Journal of Asia-Pacific Entomology 23 (2020) 36–43

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

Full length article

Peptides in the hemolymph of *Hermetia illucens* larvae completely inhibit the growth of *Klebsiella pneumonia in vitro* and *in vivo*

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ARTICLE INFO

Keywords:
Antimicrobial peptide
Hermetia illucens
Klebsiella pneumonia
High performance liquid chromatography (HPLC)
Antibiotics
Probiotics

ABSTRACT

Extensive use of antibiotics has caused the microbial resistance to rise drastically within the last few decades, and new approaches are therefore needed to develop effective antibacterial substances. In this study, we identified peptide in the hemolymph of *Hermetia illucens* larvae using reverse-phase chromatography, HPLC and Nano-LC-ESI-MS/MS system. We investigated the antibacterial effect of HP/F9 peptides against *Klebsiella pneumoniae in vitro* and *in vivo*. The peptide effectively inhibited the growth of *K. pneumoniae in vitro* and completely removed *K. pneumoniae* from the lungs of mice. Importantly, peptides (22,000 Da, HP/F9) successfully reduced lung inflammation upon *K. pneumoniae* infection. These results indicate that the HP/F9 peptide from *H. illucens* larva can effectively protect the mouse from *K. pneumoniae* infection. HP/F9 could be a new candidate for the development of effective antibacterial substance.

Introduction

Inhabitation of *H. illucens* flies have been reported across multiple continents, yet research investigating the functional role of its larvae and their properties are currently lacking (Choi and Jiang, 2014). Multiples plants, as well as insects, have been documented to possess substances that exhibit anti-microbial properties (Cowan, 1999; Yi et al., 2014). For instance, maggot therapy using *Lucila sericata* is gaining attention as it becomes re-introduced into the clinic to clear necrotic tissues (Bexfield et al., 2004) and treat chronic wounds (Daeschlein et al., 2007). Various insects have antimicrobial substances, which are produced on the surface or within their digestive tract to prevent microbial infection (Choi et al., 2012). *Klebsiella pneumoniae* is a non-motile, encapsulated rod-shaped bacterium found on the skin and in the respiratory system, including the mouth, lower respiratory tract and lungs (Goto et al., 2008; Siu et al., 2012; Struve et al., 2015; Prokesch et al., 2016). Additionally, although *K. pneumoniae* has been traditionally considered as an opportunistic pathogen causative of hospital-acquired infections, an increasing number of community-acquired invasive *K. pneumoniae* infections are being reported globally (Bouza and Cercenado, 2002; Pessoa-Silva et al., 2003; Fabbri et al., 2013). Antimicrobials have been widely used against *K. pneumoniae*. However, infections are very refractory to therapeutic interventions. Decades of heavy dependence on antibiotics in healthcare have created a recurring problem of drug-resistant microorganisms. Rising levels of multi-drug resistance have made the treatment of bacterial infections in clinical settings arduous. Consequently, controlling bacterial infection has become a major priority for hospitals, as the number of effective therapeutic options are limited (Paterson, 2001).

Detection of antimicrobial peptides in hemolymphs of insects was first reported in 1974 and over the past few decades, vast types of antimicrobial substances have been identified (Yi et al., 2014). In the case of dipterans, which encompasses *H. illucens*, antimicrobial peptides were first observed in the hemolymphs of *Drosophila melanogaster* and these insects were documented to produce increased quantities of antimicrobial products as well as possessing enhanced humoral immune response upon previous exposure to microorganisms (Robertson and Postlethwait, 1986; Muller et al., 2017). Additionally, it has also been documented that antimicrobial peptides that are synthesized in insects are eventually secreted into the hemolymph (Bulet et al., 1999; Hoffmann and Reichhart, 2002; Muller et al., 2017). Studies investigating the antimicrobial peptide isolated from *H. illucens* hemolymph continue to emerge. Previous studies have demonstrated that supplementing *H. illucens* hemolymph enhances the development of *Exorista larvarum in vitro*, whereas *Antheraea pernyi* hemolymph was unable to induce the same effect (Dindo et al., 2016). *H. illucens* have

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https://doi.org/10.1016/j.aspen.2019.10.004
Received 27 June 2019; Received in revised form 19 August 2019; Accepted 17 October 2019
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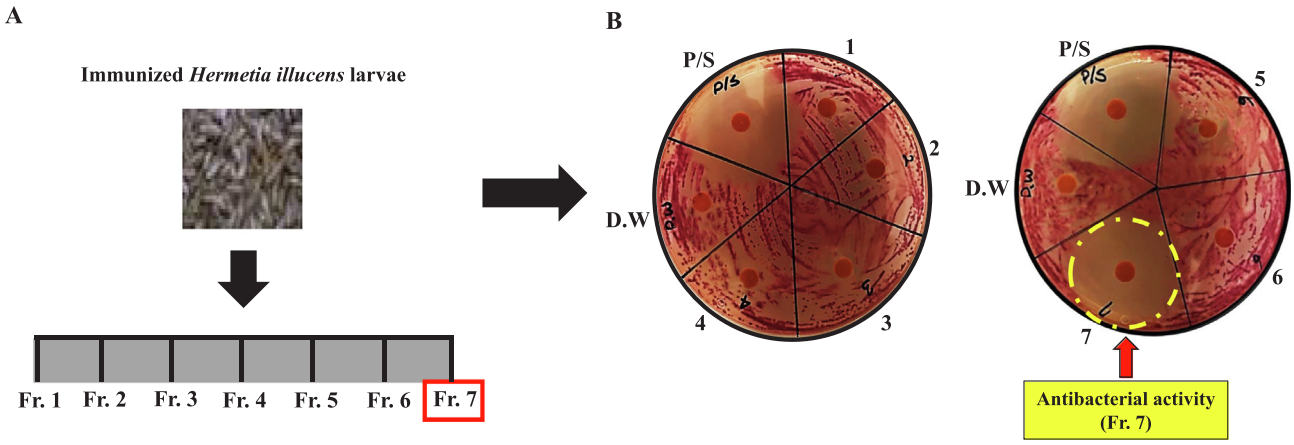

Fig. 1. Fractionation process of *H. illucens* larvae body fluids. *H. illucens* larvae body fluids extract was divided into seven fractions by C18 Cartridges chromatographic method. Then, 50 µl of the fraction solution and antibiotics were added to each 6 mm disc, which were dried at room temperature for 30 min. Diameters of the *K. pneumoniae* inhibition zones surrounding the discs were measured after 18 h.

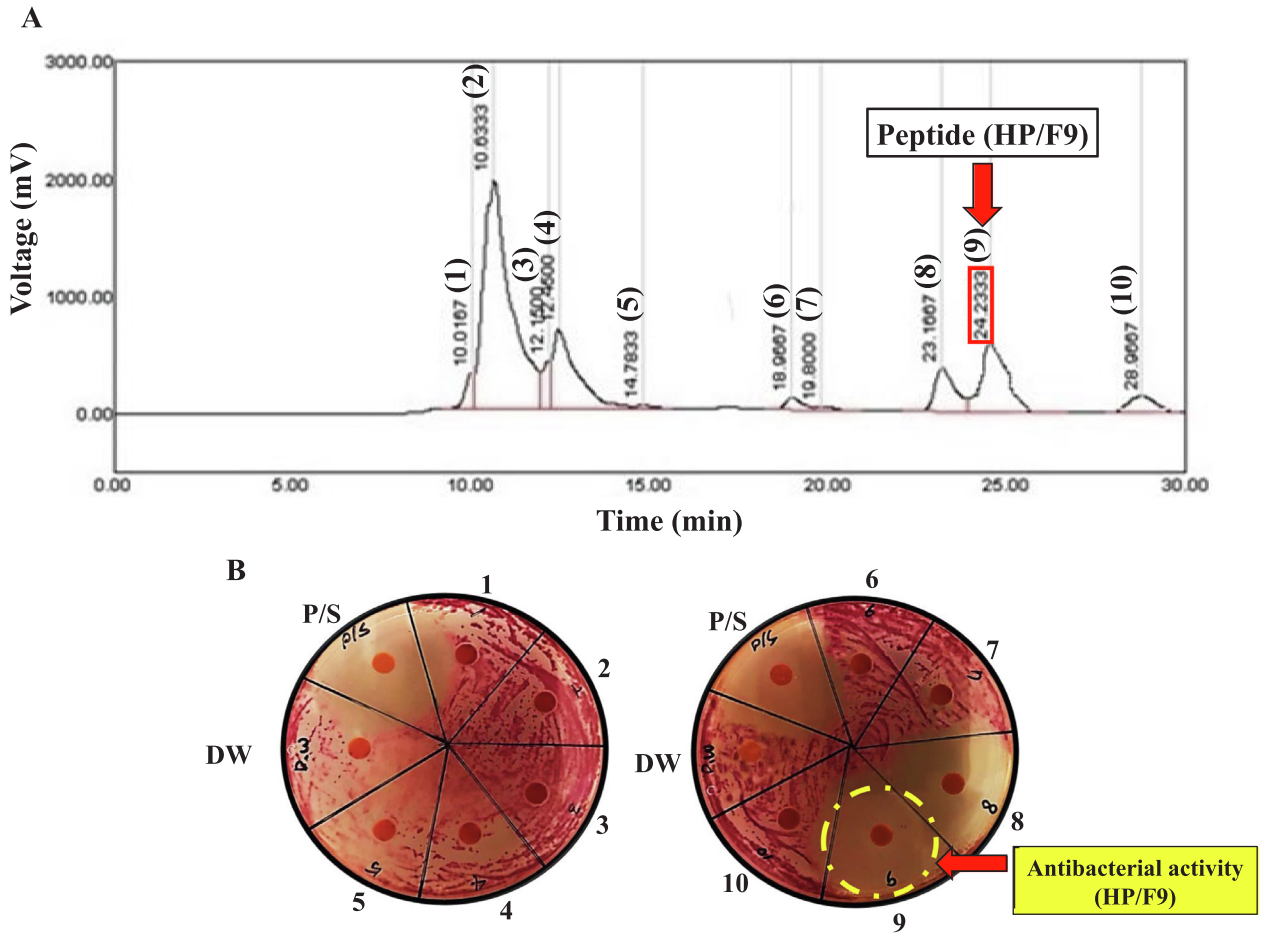

Fig. 2. High-performance liquid chromatography (HPLC) analysis. Fr.7 was analyzed by HPLC and results showed various peaks in the HPLC chromatogram. The peaks were divided into ten sub-fractions based on HPLC analysis. To identify the active substance responsible for the most effective antibacterial activity, bacteria were treated with various concentrations of HPLC sub-fractions for 18 h. Among the HPLC sub-fractions, HP/F9 strongly inhibited bacterial growth in a concentration-dependent manner compared to other HPLC sub-fractions.

been documented to possess more than 50 genes encoding numerous types of antimicrobial peptides, and the larvae reared using different dietary sources induced diverse inhibitory effect against a wide array of bacteria (Vogel et al., 2018). Several antimicrobial peptides genes from *H. illucens* have already been screened and one gene expressed in the form of thioredoxin fusion protein demonstrated inhibitory effect against various bacteria as well as fungal strains (Elhag et al., 2017). Recently identified defensin-like antimicrobial peptide from *H. illucens* hemolymph strongly inhibited antibiotic-resistant Gram-positive bacteria such as the methicillin-resistant *Staphylococcus aureus* (MRSA) (Park et al., 2015). Similarly, novel peptides DLP2 and DLP4 from *H. illucens* demonstrated strong antibacterial activity (Li et al., 2017). A novel attacin identified in *H. illucens* hindered the growth of MRSA (Shin and Park, 2019). Inoculating bacteria through piercing method

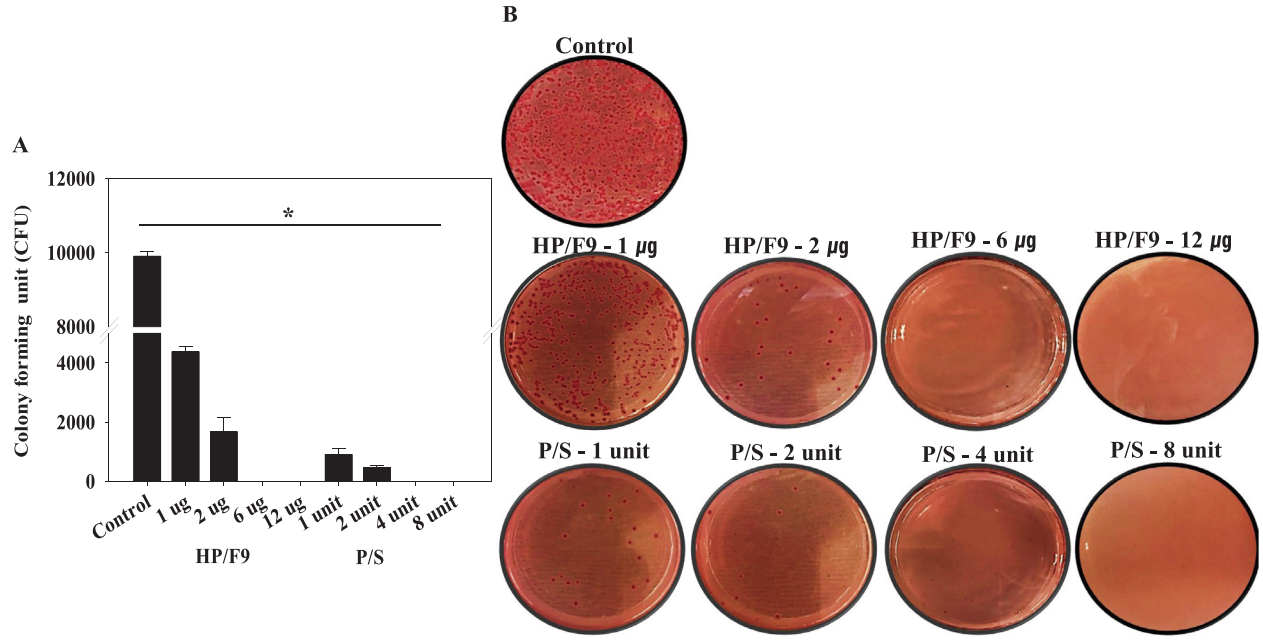

Fig. 3. Antibacterial effects of HP/F9 *in vitro*. To assess its *in vitro* antibacterial activity, *K. pneumoniae* was incubated in media containing various concentrations of HP/F9. A commercial antibiotic containing penicillin and streptomycin was used as control. HP/F9 at 6 and 12 μg/50 μl induced *K. pneumoniae* death. These results are equivalent to those of *K. pneumoniae* exposed to 4 U antibiotic (A, *P < 0.05). No growth occurred on MacConkey agar plates which involved HP/F9 at 6 and 12 μg/50 μl or P/S 4 U doses (B).

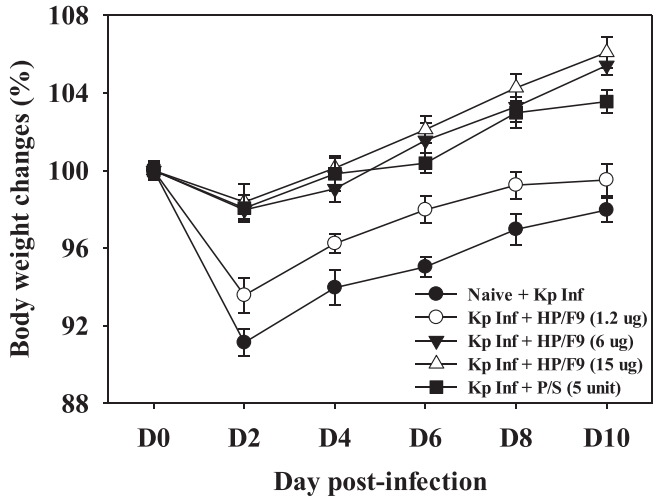

Fig. 4. HP/F9 protects mice against *K. pneumoniae* infection. HP/F9 at 6 and 15 μg protected mice against *K. pneumoniae* infection. The levels of protection were similar to those in the control group that received commercial antibiotic treatment. Compared to infection control group, lesser body weight reduction was observed from mice receiving as little as 1.2 μg HP/F9.

into *H. illucens* larvae significantly elevated antimicrobial activity within its hemolymph and their production (Zdbicka-Barabas et al., 2017; De Smet et al., 2018).

In our previous study, the hemolymph (body fluid) from *H. illucens* larvae immunized with probiotics (*Lactobacillus casei*) has been purified using reverse-phase chromatography, HPLC and Nano-LC-ESI-MS/MS system as indicated (Choi et al., 2018). The peptides k20 (HP/F8) and k22 (HP/F9) effectively inhibited the growth/proliferation of the tested bacteria, showing strong antibacterial activity against Gram-negative bacteria *in vitro* (Choi et al., 2018). However, no studies using peptides from the hemolymph from *H. illucens* for the antimicrobial activity have been reported in animal models. Since *H. illucens* larvae immunized with probiotics have been shown to induce a higher level of peptide expressions compared to non-immunized larvae (Choi et al., 2018), in our current study, we investigated the antimicrobial activity of peptides from larval hemolymph of *H. illucens* immunized with probiotics both *in vitro* and *in vivo*. We also determined the toxicity of peptides in mice and found the peptides are very safe. We found that peptides HP/F9 in hemolymph from *H. illucens* larvae completely inhibited the growth of *K. pneumoniae in vitro* and *in vivo* and protected mice from *K. pneumoniae* infection, indicating that HP/F9 is a potential peptide for the development of novel antibacterial drugs.

Materials and methods

Animals used and ethics statement

Female BALB/c mice aged 7 weeks old (n = 10 per group) were purchased from KOATECH (Pyeongtaek, Gyeonggi-do, South Korea). All animals were maintained in a facility with 12 h day and night cycle, as well as *ad lilbitum* 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 (permit number: KHUASP(SE)-18-105). Isoflurane anesthesia (BSL 2) was used to minimize suffering during surgery.

Immunization of H. illucens larvae and purification of hemolymph

Two-week-old *H. illucens* larvae were provided by the Greenteko (Gongju-si, Chungcheongnam-do, Republic of Korea). *H. illucens* larvae (600 g) were initially rinsed with distilled water three times to remove all impurities. The larvae were immunized with probiotics *Lactobacillus casei* using a needle as previously described (Robertson and Postlethwait, 1986). Small scissors were used to open the rear part of the larva one by one. Afterwards, 10 μl of hemolymph from each larva was collected and a total of 2 ml from 200 larvae were collected for use in the next step. The hemolymphs were fractionated into seven portions using C18 cartridges (Waters Co., USA), and subsequently evaporated under reduced pressure using a rotary evaporator at 50 °C as indicated previously (Chu et al., 2014; Choi et al., 2018). The seven column

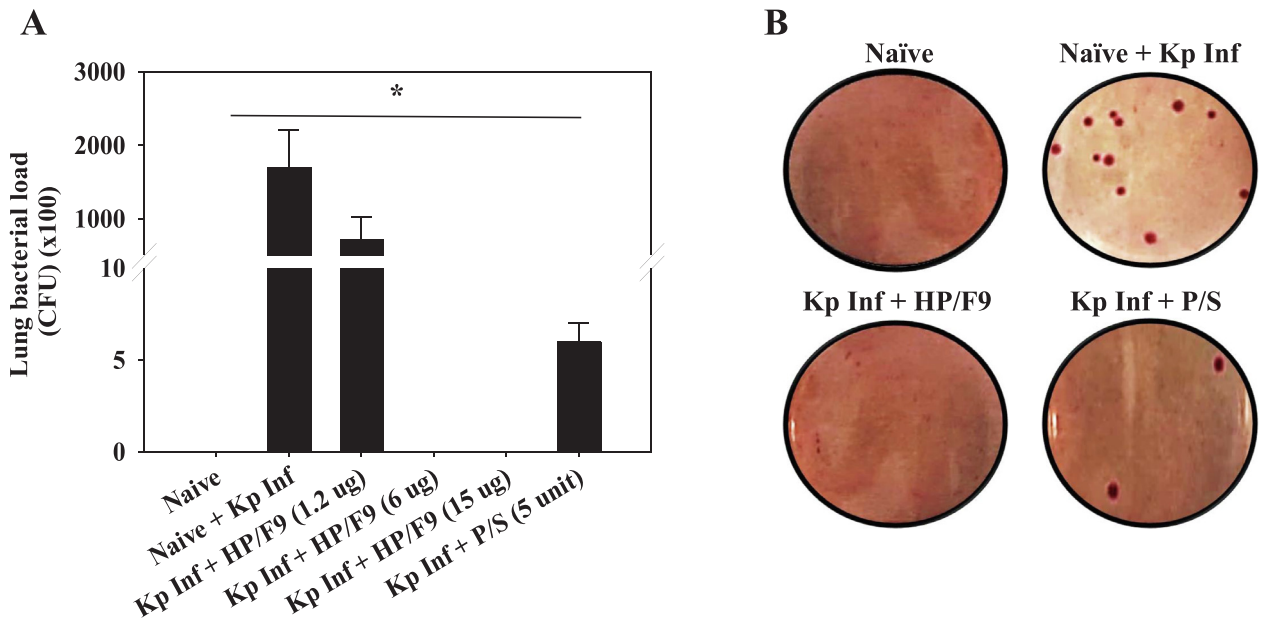

Fig. 5. HP/F9 inhibits *K. pneumoniae* replication in the murine lungs. HP/F9 significantly inhibited *K. pneumoniae* growth in the lungs. The inhibition was equivalent to that induced by the commercial antibiotic (A *P < 0.05). No growth occurred on MacConkey agar plate at HP/F9 dose of 15 μg/50 μl doses (B).

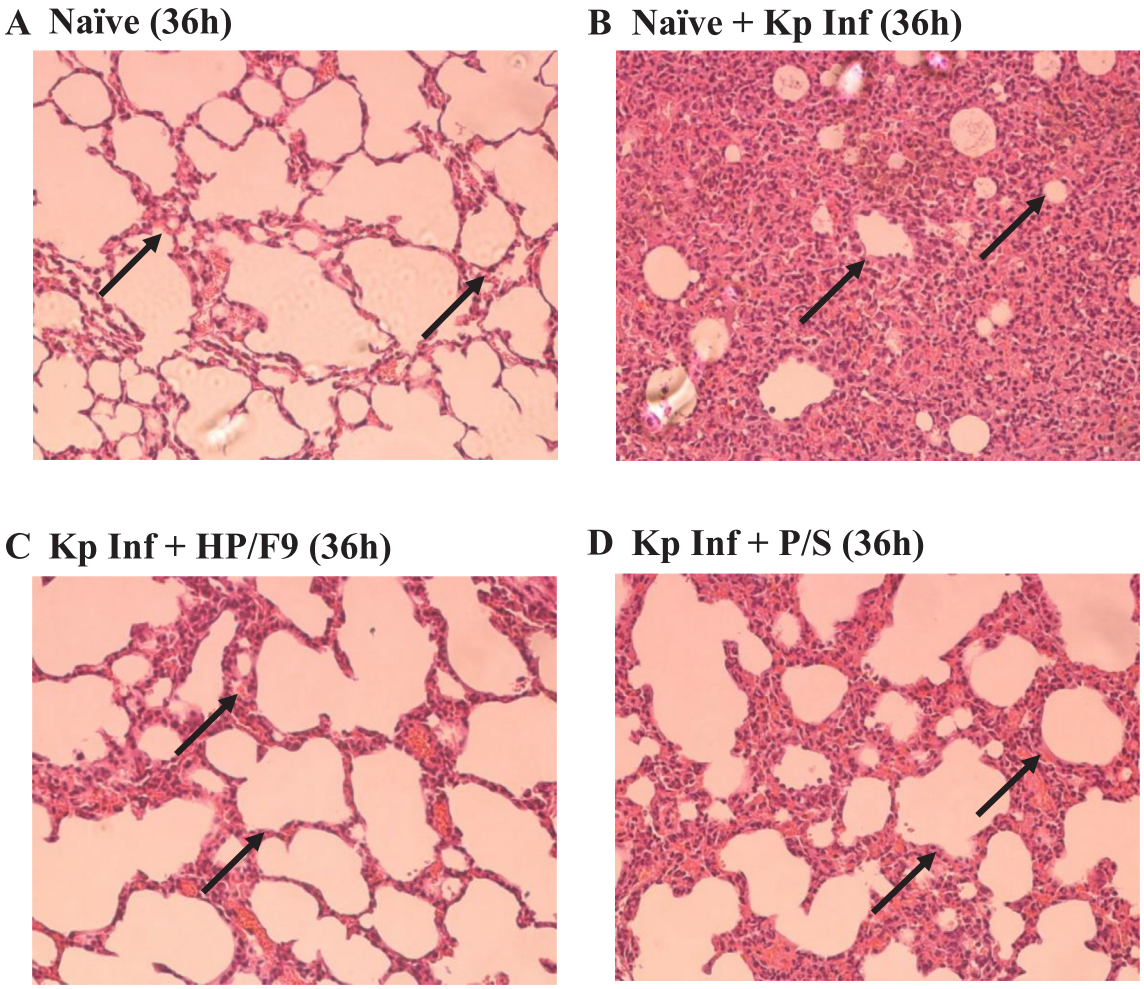

Fig. 6. Pathology study in the lungs. Pathological changes in the murine lungs were examined. Similar to control group and antibiotics-treated group, mice treated with HP/F9 had less neutrophil accumulation in the lungs (Hematoxylin & Eosin stained lung sections at 200× magnification).

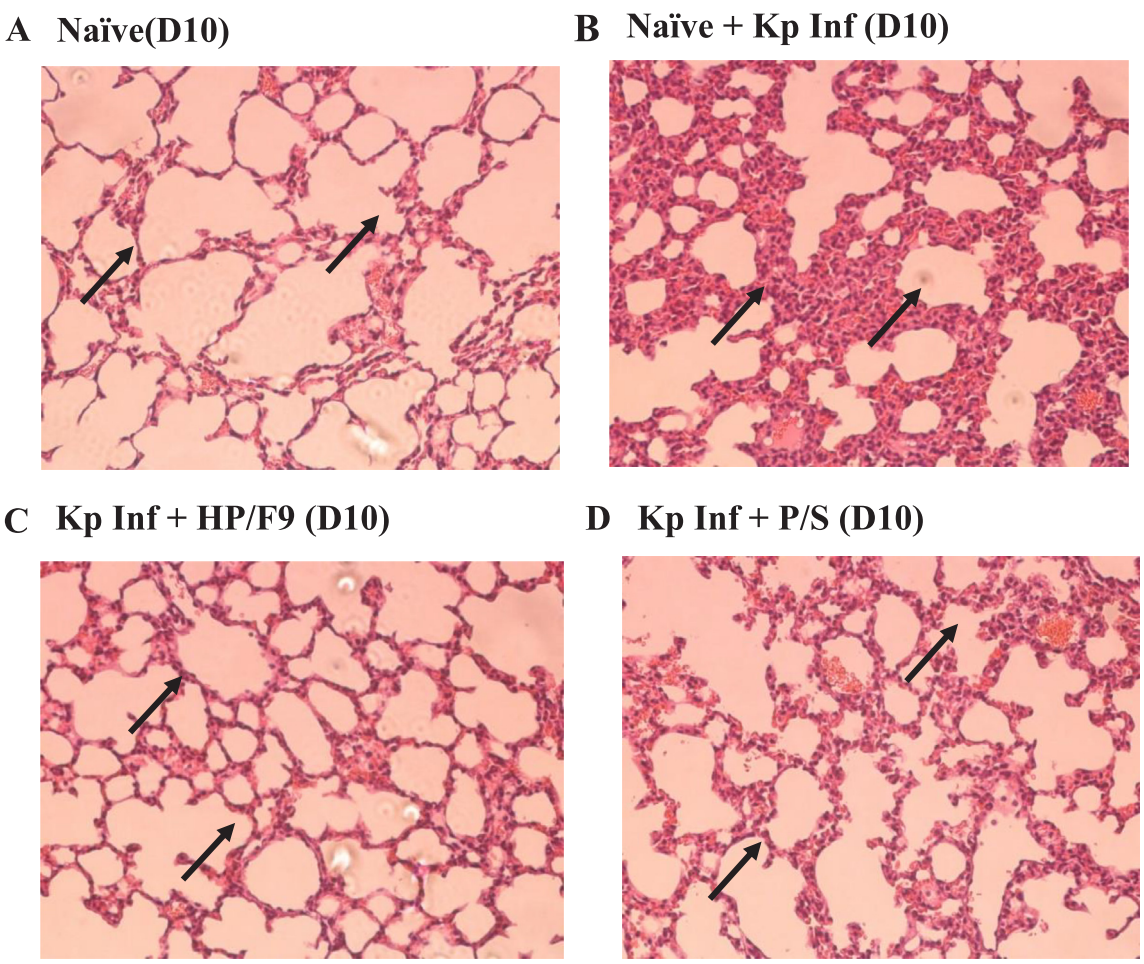

Fig. 7. Pathology study in the lung (D-10). No pathological changes were observed from mice after 10 days, which received intranasal HP/F9 treatment. In comparison to the control group, neither HP/F9-treated nor P/S-treated mice developed any pathological sings when examined under the microscope (Hematoxylin & Eosin stained lung sections at 200× magnification).

fractions were collected and each fraction was tested *in vitro* for antimicrobial effect before subjecting to further high performance liquid chromatography (HPLC) analysis. Among these fractions, fraction 7 (Fr.7) effectively inhibited the inhibition of bacteria compared to other fractions and was used for HPLC analysis. All extracts and fractions were filtered using a 0.20 mm syringe filter and stored at −80 °C until use.

HPLC analysis

Fr.7 was analyzed by YL9100 HPLC system (Young Lin Instrument Co., Ltd., Korea) containing a C18 reverse-phase column (4.5 mm i.d. × 250 mm, 5.0 μm particle diameter, Agilent Technologies Co., USA) with an injection volume of 10 μl, a column flow rate of 1.0 ml/min, and a mobile phase of 100% methanol and 100% water (the ratio of 50:50) for 60 min. The analysis was performed at 40 °C and at a UV wavelength of 215 nm. The Fr.7 was finally divided into ten HPLC fractions, including HP/F9 as indicated previously (Chu et al., 2014; Choi et al., 2018). Amino acid sequence for peptide HP/F9 was determined by using nano-LC-ESI-MS/MS system consisting of Easy-nLC 1000 (Thermo Scientific, Waltham, MA, USA) and an LTQ Orbitrap Elite mass spectrometer (Thermo Scientific) equipped with a nano-electrospray source. National Center of Biological Information (NCBI) BLAST program was used for sequence analysis. Analyzed peptide sequences were compared with known peptide using the NCBI's BLASTx algorithm.

Bacterial strain and culturing

Klebsiella pneumoniae (ATCC 13883) was purchased from ATCC (Manassas, VA, USA). The bacteria were incubated in Luria-Bertani (LB) broth at 37 °C for 24 h and then plated on MacConkey (Becton, Dickinson Co., USA) agar plates as described previously (Chu et al., 2014). Bacterial cultures were serially diluted and 50 μl from each of the dilutions were plated in triplicates. Colony-forming units (CFU) from each of the dilution plates were averaged.

In vitro antibacterial effect of HP/F9

To assess the antibacterial activity of HP/F9, *K. pneumoniae* (10^6 CFU/50 μl) was incubated at 37 °C in solutions containing various concentrations of HP/F9 (1, 2, 6 and 12 μg/50 ul). After 2 h of incubation, the *K. pneumoniae* and HP/F9 mixture was plated on MacConkey agar and bacterial growth was evaluated by calculating the CFUs. To compare the antibacterial effect of HP/F9, commercial antibiotics containing penicillin and streptomycin (P/S; WELGENE, Daegu, Republic of Korea) were used as control. Similar to the HP/F9 mixtures, *K. pneumoniae* bacterial culture treated with 1, 2, 4 and 8 units (U) of P/S were plated on MacConkey agar and colony growths were assessed. All plates were prepared in triplicates and 50 μl of each mixture was used for plating.

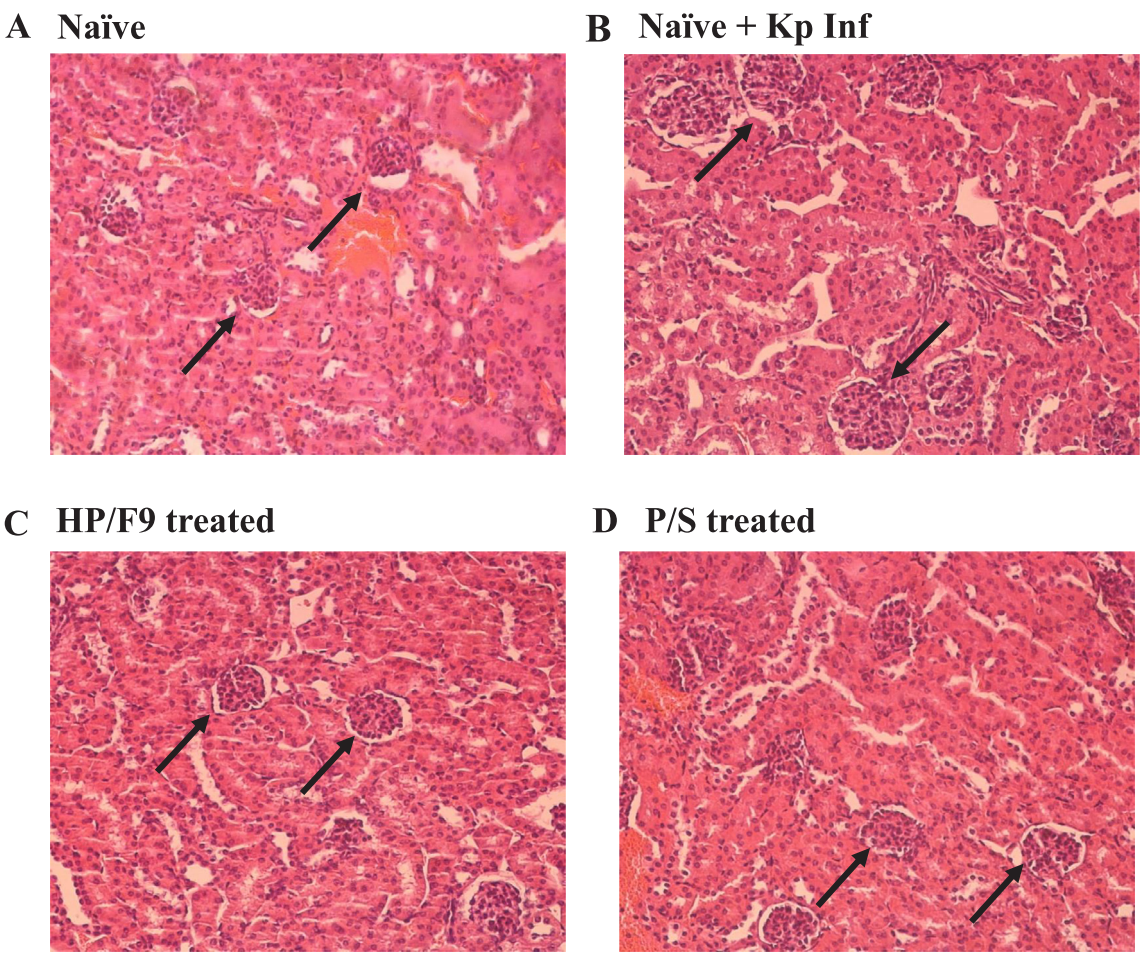

Fig. 8. Toxicity study *in vivo*. Mouse kidneys were examined for pathological changes. No pathological changes were observed in mice after 10 days in HP/F9 treated groups. Similar to control and antibiotics, mice treated with HP/F9 has less glomerulonephritis in the kidneys (Hematoxylin & Eosin stained lung sections at 200× magnification).

The effects of HP/F9 in mice against K. pneumoniae infection

After being anesthetized, mice (n = 10 per group) were intranasally infected with *K. pneumoniae* (10^7 CFU/50 µl). Antibacterial activity was determined by administering 1.2 µg, 6 µg and 15 µg of HP/F9 in 50 µl PBS 2 h after infection via intranasal route. As a control, a commercial antibiotic P/S was also intranasally injected into *K. pneumoniae*–infected mice. Of the 10 mice, 6 mice from each group were sacrificed 36 h after infection and lung bacterial loads were determined. Cervical dislocation was performed after isoflurane anesthesia to minimize suffering. Remaining 4 mice were monitored daily to determine changes in body weight. To determine lung bacterial loads, lungs were collected and homogenized in 1 ml PBS using a syringe and filtered through a 100 µm cell strainer (SPL Life Sciences, Pocheon, Korea). Serially diluted lung homogenates from each mouse were plated on MacConkey agar plates in triplicates and incubated at 37 °C for 24 h. Afterwards, CFU of individual lung homogenates were evaluated.

Lung and kidney pathological examination post-infection

At 36 h and 10 days post-infection (p.i.), mice were sacrificed and the lungs were collected for pathological examination. Harvested lung samples were exsanguinated and briefly washed in PBS for excess blood removal prior to fixation in 10% formalin. Afterwards, tissues were sectioned and stained with hematoxylin and eosin (H&E). Kidney samples were collected from sacrificed mice on day 10 p.i., which underwent similar fixation and staining procedure as the lung samples. The H&E stained tissue cross-sections were observed under a microscope (Olympus TE-200U, Olympus Optical Co., Tokyo, Japan) for signs of glomerulonephritis and peribronchial inflammation from kidneys and lungs, respectively.

Statistical analysis

All results are expressed as the mean ± SD from three individual experiments. Statistical significances were assessed by paired student's *t*-test and one-way analysis of variance (ANOVA). Data analyses were performed using SigmaPlot 11 (Systat Software Inc., Chicago, IL, USA). *P < 0.05 was considered to be statistically significant.

Results

Purification of antibacterial peptides from immunized H. illucens larvae

The antibacterial compound was extracted from the hemolymph of immunized *H. illucens* (Fig. 1A), which were subsequently purified using the C18 cartridges reverse-phase purification assay. Purified portions were subjected to rotary evaporation under reduced pressure at 50 °C. Disc diffusion assay results indicate that Fr.7 demonstrated the highest antibacterial effect against *K. pneumoniae* (Fig. 1B). The Fr.7 was subjected to further analysis using HPLC for acquisition of purified products.

Antibacterial activity of identified peptides

Analysis from HPLC revealed multiple peaks within the purified Fr.7, which were subdivided into ten HPLC fractions (Fig. 2A). Bacterial treatment with different concentrations of the HPLC fractions for 24 h was conducted for identification of specific substance embodying the most effective antibacterial effect. The sequence analysis showed that peptide HP/F9 was composed of 22 amino acid (YQASGTPLVVIAGQ-EYGTGSSR). Compared to other HPLC fractions, HP/F9 (22,000 Da, k22) was the strongest bacterial growth inhibitor (Fig. 2B).

HP/F9 peptides effectively inhibited the growth of K. pneumoniae in vitro

K. pneumoniae was incubated with various concentrations of HP/F9 for *in vitro* antibacterial activity assessment. As a control group, commercialized antibiotic P/S was used. HP/F9 treatment of 6 or 12 μg in 50 μl induced complete inhibition of *K. pneumoniae* growth, which were equivalent to the antibacterial effect induced by 4 U of antibiotics (Fig. 3A and B). Introducing 1.2 μg of HP/F9 into mice also induced significant bacterial growth reduction. Exemplified by these results, HP/F9 can effectively thwart *K. pneumoniae* growth *in vitro*.

HP/F9 peptides protected mice from K. pneumoniae infection

Upon infection with *K. pneumoniae* (10^7 CFU) in mice, HP/F9 was administered intranasally 2 h p.i.. Protection against *K. pneumoniae* was observed in mice treated with 15 μg HP/F9. After 2 days p.i., a noticeable reduction in bodyweight loss occurred in mice treated with HP/F9 compared to control groups (Fig. 4). The level of protection conferred were similar for mice treated with either 6 μg of HP/F9 or commercial antibiotics. However, 1.2 μg HP/F9 failed to confer any form of protection in mice. As demonstrated by these results, intranasal inoculation of HP/F9 can protect mice against *K. pneumoniae* infection (Fig. 4).

HP/F9 peptides successfully inhibits K. pneumoniae replication in mouse lung

Antibacterial effect of HP/F9 in the lungs was assessed by treating the mice with the HP/F9 peptides after infection, then determining the lung bacterial loads. Fig. 5 results revealed bacterial growth inhibition upon administering 6 and 15 μg of HP/F9, which were similar to the level of inhibition demonstrated by commercial antibiotics. From these results, it can be inferred that HP/F9 possesses antibacterial properties and its administration via intranasal route confers greater bacterial inhibition in the lungs of mice (Fig. 5A and B).

HP/F9 peptides significantly reduced lung pathology

In order to determine the impact of HP/F9 administration on pulmonary disease severity reduction, histopathological examination of the lungs following *K. pneumoniae* infection were performed as seen in Figs. 6 and 7. As expected, mice that received saline and HP/F9 did not exhibit any peribronchial inflammation and pulmonary parenchymal architecture was preserved. In contrast, lung histology in mice infected with *K. pneumoniae* showed focal interstitial thickening and inflammation 36 h p.i., and mild peribronchial inflammation on day 10. These results indicated that HP/F9 ameliorated the lung inflammation induced by *K. pneumoniae* infection in mice.

HP/F9 peptides significantly reduced glomerulonephritis in kidney

To assess whether HP/F9 administration lowers the severity of glomerulonephritis, murine kidneys were subjected to pathological examination following *K. pneumoniae* infection as seen in Fig. 8. As expected, kidneys remained intact and signs of glomerulonephritis were not observed from mice that received saline (naïve), commercial antibiotics P/S and HP/F9. In contrast, lung histology in mice infected with *K. pneumoniae* showed glomerulonephritis on day 10. These results indicated that HP/F9 ameliorated the kidney glomerulonephritis induced by *K. pneumoniae* infection in mice.

Discussion

The peptides in the hemolymph of *H. illucens* have effectively inhibited the growth of bacteria *in vitro* (Choi et al., 2018), which signifies the need to confirm its antibacterial effect using *in vivo* experiments. In this study, the peptide isolated from the hemolymph of *H. illucens* was used to treat mice previously infected with *K. pneumonia*. The peptide treatment significantly reduced the lung bacterial load and resulted in less body weight loss compared to control. Importantly, peptides derived from the *H. illucens* hemolymph effectively reduced lung inflammation induced by *K. pneumoniae* infection, providing important information on the potential of this peptide as a novel antibacterial drug.

In this study, we found that 6 or 12 μg of HP/F9 peptide (k22) completely inhibited the growth of *K. pneumoniae in vitro*, identical to the result illustrated by the control group which received 4 U of commercial antibiotics. One or 2 μg of HP/F9 peptides significantly reduced the CFU of *K. pneumoniae*. This is encouraging data. In our previous work, 1.25 mg of hexanedioic acid isolated from *H. illucens* larvae completely inhibited the growth of *K. pneumoniae in vitro*, which was similar to the antibacterial effect demonstrated by 5 U of commercial antibiotics including penicillin, streptomycin, and amphotericin (Chu et al., 2014). This indicates significant dose-sparing effect of HP/F9 peptide compared to the hexanedioic acid. Interestingly, HP/F9 peptide treatment contributed to lessened lung bacterial loads in comparison to the hexanedioic acid treatment used in the previous study. Resultantly, mice showed significantly reduced body weight loss upon *K. pneumoniae* infection, providing important information on potential dose-sparing effect of the HP/F9 peptide.

Our mouse study indicated that none or significantly reduced lung inflammation was observed when 6 μg or 15 μg of HP/F9 was administered, along with the complete absence of lung bacterial load. Acute glomerulonephritis can occur due to pulmonary infections (Siomou et al., 2003; Nasr et al., 2013). Kidney histopathological examinations have revealed that treatment using HP/F9 ameliorates bacteria-induced inflammatory reactions. Compared to naïve control, *K. pneumoniae* infection induced significant glomerular enlargement in the kidneys of mice (Fig. 8A and B). These features, however, were alleviated upon administration of either commercial antibiotics or HP/F9 by day 10 (Fig. 8C and D). Evidently, the presence of inflammatory cytokines such as TNF-α and IL-β have been documented (Bansal et al., 2014). Combining the reduction in glomerulonephritis upon HP/F9 treatment along with lessened bodyweight loss, we speculate that HP/F9 is not toxic since similar results were acquired from P/S-treated control groups. This might be due to the complete removal of bacteria in the lungs by the HP/F9 peptide against *K. pneumoniae* infection or no toxicity effect induced by the peptide. In sum, the HP/F9 peptide could be a candidate for a new antibiotic without side effect. Although further research on peptide characterization and systematic understanding of the antibacterial mechanism is required, we believe that the findings of this work can be useful for developing future antibacterial drugs (Nishida et al., 2017; Choi et al., 2018). Our results indicated that the peptide HP/F9 exhibited strong evidence of antibacterial action against *K. pneumoniae*, which can cause pneumonia. The results are consistent with the previous report documenting the antibacterial potential of HP/F9 peptide, which can serve to inhibit pathogenic bacteria infections (Choi et al., 2018). Therefore, this study provides the potential that HP/F9 peptide can be used as a novel antibacterial substance used for treatment of bacterial infection.

Declaration of Competing Interest

None.

Financial support

This work was supported by grants from the National Research Foundation of Korea (NRF) (2018R1A2B6003535, 2018R1A6A1A03025124), and Cooperative Research Program for Agriculture Science & Technology Development, Rural Development Administration, Republic of Korea (PJ01320501).

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