Identification and characterization of the antibacterial substances from pearl oyster Pinctada fucata by bacterial inoculation and nuclei implantation

博士学位論文の要約
Summary of doctoral dissertation

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論文題目 Title	Identification and characterization of the antibacterial substances from pearl oyster *Pinctada fucata* by bacterial inoculation and nuclei implantation （アコヤガイ由来抗菌物質の性状に関する研究）		

Pearl culturing industry started in Japan, contributing enormous value to the marine economic development. Pearl oyster *Pinctada fucata* is one of the most important bivalves for the production of seawater pearl known as Akoya pearl. They are routinely subjected to wounding and pathogen invasion as a result of nuclei implantation and pearl harvesting operations. Diseases caused by infectious organisms, including viral, bacterial, fungal, protozoan, and metazoan organisms, during the cultivation practices occur frequently, resulting in significant economic losses. In order to control disease and enhance the yields and quality of pearl, it is necessary to investigate the defense factors and to clarify their function and mechanisms. Many defense factors have been found in bivalves to prevent the colonization by microbes, including lectin (agglutinin), antimicrobial peptides (AMPs), peptidoglycan-recognition proteins, lysozymes, antibacterial proteins, and other substances (pro-phenoloxidase, protease inhibitors, lysosomal enzymes). They possess a broad-spectrum microbicidal activity and serve as a primary function in innate immunity defense. However, less information of defense factors or antibacterial substances in pearl oyster was reported. Are these traditional defense factors present in pearl oyster? Are there any novel ones instead of them playing the roles in bacteria defense?

The research objectives of this study are (1) to identify the presence of antibacterial activity in organs of pearl oyster and whether there are any antibacterial substances induced by bacterial inoculation or nuclei implantation. (2) To clarify the structural properties and antibacterial properties of these antibacterial substances. The long-term objectives of this thesis project are to clarify the roles of these antibacterial proteins in the immune defense system and the wound healing process, and to develop novel antibiotics for disease control in aquaculture and for the potential clinical application.

In the first part of this study, in order to investigate the antimicrobial activity and whether there are antibacterial substances induced by immune stimulations, pearl oysters were inoculated by injection with *Vibrio parahaemolyticus* into the adductor muscle and inserted a nucleus into the gonad by a nuclei implantation surgery, respectively. Then, acid extracts (AEs) were prepared by 0.1% trifluoroacetic acid (TFA) from different tissues, and antibacterial activity was assayed. Protein components of AEs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the antibacterial properties were investigated. In the second part, to clarify the antibacterial substances from pearl oyster after inoculation, antibacterial proteins

were separated from the gill by gel filtration chromatography, following by high-performance liquid chromatography (HPLC) using a Superdex 200 column and a TSkgel G3000 column. The protein components were analyzed by both SDS- and native-PAGEs staining with Coomassie Brilliant Blue. The antibacterial proteins were in-gel digestion and then characterized by Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). In addition, antimicrobial peptides (AMPds) were extracted by 1% TFA mixed acid medium from digestive gland with heating processing, and purified by solid phase extraction (SPE) and HPLC with a TSKgel amide-80 column and a reserved-phase (RP) C18 column. The purified peptides were characterized by MALDI-TOF MS. Finally, to clarify the antibacterial substances from pearl oyster after nuclei implantation, antimicrobial proteins were separated from digestive gland and mantle respectively. The antibacterial protein was partially characterized by MALDI-TOF MS and partial amino acid sequences were predicted by De novo sequencing.

As results, (1) the acid extracts from the gill by bacterial inoculation (AEg-H), mantle (AE2-m-6) and digestive gland (AE2-d-6) of pearl oyster by nuclei implantation showed stronger antibacterial as well as the bactericidal activity than those from the control. These experimental results support the hypothesis that the bacterial inoculation and implantation could induce antibacterial substances in pearl oyster. It provides us a new insight to study the antibacterial substances related to the bacterial defense in marine bivalves by immune stimulation. (2) It is found that various antibacterial substances, including antibacterial proteins (APg-1 of 210 kDa, APg-2 of 30 kDa, APd250 of about 250 kDa, and APm of about 114 kDa) and antibacterial peptides (AMPd-1 with *m/z* of 656.01, AMPd-2 with *m/z* of 658.32, and AMPd-3 with *m/z* of 1167.42) are present in pearl oyster accumulating in the gill, mantle, and digestive gland. These antibacterial substances are potentially induced in these tissues or released therefrom serving as a defending function against the bacterial invasion. These new finding antibacterial proteins APg-1, APg-2, and APd250 were not identified as the traditional defense factors by MALDI-TOF MS and partial amino acid sequences, suggesting that they might be novel antibacterial factors. (3) It is first found that the new antibacterial protein APg-1 possesses LAO activity, which could specifically catalyze the oxidation of L-Lys and L-Phe. APg-1 is specifically effective against *V. parahaemolyticus* and its antibacterial and bactericidal actions were mediated by the H_2O_2 producing from the oxidation of L-amino acid substrates. These results suggest that this antibacterial factor in pearl oyster is possibly different from those in other bivalves.

In this study, various antibacterial substances including antibacterial proteins and peptides, which are accumulated in the gill, mantle, and digestive gland, were identified and characterized from the pearl oysters by the bacterial inoculation and nuclei implantation.

(1) The antibacterial substances derived by bacterial inoculation

In this study, it is found that gill has a significant function in response to bacterial inoculation basing on the increasing antibacterial activity in this tissue after bacterial injection. Recent findings also showed that the gill of bivalve is one of the major portals of entries for microbes. In the normal condition, the gill is highly exposed to the microorganism in the marine environment due to its large surface and the filter-feeder character of bivalves. Bivalves have an open circulatory system that bathes the organs in hemolymph. Histological studies revealed that a lot of hemocytes and effective glycoproteins are secreted from gill filaments for immune defense. In addition, immunological studies indicated that most of the immune-related genes have a high tissue-specific expression in the gill. In this research, bacteria was inoculated by injection into the adductor muscle. It is assumed that bacteria was spread from the site of inoculation into the water flow paths and distributed to other tissues. When *V. parahaemolyticus* suspension firstly flows through the gills, which serve as the filter for expelling most of the pathogen impinging on it, a significant response to the bacterial invasion might result in the accumulation of antibacterial substances in this tissue. Therefore, gill has a significant function in immune response to the bacterial inoculation.

The antibacterial protein APg-1 was found in the gills, which are potentially released in this tissue serving as the defending function against the bacterial invasion. APg-1 possesses LAO activity and bacterial cell binding ability, and its antibacterial and bactericidal activities are mediated by the H_2O_2 producing during the oxidation of L-amino acids. It is recognized that antibacterial mechanism of LAO proteins relays on the H_2O_2 from the oxidation of L-amino acid and the depletion of amino acid required for the bacterial growth, as well as the bacterial cell binding ability. Therefore, it is a possibility that the bactericidal action of APg-1 is involved in the bacterial combination processing and the biochemical reaction, which produce H_2O_2 as a mediator for defense against the bacteria invasion. To date, numbers of LAO proteins have been reported from various vertebrate animals. In contrast, only a few examples of LAO were found in invertebrates, such as marine mollusk. To my best knowledge, the novel antibacterial protein APg-1 is the first example of marine bivalve-derived LAO protein, which presents in pearl oyster and may play the important roles in the bacterial defense. These findings also suggest that the antibacterial factor and its defense mechanism in pearl oyster are possibly different from those in other bivalves.

(2) The antibacterial substances derived by implantation

The pearl oyster is prone to operational injury followed by the pathogen invasion because of the nuclei implantation. Although the pearl oyster has been proved to possess a considerable capacity to recover from the wound and a rapid response to the acute bacterial infection, the

defense-related factors response to the nuclei implantation remained unknown. The physiological response of the nuclei implantation was considered to be composed of a comprehensive system including inflammatory response, reactive oxygen species (ROS) response, and the innate immune response. Antimicrobial proteins and peptides play a major role in innate immunity by interacting directly with bacteria and killing them. These multifunctional proteins, such as defensins, cathelicidins, and peptide LL-37, also have wound healing activity and receptor-mediated effects on eukaryotic cells. In this study, antibacterial proteins (APd250 and APm) were found in the mantle and digestive gland by nuclei implantation, whether they play the roles related to the bacteria defense and the inflammatory response still remains unclear.

Based on the above results that various antibacterial substances was present in pearl oyster and the new finding antibacterial proteins APg-1, APg-2, and APd250 were not identified as the traditional defense factors, it is a possibility that these antibacterial factors are involved in the bacteria defense and their mechanisms are possibly different from those in other bivalves.

To my best knowledge, it is first finding that various antibacterial factors are present in pearl oysters. Further studies should be carried out based on the current findings. The structures and the roles of these antibacterial proteins in the immune defense system and the wound healing process should be first clarified to contribute to the development of novel management strategies for diseases control and the long-term sustainability of pearl industry. It is anticipated that further studies could provide more information in the discovery and development of new antibiotic agents for potential use in aquaculture and in human medicine.