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Research

Identification and Physicochemical Properties of the Novel Hemolysin(s) From Oral Secretions of *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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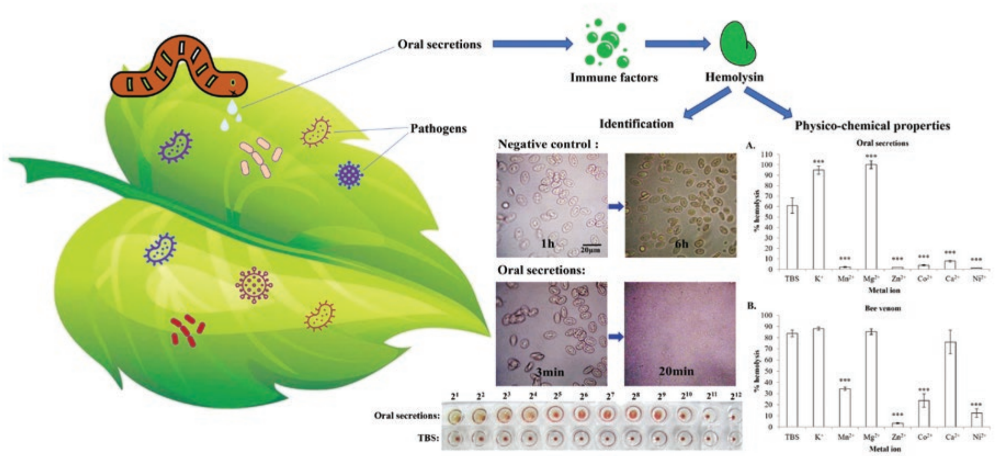
Abstract

Hemolysins cause the lysis of invading organisms, representing major humoral immunity used by invertebrates. Hemolysins have been discovered in hemolymph of *Helicoverpa armigera* larvae as immune factors. As oral immunity is great important to clear general pathogens, we presumed that hemolysins may be present in oral secretions (OS). To confirm this hypothesis, we conducted four testing methods to identify hemolysin(s) in larval OS of *H. armigera*, and analyzed physicochemical properties of the hemolysin in comparison with hemolytic melittin of *Apis mellifera* (L.) (Hymenoptera: Apidae) venom. We found hemolysin(s) from OS of *H. armigera* for the first time, and further identified in other lepidopteran herbivores. It could be precipitated by ammonium sulfate, which demonstrates that the hemolytic factor is proteinaceous. Labial gland showed significantly higher hemolytic activity than gut tissues, suggesting that hemolysin of OS is mainly derived from saliva secreted by labial glands. Physicochemical properties of hemolysin in caterpillar's OS were different from bee venom. It was noteworthy that hemolytic activity of OS was only partially inhibited even at 100°C. Hemolytic activity of OS was not inhibited by nine tested carbohydrates contrary to bee venom melittin. Moreover, effects of metal ions on hemolytic activity were different between OS and bee venom. We conclude that there is at least a novel hemolysin in OS of herbivorous insects with proposed antibacterial function, and its hemolytic mechanism may be different from melittin. Our study enriches understanding of the potential role of hemolysins in insect immunity and provides useful data to the field of herbivorous insect-pathogen research.

Graphical Abstract



Key words: hemolysin, hemolytic activity, herbivorous insect, oral secretion, physicochemical property

Many humoral immune factors have been reported in invertebrates, such as hemolysins, lectins, lysozyme, phenoloxidase, lymphokine-like substances, lipopolysaccharide-binding proteins, antibacterial peptides, and so on (Yu and Kanost 2000, Yu et al. 2006, Xylander 2009, Sasaki et al. 2010, González-Santoyo and Córdoba-Aguilar 2011, Wang et al. 2015). As pore-forming proteins to perform immunity, the hemolysins are thought to destroy invading organisms due to their lysing activity (Canicatti 1990, Potrich et al. 2009). Hemolytic activity in hemolymph of arthropods was induced increase by the parasitization of the endoparasitoid or injection with microbes (Beresford et al. 1997, Wang et al. 2015). This is inferred that the hemolytic activity is associated with insect immune response by lysing cells of invading organisms. Moreover, some lectins have lytic property of erythrocyte, which is regarded as humoral immune in response to exogenic invasive pathogens (Sasaki et al. 2010, Arockiaraj et al. 2015). Mannose-binding lectin from *Macrobrachium rosenbergii* (De Man) (Decapoda: Palaemonidae) possessed both antimicrobial and hemolytic activity, and it was induced by viruses and bacteria (Arockiaraj et al. 2015). Cytolysis is the common trick for clearance of pathogens.

The oral immunity is of great importance to initially clear general pathogens, because oral cavity is the first or easiest channel for pathogens to enter the body. It is a pity that immune factors from oral secretions (OS) have been rarely reported in herbivorous insects in the past decades. Lepidopteran herbivores release abundant amounts of OS while feeding on plants. These OS include saliva secreted from salivary glands and regurgitant derived from gut (Consales et al. 2012, Schmelz 2015), they contain many chemical compounds and proteins potentially involved in lubrication, digestion, detoxification, immunity, herbivore offense, chemoreception, and so on (Wu and Baldwin 2010, Stafford-Banks et al. 2014, Rivera-Vega et al. 2017). Some immune factors in the salivary glands or saliva of lepidopteran insects were identified based on transcriptome and proteome (de la Paz Celorio-Mancera et al. 2011; 2015, Harpel et al. 2015, Acevedo et al. 2017, Rivera-Vega et al. 2017). Bacteria or bacteria-related compounds also changed gene expressions in the salivary glands of caterpillars (de la Paz Celorio-Mancera et al. 2015). Immunity-related transcripts have been recognized in labial salivary glands of *Helicoverpa armigera*, such as lysozymes and proteinase inhibitors (de la Paz Celorio-Mancera et al. 2011). Depending on proteome analysis of *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) saliva, researchers found abundant proteins which potentially participated in immune processes, such as POX-J, apolipophorin, caspase, scolexin, and glucose dehydrogenase (Acevedo et al. 2017). In addition, some lectin transcripts had been identified in labial salivary glands of *H. armigera*, and substantiated that larval OS had hemagglutination activity (Wang et al. 2021).

By evidence of the foregoing, saliva is bound to play an important role in oral immunity of caterpillars. However, most salivary immune factors identified by omics analysis have not been directly demonstrated by specific tests for immune activity in herbivorous insect saliva. Salivary glucose oxidase (GOX), as a antibacterial factor, has been demonstrated to possess antimicrobial property in *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Eichenseer et al. 1999, Musser et al. 2005). GOX activity of labial salivary glands has been found higher in caterpillar generalists than specialists in all developmental stages (Zong and Wang 2004, Eichenseer et al. 2010, Yang et al. 2017), which is consistent with the fact that generalists often encounter a broader range of potential pathogens. Taken together, GOX appears to be a unique trait that can help generalists to overcome pathogen challenges besides as effector protein (Musser et al. 2005, Yang et al. 2017). However, some caterpillars do not have GOX enzyme (Zong and Wang 2004), and how do they protect themselves from pathogens? Are there any other immune molecules in saliva? Besides GOX known to us, there are other immune factors that have also been reported in insect saliva, such as lysozyme (Liu et al. 2004), mucin (Korayem et al. 2004), antimicrobial peptide (Lamberty et al. 2001), phenoloxidase (Satoh et al. 1999), etc. Furthermore, labial salivary gland has been reported to have noticeably higher hemolytic activity than hemocytes, fat body, epidermis, midgut, and testes in *H. armigera* (Wang et al. 2015). Therefore, we speculated that hemolysin is most likely present in OS, and its lysis activity may be an important immune pattern.

In recent years, researches on components of OS from herbivory insects have been focused mainly on the co-evolution and interaction between insects and plants, especially plant defense and insect antidefense (Rivera-Vega et al. 2018, Acevedo et al. 2019, Chen et al. 2019, Giacometti et al. 2020), but the factors involved in immunity have not been paid enough attention. Insect herbivores do not live in a sterile environment and encounter multifarious microbes (bacteria, fungi, viruses, protozoa, etc.) that may be pathogenic. Insect herbivore survival is not only dependent on circumventing plant defenses, but must have strategies to avoid the detrimental effects of insect pathogens (Musser et al. 2005, Rivera-Vega et al. 2017). So, it is worth looking into the oral immunity in herbivorous insects. Lysis of invading organisms is a major innate form of immunity used by invertebrates, we hypothesized that OS of *H. armigera* contains hemolysin to perform immune function, just like its hemolymph, and the hemolysin may be ubiquitous in lepidopteran species. If so, what are physicochemical properties of the hemolysin, and the differences between other hemolytic factors? To address these questions, we conducted four methods of testing hemolysis to identify hemolysin(s) in OS of *H. armigera* and analyzed physicochemical properties of the hemolysin in comparison with hemolytic melittin of *Apis mellifera* venom. The aim of this study was to provide useful data to the field of herbivorous insect-pathogen research based on identifying and analyzing hemolysin(s) in OS.

Materials and Methods

Insects

Larvae of *H. armigera* were originally obtained from the tobacco or pepper fields in Xuchang and Zhengzhou, Henan Province, China, in June 2014. Larvae were maintained on artificial diet made from powder of soybean (105 g), wheat germ (50 g), yeast (32 g), ascorbic acid (3.3 g), sorbic acid (1 g), methyl parahydrobenzoate (2 g), agar (14.5 g), and hot distilled water (640 ml; Wang and Dong 2001), and adults were fed with 10% honey solution. Larvae and adults were kept in manual climatic box with 28 ± 1°C, 70 ± 10% RH, and photoperiod 14:10 (L:D) h. Larvae of other lepidopteran species *Ostrinia furnacalis* (Guenée) (Lepidoptera: Pyralidae), *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), *Athetis lepigone* (Möschler) (Lepidoptera: Noctuidae), and *H. armigera* were captured from their host-plant capsicum and *Pieris rapae* (L.) (Lepidoptera: Pieridae) were captured from cabbage in a farm of Zhengzhou, and maintained on their host plant. Adults of *A. mellifera* were captured from flowering plants in the campus of Henan Agricultural University, Zhengzhou, China, and these honeybees were utilized on the same day.

Preparation of Larval OS

One hundred larvae of *H. armigera* which were in feeding period of last instar were used for OS collection. Larval head was lightly held by tweezers, then the mouthpart was gently touched with pipette tip to stimulate regurgitation, and these OS (regurgitation) was collected using pipettor. Each larva produced about 10 µl of OS which was collected and transferred into an ice-cold centrifuge tube containing phenylthiocarbamide (PTU) powder to prevent melanization. A total of 1,000 µl of OS was obtained and centrifuged at 14,000 g for 10 min at 4°C to remove fodder residue, then stored at −20°C for subsequent analysis of hemolytic activity and physicochemical properties. Other larval OS samples of 80–100 µl were respectively collected from 10 to 15 caterpillars of *O. furnacalis*, *S. exigua*, *A. lepigone*, *P. rapae,* and *H. armigera* feeding on their host plant for testing hemolytic activity in the natural host environment.

Preparation of *H. armigera* Tissue Extracts

Thirty last-instar larvae of *H. armigera* in feeding period were dissected for separately collecting fluid of foregut, midgut, and hindgut, and these fluid samples were placed into ice-cold centrifuge tubes containing PTU. Solid tissues of foregut, midgut, hindgut, and labial salivary gland were separately washed with ice-cold 0.01 M Tris buffered saline (TBS, pH 7.4) for clearing fodder residue and body fluid, and clean tissue samples were respectively placed in ice-cold homogenizer to be homogenized with 200 µl of TBS. Then, tissue homogenate was centrifuged at 14,000 g for 10 min at 4°C to collect supernatant. Moreover, protein concentration of tissue extracts was measured for correcting the hemolytic activity per 100 µg protein. Meanwhile, hemolymph was collected into an ice-cold centrifuge tube containing PTU when larvae were dissected, which was centrifuged at 14,000 g for 10 min at 4°C to remove hemocyte and collect plasma supernatant. All samples were stored at −20°C for subsequent hemolytic activity assays.

Preparation of Bee Venom

Bee's stinger together with poison sac were pulled out from 30 adults of *A. mellifera* and placed into an ice-cold centrifuge tube containing 200 µl TBS. These poison sacs were fully grinded in tubes with matched grinding rod, and then, centrifuged at 14,000 g for 10 min at 4°C. The supernatant was stored at −20°C for hemolytic activity assays and analysis of physicochemical properties.

Preparation of Vertebrate Erythrocytes

Blood of chicken, duck, or rabbit was purchased from Yuantian Farm Market (Zhengzhou, China) where have cultured animal slaughter license. These animals were healthy and maintained at a constant temperature 25 ± 2°C in cages and were fed with standard food. When consumers bought live chickens, ducks, or rabbits that need to be slaughtered, we collected blood samples and took them back to the lab. These animals' blood samples were respectively mixed with 0.8% sodium citrate for anticoagulation and centrifuged at 1,000 g for 10 min at 4°C to precipitate erythrocytes and remove supernatant. The erythrocytes were washed with ten times volumes of ice-cold TBS for three times and centrifuged at 1,000 g for 5 min at 4°C each time. Whereafter, 2% suspension of erythrocytes was diluted with 0.01 M TBS and used for hemolytic activity assay.

Hemolytic Activity Assays

V-Bottom Microtiter Plate Assay

Twenty-five microliters of OS samples were used for preparing a series of two-fold dilutions with 0.01 M TBS (pH 7.4) in 96-well V-bottom microtiter plates. Plates were then incubated for 30 min at room temperature after addition of 25 µl of 2% chicken erythrocyte suspension. In parallel, erythrocyte suspension mixed with 25 µl 0.01 M TBS was used as the negative control. After incubation, lysis or sedimentation of the erythrocytes in each well was monitored. The hemolytic activity assays of every dilution of OS sample and the TBS control were repeated three times. According to the method described by Shanmughavalli and Arumugam (2011), the hemolytic activity of larval OS was expressed as the reciprocal of the highest dilution ratio of the sample causing complete lysis of erythrocytes.

Lysis Zone Measurement

Following the method of Xylander (2009), hemolytic activity was investigated by measuring the diameter of lysis zone on agar plates containing equally distributed chicken erythrocytes. One percentage agar solution was sterilized at 120°C for 30 min, and when agar solution cooled down to 45°C, chicken erythrocytes were added to confect 5% erythrocytes-containing agar solution. Then, 10 ml erythrocytes-containing agar solution was poured into 9-cm-diameter petri dishes and stored at 4°C for hemolytic activity detecting. Five microliters of each of the twofolds serial dilutions of larval OS (prepared as previous description), or TBS (as negative control), were dripped on agar plates, and the diameters of erythrocytes lysis zones were measured after 24-h incubation at room temperature.

Optical Microscope Observation

For observing the morphological changes of erythrocytes during hemolysis, 20 µl 2% erythrocytes suspension was mixed with 20-µl eight-fold dilution of OS or TBS (negative control) in a centrifuge tube. After incubation for 3, 8, 11, 14, 17, 20, 60 min, and 6 h at room temperature, 2 µl of mixture solution was taken for microscopy observation and photographing through a Canon Power shot A650 IS under a Leica inverted phase-contrast microscope.

Spectrophotometry Analysis of Hemoglobin Release

To accurately evaluate the hemolytic activity, the spectrophotometry method of Anderson (1980) was used to measure hemolytic activity of OS samples or tissue extracts. Sixty microliters of 2% chicken erythrocytes were mixed with 60-µl TBS (as a control for correction of absorbance value) or equal volume of OS or tissue samples in a centrifuge tube. These tubes were incubated in water bath of 37°C for 60 min, then each tube was supplied with 2.5-ml TBS and centrifuged at 1,000 g for 5 min at 4°C. The supernatant was used for measuring the absorbance value at 541 nm (Abs_{541}) which was the highest absorbance for the hemoglobin released from lysed erythrocytes. For setting positive control, 60 µl of 2% erythrocytes treated with distilled water instead of TBS was performed in the same way for causing 100% lysis of erythrocytes to obtain the highest absorbance value (positive control). Each sample was tested three times. According to the formula of the corrected Abs_{541}/the highest Abs_{541} (positive control) × 100%, percentage of hemolysis was calculated to express hemolytic activity of OS or tissue extracts.

Ammonium Sulfate Precipitation

Fifty microliters of larval OS were respectively mixed with different volumes of saturated solution of ammonium sulphate [$(NH_4)_2SO_4$], and mixture was set to 20, 30, 35, 40, 45, 50, 60, 65, 70, and 80%. OS mixed with ammonium sulfate were precipitated in the tubes and kept cold in an ice bath. Precipitated protein fractions were collected by centrifuging at 14,000 g for 10 min at 4°C, and solid protein was redissolved in 0.01 M TBS and desalted by dialysis. Hemolysis activities of precipitated protein fractions were measured by spectrophotometry method.

Protein Concentration Determination

Protein concentrations of OS or tissue extracts were measured by the method described by Bradford (1976). Protein Assay Kit (DingGuo Biotech. Co., Beijing, China) was used to complete measurement of protein concentration with bovine serum albumin as the protein standard.

Physico-Chemical Property Assays

Temperature and Concentration

OS or bee venom samples were distributed in several test tubes and treated in a water bath of 4, 37, 45, 50, 60, 70, 80, and 100°C, respectively, for 30 min, then post-treated samples were centrifuged at 14,000 g for 10 min at 4°C to obtain the supernatant for measurement of hemolytic activity. Meanwhile, original OS or bee venom were performed a series of twofold dilutions with 0.01 M TBS (pH 7.4) for obtaining different concentration samples. According to spectrophotometry method described above, the effects of temperatures and concentration on hemolytic activity of OS or bee venom was detected.

Carbohydrates and Metal Ions

Carbohydrate solutions (0.3 M) were prepared in TBS, including lactulose, raffinose, mannose, galactose, glucose, lactose, trehalose, p-Nitrophenyl-β-D-Galactopyranoside (PNPG), and sialic acid. Meanwhile, metal ion salts (KCl, $CaCl_2$, $MgCl_2$, $CoCl_2$, $NiCl_2$, $MnSO_4$, and $ZnSO_4$) were, respectively, dissolved with distilled water to obtain 0.5 M solution. Then, OS or bee venom samples were mixed respectively with different carbohydrate solutions (volume ratio 1:1) or metal ion solutions (volume ratio 3:1) for incubating 3 h at room temperature. We used TBS instead of carbohydrates and metal ions solutions to mix OS or bee venom as control. Meanwhile, negative control was conducted by using TBS instead of OS or bee venom samples to exclude the influence of carbohydrate and metal ions themselves on hemolysis. Post-treated samples were used to measure hemolytic activity by spectrophotometry method.

Statistical Analysis

All data of hemolysis percentage was transformed by arcsin square root before statistical analysis, and was back transformed to percentage for presentation. Variation analysis of two treatments was performed by Student's *t*-test. Tukey HSD test of ANOVA was used for variation analysis of multiple treatments. All of the statistical tests were performed by SPSS version 17.0 software.

Results

Authentication of Hemolytic Factor in OS

Four different methods of hemolytic activity assay described above were performed to demonstrate the presence of hemolytic factor in OS. According to V-bottom microtiter plate assay, chicken erythrocytes could be completely lysed by larval OS in wells of 2^0–2^4, partially lysed in well of 2^5. Erythrocytes were agglutinated in wells of 2^6–2^9, and fully precipitated in wells of 2^{10}–2^{12} (Fig. 1A). While erythrocytes were fully precipitated in all of wells by TBS control (Fig. 1B). When using different vertebrate erythrocytes to test, hemolytic activity titer of OS was different. Rabbit erythrocytes were easiest to be lysed (titer: 2^5), followed by chicken and duck erythrocytes, their titers were both 2^4 (Table 1).

Moreover, hemolytic activity of OS could be observed visually on the blood agar plate, two-fold serial dilutions of OS could produce lysis zone of different diameter, whereas control TBS could not cause lysis zone (Fig. 2). Undiluted OS sample could generate the maximal diameter of lysis zone, diameters of lysis zone gradually dwindled with the decrease of OS concentration (Table 2). Sixty-four-fold and over 64-fold dilution of OS could not produce hemolysis zone on the blood agar plate (Fig. 2 and Table 2). In addition, hemolytic activity could be evaluated accurately by spectrophotometry method depending on hemoglobin released from lytic erythrocytes, just as the percentage hemolysis of OS (69.8%) was higher than plasma (41.9%) of *H. armigera* (Table 3).

Through optical microscope observation, oval chicken erythrocytes exposed to OS could be observed to transform into round smaller spherocytes. After 20 min incubation, all erythrocytes had changed in size and shape (Fig. 3B), while erythrocytes that were not exposed to OS still keep their original state after 6 h (Fig. 3A). These results indicate that OS have the ability of cytolysis to lyse erythrocyte.

Ubiquity of Hemolytic Factor in Lepidopteran Herbivores

Larval OS samples of *O. furnacalis*, *S. exigua*, *A. lepigone*, *P. rapae,* and *H. armigera* feeding on their host plant were tested hemolytic activity by V-bottom microtiter plate assay method. Hemolytic activity of OS from *A. lepigone* was highest (titer: 2^3), the sequence decreasingly was *H. armigera* (titer: 2^2), *P. rapae* (titer: 2^1-2^2), *O. furnacalis* (titer: 2^1), and *S. exigua* (titer: 2^0–2^1; Table 4). The result suggests that hemolytic factors are widespread in lepidopteran species living in the natural host-plant environment.

Chemical Nature of Hemolytic Factor in OS

The protein fraction of OS precipitated in 20% saturation of ammonium sulfate had no hemolytic activity. Whereas hemolytic activity of precipitated protein fraction was higher and higher with the increasing of ammonium sulfate saturation from 30 to 65%, and hemolytic activity reached the maximum in 65% saturation, while hemolytic activity no longer increased in 70 and 80% saturation. The result suggests that hemolytic factor of OS is precipitated by saturation of ammonium sulfate from 30 to 65%, and demonstrates that the hemolytic factor is proteinaceous.

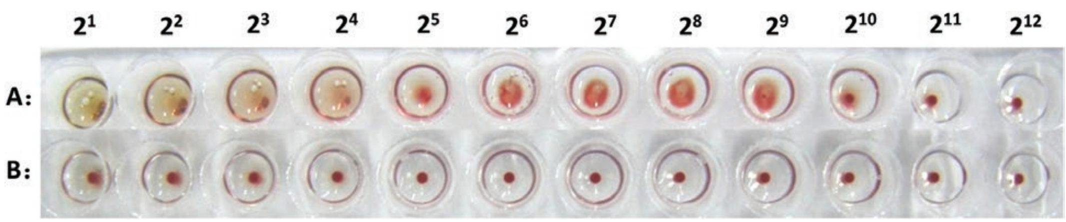


Fig. 1. Larval OS of *H. armigera* caused hemolysis of chicken erythrocytes in V-bottom microtiter plates. Row A: 2-fold serial dilutions (2^1–2^{12}) of the OS, the concentration of OS decreased from the left to the right in a 2-fold. Row B: TBS control.

Source of Hemolytic Factors

Hemolytic activity of tissue samples was detected by spectrophotometry method. The result showed that fluid of foregut, midgut, and hindgut all had hemolytic activities, and hemolytic activity of midgut fluid was highly significantly highest ($P < 0.01$), followed by OS and foregut fluid, and hindgut fluid was significantly lower than others ($P < 0.01$; Fig. 4A). For tissue extracts, labial salivary gland had extremely significantly higher hemolytic activity (67.4% hemolysis) than that of foregut (18.3%), midgut (31.1%), and hindgut (7.4%; $P < 0.01$). Although midgut was slightly higher than foregut, the difference was not significant. Hemolytic activity of hindgut was significantly the lowest (Fig. 4B). The result suggests that labial salivary gland is the main site of hemolytic factor (hemolysin) synthesis and secretion.

Table 1. Hemolytic titer of larval OS of *H. armigera* against various vertebrate erythrocyte types

Erythrocyte	Hemolytic titer
Chicken	2^4
Duck	2^4
Rabbit	2^5

Based on 3 determinations for each erythrocyte type. Values represent median values.

Fig. 2. Larval OS of *H. armigera* caused hemolysis zones on a chicken erythrocyte agar plate. Number 1: 5 μl of OS; Number 2–8: 5 μl of 2- to 128-folds serial dilutions of the OS; Number 9: TBS control.

Physico-chemical Properties

Hemolytic Activity in OS Is Slightly Temperature Sensitive

Tested temperature showed different effects on hemolytic activity of OS and bee venom. Hemolytic activity of OS treated at 37°C would slightly decrease as compared with activity at 4°C ($P > 0.05$; Fig. 5). OS treated at 45 or 50°C had significantly higher hemolytic activity than other temperature treatments ($P < 0.05$; Fig. 5). However, hemolytic activity of OS appeared a gradually downward trend with temperature increasing from 60 to 100°C, hemolytic activity was numerically lower at 60–80°C ($P > 0.05$) and significantly lower at 100°C ($P < 0.05$) than that at 4°C. Percentage of hemolysis declined from 65.4% (at 4°C) to 52.6% (at 100°C; $P < 0.05$; Fig. 5). The result suggests that hemolytic activity in OS has a certain degree of temperature sensitivity. It was worth noting that hemolytic activity of OS was not destroyed completely at 100°C, which indicates that hemolysin of OS is resistant to heat to some degree. For bee venom, it had higher level of heat resistance, as hemolytic activity of bee venom polypeptide melittin had no obvious change regardless of high-temperature treatment, percentage of hemolysis only declined from 80.1% (at 4°C) to 78.2% (at 100°C) ($P > 0.05$), even up to 100°C (Fig. 5).

Hemolytic Activity Is Not Positive Related to OS Concentration

Different concentrations of samples were tested, and the results revealed that hemolytic activity of 1- to 4-fold dilutions of OS did not change obviously ($P > 0.05$), while OS of 16-fold dilution possessed significantly highest hemolytic activity than other concentrations of OS ($P < 0.01$). Hemolytic activity of OS revealed a highly significant drop when fold dilution was greater than or equal to 32 ($P < 0.001$). By contrast, bee venom had similar character as OS, hemolytic activity of 1- to 8-fold dilutions of bee venom was no significant change ($P > 0.05$), 16-fold dilution of bee venom reach maximum hemolysis. While fold dilution was greater than or equal to 64, the hemolytic activity of bee venom was highly significantly lower than other fold dilutions ($P < 0.001$; Fig. 6).

Hemolysis Is Not Inhibited by Tested Carbohydrates

All of OS samples which were incubated with different carbohydrates had no significant change in hemolytic activity compared with TBS control ($P > 0.05$; Fig. 7A). However, hemolytic activity of bee venom could be highly significantly inhibited by lactulose ($T = -61.345$; df = 4; $P < 0.001$) and lactose ($T = -137.171$; df = 4; $P < 0.001$) compared with TBS control, while other carbohydrates had no inhibiting effects ($P > 0.05$; Fig. 7B).

Influence of Metal Ions on Hemolytic Activity of OS

Effect on hemolytic activity of OS caused by metal ions was different from bee venom. Compared with the TBS control, hemolytic activity of OS was highly significantly promoted by K^+ ($T = 10.085$; df = 4; $P < 0.001$) and Mg^{2+} ($T = 13.687$; df = 4; $P < 0.001$), and inhibited by Ca^{2+} ($T = -17.708$; df = 4; $P < 0.001$), but these ions had no effect on bee venom ($P > 0.05$). In addition, Mn^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} caused the similar effect on both OS and bee venom, all of them highly significantly inhibited hemolytic activity of OS and bee venom

Table 2. Hemolysis-zone diameter in chicken erythrocyte agar plate caused by larval OS of *H. armigera*

OS dilution	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	TBS
Dimeters (mm)	8.3 ± 0.2	7.2 ± 0.2	6.5 ± 0.2	5.9 ± 0.2	4.7 ± 0.2	3.5 ± 0.2	0	0	0

Each measurement was replicated three times. The data were presented as the mean ± SD.

($P < 0.001$), but these inhibitions were partial in bee venom and almost complete in OS (Fig. 8).

Discussion

The hemolysin(s) was firstly identified in OS from insect herbivores in our study. OS comprise not only saliva produced by salivary gland but also regurgitant derived from gut (Felton 2008, Peiffer and Felton 2009). Our result shows that whole digestive tract presents hemolysin, and salivary gland extracts have significantly higher hemolytic activity than that of three gut extracts (Fig. 4B), which suggests that salivary gland is the main tissue for synthesizing and secreting hemolysin. Hemolytic activity in fluid of gut may be derived from saliva influx. The hemolysin in OS had a certain degree of thermostability, even with treatment of high temperature up to 100°C (Fig. 5). Whereas, hemolytic activity of hemolymph was almost completely lost in high temperature (Wang et al. 2019). It can be inferred that these hemolysins with different origins are different kinds having varied functions.

Table 3. Percentage of hemolysis caused by larval OS and plasma of *H. armigera*

Sample	Abs_{541}	Hemolysis (%)
OS	0.120	69.8
Plasma	0.072	41.9
Positive control	0.172	100

Incubation of 2% chicken erythrocytes in distilled water was deemed 100% lysis to as positive control.

The biological functions of hemolytic factors are diverse. Hemolysins had been reported in saliva of blood-feeding insects, these hemolysins were considered to play digestive role to assist blood-feeding (Dorrah et al. 2021). Bee venom also possessed hemolytic activity (Babaie and Ghaempanah 2020), but the hemolysis was an assault weapon against violator rather than immune factor against pathogen in hymenopteran. Moreover, hemolytic factors also play an antimicrobial role. For example, salivary trialysin, a pore-forming protein, had both hemolytic and antibacterial activity in hematophagous insect *Triatoma infestans* (Klug) (Hemiptera: Reduviidae) (Amino et al. 2002). Bee venom had also been widely reported to be an antibacterial factor (Asthana et al. 2004, Hegazi et al. 2014). Some lectins known as immune factors also had hemolytic activity (Sasaki et al. 2010, Rotskaya et al. 2021). Hemolysin that was from hemolymph of *H. armigera* was induced by endoparasitoid parasitism or bacterial infection, which indicated that it was associated with immunity and antibacterial (Wang et al. 2015). So, we deduce that OS hemolysins of herbivores also have antimicrobial activity and hemolysins derived from OS or hemolymph might have different antibacterial spectrums, which requires further research.

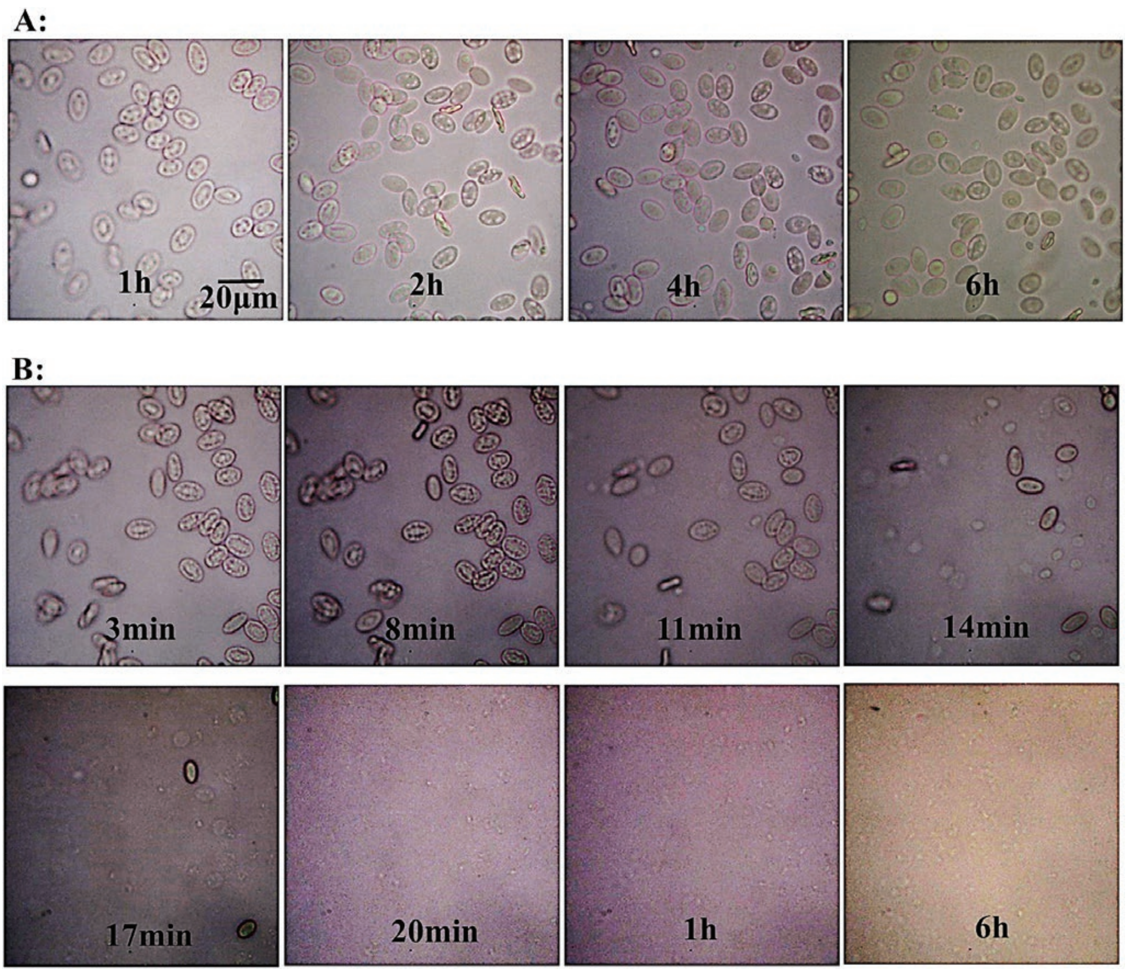


Fig. 3. Morphological changes of chicken erythrocytes caused by larval OS of *H. armigera.* (A) TBS control erythrocytes. (B) OS-treated erythrocytes, with incubation time.

Table 4. Comparison of hemolytic activity in larval OS of different lepidopteran herbivores

Insect species	Diets	Hemolytic titer
Helicoverpa armigera	Capsicum	2^2
Ostrinia furnacalis	Capsicum	2^1
Spodoptera exigua	Capsicum	2^0–2^1
Athetis lepigone	Capsicum	2^3
Pieris rapae	Cabbage	2^1–2^2

Based on 3 determinations for each species. Values represent median values.

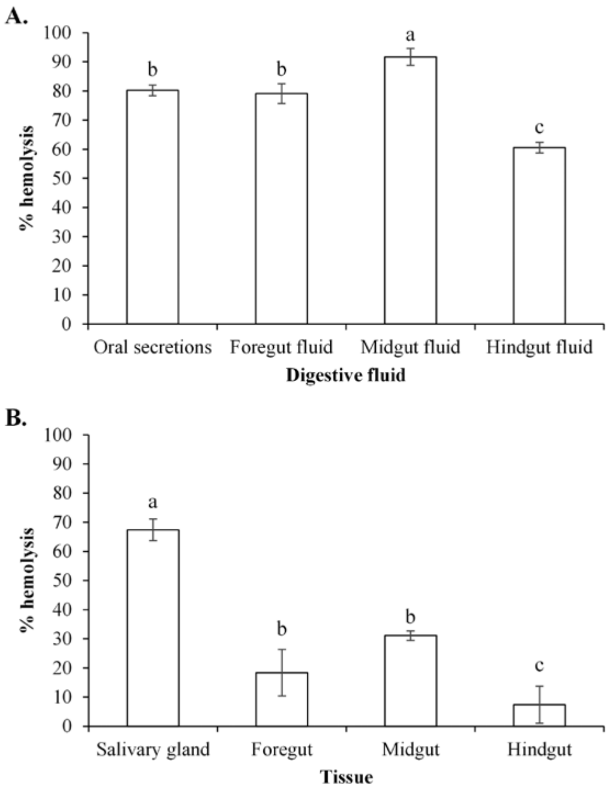


Fig. 4. Hemolytic activity of digestive fluid (A) and tissue extracts (B) in the last instar larvae of *H. armigera.* Hemolysis of tissue extracts is expressed as the percentage hemolysis per 100 µg protein. Experiments were performed in triplicate and mean ± SD of percent hemolysis are shown. Different lowercase letters above the bars indicate statistically significant differences (Tukey HSD test of ANOVA, $P < 0.05$, $n = 3$).

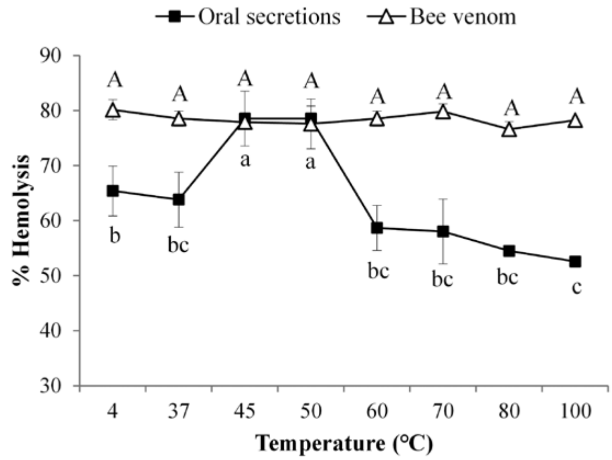


Fig. 5. Hemolytic activity of larval OS of *H. armigera* and bee venom after pre-incubation at different temperatures. Experiments were performed in triplicate and mean ± SD of percent hemolysis are shown, some error bars are within the symbols. Different uppercase letters above symbols of bee venom and lowercase letters under symbols of OS indicate statistically significant differences, respectively (Tukey HSD test of ANOVA, $P < 0.05$, $n = 3$).

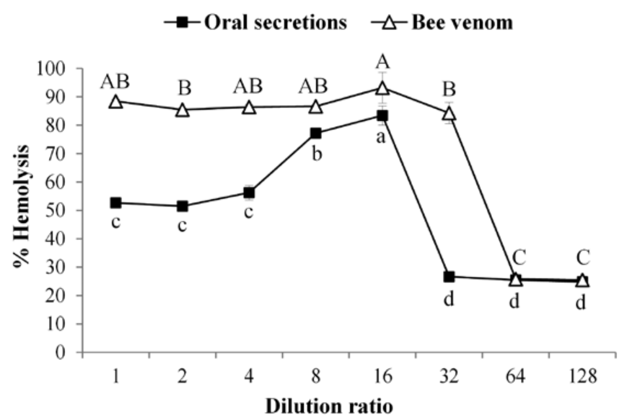


Fig. 6. Effect of sample concentration on hemolytic activity of larval OS of *H. armigera* and bee venom. Experiments were performed in triplicate and mean ± SD of percent hemolysis are shown, some error bars are within the symbols. Different uppercase letters above symbols of bee venom and lowercase letters under symbols of OS indicate statistically significant differences, respectively (Tukey HSD test of ANOVA, $P < 0.05$, $n = 3$).

In order to verify that hemolysin is ubiquitous as a kind of OS protein in lepidopteran species in the natural host-plant environment, not just in the artificial environment. Larvae of *O. furnacalis*, *S. exigua*, *A. lepigone*, *P. rapae*, and *H. armigera* were captured from their host plant in filed for hemolysis testing. Our result shows that all of tested insects' OS have hemolytic activity (Table 4). This indicates that hemolysin(s) is essential immune factor in OS of insect herbivores living in natural environment. Remarkably, hemolytic activity of larval OS of *H. armigera* feeding on artificial diet (titer: 2^4) was higher than those feeding on host plant (titer: 2^2; Tables 1 and 4). This suggests that feeding medium might affect the hemolytic activity of OS.

The mechanisms are different between immune hemolysis and venom hemolysis. Immune factors recognize glycosyl group on the surface of the pathogen for immune surveillance, like lectins (Yu and Kanost 2000, Yu et al. 2006). But hemolytic activity of OS was not inhibited by tested carbohydrates in this study (Fig. 7A), and hemolymph (Wang et al. 2019) and coelomic fluid of *Marthasterias glacialis* (L.) (Forcipulatida: Asteriidae) (Canicatti 1989) also were not inhibited. However, hemolysis of *Apis* venom was inhibited by two tested carbohydrates (Fig. 7B), and Hawaiian box jellyfish (*Carybdea alata* (Reynaud) (Carybdeida: Alatinidae)) venom also was inhibited by carbohydrates (Chung et al. 2001). All these evidences indicate that the mechanism of immune hemolysis (from OS or hemolymph) might be different from venom hemolysis. Hemolysis mechanism of salivary hemolysin might be related to lipid on the cell membrane. Hemolytic activity of *Eisenia foetida* (Savigny) (Opisthopora: Lumbricidae) coelomic fluid could be inhibited by sphingomyelin (Roch et al. 1989, Yamaji et al. 1998), and pore-forming of erythrocyte was caused by interaction between hemolysins and lipids (Saha and Banerjee 1997). The specific hemolysis mechanism of different hemolytic factors needs to be further studied.

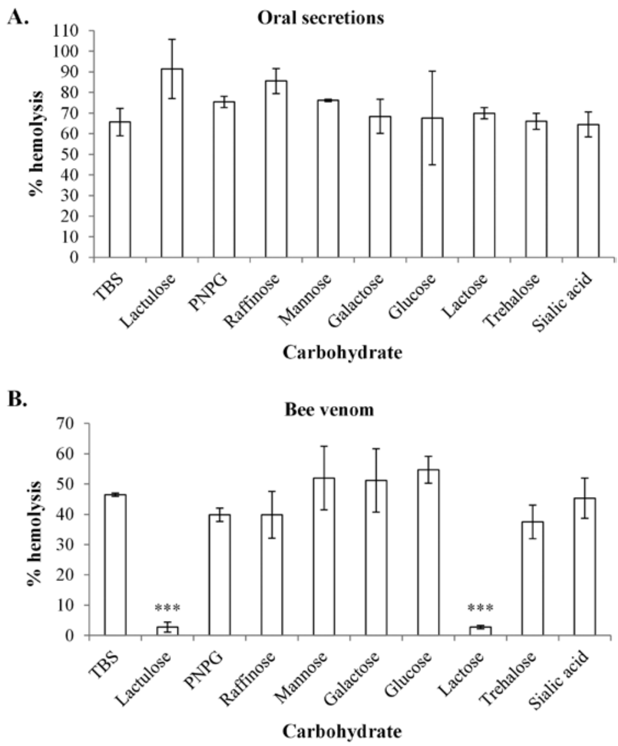


Fig. 7. Effect of carbohydrates on hemolytic activity of larval OS of *H. armigera* (A) or bee venom (B). Experiments were performed in triplicate and mean ± SD of percent hemolysis are shown. '***' above the bars indicates statistically significant differences with TBS control (t-test, $P < 0.001$, $n = 3$).

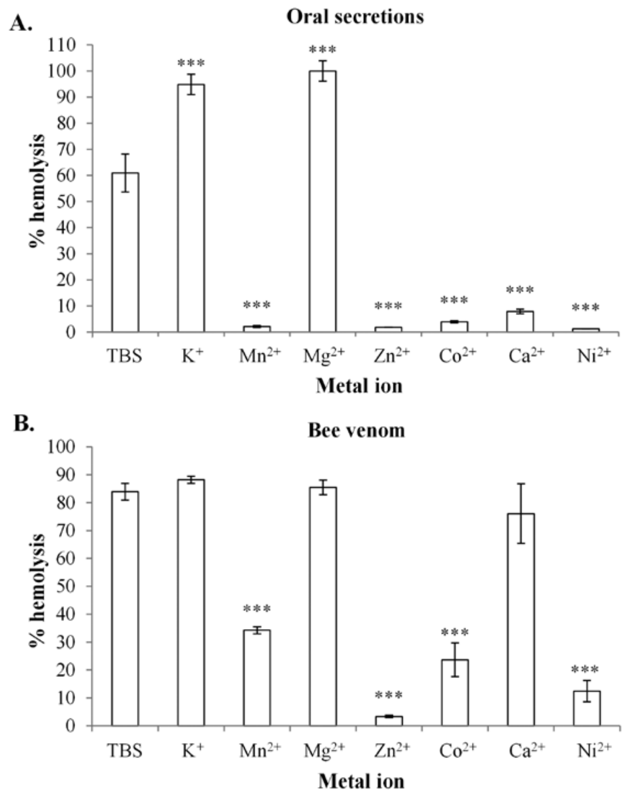


Fig. 8. Effect of metal ions on hemolytic activity of larval OS of *H. armigera* (A) and bee venom (B). Experiments were performed in triplicate and mean ± SD of percent hemolysis are shown. '***' above the bars indicates statistically significant differences with TBS control (*t*-test, $P < 0.001$, $n = 3$).

Effects of metal ions on hemolytic activity of different hemolytic factors are various. Hemolysin of hemolymph could be fully inhibited by Mn^{2+}, Zn^{2+}, and Ca^{2+}, partially inhibited by Co^{2+}, but could not be inhibited by Ni^{2+} (Wang et al. 2019). Which is partially different with salivary hemolysin (Fig. 8A). This means that the hemolysins in OS and hemolymph are not the same type. Hemolysins as immune factors in both OS and hemolymph of *H. armigera* were inhibited by Ca^{2+}, but bee venom was not. There have been many reports about the influence of Ca^{2+} on hemolytic activity in bacteria, but this influence is inconsistent (Chu et al. 1991, Shinoda et al. 1993, Beecher and Wong 1994, Park et al. 1994, Billson et al. 2000, Chung et al. 2001, Ochi et al. 2003). Some researchers explained that inhibitory mechanism of Ca^{2+} on the hemolysis as Ca^{2+} is an osmotic protectant to prevent hemolysis (Park et al. 1994). However, Ca^{2+} is considered necessary for lysis in some cases (Billson et al. 2000, Chung et al. 2001, Ochi et al. 2003). The inhibitory or promotion mechanism of metal ions on hemolysis is still unclear.

We found a novel hemolytic factor (hemolysin) in OS of herbivorous lepidopterans. The hemolysin is a ubiquitous proteinaceous factor with a labial salivary gland origin in lepidopteran species. Its physicochemical properties are different from bee venom, which suggests they are not the same class of hemolytic factors and are presumed to have different biological functions. The work on physicochemical properties would provide valuable information for separation, purification, and further research of OS hemolysin(s) in the future. Even though the potential role in immunity acted by hemolysin(s) was proposed here, actual physiological functions of hemolysin(s) in OS of caterpillar need to be further experimentally proven. This study can provide a basis for better understanding of immune factors for these herbivorous pests to thrive under the biotic stress caused by insect pathogens.

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Author Contributions

S.-F.B. and F.-M.Y. conceived and designed the experiments. and X.-Y.W. performed the experiments. X.-Y.W., D.-Z.C., X.L., S.-F.B. and F.-M.Y. wrote the manuscript.

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