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### RESEARCH ARTICLE

#### STUDY ON ANTIBACTERIAL ACTIVITY OF BEE VENOM.

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#### Abstract

The purpose of this study was to investigate the antimicrobial activity against *Salmonella* infection which causes intestinal diseases from bee venom which is one of the social insects, and to find a way which use ghost vaccine. The minimum inhibitory concentration (MIC) of bee venom against *Salmonella* Typhimurium  $\chi$ 3339 was 101.81  $\mu$ g/ml. Based on the result of MIC, the antimicrobial activity according to amount of the cells showed strong activities below  $10^6$  CFU/ml, but exhibited no and low activity at  $10^8$  and  $10^7$  CFU/ml, respectively. In addition, the antimicrobial activity assay according to the cultural temperature showed the strongest activity at 37°C. The thermal stability of bee venom appeared to be very stable at temperatures below 60°C, but gradually decreased at more than 60°C. The protease inhibitor mixture was shown to greatly enhance the antimicrobial activity of bee venom. Since most of the activity is lost due to the treatment with Proteinase K, it is estimated that the antimicrobial activity of bee venom is induced by peptides. Therefore, we suggest that bee venom can be used as an antimicrobial agent for ghost vaccine production.

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#### Introduction:-

Bee venom is a colorless transparent and viscous liquid to maintain strong aromatic bitter taste, and an off-white or yellowish white mass or powder in dry state. The specific gravity of the bee venom solution is 1.13 and the acidity is in the range of 5.2 ~ 5.5. Bee venom is easy to dissolve in water and acids, but hardly soluble in alcohol. The bee venom solution loses 70% of its liquid weight by quickly drying when exposed to air at room temperature. Bee venom maintains its long-term activity in the frozen state, but tends to be easily destroyed by oxidizing substances (Koh, 1993; Kim, 1996).

Bee venom is composed of about 40 components, which consists of peptide components such as melittin, apamin, and mast cell degranulating (MCD) peptide, enzyme components such as phospholipase A2, hyaluronidase, and acid phosphomonoester, and amine components such as histamine, dopamine, and noradrenaline (Chmielewska and Szczęśna, 2004; Kokot and Matysiak, 2009). Among them, melittin is a major component that accounts for about 50% of the dry bee venom, and it is known that it works very well in pain and inflammation (Piek, 1986; Curcio-Vonlanthen V et al., 1997). Melittin was first discovered as a hemolytic element in 1954, and observed by cell membrane activity during erythrocyte lysis in 1972 (Lee et al., 2000). Structurally, it consists of 26 amino acids, which strongly inhibits macrophage migration and synergizes with phospholipase A2 during hemolysis to increase mutual activity. In the enzyme action, it stimulates catecholamine and cortisone secretion by stimulating the pituitary and adrenal cortex system, induces an increase of serotonin in the hypothalamus, and has anti-

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inflammatory action via stabilization of the lysosomal membranes (Kwan et al., 2000; Lee, 2000). There are also dissolution, enzymes, pain induction, radiation-resistant action, and antibacterial action (An et al., 2010).

To date, many AMPs (antimicrobial peptides) have been obtained from nature, but the most promising candidates in recent years have been originated from poisonous animals such as snakes, scorpions, spiders, ants, wasps, bees, and centipede (Wang et al., 2016). Peptide toxins such as androtonin, parbutopirin, opistoporins, TstH, and vpAmp 1.0, which are derived from scorpion venom, show strong antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria or fungi (Hetru et al., 2000; Powers and Hancock, 2003; Ramirez-Carreto et al., 2015; Machado et al., 2016). Cardiotoxin and crotoamine of snake venom show strong activity against bacteria and fungi (Chen et al., 2011; Oguiura et al., 2011). In particular, bee and spider poisons yield AMPs more than 20,000 and 40,000 members, respectively (Wang et al., 2016).

In this study, the antimicrobial activity against *Salmonella* Typhimurium  $\chi$ 3339, a causative organism of *Salmonella* infection, was evaluated by the bee venom and examined for the possibility to apply the venom for preparation of ghost vaccine.

## Materials and Methods:-

### Bee venom and bacterial strain:-

The bee venom powder used in this study was purchased from Chung Jin Biotech Co., Ltd (South Korea), and applied as a concentration of 10  $\mu$ g/ $\mu$ l. The test strain was examined by *Salmonella* Typhimurium  $\chi$ 3339.

### Analysis of minimal inhibitory concentration (MIC):-

MIC analysis of bee venom against *Salmonella* Typhimurium  $\chi$ 3339 was performed with a microtiter plate method. Bee venom powder was dissolved in an appropriate concentration (10  $\mu$ g/ $\mu$ l) and used for antimicrobial activity analysis. The used strain was employed for MIC assay by adjusting the precultured broth to 10<sup>6</sup> CFU/ml or an appropriate concentration. The concentrations of bee venom used in the analysis were 0, 25, 50, 100, and 250  $\mu$ g/ml. Each 200  $\mu$ l of the reaction solution was added into a microtiter plate, and the antimicrobial activity was observed after culturing at 37°C for 16 h. The results were measured at 600 nm with an ELISA leader (Multiscan GO, Thermo Scientific Co. Ltd., Rochester, NY, USA).

### Antibacterial activity of bee venom according to the amount of bacterial cells:-

The antimicrobial activity of *Salmonella* Typhimurium  $\chi$ 3339 for the amount of bacterial cells was assayed by a microtiter plate method. Concentration of bee venom according to MIC results obtained from 10<sup>6</sup> CFU/ml were used for the subsequent analysis of antimicrobial activity. The used strains were employed for the evaluation depending on the amount of bacterial cells by adjusting the cultured broth at concentrations of 10<sup>8</sup>, 10<sup>7</sup>, 10<sup>6</sup>, 10<sup>5</sup>, 10<sup>4</sup>, and 10<sup>3</sup> CFU/ml. Each 200  $\mu$ l of the reaction solution was added into a microtiter plate, and the antimicrobial activity was observed after culturing at 37°C for 16 h. The results were measured at 600 nm with an ELISA leader (Multiscan GO, Thermo Scientific Co. Ltd., Rochester, NY, USA).

### Analysis of antimicrobial activity for bee venom by temperature

The antimicrobial activity of *Salmonella* Typhimurium  $\chi$ 3339 by temperature was assayed by microtiter plate method. The bee venom was used for the antimicrobial activity analysis according to the MIC result. The used strain was employed for MIC assays by adjusting the precultured broth to 10<sup>6</sup> CFU/ml or an appropriate concentration. Each 200  $\mu$ l of the reaction solution was added into a microtiter plate, and the antimicrobial activity was observed after culturing at 37°C for 16 h. The results were measured at 600 nm with an ELISA leader (Multiscan GO, Thermo Scientific Co. Ltd., Rochester, NY, USA).

### Evaluation of thermal stability for bee venom:-

Analysis of the thermal stability of bee venom for *Salmonella* Typhimurium  $\chi$  3339 was performed using a microtiter plate method. The bee venom was used for the antimicrobial activity analysis according to the MIC result. The used strain was employed for evaluation of thermal stability by adjusting the precultured broth to 10<sup>6</sup> CFU/ml or an appropriate concentration. The bee venom used in the evaluation was examined after heating for 10 minutes at 0, 30, 40, 50, 60, 70, and 80°C, respectively. Each 200  $\mu$ l of the reaction solution was added into a microtiter plate, and the antimicrobial activity was observed after culturing at 37°C for 16 h. The results were measured at 600 nm with an ELISA leader (Multiscan GO, Thermo Scientific Co. Ltd., Rochester, NY, USA).

### Antibacterial activity of bee venom according to protease inhibitor mixture:-

Antimicrobial activities of *Salmonella* Typhimurium  $\chi$ 3339 according to protease inhibitor mixture of bee venom were analyzed by microtiter plate method. The bee venom was used for the antimicrobial activity analysis according to the MIC result. The used strains were employed for evaluation of an effect for protease inhibitor mixture by adjusting the precultured broth to 10<sup>8</sup> CFU/ml or an appropriate concentration. The protease inhibitor mixture used in the assay was Protease inhibitor P8465 (Sigma-Aldrich, Milwaukee, WI, USA) and the concentrations were 0, 0.5, 1, 2, 5, and 10  $\mu$ l. Each 200  $\mu$ l of the reaction solution was added into a microtiter plate, and the antimicrobial activity was observed after culturing at 37°C for 16 h. The results were measured at 600 nm with an ELISA leader (Multiscan GO, Thermo Scientific Co. Ltd., Rochester, NY, USA).

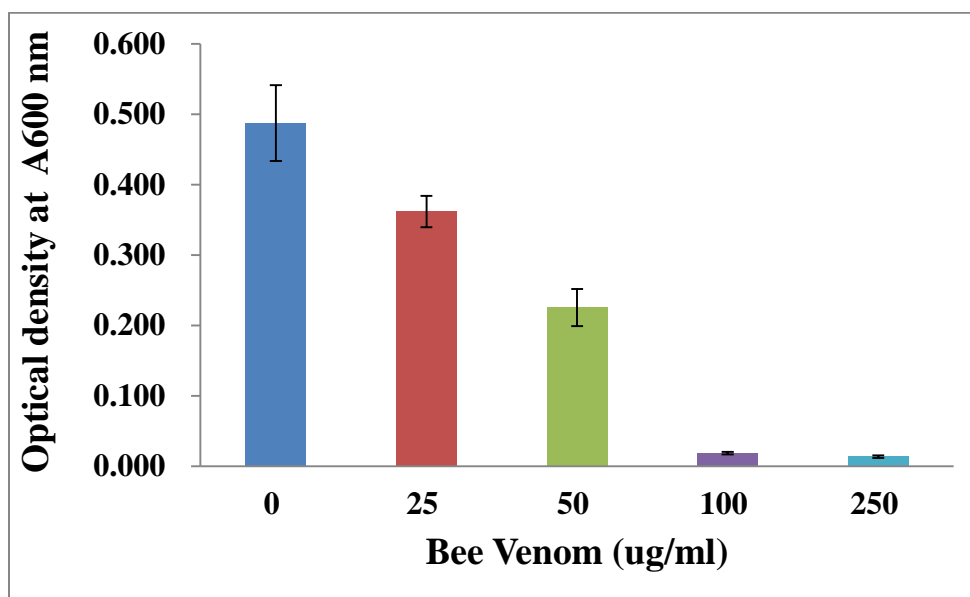
### Analysis of antimicrobial activity of bee venom by simultaneous treatment with Proteinase K:-

The antimicrobial activities of *Salmonella* Typhimurium  $\chi$ 3339 by simultaneous treatment with Proteinase K of bee venom were analyzed by microtiter plate method. The used strain was employed for evaluation of an effect for protease by adjusting the precultured broth to  $10^6$  CFU/ml or an appropriate concentration. Proteinase K (Sigma-Aldrich, Milwaukee, WI, USA) was used by 80  $\mu$ g/ml concentration for the analysis. Each 200  $\mu$ l of the reaction solution was added into a microtiter plate, and the antimicrobial activity was observed after culturing at 37°C for 16 h. The results were measured at 600 nm with an ELISA reader (Multiscan GO, Thermo Scientific Co. Ltd., Rochester, NY, USA). Proteinase K (Sigma-Aldrich, Milwaukee, WI, USA) was used by for the analysis.

### Results and Discussion:-

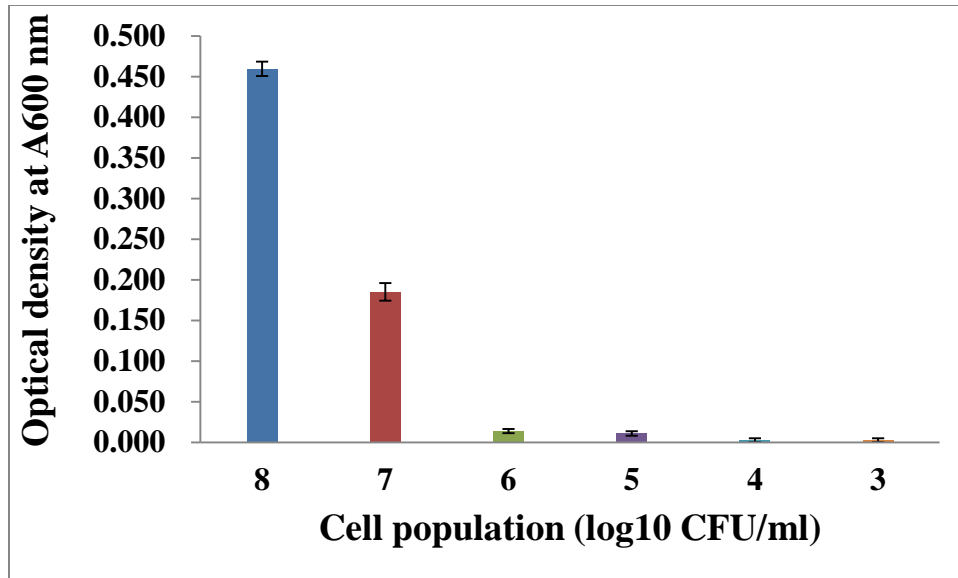
#### Antimicrobial activity against *S. Typhimurium*:-

This study was done to evaluate the efficacy of commercially available bee venom through *S. Typhimurium*. It is known that the antibacterial activity of commercial bee venom is 20 to 40  $\mu$ g / ml (Leandro et al., 2015). However, it was presumed that some activities were changed during addition of excipient and drying process in the process of powdering bee venom. As a result of this study with the purchased bee venom, the minimal inhibitory concentration (MIC) for *S. Typhimurium*  $\chi$ 3339 was observed by 101.8085  $\mu$ g/ml (Fig. 1). This result is 2.5 ~ 5 times lower than the previous research results. Therefore, it is judged that the excipient is mixed with the corresponding concentration or some activity is lost in the drying process.



**Fig. 1:-** Evaluation of antibacterial activity for bee venom. *Salmonella* Typhimurium  $\chi$ 3339 strain was diluted to  $10^6$  CFU/ml and applied for the study. Bee venom was used by concentration of 10  $\mu$ g/ $\mu$ l and added at concentrations of 0, 25, 50, 100, and 250  $\mu$ g/ml. X- and Y-axes indicate optical density at 600 nm and concentration of bee venom, respectively.

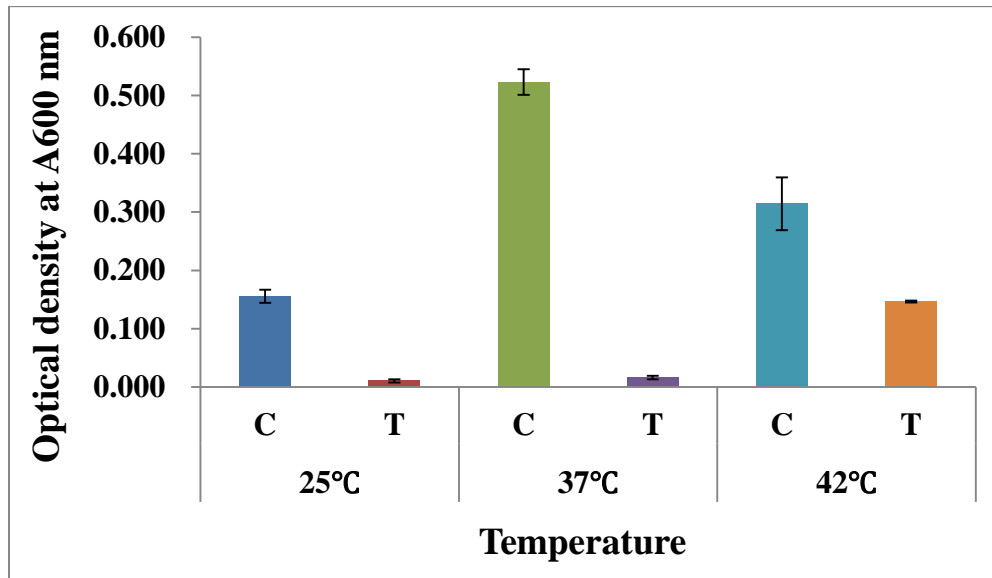
One of the purposes of this study is to produce *Salmonella* ghost vaccine with treatment of bee venom. Therefore, we investigated whether the bee venom activity is a good function in high population of the strain. The antimicrobial activity was evaluated by the amount of MIC value obtained from  $10^6$  CFU/ml to investigate the antimicrobial activity against the high concentration of the bacterial mass. As shown in Fig. 2, higher activity was observed below  $10^6$  CFU/ml, whereas the activity was markedly lowered at  $10^7$  CFU/ml and little activity at  $10^8$  CFU/ml. Therefore, it is suggested that a higher content of bee venom must be used in order to exhibit high antimicrobial activity at  $10^8$  CFU/ml. Although it is a disadvantage of using a high concentration of bee venom, it is assumed that bee venom powder employed in this study is a possibility of application as a raw material for production of ghost vaccine.



**Fig. 2:-** Evaluation of antimicrobial activity according to the amount of cells. *Salmonella* Typhimurium  $\chi$ 3339 strain was diluted to  $10^8$ ,  $10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$ , and  $10^3$  CFU/ml, respectively, and employed for the study. Bee venom was used as a stock at a concentration of  $10 \mu\text{g}/\mu\text{l}$ , and added at the concentration of the MIC of  $10^6$  CFU/ml. X- and Y-axes indicate optical density at 600 nm and amount of the cells, respectively.

**Evaluation of thermal stability:-**

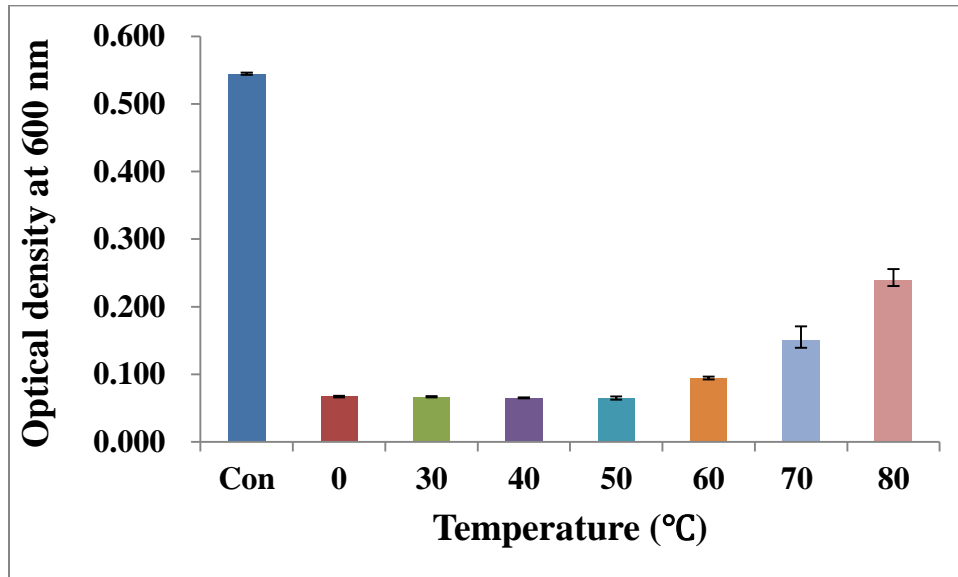
To investigate the suitable temperature for bee venom to be used for ghost vaccination, we evaluated the antimicrobial activity according to each cultural temperature of *S. Typhimurim*. As shown in Fig. 3, high activity was observed at  $25^\circ\text{C}$  and  $37^\circ\text{C}$ , but remarkably low activity was observed at  $42^\circ\text{C}$ . Bee venom treated at  $25^\circ\text{C}$  showed high antimicrobial activity, but overall growth was significantly reduced. Therefore, it is suggested that treatment at  $37^\circ\text{C}$  was appropriate for ghost formation of *S. Typhimurium*.



**Fig. 3:-** Examination of antimicrobial activity according to cultural temperature. *Salmonella* Typhimurium  $\chi$ 3339 strain was diluted to  $10^6$  CFU/ml and applied for the study. Bee venom was used by concentration of  $10 \mu\text{g}/\mu\text{l}$ , and added at the concentration of the MIC of  $10^6$  CFU/ml. X- and Y-axes indicate optical density at 600 nm and cultural temperature, respectively. C; control without bee venom, T; treatment with bee venom.

In order to increase the possibility of commercial application, the used AMPs must have high thermal stability. Therefore, in this study, heat stability was evaluated at each temperature for 10 minutes. As shown in Fig. 4, the activity was remained by treatment

at 0°C, 30°C, 40°C, and 50°C for 10 minutes, but the activity was gradually decreased when the temperature was further increased. Therefore, it is presumed that most activity of the bee venom is retained when it is treated at a temperature of 60°C or lower in the processing process for powder.

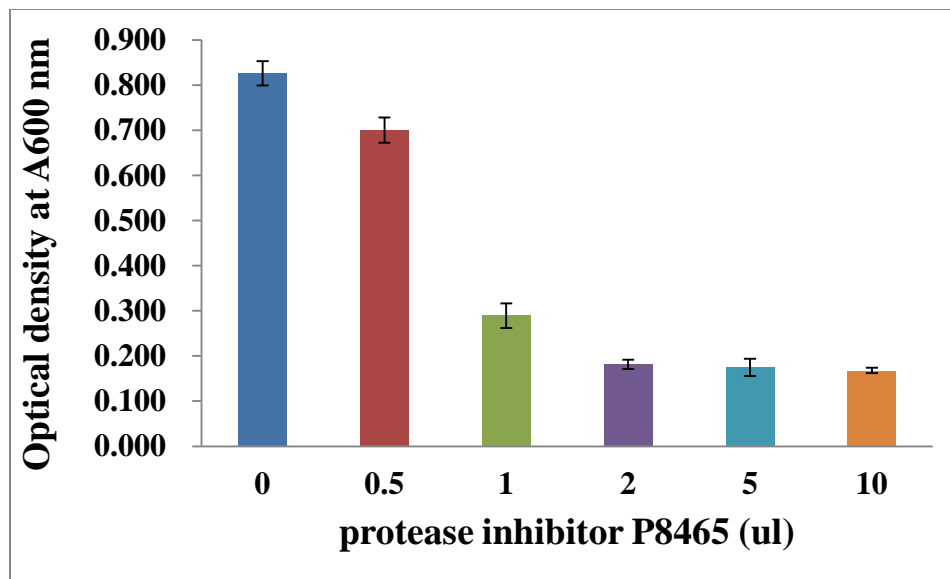


**Fig. 4:-** Evaluation of thermal stability of bee venom. *Salmonella* Typhimurium  $\chi$ 3339 strain was diluted to  $10^6$  CFU/ml and applied for the study. Bee venom was used by concentration of  $10 \mu\text{g}/\mu\text{l}$ , and added at the concentration of the MIC of  $10^6$  CFU/ml. Bee venom was added after heating for 10 minutes at 0 ~ 80°C X- and Y-axes indicate optical density at 600 nm and cultural temperature, respectively. Con; control without bee venom.

**Analysis of association between antimicrobial activity and peptide:-**

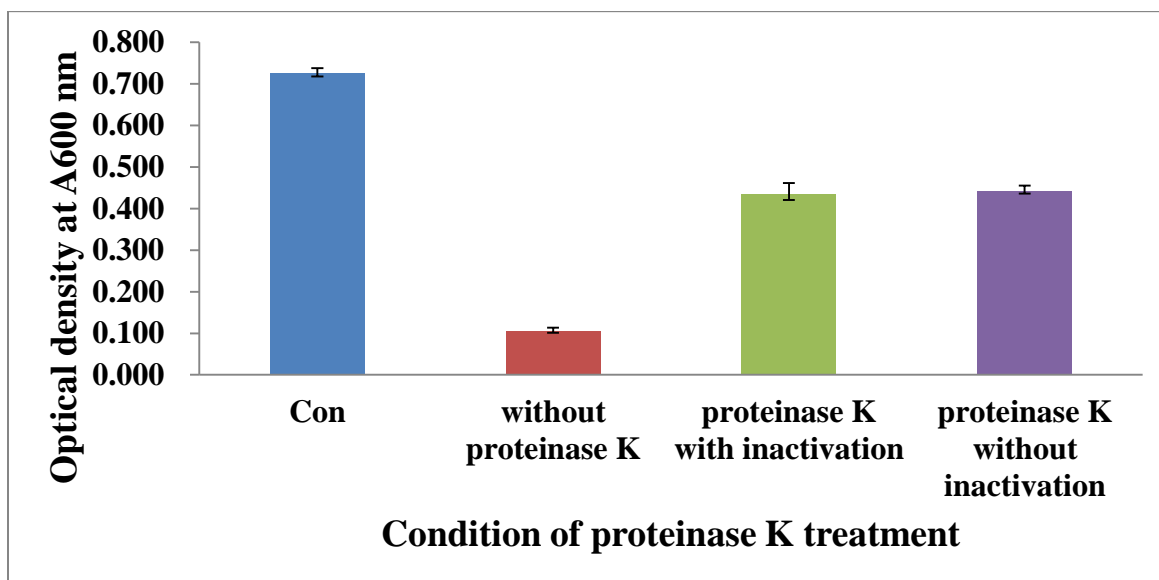
Treatment with high-concentration of *Salmonella* at MIC value of  $10^6$  CFU/ml showed a tendency to significantly decrease activity at an amount of the cells higher than  $10^7$  CFU/ml (Fig. 1). Therefore, to overcome these drawbacks, we analyzed the activity with a protease inhibitor mixture. It is well known that protease inhibitor plays a role in inhibiting the activity of protease in the cultural medium and correcting the MIC value (Ha et al., 2017).

Protease inhibitor mixture was added in each concentration to analyze its activity. As a result, the treatment with protease inhibitor mixture depending on concentration showed that the activity of the mixture was increased by 50% or more when 1  $\mu\text{l}$  of the mixture was treated and increased to the maximum value at 2  $\mu\text{l}$  (Fig. 5). These results are consistent with the results of mastoparan V1 originated from a wasp venom toxin (Ha et al., 2017).



**Fig. 5:-** Examination of antimicrobial activity depending on concentration of protease inhibitor mixture. *Salmonella* Typhimurium  $\chi$ 3339 strain was diluted to  $10^8$  CFU/ml and applied for the study. Bee venom was used by concentration of  $10 \mu\text{g}/\mu\text{l}$ , and added at the concentration of the MIC of  $10^6$  CFU/ml. Protease inhibitor mixture was added in an amount of  $0 \mu\text{l}$ ,  $0.5 \mu\text{l}$ ,  $1 \mu\text{l}$ ,  $2 \mu\text{l}$ ,  $5 \mu\text{l}$ , and  $10 \mu\text{l}$ . X- and Y-axes indicate optical density at 600 nm and concentration of protease inhibitor mixture, respectively.

We treated proteinase K in order to confirm that the antimicrobial activity of bee venom is derived from the peptide. As a result, high activity was observed when proteinase K was not treated, but proteinase K treatment showed low activity regardless of activation and inactivation states of proteinase K (Fig. 6). The MIC of melittin and phospholipase A2 were  $4 \sim 40$  and  $400 \mu\text{g}/\text{ml}$ , respectively, whereas in the case of treatment at the same time, the MIC was  $6 \sim 80 \mu\text{g}/\text{ml}$  (Leandro et al., 2015). Therefore, it is assumed that most of the antimicrobial activity of bee venom is derived from the peptide melittin and phospholipase A2.



**Fig. 6:-** Analysis of antimicrobial activity according to simultaneous treatment with proteinase K. *Salmonella* Typhimurium  $\chi$ 3339 strain was diluted to  $10^6$  CFU/ml and applied for the study. Bee venom was used by concentration of  $10 \mu\text{g}/\mu\text{l}$ , and added at the concentration of the MIC of  $10^6$  CFU/ml. X- and Y-axes indicate optical density at 600 nm and concentration of protease inhibitor mixture, respectively. Con; non treated.

In summary, these results indicate that bee venom purchased from the market is slightly lower than the general activity, but it can be used for the production of ghost vaccine when mixed with a protease inhibitor mixture. In addition, since it has a temperature stability up to  $60^\circ\text{C}$ , it has been found that there is no problem in making the powder into industrial use.

### Acknowledgements:-

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