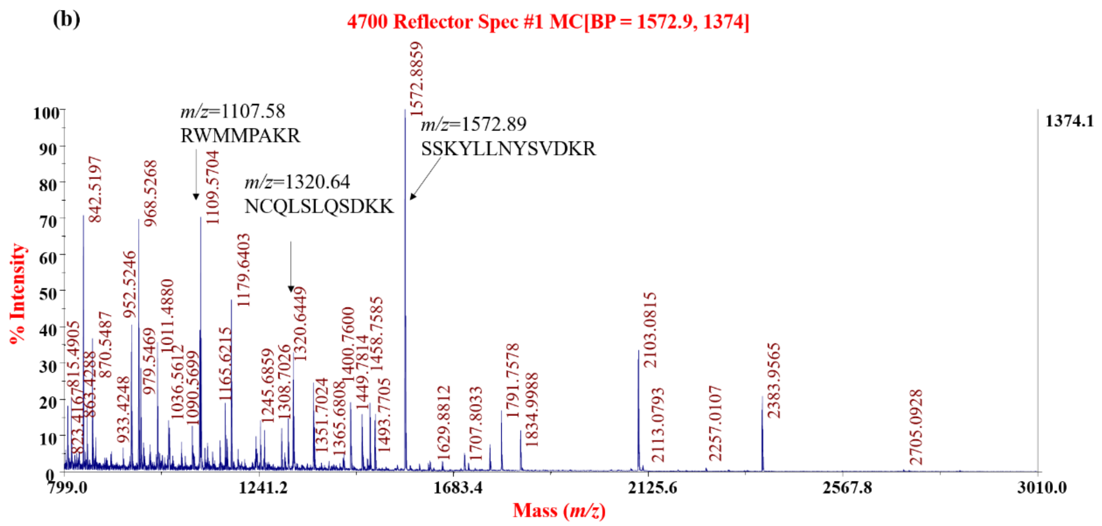
(b)



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37 Fig. 3

38

Table 1 Antibacterial activity against *V. parahaemolyticus* of acidic extracts

Groups	Gill		Mantle		Adductor muscle		Mucus		Hemolymph		Digestive gland	
	IT	BT	IT	BT	IT	BT	IT	BT	IT	BT	IT	BT
Control	8	<2	128	64	2	<2	<2	<2	<2	<2	16	<2
Low dose	16	8	128	64	8	2	<2	<2	<2	<2	16	<2
Mid dose	32	16	128	64	4	2	<2	<2	<2	<2	16	<2
High dose	32	32	64	32	4	2	<2	<2	<2	<2	16	<2

Control: acidic extracts from non-inoculated pearl oyster. Low dose: acidic extracts from pearl oyster inoculated by 1×10^4 CFU/ml *V. parahaemolyticus*. Mid dose: acidic extracts from pearl oyster inoculated by 1×10^8 CFU/ml *V. parahaemolyticus*. High dose: acidic extracts from pearl oyster inoculated by 1×10^{12} CFU/ml *V. parahaemolyticus*. Inhibitory titer (IT) was defined as the reciprocal of the highest dilution to inhibit the growth of bacteria (incubated for 24 h). Bactericidal titer (BT) was defined as the reciprocal of the highest dilution to inhibit the growth of bacteria (incubated for 72 h). The protein content of each test sample was 5.0 mg/ml.

Table 2 Antibacterial activity against *V. parahaemolyticus* of neutral extracts

Groups	Gill		Mantle		Adductor muscle		Mucus		Hemolymph		Digestive gland	
	IT	BT	IT	BT	IT	BT	IT	BT	IT	BT	IT	BT
Control	4	4	2	2	<2	<2	<2	<2	<2	<2	<2	<2
Low dose	4	4	4	2	<2	<2	<2	<2	<2	<2	<2	<2
Mid dose	8	8	2	2	<2	<2	<2	<2	<2	<2	<2	<2
High dose	4	4	4	2	<2	<2	<2	<2	<2	<2	<2	<2

Control: neutral extracts from non-inoculated pearl oyster. Low dose: neutral extracts from pearl oyster inoculated by 1×10^4 CFU/ml *V. parahaemolyticus*. Mid dose: neutral extracts from pearl oyster inoculated by 1×10^8 CFU/ml *V. parahaemolyticus*. High dose: neutral extracts from pearl oyster inoculated by 1×10^{12} CFU/ml *V. parahaemolyticus*. Inhibitory titer (IT) was defined as the reciprocal of the highest dilution to inhibit the growth of bacteria (incubated for 24 h). Bactericidal titer (BT) was defined as the reciprocal of the highest dilution to inhibit the growth of bacteria (incubated for 72 h). The protein content of each test sample was 5.0 mg/ml.

12 Table 3 Antibacterial activity of AEg by radial diffusion assay

Microbe	Inhibition zone diameter (mm)
Gram-negative	
Vibrio parahaemolyticus	6.5±0.3
Vibrio alginolyticus	5.3±0.3
Vibrio anguillarum	6.5±0.3
Edwardsiella tarda	-
Edwardsiella hoshinae	-
Shewanella putrefaciens	-
Escherichia coli	-
Gram-positive	
Lactococcus garvieae	4.5±0.3
Bacillus subtilis	3.0±0.3
Micrococcus luteus	-

"-" no inhibitory effect. The antibacterial activity was evaluated by the diameter of inhibition zone (2.0 mm of the well). The protein concentration of the acidic extract was 50 mg/ml.

13