Prospecting Peptides Isolated From Black Soldier Fly (Diptera: Stratiomyidae) With Antimicrobial Activity Against Helicobacter pylori (Campylobacterales: Helicobacteraceae)

Daniela Alvarez,1 Kevin A. Wilkinson,2,5,* Michel Treilhou,3 Nathan Téné,3 Denis Castillo,1 and Michel Sauvain1,4

1Laboratorio Mixto Internacional Andino Amazónico de Química de la Vida LMI-LAVi, Laboratorios de Investigación y Desarrollo LID, Universidad Peruana Cayetano Heredia, Av. Honorio Delgado 430, Urb Ingeniería, Lima, Peru, 2Universidad Privada Antenor Orrego (UPAO), Av. América Sur 3145 Urb. Monserrate, Trujillo, La Libertad, Peru, 3Equipe BTSB-EA 7417, Université de Toulouse, Institut National Universitaire Jean-François Champollion, Place de Verdun, 81 012 Albi, France, 4Institut de Recherche pour le Développement (IRD), Université Paul Sabatier, 118 route de Narbonne 31062, Toulouse, France, and 5Corresponding author, e-mail: kevinawilk@gmail.com

*Author is no longer associated with UPAO.

Subject Editor: Phyllis Weintraub

Received 29 August 2019; Editorial decision 18 November 2019

Abstract

Helicobacter pylori (Marshall & Goodwin) is a widespread human pathogen that is acquiring resistance to the antibiotics used to treat it. This increasing resistance necessitates a continued search for new antibiotics. An antibiotic source that shows promise is animals whose immune systems must adapt to living in bacteria-laden conditions by producing antibacterial peptides or small molecules. Among these animals is the black soldier fly (BSF; Hermetia illucens Linnaeus), a Diptera that colonizes decomposing organic matter. In order to find anti-H. pylori peptides in BSF, larvae were challenged with Escherichia coli (Enterobacteriales: Enterobacteriaceae). Small peptides were extracted from hemolymph and purified using solid-phase extraction, molecular weight cutoff filtration and two rounds of preparative high performance liquid chromatography (HPLC). The anti-H. pylori fraction was followed through the purification process using the inhibition zone assay in brain-heart infusion agar, while peptides from uninoculated larvae had no activity. The inhibition halo of the active sample was comparable to the action of metronidazole in the inhibition zone assay. The purified sample contained four peptides with average masses of approximately 4.2 kDa that eluted together when analyzed by HPLC-mass spectrometry. The peptides likely have similar sequences, activity, and properties. Therefore, BSF produces inducible antibacterial peptides that have in vitro activity against H. pylori, which highlights BSF’s position as an important target for further bioprospecting.

Key words: Hermetia illucens, bioprospecting, Helicobacter pylori, antimicrobial peptides
A recent study that investigated the effect of diet on the BSF immunity-related transcriptome identified more than 50 genes encoding putative antimicrobial peptides (AMPs) (Vogel et al. 2018). At present only two AMPs have been isolated and characterized from BSF larvae: one displays activity against Gram-positive bacteria (Park et al. 2015), the other acts against Gram-negative bacteria like Escherichia coli, Enterobacter aerogenes Hormaeche & Edwards (Enterobacteriales: Enterobacteriaceae), and Pseudomonas aeruginosa Migula (Pseudomonadales: Pseudomonadaceae) (Park and Yoe 2017). Considering the need for new anti-\textit{H. pylori} compounds and that BSF seems to be a good source for novel antibacterials, we prospect for and characterize inducible AMPs that inhibit \textit{H. pylori} using bioassay-directed isolation and mass spectrometry.

Materials and Methods

Insects and Inoculation

BSF larvae were grown and maintained under a lighting schedule of 12 h light and 12 h dark at 28 ± 2°C and at 70% relative humidity. An actively growing overnight culture of nonpathogen \textit{E. coli} (ATCC 25922, Manassas, VA; 1 × 10^3 cells/ml) was injected into the hemolymph of lots of approximately 750 rinsed and dried fourth instar larvae using a tuberculin needle. Cell count was confirmed using a hemocytometer. Inoculated larvae were incubated for an additional 36 h. The experimental control consisted of untreated BSF larvae.

Hemolymph Extraction

Hemolymph was collected following an established protocol (Hetrue and Bulet 1997). In brief, larvae abdomens were sectioned with a scalpel, hemolymph was collected with a tuberculin syringe and deposited in precooled tubes containing aprotinin and phenylthiourea residues and filtrates were tested for activity.

Solid-Phase Extraction

Chromabond Sep-Pak C18 cartridges (Macherey-Nagel, Düren, Germany) were equilibrated with methanol and 0.05% TFA(aq). Chromabond Sep-Pak C18 cartridges (Macherey-Nagel, Düren, Germany) were equilibrated with methanol and 0.05% TFA(aq).

Preparative HPLC of the Active Fractions

Peptides were eluted from an Acris Peptides XB-C18, 150 × 2.1 mm; 2.6 µm, 100 Å HPLC column (Phenomenex, Torrance, CA) on a gradient at a flow of 0.5 ml/min from 10 to 45% ACN in 0.1% TFA over 45 min using an HPLC Dionex Ultimate 3000 UHPLC system (Thermo Scientific, Waltham, MA). Six fractions were collected (Fig. 1) by monitoring absorbance at 215 nm. Fractions were concentrated using a rotary evaporator. The active fraction was subjected to an additional purification step using a modified gradient from 25 to 40% ACN in 0.1 TFA over 45 min, where an additional nine fractions were collected.

Sample Analysis by Mass Spectrometry

Intermediately purified peptide samples were separated and analyzed using a UPLC Acquity coupled to a Xevo G2-XS qTOF (Waters, Milford, MA) by injection on a UPLC BEH C18 (2.1 × 50 mm, 1.7 µm) column and eluted using 0.1% aqueous formic acid and ACN/0.5% formic acid on a gradient. Sequence, mass, and disulfide analyses (Touchard et al. 2015) of the final purified product were carried out on an LTQ-Velos Pro Orbitrap (Thermo Scientific, Danforth Plant Science Center, St. Louis, MO); the masses were confirmed on the Xevo G2-XS qTOF.

Results

Figure 1 tracks the purification of BSF AMPs and reports anti-H. pylori activity measured at each step. The first inhibition zone assay indicated that the 10% ACN fraction had no activity, while the 40 and 80% fractions had inhibition halos of 11 mm and 3 mm (5 µg/well). Noninoculated fractions did not have anti-H. pylori activity. The 40% fraction (F40) had a recovery of 11 mg of protein and was selected for size-
exclusion filtration to remove proteins that were not in the mass range of other known BSF AMPs. The filtered F40 had a halo of 18.5 mm (5 µg/well) and a recovery of 430 µg.

Filtered F40 was then analyzed using mass spectrometry, resulting in a catalogue of over 93 unique cationic, multiply charged peptides with a mass range of 1.6–7.3 kDa; which made necessary additional purification to find the active peptide(s).

Filtered F40 was separated into six fractions by preparative HPLC (Figs. 1 and 2A). The sample contained many components, some of which were inducible by E. coli inoculation (compare orange and blue lines, Fig. 2A). Each fraction was evaluated for anti-H. pylori activity, with only fraction 5 (F5, 40 µg recovery) having activity (Fig. 2B, 20 mm, 5 µg/well; Fig. 2A, blue shaded area). Because F5 also had several peptide components of different mass, as exhibited by multiple chromatographic peaks, F5 was subjected to a second round of preparative HPLC (Fig. 2C). The most potent fraction (F5.8, 2 µg recovery) had an inhibition halo of 8 mm (Fig. 2D, 0.63 µg/well; Fig. 2C, blue shaded area).

Fraction 5.8 was then analyzed by UPLC-MSqTOF to determine the exact masses of the peptides in the sample. One chromatographic peak was observed that had masses consistent with peptides. This peak was found to contain four separate peptides with slightly different masses (Table 1 and Fig. 2E). The two most abundant peptides (4180 and 4199 Da) were analyzed for disulfide bridges, and none were found. Tandem mass spectrometry analysis of these two peptides did not provide comprehensive sequence information; however, both tandem mass spectra had prominent peaks at 120.0808 and 157.1337 m/z and greatly resembled each other in the low-mass range.

**Discussion**

We find that BSF larval hemolymph is a source of many AMPs expressed both constitutively and by induction. These peptides range from about 20 to 50 amino acids (1.6–7.3 kDa) and most likely function as part of the immune system to combat infection (Bahar and...
Ren 2013, Park et al. 2014, Elhag 2017). Through bioassay-directed purification, we were able to identify a set of four coeluting peptides with length of about 40 amino acids that have potent anti-H. pylori activity. These peptides are not the most abundant AMPs in hemolymph; combined they represent less than 10% of the putative AMPs in F40. The production of anti-H. pylori peptides is enhanced when the larvae are challenged with E. coli; expression increases by about 3–5 times based on chromatographic peak height (Fig. 2A). The anti-H. pylori activity of the peptides is comparable to metronidazole in the gel diffusion test, where a 21 mm zone of inhibition with metronidazole (5 µg) indicates susceptibility (DeCross et al. 1993, Chaves et al. 1999). This equates to an area of 346 mm². When inhibition areas and amounts added to the plate are compared, F5.8 should surpass these thresholds if sufficient sample was used. Since a 0.63 µg sample inhibited an area of 50 mm², a sample quantity of 5 µg should inhibit about 400 mm² of agar, or a radius of 22 mm.

Some characteristics of the isolated peptides can be inferred from their mass and charge. Given that average charge (Table 1) is a function of the surface area of a protein, the surface area of the peptides should be about 8 Å². Other common proteins that have similar surface areas tend to have greater masses than the measured AMP masses. Therefore, it is likely that these AMPs have relatively high solvent accessibility when compared with other proteins of similar mass (Kaltashov and Mohimem 2005).

Some sequence information can be obtained from mass spectrometry. First, the sequence of these peptides does not contain cysteine. Second, the tandem mass spectra contain some interpretable sequence information. Tandem mass spectra of the two most abundant peptides contain a prominent peak at m/z 120.0808 that can be assigned as a phenylalanine immonium ion as well as peaks at 157.1337 and 272.1607 that may be b2 and b3 ions containing N-terminal glycine and valine followed by aspartic acid. In addition, there are several other low-mass peaks in both spectra (at least 17 intense peaks shared between m/z 100 and 450), indicating that these peptides may have sequence homology. Nonetheless, comprehensive sequence analysis was hindered because sample size was small and because the sample was a mixture of different peptides of similar masses. Revisiting this process with a concentrated pure sample of sufficient size may simplify mass spectra enough to extract interpretable sequence information.

BSF produces a potent antibiotic against H. pylori when challenged with E. coli. In effect, this assay measured cross reactivity of the peptides, which may indicate that the described AMPs may have a wide spectrum of action (Bahar and Ren 2013, Chung and Khanum 2017). To the best of our knowledge, this is the first time an anti-H. pylori peptide has been isolated from insect larvae. Additionally, the mass of the peptides described here compares well with the previously reported mass range of 2.0–4.5 kDa for other anti-H. pylori AMPs (Neshani et al. 2019). Given that these peptides coelute during HPLC, and that high sequence homology is expected, it is likely that the purified peptides have very similar antimicrobial, physical and chemical properties.

We hope that these peptides may provide a basis for development of safe and effective medicines to treat H. pylori infection. Furthermore, our work underlines the bioprospecting potential of insects in the search for new antibiotic compounds. It is likely that other saprophagous insects will harbor additional AMPs effective against a wide range of important human pathogens, including those that are not normally present in their environment. Potential future investigation could include improving the yield of peptide production in BSF hemolymph, testing resistant strains of H. pylori, and additional mass spectrometric analysis to complete the sequence of the peptides.

Acknowledgments
This work was funded by Fondo Nacional de Desarrollo Científico, Tecnológico y de Innovación Tecnológica del Perú grants 126-2013-CONCYTEC-P and 108-2015-FONDECYT.

References
Abkar, N., R. Siddiqui, K. A. Sagathevan, and N. A. Khan. 2019. Gut bacteria of animals/pests living in polluted environments are a potential source of antibacterials. Appl. Microbiol. Biotechnol. 103: 3955–3964.
Bahar, A. A., and D. Ren. 2013. Antimicrobial peptides. Pharmaceuticals (Basel) 6: 1543–1575.
Chaves, S., M. Gadanho, R. Tenreiro, and J. Cabrita. 1999. Assessment of metronidazole susceptibility in Helicobacter pylori: statistical validation and error rate analysis of breakpoints determined by the disk diffusion test. J. Clin. Microbiol. 37: 1628–1631.
Choi, W. H., H. J. Choi, T. W. Goo, and F. S. Quan. 2018. Novel antibacterial peptides induced by probiotics in Hermetia illucens (Diptera: Stratiomyidae) larvae. Entomol. Res. 48: 237–247.
Chung, P. Y., and R. Khanum. 2017. Antimicrobial peptides as potential anti-biofilm agents against multidrug-resistant bacteria. J. Microbiol. Immunol. Infect. 50: 405–410.
DeCross, A. J., B. J. Marshall, R. W. McCallum, S. R. Hoffman, L. J. Barrett, and R. L. Guerrant. 1993. Metronidazole susceptibility testing for Helicobacter pylori: comparison of disk, broth, and agar dilution methods and their clinical relevance. J. Clin. Microbiol. 31: 1971–1974.
Elhag, O., D. Zhou, Q. Song, A. A. Soomro, M. Cai, L. Zheng, Z. Yu, and J. Zhang. 2017. Screening, expression, purification and functional characterization of novel antimicrobial peptide genes from Hermetia illucens (L.). PLoS One 12: e0165982.

Table 1. Peptides in F5.8 analyzed by mass spectrometry

| Average mass (Da) | Relative abundance | Average charge | m/z | Charge (+) | Calculated mass (Da) | Relative peak height |
|-------------------|-------------------|----------------|-----|-----------|---------------------|---------------------|
| 4179.8696         | 14.3              | 4.7            | 697.6521 | 6         | 4179.869            | 14.8                |
|                   |                   |                | 836.9813 | 5         | 4179.8701           | 105                 |
|                   |                   |                | 1045.9747 | 4        | 4179.8697           | 54.9                |
|                   |                   |                | 840.7896 | 5         | 4198.9116           | 10.0                |
|                   |                   |                | 1050.7534 | 4        | 4198.9845           | 33.0                |
| 4198.9358         | 8.78              | 4.8            | 700.8258 | 6         | 4198.9111           | 12.0                |
|                   |                   |                | 847.5647 | 5         | 4232.7871           | 7.17                |
|                   |                   |                | 1059.2036 | 4        | 4232.7853           | 7.12                |
| 4232.7873         | 1.70              | 4.7            | 706.4722 | 6         | 4232.7895           | 1.54                |
|                   |                   |                | 847.5647 | 5         | 4232.7871           | 12.0                |
|                   |                   |                | 1059.2036 | 4        | 4232.7853           | 7.17                |
| 4251.8278         | 1.00              | 4.8            | 709.6456 | 6         | 4251.8299           | 1.00                |
|                   |                   |                | 851.3734 | 5         | 4251.8306           | 7.12                |
|                   |                   |                | 1063.9630 | 4        | 4251.8229           | 4.06                |

Table 2. Characteristics of the peptides identified in helicobacter pylori hemolymph

| Average mass (Da) | Relative abundance | Average charge | m/z | Charge (+) | Calculated mass (Da) | Relative peak height |
|-------------------|-------------------|----------------|-----|-----------|---------------------|---------------------|
| 1063.9630         | 4                 | 4.03           | 851.3734 | 5         | 4251.8299           | 1.00                |
|                   |                   |                | 847.5647 | 5         | 4232.7871           | 12.0                |
|                   |                   |                | 1059.2036 | 4        | 4232.7853           | 7.17                |
| 4251.8306         | 7.12              | 3.78           | 1063.9630 | 4        | 4251.8229           | 4.06                |
Hetru, C., and P. Bulet. 1997. Strategies for the isolation and characterization of antimicrobial peptides of invertebrates, pp. 35–49. In W. M. Shafer (ed.), Methods in molecular biology, vol 78: antibacterial peptide protocols. Humana Press Inc, Totowa, NJ.

Kaltashov, I. A., and A. Mohiemen. 2005. Electrospray ionization mass spectrometry can provide estimates of protein surface areas in solution. Anal. Chem. 77: 5370–5379.

Neshani, A., H. Zare, M. R. Akbari Eidgahi, A. Hooshyar Chichaklu, A. Movaghar, and K. Ghazvini. 2019. Review of antimicrobial peptides with anti-Helicobacter pylori activity. Helicobacter 24: e12555.

Park, S. I., and S. M. Yoe. 2017. A novel cecropin-like peptide from black soldier fly, Hermetia illucens: isolation, structural and functional characterization. Entomol. Res. 47: 115–124.

Park, S. I., H. S. An, B. S. Chang, and S. M. Yoe. 2013. Expression, cDNA cloning, and characterization of the antibacterial peptide cecropin D from Agrina convolvuli. Anim. Cells Syst. 17: 23–30.

Park, S. I., B. S. Chang, and S. M. Yoe. 2014. Detection of antimicrobial substances from larvae of the black soldier fly, Hermetia illucens (Diptera: Stratiomyidae). Entomol. Res. 44: 58–64.

Park, S. I., J. W. Kim, and S. M. Yoe. 2015. Purification and characterization of a novel antibacterial peptide from black soldier fly (Hermetia illucens) larvae. Dev. Comp. Immunol. 52: 98–106.

Peek, R. M., Jr., and J. E. Crabtree. 2006. Helicobacter infection and gastric neoplasia. J. Pathol. 208: 233–248.

Sheppard, D. C., J. K. Tomberlin, J. A. Joyce, B. C. Kiser, and S. M. Sumner. 2002. Rearing methods for the black soldier fly (Diptera: Stratiomyidae). J. Med. Entomol. 39: 695–698.

Tortorella, E., P. Tedesco, F. P. Esposito, G. G. January, R. Fani, M. Jaspars, D. de Pascale. 2018. Antibiotics from deep-sea microorganisms: current discoveries and perspectives. Mar. Drugs 16: 355.

Touchard, A., J. M. Koh, S. R. Aili, A. Dejean, G. M. Nicholson, J. Orivel, and P. Escoubas. 2015. The complexity and structural diversity of ant venom peptidomes is revealed by mass spectrometry profiling. Rapid Commun. Mass Spectrom. 29: 385–396.

Vogel, H., A. Müller, D. G. Heckel, H. Gutzeit, and A. Vilcinskas. 2018. Nutritional immunology: diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly Hermetia illucens. Dev. Comp. Immunol. 78: 141–148.

Wroblewski, L. E., R. M. Peek, Jr., and K. T. Wilson. 2010. Helicobacter pylori and gastric cancer: factors that modulate disease risk. Clin. Microbiol. Rev. 23: 713–739.

Zamani, M., F. Ebrahimtabar, V. Zamani, W. H. Miller, R. Alizadeh-Navaei, J. Shokri-Shirvani, and M. H. Derakhshan. 2018. Systematic review with meta-analysis: the worldwide prevalence of Helicobacter pylori infection. Aliment. Pharmacol. Ther. 47: 868–876.