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

［課程博士・論文博士共通］

# 博士学位論文内容要旨
# Abstract

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


Marine organisms live in a microbe-rich environment and they are under persistent threat of infection by resident pathogenic microbes. Because of the characteristic of filter feeding, the bivalve mollusks are continuously and markedly exposed to potential pathogens. To prevent the colonization by microbes, they developed a number of biologically active compounds, such as peptides and proteins, which possess a broad-spectrum microbicidal activity to defense against the invading pathogens. Pearl oyster *Pinctada fucata* is primarily cultured for the production of seawater pearl known as Akoya pearl. The Pearl culturing industry started in Japan by Kokichi Mikimoto, contributing enormous economic value in aquaculture. Due to the nuclei implantation during the pearl production, pearl oysters are prone to operational injury followed by bacterial infection. Diseases outbreaks caused by various pathogens occur frequently during the cultivation practices, 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 in the innate immune system. As the vital characteristic of antimicrobial activity of defense factors, the antibacterial substances including antibacterial proteins and peptides were identified and characterized from the pearl oysters by the bacterial inoculation and nuclei implantation.

In chapter 2, 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* and nuclei implantation, 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. As results, (1) most of the antibacterial substances were found to be present in the gill, mantle, and digestive gland. (2) The gill extracts from inoculated pearl oysters showed stronger antibacterial as well as the bactericidal activity than those from the control, suggesting that some antibacterial substances were potentially induced in the gill or released therefrom to defend against the bacterial invasion. (3) Nuclei implantation could potentially induce some antibacterial proteins in the gill, mantle, and digestive gland at 0~6 hours. (4) The bacteria sensitive test showed that antibacterial substances in gill (AEg-H, acidic extract from the gill of high dose inoculated oyster) had specific antibacterial activity against *Vibrio* strains, but those in mantle (AE2-m-6, acidic extract from the mantle after 6 h post-implantation) and digestive gland (AE2-d-6, acidic extract from the digestive gland after 6 h post-implantation) possessed a broad inhibition spectrum, revealing that the effective components in these three tissues were different. (5) The presence of catalase inhibited both the antibacterial and bactericidal activities of the acidic extracts, indicating that the antibacterial action was mediated by the hydrogen peroxide ($H_2O_2$).

In chapter 3, in order to clarify the antibacterial proteins from the gill, AEg-H was separated by gel filtration chromatography, then the antibacterial proteins were isolated 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 target proteins were in-gel digestion and then characterized by Matrix-assisted laser desorption/ionization time of flight mass spectrometry

(MALDI-TOF MS). To investigate whether there are antibacterial peptides in pearl oyster derived by bacterial inoculation, acidic extract (AEdi) was prepared by 1% TFA mixed acid medium from digestive gland with heating processing. Then antibacterial peptides were purified by solid phase extraction (SPE) and HPLC with a TSKgel amide-80 column and a reserved-phase (RP) C18 column. As results, (1) two antibacterial proteins (named as APg-1 of 210 kDa and APg-2 of 30 kDa) were found in the gill of pearl oyster derived by bacteria inoculation. Database similarity search using peptide mass fingerprints (PMFs) and partial amino acids revealed that APg-1 and APg-2 might be novel antibacterial proteins. APg-1 shows L-amino acid oxidase (LAO) activity and could specifically catalyze the oxidation of L-Lys and L-Phe. Antibacterial and bactericidal activities of the APg-1 were mediated by the $H_2O_2$ producing from the oxidation of amino acid substrates. These antibacterial proteins might potentially play the roles in bacteria defense in the gill. (2) Three antimicrobial peptides, named as AMPd-1 (*m/z* 656.01), AMPd-2 (*m/z* 658.32), and AMPd-3 (*m/z* 1167.42), were purified from digestive gland derived by bacteria inoculation. The predicted amino acid sequences were with high hydrophobic ratio and similarity with the AMPds reported in the Antimicrobial Peptide database.

In chapter 4, in order to clarify the antimicrobial proteins in digestive gland and mantle by nuclei implantation, (1) antibacterial protein was separated from AE2-d-6 by gel filtration chromatography and ion exchange chromatography, following by HPLC using a Mono Q column. The antibacterial protein was characterized by MALDI-TOF MS and partial amino acid sequences were predicted by De novo sequencing. (2) Antibacterial protein was separated from AE2-m-6 by gel filtration chromatography and HPLC using a using a Mono Q column and a TSKgel G3000 SWxl column. As results, an antibacterial protein of about 250 kDa, named as APd250, was obtained from digestive gland by nuclei implantation. The partial amino acid sequence of APd250, RGRNMLKKLNE derived by De novo sequencing, showed high similarity with an immunoglobulin-like domain repeat protein. Another antibacterial protein of about 114 kDa, named as APm, was found in the mantle by nuclei implantation. These results lay a foundation for further study of their roles in the defense system and wound healing.

In this study, experimental results support my 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 in marine bivalves by immune stimulation. Various antibacterial substances including antibacterial proteins (APg-1, APg-2, APd250, and APm) and antibacterial peptides (AMPds) 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. It is first found that the novel antibacterial protein APg-1 possesses LAO activity, which suggests that this antibacterial factor in pearl oyster is possibly different from those in other bivalves. This study lays a foundation for further study of the role of these antibacterial proteins in pearl oyster, which might be related to both the immune defense and wound healing. In addition, the study also provides important information for the development of new efficient antimicrobial agents for potential use in disease control in aquaculture and in human medicine.