Iraqi Journal of Veterinary Sciences

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Use of black soldier fly (*Hermetia illucens*) prepupae amino acids as anti *Aeromonas hydrophila* enterotoxin in vivo

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Article information

Article history:
Received March 22, 2022
Accepted August 02, 2022
Available online August 03, 2022

Keywords:
Aeromonas hydrophila
Prepupae
Insects
Protein
in silico

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Abstract

Aeromonas hydrophila is an opportunistic freshwater. These bacteria cause gastroenteritis and septicemia in animals and humans. Hemolysin and aerolysin, are important in the pathogenesis of *A. hydrophila*. Prepupae Black soldier fly (BSF) can be used as an antibacterial using its active substance against hemolysin and aerolysin. This study aimed to determine how the interaction of prepupae BSF amino acids with *A. hydrophila* enterotoxin in silico and protein level in various substrates in vivo. The study consisted of BSF larva of T1 (fed fruit waste), T2 (fed fermented fruit waste), T3 (fed tofu waste), T4 (fed fermented tofu waste), and T5 (fed fermented fruit waste and tofu waste). Data on the difference of protein level of prepupae among groups were analyzed statistically using the ANOVA test. The study showed that the highest protein content of BSF prepupae was found in treatment T3 dan T4. Protein docking analysis showed that L-arginine had the most hydrogen interaction (11 H-bonds) with aerolysin and 10 H-bonds against hemolysin, indicating an antibacterial role. The most favorable interacting residues of 17 amino acids against hemolysin were ARG73, ASP74, THR541, ALA523, and ASN483, while the residues of the active site against aerolysin were ASP92, ARG394, SER354, TYR348, ARG356, VAL396, PRO395, and ASP350. Amino could inhibit the hemolytic toxin of *Aeromonas* by interacting with binding site residues. The better the nutritional value of the substrate given to BSF larvae, the higher the protein content of BSF prepupae. Proteins from BSF prepupae can be antibacterial candidates against *A. hydrophila*.

DOI: 10.33899/ijvs.2022.133332.2205,

Introduction

Aeromonas hydrophila (*A. hydrophila*) is a Gram-negative freshwater bacterium and an opportunistic pathogen which causes gastroenteritis, necrotizing fasciitis, and septicemia in fish, mammals, birds, amphibians, and reptiles as well as humans (1). Hence the name "jack-of-all-trades" is an important disease-causing fish-animal-human pathogens. Infections in fish bringing about high mortality and morbidity rates (2). Factors, contributing to bacterial virulence, include cytotoxin, proteases, hemolysin, lipase, adhesins, agglutinins, and various hydrolytic enzymes. As for haemolytic toxins, such as hemolysin and aerolysin, they play a crucial in the pathogenesis of *A. hydrophila* (1). Hemolysin and aerolysin are cytotonic and cytotoxic, that cause diarrhea to the host (3). The current treatment for *A. hydrophila* infection is using antibiotics, but many strains of bacteria are multiple drug-resistant, resulting in health problems for humans and animals (1,2). The role of protein as an antibacterial is still rarely researched and the molecular

modeling of the interaction between amino acids and *A. hydrophila* is not yet available. Therefore, it prompted the search for new alternative protein resources that are safe and economical (4). Insects are a protein source that could be administered during *A. hydrophila* infection. Insects, that can be used as food, provide significant advantages as a source of nutrients including high protein, amino acids, lipids, energy, and various other micronutrients (5) (e.g. copper, iron, zinc) (6). Black soldier fly larvae (BSFL) contain 45.2% crude protein (7) and essential amino acids that match the amino acid profile required for growing livestock (such as pigs and poultry). BSFL contains a high amount of lysine, threonine, and methionine (6). Amino acids are useful for modulating immune responses (T cell receptors activation, lymphocyte proliferation, production of cytokines and antibodies, polarizing macrophages, and killing pathogens by free radicals) and prevention of infectious diseases as well as production of antioxidants (8). BSFL development takes more than three weeks; longer than that of house flies and carrion flies (< 5 days), which allows the larvae to consume a larger amount of substrate and produce larger prepupae (9). BSF larvae can consume a variety of organic materials such as: fruits, vegetables, and livestock feces (9,10). BSF larvae accumulate food in body fat so that when they become adults, BSF can survive without eating as they rely on their larval stage nutrition stock. Also, BSF larvae do not carry diseases of bacterial origin (9), which makes BSFL also suitable for use in animal feed, biodiesel production, and also for the cosmetic or pharmaceutical industry (11). Currently, there are restrictions on the use of antibiotics because they cause bacterial resistance. Various drug candidates have been tested for hemolytic toxins but the use of antimicrobial peptides against hemolytic activity is still rare. Amino acid-based drugs; however, have good bioavailability, low toxicity, and good pharmacokinetic properties (12).

As stated before, protein usage as an antibacterial is scarcely researched and the modeling of interaction between amino acids of BSF larvae and *A. hydrophila* is not yet available. Thus, this study aimed to evaluate the protein content and weight of BSF prepupae reared on different organic substrates and to analyze the interaction between amino acids contained in BSF and hemolytic proteins (aerolysin and hemolysin) through molecular docking analysis.

Materials and methods

Ethical approve

This study was approved by the ethics committee (certified no. 035-KEP-UB-2022) of University of Brawijaya.

Preparation of experimental diets

The study consisted of 5 treatments and 3 replications, namely: T1 (larva that fed with fruit waste), T2 (larva that fed with fermented fruit waste), T3 (larva that fed with tofu waste), T4 (larva that fed with fermented tofu waste), and T5 (larva that fed with fruit waste and fermented tofu waste, by a ratio of 1:1) respectively. Every substrate weighed 300 grams. As much as 150 grams of tofu waste and 150 grams of fruit mixture (papaya, guava, star fruit, avocado, watermelon, apple, and pear) were put in a container (27 cm x 21,5 cm x 9 cm), and then, fermented using a mixture of EM4: molasses: water: medium in a ratio (ml) of 2:1:50:500 for 2x24 hours in an anaerobic environment. The container was placed at a temperature of 33-40°C and relative humidity of 65 ± 5% (6). As much as 100 grams of coconut pulp was added into the plastic container to keep the larval substrate from getting damp which would have resulted in death and larvae migrating out of the container. Larvae were fed every 2 days (12). Media reduction was carried out every 2 days so that the media would not be too thick as well as to reduce humidity and accumulation of ammonia which affected the growth and survival of larvae.

Rearing of black soldier fly prepupae

The study used 150 grams of BSFL aged 7 days. The rearing period was 18 days. BSF prepupae were harvested on day 25 by separating the larvae and the rest of the growing medium. Prepupae BSF weight gain was calculated by subtracting the final weight from the initial weight. The BSF prepupae was then washed and rinsed with sterile water (13). As much as 250 g of BSF prepupae were left overnight. Then, the drying process was carried out using a microwave with 1000 watts of power for 5 minutes. The BSF prepupae were removed after 5 minutes, aired for 1 minute, and then microwaved again for 5 minutes. The drying process was to be deemed successful if the texture characteristics of the BSF prepupae were crispy and dry. The dried prepupae were then blended and made into powder, stored in a dry place until used.

Proximate analysis of BSF prepupae

The crude protein and crude fiber content of the prepupae powder was measured (6). The test was carried out at the Food Nutrition laboratory, Husbandry Faculty, University of Brawijaya. Parameters for determining the proximate composition were calculated based on the percentage (%) weight per weight (w/w) (14).

Amino acid docking of BSF prepupae with aerolysin and hemolysin proteins: 3D structure capture of target compounds and prediction of target compound's bioactivity

The amino acid structures contained in the BSF were downloaded from the NCBI PubChem database. Amino acids consisted of glycine (CID 750), cysteine (CID 5862), L-lysine (CID 5950), serine (CID 5951), aspartic acid (CID 5960), lysine (CID 5962), tyrosine (CID 6057), proline (CID 145742), leucine (CID 6106), methionine (CID 6137),

phenylalanine (CID 6140), histidine (CID 6274), valine (CID 6287), threonine (CID 6288), isoleucine (CID 6306), L-arginine (CID 6322) and glutamic acid (CID 33032). The bioactivity of amino acids was predicted by the PASS-Two-way drug online program (15).

Molecular docking and data analysis

Ligands in the form of amino acids and antibacterial target proteins were imported into Molegro virtual Docker program version 5.0. Aerolysin and hemolysin target proteins were predicted to be active sites (binding cavity) with the Molegro virtual Docker program version 5 with van der Waals parameters 5 (15). Docking of amino acids with aerolysin was carried out on specific grids X=17.74, Y=53.13, Z=31.37, and radius 24. The specific grids for hemolysin were X=-11.97; Y=-8.61; Z=17.23, and radius 12. Other docking parameters are Moldock Grid 0.3A, RMSD less than 2, Binding pose 5, running 10 times. Docking results were analyzed with PyMol 2.2 and Discovery studio version 21.1.1 (16).

Statistical analysis

Analysis data on protein content were tabulated using excel, presented in the form of mean±standard deviation and statistically analyzed using One way ANOVA (Analysis of Variance) with SPSS version 21.0 software.

Result

The highest average weight gain of BSF prepupae was found in treatment T5 (fermented of tofu waste and fruit waste), which was significantly different from the other treatments. The results of the study were that the BSF prepupae weight gains from T1-T5 were 233±10.44; 233.33±15.31; 222±8.66; 244.67±21.46 and 259.33±16.17, respectively. All treatments showed the transition from larvae to prepupae at the age of 18-19 days, but prepupae changes were more dominantly observed in T5 treatment.

The protein content of the BSF prepupae was significantly different amongst the treatment groups, with an average of above 43% w/w. The highest protein contents were found in BSF prepupae grown on tofu waste media (T3) and fermented tofu waste (T4), compared to T1, T2, and T5 treatments (Table 1). The average protein content of BSF prepupae using fermented tofu waste 45.96% was higher than that of unfermented tofu waste 45.73%. The results showed that the protein content on the use of fruit waste substrate (T1) was 45.32%, while fermented fruit waste (T2) was 43.14%. The results also indicated that the fermentation process affected reducing crude fiber of BSF prepupae. The crude fiber content of BSF prepupa, grown on fermented tofu waste substrate (T2), was lower than that of unfermented tofu waste (T1), as well as BSF prepupa grown on fermented fruit waste substrate (T4) was lower than that of unfermented tofu waste. Although fermentation (T3) not significantly different.

Table 1: Results of proximate analysis of BSF prepupae in all treatment groups

Treatment	Crude Protein	Crude fiber
T1	45.32±0.47^{ab}	8.74±0.30^{ab}
T2	43.14±1.31^{a}	7.91±0.52^{a}
T3	45.73±0.47^{b}	10.39±0.73^{b}
T4	45.96±0.77^{b}	10.15±0.64^{b}
T5	44.65±0.85^{ab}	10.24±0.77^{b}

BSF has 17 amino acids (Cysteine, L-Lysin, Serine, Aspartic acid, Lysine, Tyrosine, Leucine, Methionine, Phenylalanine, Histidine, Valine, Threonine, Isoleucine, L-Arginine, Glutamic acid, and Proline). The essential amino acids are Lysine, Leucine, Methionine, Valine, Threonine, and Isoleucine. The 17 amino acids of the BSF were bound to aerolysin and hemolysin active sites but in different regions. The binding energy (kJ/mol) of the 17 tested amino acids was L-arginine (-228 kJ/mol) so L-arginine had the strongest binding to the hemolysin target. The types of bonds are hydrogen (Conventional Hydrogen Bond and Carbon Hydrogen Bond), unfavourable (Unfavourable Donor-Donor), and hydrophobic bonds (Pi-Alkyl, Alkyl, Pi-Sulfur, and Pi-Sigma). The residues predominantly identified on the interaction between amino acids and hemolysin were ARG73, ASP74, THR541, ALA523, and ASN483 (Figure 1).

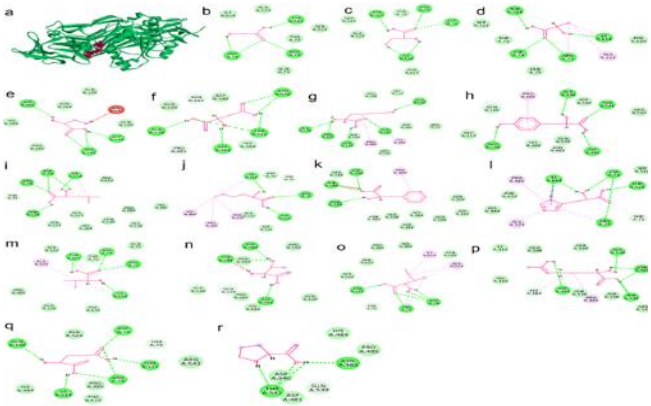

Figure 1: Interaction between amino acids and hemolysin proteins, a. superimposed BSF amino acid against hemolysin protein, the 2D structure of amino acid interaction with hemolysin shown in b-r, b. Glycine, c. Cysteine, d. L-Lysin, e. Serine, f. Aspartic acid, g. Lysine, p. Tyrosine, i. Leucine, j. Methionine, k. Phenylalanine, l. Histidine, m. Valine, n. Threonine, o. Isoleucine, p. L-Arginine, q. Glutamic acid, r. Proline.

Within the 17 amino acids in BSF, tyrosine was found to have the lowest binding energy (kJ/Mol) against aerolysin, which means tyrosine (-226 kJ/mol) has the strongest bond to aerolysin. The bond types are hydrogen, unfavourable, and hydrophobic (Pi-Alkyl, Pi-Sulfur) (Figure 2). Residues predominantly identified the interaction between amino acid and aerolysin are ASP92, ARG394, SER354, TYR348, ARG356, VAL396, PRO395, ASP350.

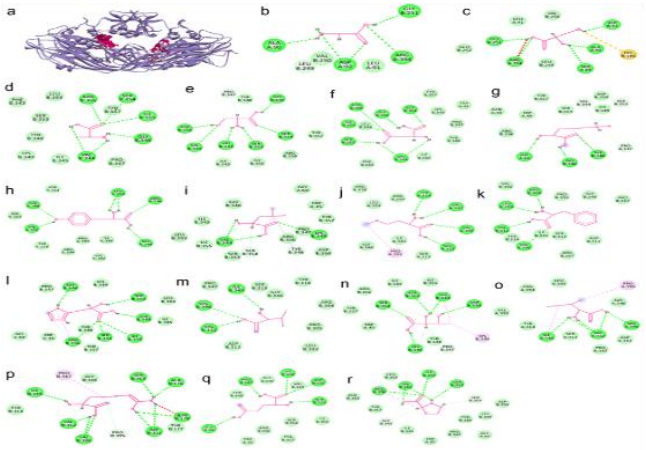

Figure 2: Interaction between amino acids and aerolysin protein. a. superimposed BSF amino acids against hemolysin protein, the 2D structure of amino acid interaction with hemolysin shown in b-r, b. Glycine, c. Cysteine, d. L-Lysin, e. Serine, f. Aspartic acid, g. Lysine, p. Tyrosine, i. Leucine, j. Methionine, k. Phenylalanine, l. Histidine, m. Valine, n. Threonine, o. Isoleucine, p. L-Arginine, q. Glutamic acid, r. Proline.

Discussion

In this study, the prepupae phase of BSF was applied. The BSF prepupae stage has advantages over the larval stage; namely the prepupae empty their digestive tract and thereby reduce the risk of carrying pathogenic microorganisms, as well as migrating from the substrate to dry and higher location (9) which facilitates harvesting in large-scale rearing (6). They also tend to be clean. Larvae were reared on standard feed for 7 days (chicken feed) and then transferred to each substrate treatment. Substrate feed, in the study, was given every two days. Feed supplementation, during larval growth, depends on larval density, amount of initial feed, initial substrate residue, and industrial requirements (5).

In this study, BSF prepupae weight was also measured at harvest. Notably, prepupae weight greatly influences growth, survival, and biological traits related to the reproduction of adult BSF. Low prepupae weight can inhibit the sustainability of the bioconversion process because they will produce mature BSF with lower reproductive abilities. The duration of BSF prepupae development in this study was faster than those used in studies using meat meal (33 days), rice straw (38-52 days), and cassava peel (20-54 days) (17), which means the substrate used in this study has the potential to be used as BSF larval cultivation substrate.

The crude protein content of the BSF prepupae produced from this study was higher than that produced in the study conducted by Azizah *et al.* (18) which was 39.87%, using fermented tofu waste as a substrate, with the same BSF prepupae rearing period of 25 days. Larvae, reared with high protein content in the substrate, will have a higher larval protein content and vice versa (10). This statement contradicts with Fuso *et al.* (11), who stated that larvae, feeding on protein-rich substrates, have a higher lipid content and thus, the protein content is reduced (in % dry mass) when turned to being prepupae. Therefore, to get prepupae with high protein content, they need to be grown on a substrate containing a minimum of 7% protein. This means low nutrients in the substrate affect the decrease in BSF prepupae biomass which will result in a decrease in protein value.

Tofu waste was used as a substrate for the development of BSF larvae into prepupae. According to Azizah *et al.* (18), the protein content of tofu waste is 21.3% - 27%. The use of fermented tofu waste as a substrate resulted in higher protein content of BSF prepupae than that obtained from unfermented tofu waste. The fermentation process can change the substrate or raw material into a product with better nutritional quality, reduce crude fiber content, increase digestibility, and increase dissolved protein and crude protein content of raw materials (17). The protein content of the BSF prepupae grown in fruit waste in another study was 3.85%, lower than the protein content produced in this research. Fruit waste is an ideal substrate for the bioconversion of insects. The average water content of fruit waste such as apples is around 90%. It should be noted that protein and lipid content in dry matter is optimal for the maintenance of BSFL (5).

BSF prepupae drying method in this study was microwave heating at a voltage of 1000 V for 15 minutes. Crude protein content in all substrates used was higher than that of the study conducted by Adebayo *et al.* (13), which showed 13-20% protein content in BSF prepupae grown on chicken feed substrates, brewery waste, food waste, and fruit waste, harvested at 2-4 weeks of age and dried using the freeze-drying method. Protein content while using microwave drying is higher than freeze-drying. These results are instead the opposite of the statement of Spranghers *et al.* (6), which said freeze-drying can result in less complete moisture removal than oven drying but guarantees better preservation of nutrients.

A good protein value will result in a better amino acid profile even if they come from different substrates. Amino acids have the main effect on controlling protein metabolism and since they are a component of tissues, their insufficient availability in the body leads to reduced protein synthesis.

Amino acids are important molecular precursors. The amino acid cysteine is required for the synthesis of glutathione and taurine, which are important compounds for host defense against oxidative stress such as bacteria (19).

When invading, bacteria produce exotoxins such as: hemolysin and aerolysin. The two exotoxins cause rupture of cell membranes of the erythrocytes, anemia in the host. Aerolysin can enter the target cell membrane after forming heptamers with transmembrane pores. Then, heptamer channel pores disrupt the permeability of the cell membrane which results in host cell death (20). Hemolysin is an enterotoxin causing inflammation in the gastrointestinal tract. This further causes an increase in intestinal transit time (dysmotility); making water and diluted compound unable to be absorbed adequately. Electrolyte retention within the intestinal lumen causes water accumulation and initiates diarrhea (21).

Protein-ligand docking using virtual screening and molecular dynamics simulations are useful for predicting the binding affinity of the ligand to its receptor (22). Antimicrobial activity depends on the functional group of the amino acid side chain and the hydrophobicity, aromaticity, and amphipathicity of the molecule. The more hydrophobic and aromatic amino acids present in the molecule of a material, the more biologically active the compound will be. The hydrophobic effect causes damage to cell membranes which will result in bacterial cell death. Tyrosine is one of the aromatic amino acids, in addition to tryptophan, and phenylalanine which are crucial as an antimicrobial compound through binding to bacterial membranes (11). Arginine is a positively charged amino acid in proteins that have high aqueous pKa's (~13.8 for Arg2), so it has a strong tendency to carry charge at physiological pH. These amino acids are important for protein structures and functions which involve electrostatic interactions and protein solvation (23). The results of the docking molecule measurement are described by the binding affinity score. The lower the binding affinity score, the lower the strength between the ligand and protein, but the more stable the resulting complex interaction (22). Factors that influence the strength of the protein-ligand complex include binding partners, solvent effects, and dynamics molecular. Based on the results of molecular docking, the highest number of bonds is hydrogen bond. Hydrogen bond (H-bonds) strength is required for most high-affinity ligands (24). Some types of interactions contribute to the binding energy. Hydrogen bonding and position of active ligand interactions; the type of interaction between the ligand and the protein affect the calculation of the binding energy. Hydrogen bonding can also increase the binding affinity. Phytochemistry is crucial in adjusting drug activity (25).

Amino acids from BSF larvae may potentially be an antibacterial compound by inhibiting aerolysin and hemolysin from *Aeromonas hydrophila* bacteria. The results of other studies have shown that D-amino acid (D-Leucine, D-Methionine, D-Tyrosine, D-Tryptophane) can inhibit bacterial growth, biofilm formation, and bacterial attachment to eukaryotic cells, as well as protect alveolar cells from *Pseudomonas aeruginosa* infection (26). Dong (20), demonstrated that luteolin, isolated from herbs, acted as an anti-*A. hydrophila*, by reducing aerolysin-induced hemolysis through molecular dynamics simulations. Research conducted by Venkatasamy *et al.* (1), stated that the subtilosin, a peptide from *Bacillus subtilis*, has the antimicrobial activity to inhibit aerolysin and hemolysin from *A. hydrophila* through the interaction of binding site residues to prevent extracellular division.

The results showed that the substrate with the addition of probiotic EM4 (content such as *Lactobacillus sp*) could increase the protein content and decrease the crude protein content. According to He (27) that lactic acid bacteria play a role in inhibiting the growth of pathogenic bacteria by stimulating acid production and decreasing pH. Besides, it also hydrolyzes protein and reduces fiber. BSF prepupae can be used as a candidate for feed supplements to improve antibacterial activity in animals because it produces high protein content, easier to manufacture and process, and can be produced on a large scale in a short time.

Conclusions

Based on the proximate analysis, it is showed that the highest protein content of BSF prepupae was found on those grown on tofu waste (T3) and fermented tofu waste (T4). BSF prepupa grown on the substrate with the addition of probiotics can increase protein and decrease crude fiber. The results of molecular docking analysis showed an interaction between 17 amino acids from BSF larvae and hemolytic toxins from *A. hydrophila*. Protein docking analysis illustrated that L-arginine had the most interaction (11 H-bonds) with aerolysin and (10 H-bonds) with hemolysin; thus, indicating its function as antibacterial. L-arginine has the strongest binding to hemolysin, while tyrosine has the strongest binding to aerolysin. The protein of BSF prepupae can be used as an antibacterial against *A. hydrophila*.

Acknowledgments

Many thanks to the doctoral study program in medical science, Faculty of Medicine, University of Brawijaya, and Faculty of Veterinary Medicine, University of Brawijaya for their support.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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استخدام المعلومات الحيوية وتحديد المكونات الغذائية لطور ما قبل العذراء لذبابة الجندي الاسود ضد سموم جراثيم ايروموناس هيدروفيليا

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الخلاصة

تعد جراثيم ايروموناس هيدروفيليا *Aeromonas hydrophilia* حرة المعيشة في الماء وانتهازية وتسبب التهاب المعدة والامعاء والسمدمية، ويلعب كل من محلل الدم Hemolysin ومحلل الغاز Aerolysin دوراً رئيسياً في امراضية هذه الجراثيم. لوحظ امكانية اســـتعمال الطور قبل العذراء لذبابة الجندي الاسود (BSF) كمضاد بكتيري ضد محلل الدم ومحلل الغاز. كان الهدف من الدراسة الحالية هو ايجاد التداخل بين الاحماض الامينية لطور قبل العذراء لذبابة الجندي الاسود مع السموم الداخلية لبكتريا الايروموناس هيدروفيليا وذلك في السيليكو (افتراضياً) وكذلك داخل الجسم الحي. شملت الدراسة يرقات BSF مجموعة T1 (غذيت على مخلفات الفاكهة) و مجموعة T2 (غذيت على مخلفات الفاكهة المتخمرة) و مجموعة T3 (غذيت على مخلفات الصويا) و مجموعة T4 (غذيت على مخلفات الصويا المتخمرة) و مجموعة T5 (غذيت على مخلفات الفاكهة ومخلفات الصويا). تم تحليل النتائج احصائياً لايجاد اختلافات مستويات البروتين بين المجاميع باستخدام اختبار ANOVA. اظهرت النتائج أن اعلى مستوى بروتين كان في مجموعتي T3 و T4. تحليل البروتين أظهر أن الحامض الاميني L-arginine كان الاكثر تفاعلا (١١ اصرة هيروجينية مع المحلل الغازي، ١٠ اواصر هيدروجينية مع محلل الدم) مما يشير الى الفعالية المضادة للجراثيم. الاحماض الامينية الاكثر تفاعلا مع محلل الدم فكانت ARG73, ASP74, THR541, ALA523, ASN483، أما الأحماض الأمينية الأكثر تفاعلاً مع المحلل الغازي فكانت ASP92, ARG394, SER354, TYR3428, ARG356, VAL396, PRO395, ASP350. اذ بامكان الحامض الاميني تثبيط البروتين المحلل للدم لهذه الجراثيم بالارتباط بالموقع الفعال. وكلما كانت القيمة الغذائية عالية لغذاء يرقات BSF كلما كان المحتوى البروتيني لطور قبل العذراء عاليا. وهذا البروتين من طور قبل العذراء يمكن استخدامه كمضاد بكتيري.