Optimization of Antibacterial Protein against *E. tarda* Producing from Marine Bacteria

Hyun Sol Jo*, Youl Rae Jo**, Sun Mee Hong*
*Dep. Research and Development, Inst. MIRE,
**Inst Environmental Research, YeungNam University
e-mail:hongsunmee@mire.re.kr/joyr@yunui.ac.kr

해양 미생물이 생산하는 에드워드 항균 단백질 효능의 최적화

조현솔*, 조윤래**, 홍선미*
*환동해산업연구원 기술개발부 소재산업화팀
**영남대학교 생명과학부

Abstract

This study was aimed to isolate antibacterial potential compound against fish pathogen. Marine bacterium has been recognized as important antimicrobial substances producers which have an exceedingly bright future in the discovery of life saving drugs. Deep sea water and fish were collected from resource of Korea East sea for the isolation of bacteria. They were screened for antibacterial activity against fish pathogens by agar well diffusion method. The marine isolates were identified as *Pseudomonas extremorientalis* by 16s rRNA sequences. The fish antimicrobial protein from the supernatant of *P. extremorientalis* had high antibacterial activity against *Edwardsiella tarda*. A 37 kDa thermostable protein was found responsible for the antibacterial activity by SDS-PAGE and agar diffusion method against *Edwardsiella tarda*. The findings will serve as a basis for future development of new antibacterial substance or resistance against fish pathogens.

1. Introduction

Aquaculture is the cultivation of aquatic animals, such as fish of shellfish in a controlled and sometimes enclosed body of water. During the past three decades, aquaculture has developed to become the fastest growing food producing sector in many parts of the world and has become the characteristic feature of today's fishery industry (1). Of them *Edwardsiella tarda* (*E. tarda*) infections are frequent causes of severe outbreaks in the fish farming industry besides representing possible zoonotic risks. However naturally occurring outbreaks that affect various species besides fishes are seldom described (2). The marine microorganisms also ensure the safety and prolong the shelf-life of the product by the secretion of antimicrobial compunds such as bacteriocins, organic acids and hydrogen peroxide (3). The present study was conducted to investigated the anti *E. tarda* potential from marin bacteria and also to isolate the antibacterial compound produced by them. The potential isolated were further explored for the preparation of safety antibacterial materials.

2. Materials and Methods

2.1 Materials

Isolation of antibacterial strain against *E. tarda* was done from fish intestin and marine water samples by spread plate method. Selected dilutions (10^{-2}~10^{-6}) of each samples were spread on LB agar medium. Identification of the isolates was done by morphological, biochemical tests and antibacterial assay.

2.2 Biochemical Characterization

Growth of strain isolated was assessed in LB broth at 20, 25, 30, 37, 40 and 50 ℃ and by adding carbon (1% galactose), nitrogen (1 % yeast extract).

2.3 Assay for antibacterial activity

The antibacterial activity of the isolate for antibacterial potential against *E. tarda* was evaluated by agar well diffusion assay.

The isolates were inoculated in LB broth and

incubated on a rotary shaker at 100 rpm and 25 ℃ for 24 h. After incubation, the cultures were centrifuged at 10,000 rpm for 10 min to obtain cell-free culture supernatant. The supernatant were examined for antibacterial activity against *E. tarda*. The plates were incubated for 24 h at 25 ℃ and then examined for the appearance of the zone of inhibition around each well.

2.4 Isolation and Analysis of the antibacterial protein

After incubation, the cells were removed from the broth culture by centrifugation at 10,000 rpm for 10 min. Fractions of heat treated proteins of supernatant obtained were assayed for the antibacterial activity by the agar well diffusion method. The supernatant protein fractions showing antibacterial activity against *E. tarda* were seperated by SDS-PAGE using 12% separating gel. After electrophoresis, the was stained with Coomassie Brilliant Blue R-250 and destained overnight.

2.5 Assay of physicochemical parameters of antibacterial proteins

The metal ions ($MgSO_4$, $ZnSO_4$, $CaCl_2$, $CoCl_2$, $FeSO_4$, $CuSO_4$)and surfactant (SDS, sodium azide, Tween-40, TritonX-100, urea) stability of the supernatant protein was observed. After treatment, the supernatant were again assay for antibacterial activity as described above.

3. Results and Discussion

3.1 Isolation and identification of antibacterial against *E. tarda*

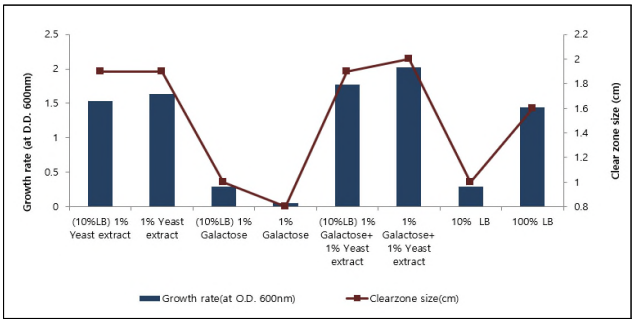
All of 100 colonies isolated from fish intestin and marine water samples were screened to be antibacterial effect, but only one isolated showing potent antibacterial activity against *E. tarda*. The selected colony was found to be Gram-negative, rod shape, aerobic, catalase, oxidase positive, gelatin hydrolysis, urease positive (Table 1). Based on the 16s rDNA gene sequence analysis, selected colony represented 99% similarity with *Pseudomonas extremorientalis* strain but not similar the character. This noble colony is named PEY1.

[Table 1] Characteristics of the strain *P. extremorientalis* KMM 3447 and PEY1 Table title

Test	PEY1	*Pseudomonas extremorientalis* KMM 3447
Gram stain	-	-
Cell shape	rod	rod
Aerobic	+	+
Catalase test	W(+)	+
Oxidase test	+	+
Casein test	+	+
Gelatin hydrolysis	+	-
Urease test	W(+)	ND
MR test	+	ND
Antibiotic resistant		
Ampicillin 100μg/ml	+	+
Gentamycin 100μg/ml	-	-
Kanamycin 100μg/ml	-	-
Penicillin 100μg/ml	+	+
Streptomycin 100μg/ml	-	-
Vancomycin 100μg/ml	+	ND
+,Positive; -,Negative; W,weak; ND,No Date available		

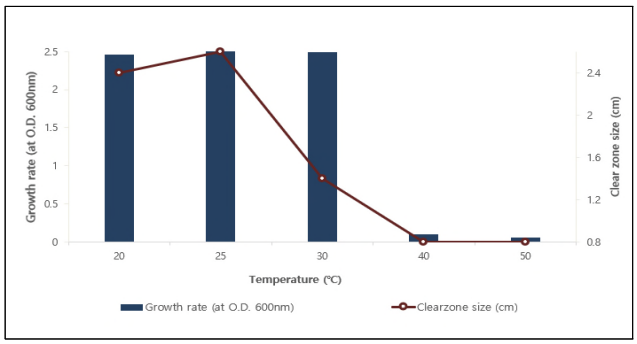
3.2 Antibacterial activity of PEY1 against *E. tarda*

The PEY1 was inoculated in different culture conditions (carbons source, nitrogen source, temperature, and pH) to optimize for effective antibacterial activity. It was observed that medium used of 1% yeast extract and 1% galactose as carbon and nitrogen sources showed strong antibacterial effect against *E. tarda*. (Fig 1).



[Fig. 1] Growth and antimicrobial activity of PEY1 according to medium composition

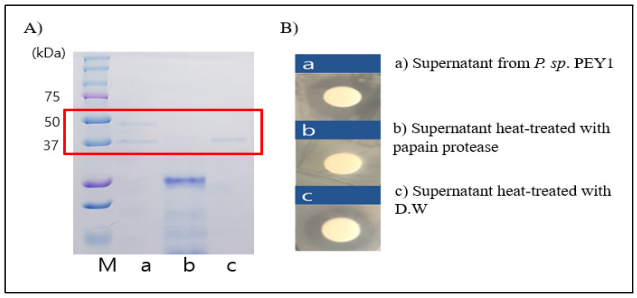
The growth and antibacterial activity according temperature was found to be maximum at the 20~30℃ (Fig 2).



[Fig. 2] Growth and antimicrobial activity of PEY1 on various temperature

3.3 Isolation of active protein fraction

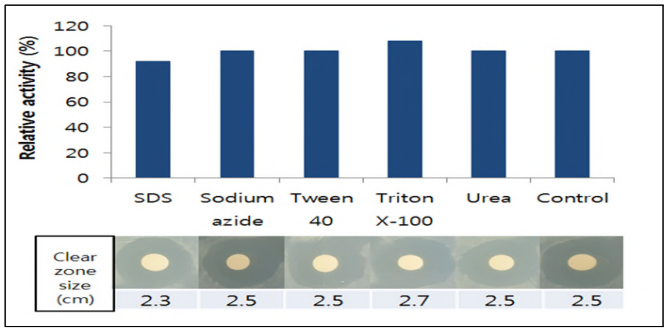
The protein of PEY1 observed 37 and 50 kDa MW in the supernatant. Active antibacterial protein 37 kDa band retained its antibacterial activity when heat-treatmented with papain protease at 65 ℃ for 5h (Fig 3). Both bands have the antibacterial activity against *E. tarda* but only 37 kDa protein have a stability for heat and enzyme treatment.



[Fig. 3] Verification of antibacterial protein by SDS-PAGE

3.4 Effect of physicochemical parameters

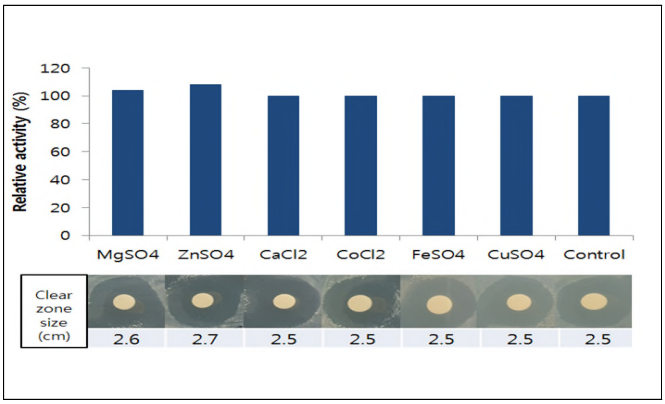
The PEY1 antimicrobial agent showed more than 100% relative antibacterial activity against various metal ions such as $FeSO_4$, $CaCl_2$, $CoCl_2$, $CuSO_4$, $MgSO_4$ and $ZnSO_4$ (Fig 4).



[Fig. 4] Effect of the antibacterial activity of protein produced from PEY1 according to various surfactants.

It showed a relative antibacterial activity of more than 90% for various inhibitors such as SDS, sodium azide, Tween 40, Triton X-100 and urea (Fig 5).

This confirmed that the PEY1 protein was stable to various metal ions and surfactants.



[Fig. 5] Change of the antimicrobial activity of protein produced from PEY1 according to various metal ions.

This study confirms that the proteinaceous antibacterial substance produced from PEY1 has strong antibacterial activity against *E. tarda*.

In addition, temperature and enzyme treatment were stable, and various surfactants and metal ions showed high antibacterial activity. Therefore, this study suggests that the proteinaceous antimicrobial substance produced by PEY1 inhibits the growth of *Edwardsiella tarda*, a fish pathogen that causes bacterial diseases in fish and significant economic loss to the aquaculture industry.

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