Short communication KOREAN JOURNAL OF APPLIED ENTOMOLOGY

한국응용곤충학회지
Korean J. Appl. Entomol. 58(4): 283-289 (2019)
DOI: https://doi.org/10.5656/KSAE.2019.11.0.047

pISSN 1225-0171, eISSN 2287-545X

Peptide isolated from *Hermetia illucens* larvae inhibits mice from *Klebsiella pneumoniae* infection in the kidney

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동애등애유충에서 분리된 펩타이드의 신장에서의 폐렴간균 감염 억제 효능

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ABSTRACT: Overuse of antibiotics has significantly contributed to an increase in microbial antibiotic resistance, causing difficulties in the suppression of microbe-borne infectious diseases. In this study, we determined the anti-*Klebsiella pneumoniae* effect in the kidneys of mice induced by peptides isolated from *H. illucens* larvae. Mice were intranasally infected with a high dose of *K. pneumoniae* and 1 day later, peptides were introduced through the intramuscular route. Mice were sacrificed on day 10 upon *K. pneumoniae* infection to determine the bacterial loads in the kidneys. Mice receiving peptide treatment demonstrated significantly reduced bacterial loads, reduced bodyweight loss, and higher survival in a dose-dependent manner compared to control. These results indicate that peptide isolated from *H. illucens* larva inhibits *K. pneumoniae* infection in the kidney. The peptide from *H. illucens* larva could be a potential candidate for the development of an effective antibacterial drug.

Key words: *Hermetia illucens*, Peptide, *Klebsiella pneumoniae*, Kidney

초 록: 항생제의 오남용은 세균의 항생제 내성을 증가시켜 세균감염에 의한 질병 치료에 어려움을 초래한다. 본 연구에서는 동애등애유충으로부터 분리된 펩타이드의 신장에서의 폐렴간균 감염 억제 효능을 관찰하였다. 마우스는 비강을 통해 폐렴간균을 감염시키고 1일 후 펩타이드를 마우스에 근육 주사로 투여하였다. 10일 후 마우스를 희생하여 신장에서 세균 감염증을 조사하였다. 대조군에 비해 펩티드를 투여한 마우스의 신장에서 세균 감염증상, 몸무게의 감소가 유의하게 억제되었고 생존률이 농도 의존적으로 증가하는 것으로 나타났다. 이러한 결과들은 동애등애로부터 분리된 펩타이드가 폐렴 간균의 신장에서의 감염증상을 억제할 수 있음을 보여준다. 따라서 동애등애로부터 분리된 펩타이드는 효과적인 항생제 개발에서 가능성 높은 후보물질이 될 수 있을 것이다.

검색어: 동애등애, 펩타이드, 폐렴간균, 신장

Antimicrobial resistance has become a global challenge, with approximately 500,000 patients from 22 different countries being infected by antimicrobial-resistant pathogens (WHO, 2018). ESKAPE pathogens, an acronym for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., are of significant importance as they are frequently associated with nosocomial infections (Santajit and

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Received October 6 2019; **Revised** November 5 2019
Accepted November 7 2019

Indrawattana, 2016). Among them, *K. pneumoniae* is of particular interest due to their frequent outbreaks in the Neonatal Intensive Care Units (NICUs) (Fabbri et al., 2013). With the emergence of several drug-resistant bacterial strains such as carbapenem-resistant *K. pneumoniae* (Kamaruzzaman et al., 2019) and the hypervirulent *K. pneumoniae* which can be life-threatening even in healthy hosts (Shon et al., 2013), alternative antimicrobials to control these pathogens are urgently needed since drug-resistant bacteria are becoming increasingly difficult to treat in the clinical settings (Sharma et al., 2016).

Insects are capable of producing a variety of antimicrobial peptides (AMPs) and these are frequently used as major sources of AMPs (Yi et al., 2014). The black soldier fly *Hermetia illucens* larvae are of strong interest since they thrive in an environment ingesting decomposing organic matter while being surrounded by a wide variety of microorganisms within its habitat (Muller et al., 2017). Under these circumstances, the presence of antimicrobial substances would be essential for survival of the larvae. Recent investigations have reported that *H. illucens* larval extracts contain various types of AMPS (Muller et al., 2017), which are encoded by more than 50 genes (Vogel et al., 2018). Earlier studies have reported the potential of *H. illucens* larvae for microbial pathogen control. For example, it has been documented that introducing *H. illucens* larvae to animal manures results in a significant reduction of microorganisms such as *Escherichia coli* and *Salmonella* spp. (Erickson et al., 2004; Lalander et al., 2013; Liu et al., 2008). Screening and characterizing multiple AMPs of *H. illucens* appear to be promising as these effectively controlled a wide array of microorganisms, including the multidrug-resistant microbes (Elhag et al., 2017; Li et al., 2017; Shin and Park, 2019). Additionally, our previous investigations using *H. illucens* larval extract demonstrated an antibacterial effect against *K. pneumoniae* (Choi et al., 2012; Choi et al., 2018). Nevertheless, the antibacterial effect demonstrated in the aforementioned works were solely based on *in vitro* studies, which signifies the need for *in vivo* studies to validate their antimicrobial effects.

H. illucens peptide analysis in our previous investigations revealed that the peptides k22 (HP/F9) demonstrated strong antimicrobial activity against Gram-negative bacteria *in vitro* (Choi et al., 2018). In the current study, *K. pneumoniae*-infected mice were treated with peptides isolated from the *H. illucens* larvae to confirm antibacterial effects *in vivo*. We found that administering the peptides into infected mice protected them by reducing the bacterial loads, even in non-target organs such as the kidneys in a dose-dependent manner. Findings herein may contribute to identifying alternative AMPs for controlling *K. pneumoniae* infections.

Materials and methods

Animals and ethics statement

Seven-week old female Balb/c mice were purchased from KOATECH (Pyeongtaek, Gyeonggi-do, South Korea). A total of 60 mice were randomly grouped (n = 10 per group) and maintained under specific pathogen free conditions with easy access to food and water. All of the experimental procedures involving animals have been approved and conducted under the guidelines set out by Kyung Hee University IACUC (KHUASP(SE)-18-105).

High-performance liquid chromatography (HPLC)

HPLC was performed to isolate the peptide from *H. illucens* larvae after purification through open column systems as previously described (Choi et al., 2018). The peptide was analyzed using the nano-LC-ESI-MS/MS system consisting Easy-nLC 1000 (Thermo Scientific, Waltham, MA, USA) and an LTQ Orbitrap Elite mass spectrometer (Thermo Scientific) equipped with a nano-electrospray source as described previously (Choi et al., 2018).

K. pneumoniae bacteria culture

K. pneumoniae (ATCC 13883) was purchased from ATCC (Manassas, VA, USA) and the bacteria were cultured in Luria-Bertani (LB) broth at 37℃ for 24 h. Afterward, 50 ul of the cultured bacteria were plated on MacConkey agar plates (Becton, Dickinson Co., USA) in triplicates as previously described (Chu et al., 2014). Bacterial concentrations were enumerated by determining the colony-forming units from the

plated agar plates.

Assessment of antibacterial effect *in vitro*

In solutions containing different peptide concentrations (7.5, 15, 30, and 60 ug), 10^6 CFUs of *K. pneumoniae* were inoculated and cultured for 24 h at 37℃. A commercial antibiotic solution composed of 10,000 U/ml of penicillin and 10,000 ug/ml of streptomycin (P/S) was purchased from WELGENE (Daegu, Republic of Korea). As a control, *K. pneumoniae* was cultured with 1, 2, 4, and 8 units of P/S. All of the bacterial cultures were plated on MacConkey agar plates in triplicates and incubated overnight at 37℃ for CFUs calculation.

In vivo antibacterial effect

Mice were anesthetized and intranasally infected with 10^7 CFUs of *K. pneumoniae*. Antibacterial activity of HP/F9 peptide was determined by injecting 6, 30, and 75 ug of the peptides in PBS through the intramuscular (IM) route 24 h after infection. P/S was intramuscularly administered to control group mice following *K. pneumoniae* infection. All of the mice were sacrificed 10 days post-infection (dpi) to determine bodyweight loss and bacterial load. Individual kidney bacterial loads were determined by homogenizing the tissues in 1ml PBS using a syringe. Homogenates were filtered through a 100 um cell strainer (SPL Life Sciences, Pocheon, Korea) and serially diluted for plating on MacConkey agar in triplicates. Plates were incubated overnight at 37℃ and CFUs were calculated the next day.

Pathology study of the lungs and kidneys

Mice were sacrificed on 10 dpi and the collected kidney samples were fixed in 10% formalin, sectioned, and stained with Periodic Acid-Schiff (PAS). Tissues were observed under a microscope (Olympus TE-200U, Olympus Optical Co., Tokyo, Japan).

Statistical analysis

All results are expressed as mean ± standard deviation and the data sets were analyzed using the GraphPad Prism 5 software (San Diego, CA, USA). Statistical significance was determined by paired student's *t*-test and one-way analysis of variance (ANOVA). *P values < 0.05 were considered statistically significant.

Results

Peptide isolated from *H. illucens* larva inhibited *K. pneumoniae* growth *in vitro*

The peptide was isolated from *H. illucens* larva. To assess its antibacterial activity *in* vitro, *K. pneumoniae* was incubated in media containing various concentrations of the HP/F9 peptide or P/S. As seen in Fig. 1A, peptide at a concentration of 30 ug and 60 ug completely inhibited *K. pneumoniae* growth. Inoculating 4 and 8 units of P/S had the same effect on bacterial growth as colony formation was not observed under these conditions. Although weakly effective, administering 7.5 and 15 ug of HP/F9 peptide into bacterial culture hindered *K. pneumoniae* growth when compared to the untreated control group. Exemplified by these results, HP/F9 peptide can effectively thwart *K. pneumoniae* growth *in vitro* in a dose-dependent manner.

Peptides protected mice from *K. pneumoniae* infection

To confirm whether the isolated peptide induces antibacterial effect *in vivo*, female BALB/c mice were infected with *K. pneumoniae* followed by intramuscular injection of the *H. illucens* peptide 24 h later. Compared to the infection control group, mice treated with the peptide showed lesser bodyweight reduction (Fig. 1B) whereas all of the infection control mice died by day 4. Inoculating 75 ug of the *H. illucens* peptide protected against *K. pneumoniae* infection, as indicated by the 100 % survival rate (Fig. 1C). At 6 ug, there was no significant protection against infection and only 40% of the mice survived. Similar to control mice receiving 5 units of P/S, mice treated with 30 ug of *H. illucens* HP/F9 peptide demonstrated 60% survival rate which implies protection induced in a dose-dependent manner. As a result, it was confirmed that

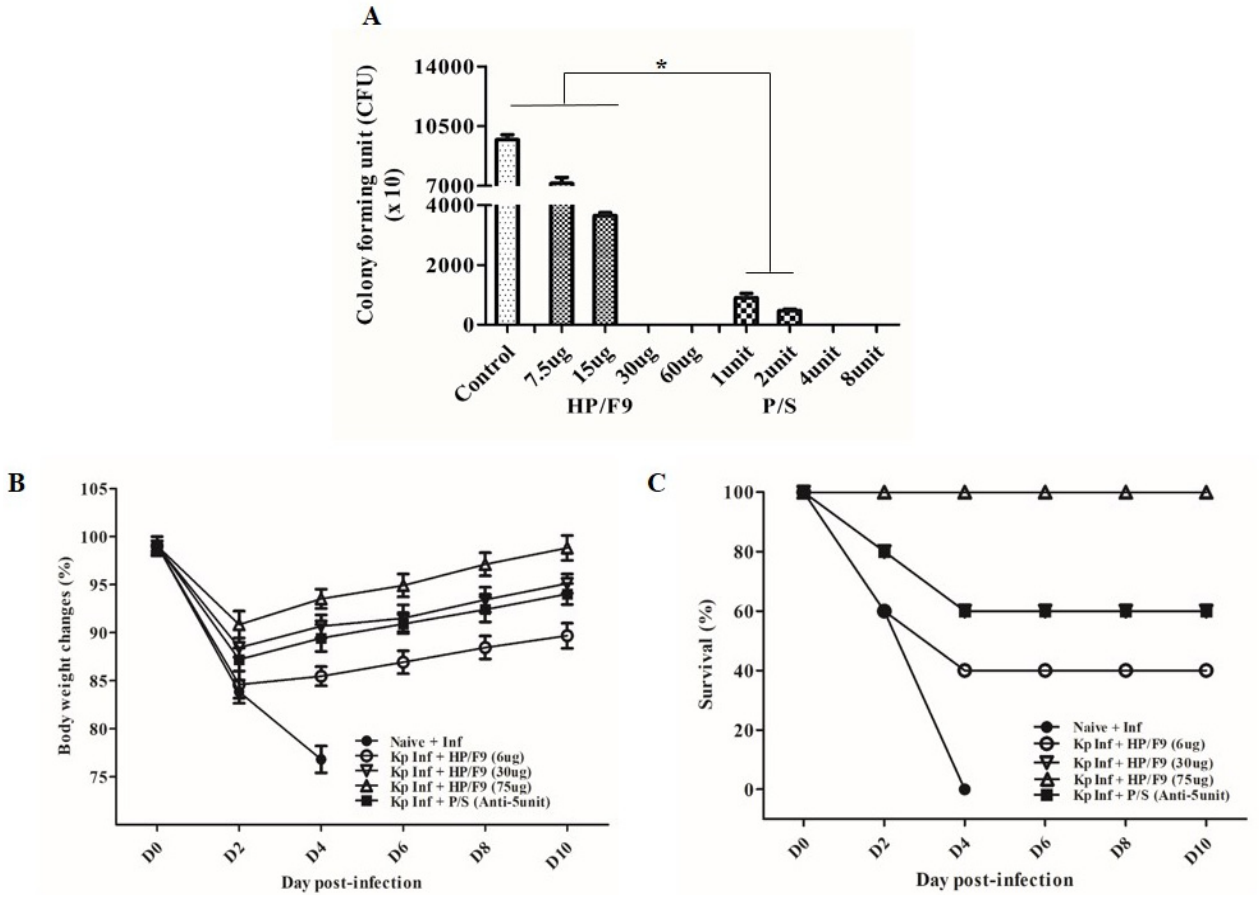

Fig 1. Antibacterial effect of the peptide on *K. pneumoniae in vitro* and murine protection. To evaluate the antimicrobial activity of the peptide, *K. pneumoniae* were treated with various concentrations of peptide *in vitro*. *K. pneumoniae* was inhibited by 30 ug and 60 ug of HP/F9, which were identical to that induced by 4 U of P/S (Fig. 1A). Peptide protected the *K. pneumonia*-infected mice from experiencing weight loss and promoted survival. Mice receiving 75 ug of HP/F9 peptide underwent significantly less bodyweight loss than the P/S control groups, and the protection induced by 30 ug of HP/F9 was similar to that of the P/S control group (Fig. 1B). Inoculating 75 ug of HP/F9 peptide into mice ensured 100% survival, whereas the survival rates of mice receiving 30 ug of HP/F9, 6 ug HP/F9, and P/S were 60%, 40%, and 60%, each respectively (Fig. 1C). Statistical significance between the groups was determined by using one-way ANOVA. An asterisk(*) denotes statistical significance where P value < 0.05.

intramuscular administration of the peptide protected the mice against *K. pneumoniae* infection.

Peptides inhibited *K. pneumoniae* replication in kidney

To assess whether HP/F9 peptide is capable of reducing bacterial infection in other organs, murine kidneys were collected. Plating of kidney homogenates from mice receiving various doses of HP/F9 peptide exhibited significant bacterial load reduction (Fig. 2A). Inhibition induced by 30 ug of the peptide was comparable to those of 5U P/S. Highest bacterial load reduction was observed from mice treated with 75 ug HP/F9 peptide. These results confirm that peptide isolated from *H. illucens* inhibits *K. pneumoniae* in multiple murine organs.

Peptide inhibited glomerulonephritis in the kidney

To assess the severity of glomerulonephritis, kidneys were pathologically examined. As illustrated in Fig. 2B, glomerulonephritis was not detected in any of the groups, except for mice receiving 6 ug of the peptide. No significant differences were observed from mice receiving either 30 ug or 75 ug of HP/F9 peptide compared to control. Findings suggest that *K. pneumoniae* inhibition through antibiotic treatment prevents nephrotic

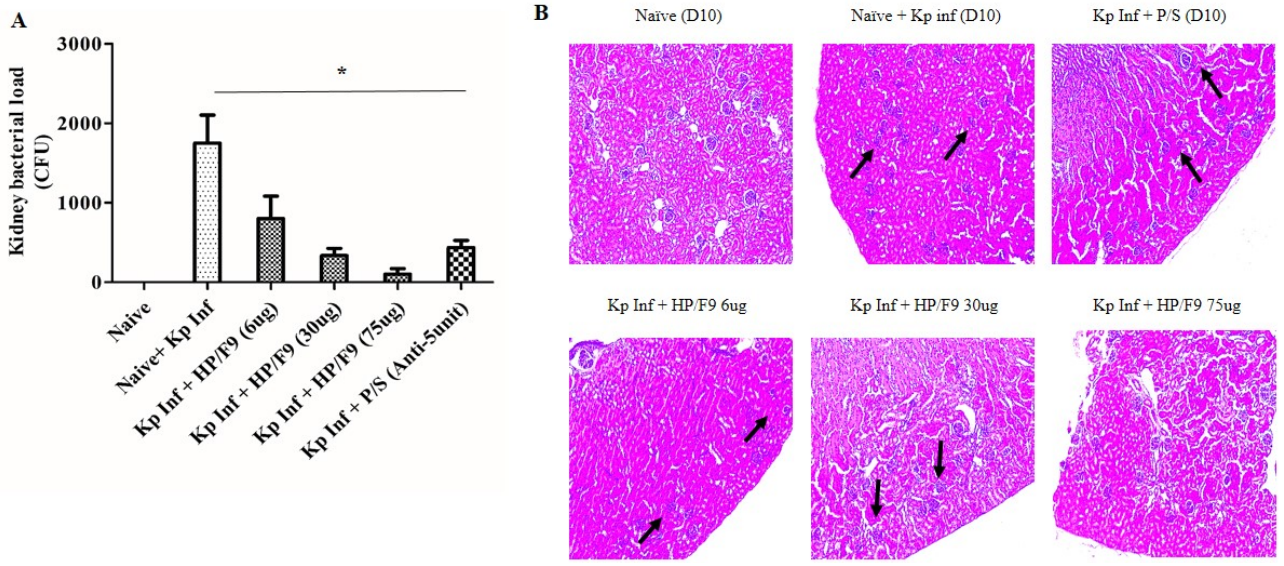

Fig 2. Peptide inhibits the growth of *K. pneumoniae* in the kidney. Peptide significantly inhibited *K. pneumoniae* growth in the kidneys of mice. Greater inhibition was observed from mice receiving 75 ug of peptide than mice receiving P/S, whose inhibition levels were similar to those treated with 30 ug of the peptide. Mice receiving 6 ug of HP/F9 peptide demonstrated weak inhibition of *K. pneumoniae* (Fig. 2A). Pathological changes in the kidneys of mice were observed at 10 dpi. No signs of glomerulonephritis were detected in naïve mice and P/S antibiotics group, which were evident in the case of infection control mice. Minor signs of glomerulonephritis were observed from mice receiving 6ug HP/F9 peptide, whereas its signs were less evident in kidneys of mice which had received 30 ug and 75 ug of HP/F9 (Fig. 2B). Statistical significance between the groups was determined by using one-way ANOVA. An asterisk(*) denotes statistical significance where P value < 0.05. Arrows indicate influx of inflammatory cells. All images are shown at 200X magnification after Periodic acid-Schiff staining.

inflammation and administering HP/F9 had a similar effect.

Discussion

Peptide extracted from the hemolymph of *H. illucens* effectively inhibits bacterial proliferation *in vitro*, but its antibacterial effect *in vivo* has not been investigated. Additionally, although several defensin and cecropin AMPs from *H. illucens* have been characterized to date (De Smet et al., 2018), a *H. illucens*-derived peptide demonstrating antibacterial effect against *K. pneumoniae* has not been reported. In this study, a peptide isolated from the hemolymph of *H. illucens* larvae was used to treat *K. pneumoniae*-infected mice. Inoculating this peptide into infected mice significantly reduced bacterial loads, as well as preventing drastic weight loss compared to the control group. These results provide insights into the development of a potentially new AMP to deal with increasing drug-resistant microbes.

In our previous study, the lowest bodyweights in *K. pneumoniae*-infected mice were observed at 2 dpi (Chu et al., 2014). However, drastic changes to bacterial load reduction or bodyweight were not observed in the aforementioned study due to the early administration of antibacterial agents. Therefore, in the current study, mice were treated with antimicrobial HP/F9 peptide 1 dpi to ensure severe disease progression prior to introducing the peptides. Confirmation of greater bacterial burden reduction and lesser bodyweight loss under these conditions would imply that the HP/F9 peptide possesses strong antibacterial properties. As anticipated, administering HP/F9 peptide into mice conferred protection against *K. pneumoniae* in a dose-dependent manner.

Bacterial infection-related glomerulonephritis (IRGN) has been documented in numerous clinical settings, which were associated highly with infection of the upper respiratory tract, lung, urinary tract, etc (Nasr et al., 2013). Although bacteria belonging to *Staphylococcus* and *Streptococcus* genera are the predominant cause, approximately 10% of the IRGN arises from infection due to Gram-negative bacteria, which involves members of the genera *Escherichia, Yersinia, Pseudomonas, Klebsiella,* etc (Nasr et al., 2013). Evidently, patients who died of pneumonia due to *K. pneumoniae* infection had glomerulonephritis when autopsied using light microscopy (Forrest et

al., 1977). Signs of acute glomerulonephritis in the kidneys of mice, which may be attributed to IRGN were found and HP/F9 peptide effectively inhibited the *K. pneumoniae*-induced glomerulonephritis. Pathological examinations have revealed that kidneys of mice treated with 75 ug were identical to those of naïve mice. Similar results were acquired from kidneys of mice treated with 30 ug peptide, which were comparable to those from mice receiving commercial antibiotics. Combined, suppression of *K. pneumoniae* by the peptide as demonstrated in our results signifies its potential as a new candidate for antimicrobial drug development.

A systematic study conducting a thorough characterization of the peptide and identifying its antimicrobial mechanism against *K. pneumoniae* is highly desired. However, the results of this study may be useful for the future development of antimicrobial agents. Our results have confirmed that the peptide possesses strong antibacterial activity, specifically against *K. pneumoniae* (Choi et al., 2018). These results are consistent with the previous findings, which documented its potential as a novel antimicrobial compound. Thus, this study suggests that the peptide is a novel antimicrobial active substance useful for treating bacterial infections.

Acknowledgments

The authors declare that no competing interests exist. This work was supported by grants National Research Foundation of Korea (NRF) (2018R1A2B6003535, 2018R1A6A1A03025124) and the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01320501), Rural Development Administration, Republic of Korea

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