

RESEARCH ARTICLE

ANTIMICROBIAL ACTIVITY OF INTESTINAL MICROORGANISMS DERIVED FROM PROTAETIA BREVITARSIS SEULENSIS LARVAE.

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Abstract

..... This study was done to analyze the antimicrobial activity of gut-derived microbes of Protaetia brevitarsis seulensis larvae. Among the lactic acid bacterial strains isolated from the intestinal microbes of P. brevitarsis seulensis larvae, 10 strains were firstly selected from results of antimicrobial activity against Salmonella Typhimurium $\chi 3339$ strain as a susceptible strain. As a result, since the Minimum Inhibitory Concentration (MIC) was the best result in the K9 strain, further tests were performed on the K9 strain in subsequent studies. The K9 strain was identified as Lactobacillus plantarum from 16 SrDNA sequencing, named as L. plantarum K9. The antibacterial activity according to the amount of susceptible strain showed no or lower activity at 10^8 and 10^7 CFU/ml, whereas stronger antibacterial activity below 10⁶ CFU/ml. The antibacterial activity test according to the cultural temperature showed the strongest antibacterial activity at 37°C. In the thermal stability test, it was confirmed that the antimicrobial activity was maintained up to 50°C but significantly decreased from 60°C or higher. However, some of the AMPs exhibited thermal stability because of remaining the activity above 60°C or higher. Since proteinase K treatment decreases the antibacterial activity, we confirm that antibacterial activity is done by peptide-based bioactive substances. In summary, we suggest that AMPs in L. plantarum K9 strain are a potential of application as a natural antibacterial agent owing to thermal stability.

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Introduction:-

Numerous microorganisms in natural ecosystems have a wide and complex relationship of symbiosis with other organisms such as animal, plant and microorganism (Margulis and Fester, 1991; Ruby, 2004). These symbiotic relationships also play a beneficial role in the host organism but are involved in lethal pathogens or harmful roles (Buchner, 1965; Moran, 2006). A bacterium that lives in the host's body and maintains a close relationship with the host among these symbiotic bacteria is called an internal symbiotic, whereas a bacterium that affects the outside is called an external symbiotic (Kikuchi, 2009).

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Internal symbiosis is essential to the flexible feeding of insects. In fact, internal symbiosis is commonly found near insect digestive organs, and widely known common internal symbiosis plays an important role in the digestion and nutrition of host insects (Bourtzis and Miller, 2003; 2006; Buchner, 1965; Genta, et al., 2006). Many insect gut symbiosis, especially cell symbiosis, have been shown to affect genetic elements (Ishikawa, 1989). Insects and many other microbes living in the intestine coexist in a variety of ways.

Insects, which make up a large part of all living things on earth, have been used in daily life in human food, agriculture, industry and medicine. A number of bioactive substances in insects and high nutritional value have been reassessed as undeveloped biological resources. Insects are resistant to external infections by methods such as immune cells, insect blood cells, and continuous reactions of enzymes or antibacterial proteins/peptides. Antimicrobial peptides (AMPs) are one of the major components of the innate immune system of hemolymph of insects, and AMPs frequently attracts attention as a potential candidate for a urgently solution according to the emergence of antibiotic-resistant strains. More than 150 insect-derived AMPs have been isolated and classified largely in secropin, defensin and glycine/proline-rich peptides (Kim et al., 2017). These peptides have an antimicrobial effect and, except melittin, do not show hemolysis of erythrocytes, and mainly inhibit the growth of pathogenic microorganisms by disrupting cell membranes or inducing apoptosis (Reddy, et al., 2004; Zelezetsky and Tossi, 2006).

Bioactive substances such as insect-derived peptides have tremendous potential in terms of their applicability. In this study, in order to obtain excellent AMPs and induce industrial application, we isolated and identified LABs to maintain excellent antimicrobial activity from gut of *P. brevitarsis* seulensis larvae. The isolated strain examined antibacterial activity and physicochemical characteristics.

Materials and Methods:-

Bacterial strains used for this study

The antimicrobial susceptible bacterial strain used in this study was *Salmonella* Typhimurium $\chi 3339$ strain stored in the laboratory. LB broth or agar (Sigma-aldrich, USA) was used for the culture of *S*. Typhimurium strain. Lactic acid bacteria isolated from gut of *P. brevitarsis* seulensis larvae were cultured by MRS broth or agar (Sigma-aldrich, USA).

Isolation of LAB from gut in P. brevitarsis seulensis larvae

For use in the experiment, the 5th instar of *P. brevitarsis* seulensis larvae was obtained from Dodaum Co., Ltd. in South Korea, and then stabilized for one week. Gut in the larvae was separated from the individual larva, diluted to 100 fold in sterile water, and then spread on a MRS agar plate to separate single colony.

Preparation of centrifugated supernatant of isolated LAB

The isolated LAB was inoculated in 10 ml of MRS broth and cultured at 37° C for 16 h, followed by centrifugation at 12,000 x g to recover the supernatant. The recovered supernatant was filtered through a 0.22 um syringe filter and then the aliquoted supernatant in 1 ml each was applied directly or stored at -20°C until use.

Evaluation of antibacterial activity for LAB supernatants

Antibacterial activity of the LAB supernatant against *S*. Typhimurium $\chi 3339$ was done using the microtiter plate method. The precultured broth of susceptible bacteria was adjusted to 10^6 CFU/ml or an appropriate concentration and used for the study. The reaction solution was composed of pre-cultured broth, LAB supernatant, and fresh medium, which the final 50 ul of LAB supernatant was used for assay of antibacterial activity. The reaction solution was added to the microtiter plate, and then the antimicrobial activity was observed after incubation at 37°C for 16 h. The results were obtained via measurement at 600 nm with an ELISA leader (Multiscan GO, Thermo Scientific Co. Ltd., Rochester, NY, USA).

MIC of selected LABs and identification of LAB K9

The assay of MIC for the LAB supernatant against *S*. Typhimurium $\chi 3339$ was done using the microtiter plate method. The precultured broth of susceptible bacteria was adjusted to 10^6 CFU/ml or an appropriate concentration and used for the study. The concentrations of supernatant used for analysis were 0, 5, 10, 25, 50, 100 µl. The reaction solution was added to the microtiter plate, and then the antimicrobial activity was observed after incubation at 37°C for 16 h. The results were obtained via measurement at 600 nm with an ELISA leader (Multiscan GO, Thermo Scientific Co. Ltd., Rochester, NY, USA).

To identify the lactic acid bacteria strain, genomic DNA was isolated using genomic DNA kit (Bioneer, South Korea), PCR-amplified, and confirmed by 16S rDNA sequencing in Macrogen (South Korea).

Antibacterial activity of LAB supernatant according to susceptible bacterial amount

The antibacterial activity of LAB supernatant depending on *S*. Typhimurium $\chi 3339$ cell amount was done using the microtiter plate method. The amount of LAB supernatant determined to MIC concentration in 10⁶ CFU/ml was used for the analysis of antibacterial activity depending on susceptible bacterial amount. *S*. Typhimurium $\chi 3339$ was used for the evaluation of the antibacterial activity by adjusting the cultured bacterial solution to 10⁸, 10⁷, 10⁶, 10⁵, 10⁴, and 10³ CFU/ml concentration. The reaction solution was added to the microtiter plate, and then the antimicrobial activity was observed after incubation at 37°C for 16 h. The results were obtained via measurement at 600 nm with an ELISA leader (Multiscan GO, Thermo Scientific Co. Ltd., Rochester, NY, USA).

Antimicrobial activity by LAB supernatants depending on cultural temperature

L. plantarum K9 was incubated for 16 h at 25, 37, and 42°C. The antibacterial activity of LAB supernatant depending on cultural temperature was done using the microtiter plate method. The amount of LAB supernatant determined to MIC concentration in 10^6 CFU/ml was used for the analysis of antibacterial activity depending on susceptible bacterial amount. The precultured broth of susceptible bacteria was adjusted to 10^6 CFU/ml or an appropriate concentration and used for the study. The reaction solution was added to the microtiter plate, and then the antimicrobial activity was observed after incubation at 37°C for 16 h. The results were obtained via measurement at 600 nm with an ELISA leader (Multiscan GO, Thermo Scientific Co. Ltd., Rochester, NY, USA).

Evaluation of thermal stability of LAB supernatant

The antibacterial activity of LAB supernatant depending on heat treatment was done using the microtiter plate method. The amount of LAB supernatant determined to MIC concentration in 10^6 CFU/ml was used for the analysis of antibacterial activity depending on susceptible bacterial amount. The precultured broth of susceptible bacteria was adjusted to 10^6 CFU/ml or an appropriate concentration and used for the study. LAB supernatant used in the evaluation was used after heating for 10 min at non-treated, 30, 40, 50, 60, 70 and 80°C each. The reaction solution was added to the microtiter plate, and then the antimicrobial activity was observed after incubation at 37°C for 16 h. The results were obtained via measurement at 600 nm with an ELISA leader (Multiscan GO, Thermo Scientific Co. Ltd., Rochester, NY, USA).

Evaluation of antibacterial activity depending on proteinase K treatment

The antibacterial activity of LAB supernatant depending on proteinase K treatment was done using the microtiter plate method. The precultured broth of susceptible bacteria was adjusted to 10⁸ CFU/ml or an appropriate concentration and used for the study. Proteinase K (Sigma-Aldrich, Milwaukee, WI, USA) was treated by final concentration of 200 ug/ml, and then reacted at 37°C for 30 min. The reaction solution was added to the microtiter plate, and then the antimicrobial activity was observed after incubation at 37°C for 16 h. The results were obtained via measurement at 600 nm with an ELISA leader (Multiscan GO, Thermo Scientific Co. Ltd., Rochester, NY, USA).

Results and Discussion:-

Antibacterial activity of LABs originated from gut in P. brevitarsis seulensis larvae

Among various LABs in gut of *P. brevitarsis* seulensis larvae, 10 LABs were selected with excellent antibacterial activity (Fig. 1). Antibacterial activity was observed in all LAB strains, but K3, K4, K7, K8, K9 and K10 strains were higher than K1, K2, K5 and K6 strains. As a result, K3, K4, K7, K8, K9 and K10 strains were selected for the further study.

The selected LABs were evaluated for MIC concentrations (Fig. 2). The concentration of MIC was 47, 48.25, 47.75 47.83, 46.8 and 46.82 for K3, K4, K7, K8, K9 and K10 strains, respectively. MIC values of the compared six strains were found to be similar, but the highest activity among them was shown in K9 strain. Based on this result, only the K9 strain was performed from the further study. 16S rDNA sequencing of K9 strain resulted in *Lactobacillus plantarum* (Fig. 3). Therefore, K9 strain was named as *L. plantarum* K9.

Antibiotics are commonly produced to gain a competitive advantage to other microorganisms in the same community (Ueda and Beppu, 2017). All the LABs introduced in this study had high antimicrobial activity, and especially *L. plantarum* K9 strain showed the highest activity.

Physicochemical properties of AMPs

The antimicrobial activity of K9 supernatant was observed by high activity below 10^6 CFU/ml the susceptible bacterial amount (Fig. 4). However, no activity or significantly lower activity was observed at 10^8 CFU/ml and 10^7 CFU/ml. As the results of the antimicrobial activity for K9 supernatant according to cultural temperature, high activity was observed at 37° C, but significantly low activity was observed at 25° C and 42° C (Fig. 5). The thermal stability of the K9 supernatant was shown in Fig. 6. When heated for 10 min at 30, 40, and 50° C, it showed the same results with only supernatant. However, the results of heating for 10 min at 60, 70 and 80° C were observed to decrease the activity due to protein denaturation (Fig. 6). Although the activity was partially lowered, the relative activity was maintained by 87.4, 77.9 and 62.9% when treated at 60, 70 and 80° C compared to only supernatant. Therefore, some of the AMPs present in the culture are estimated to have thermal stability.

When treated simultaneously with LABs and proteinase K, in the case of proteinase K treatment, low activity was observed in both with and without inactivation of proteinase K (Fig. 7). Bacteriocin has been shown to be almost completely degraded by protease treatment and lose antimicrobial activity (Kaur and Tiwari, 2018). In this study, since the most activity was lost by proteinase K treatment, it was assumed that most of the antimicrobial activity is done by AMPs.

In summary, the most substances to show antibacterial activity in *L. plantarum* K9 consisted of peptides, maintained partially stable to heat, and showed concentration-dependent activity of susceptible bacteria. Therefore, it is suggested that these AMPs are highly usable for future industrialization because of their high activity and thermal stability.

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Fig 2:-MIC of LAB supernatants. *S*. Typhimurium χ 3339 of the susceptible bacteria was applied by 10⁶ CFU/ml. X- and Y-axes indicate LAB supernatant and absorbance at 600 nm, respectively.



Supernatant (ul) and strain name

Fig 3:-Identification of the isolated K9 strain, SMA290418-010-1-K9-27F. The dendrogram was obtained from result of blast from NCBI blast program. K9 strain was named as SMA290418-010-1-K9-27F during 16S rDNA sequencing at Macrogen company.



Fig 4:-Antibacterial activity depending on susceptible bacterial amount. S. Typhimurium $\chi 3339$ of the susceptible bacteria was applied by 10^8 , 10^7 , 10^6 , 10^5 , 10^4 and 10^3 CFU/ml. X- and Y-axes indicate LAB supernatant and absorbance at 600 nm, respectively.



Fig 5:-Antibacterial activity according to cultural temperature. *S*. Typhimurium χ 3339 of the susceptible bacteria and K9 supernatant were applied by 10⁶ CFU/ml and MIC in 10⁶ CFU/ml, respectively. X- and Y-axes indicate K9 supernatant and absorbance at 600 nm, respectively.



Fig 6:-Evaluation of thermal stability. *S*. Typhimurium $\chi 3339$ of the susceptible bacteria and K9 supernatant were applied by 10⁶ CFU/ml and MIC in 10⁶ CFU/ml, respectively. The LAB supernatant was treated for 10 min at each temperature. X- and Y-axes indicate K9 supernatant and absorbance at 600 nm, respectively.



Fig 7:-Evaluation of antibacterial activity according to proteinase K treatment. *S.* Typhimurium χ 3339 of the susceptible bacteria and K9 supernatant were applied by 10⁸ CFU/ml and MIC in 10⁶ CFU/ml, respectively. X- and Y-axes indicate K9 supernatant and absorbance at 600 nm, respectively.



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