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Histological study of SlNPV infection on body weight and peritrophic membrane damage of *Spodoptera litura* larvae

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Abstract. *Sanjaya, Machmudin D, Kurniawati ND. 2010. Histological study of SlNPV infection on body weight and peritrophic membrane damage of* Spodoptera litura *larvae. Nusantara Bioscience 2: 135-140.* The effect of SlNPV infection on body weight and peritrophic membrane damage of *Spodoptera litura* Fab. larvae has been carried out. The method was used Probit analysis, and based on LD 50 the virus was infected to know body weight and post infection damage.The damage of histological structure caused by SlNPV (0, 315, 390, 465, 540 dan 615 PIB/mL) was investigated after 0, 12, 24, 72 and 96 hours post infection. The histological material was prepared by using parafin method after fixation with Bouin Solution, then slice into 7 um and colored with Hematoxilin-Eosin. The result showed that the exposure SlNPV cause decreasing food consumption especially on 540 PIB/mL give average rate as amount of 0.1675 mg. The descriptive obsevation on structural intact of peritrophic membrane histology caused by SlNPV infection shows a tendency to decrease, while in control, there was no damage at all. The longer the exposition of virion in the midgut lumen the more damage on peritrophic membrane occurred. The severest damage occurred 96 hour after infection. The result prove that haNPV virion can destroy hystological structure of midgut.

Key words: SlNPV, *Spodoptera litura*, LD50, consumption rate, peritrophic membrane.

Abstrak. *Sanjaya, Machmudin D, Kurniawati ND. 2010. Kajian histologis infeksi SlNPV terhadap berat badan dan kerusakan membran peritrofik larva* Spodoptera litura. *Nusantara Bioscience 2: 135-140*. Pengaruh infeksi SlNPV pada berat badan dan kerusakan membran peritrofik larva *Spodoptera litura* Fab. telah dilakukan. Metode yang digunakan adalah analisis probit, dan berdasarkan LD 50 virus yang terinfeksi untuk mengetahui berat badan dan kerusakan pasca infeksi. Kerusakan struktur histologi yang disebabkan oleh infeksi SlNPV (0, 315, 390, 465, 540 dan 615 PIB/mL) diamati setelah 0, 12, 24, 72 dan 96 jam pasca infeksi. Preparasi histologis dibuat dengan metode parafin setelah fiksasi dengan larutan Bouin, kemudian diiris setebal 7 um dan diwarnai dengan Hematoxilin-Eosin. Hasil penelitian menunjukkan bahwa paparan SlNPV menyebabkan penurunan konsumsi pangan terutama pada 540 PIB/mL dengan rata-rata 0.1675 mg. Pengamatan deskriptif pada struktur histologi membran peritrophic yang terkena infeksi SlNPV menunjukkan kecenderungan kerusakan, sementara pada kontrol tidak ada kerusakan sama sekali. Semakin lama paparan virion di dalam lumen midgut maka semakin tinggi terjadinya kerusakan membran peritrofik. Kerusakan paling parah terjadi 96 jam setelah infeksi. Hasilnya membuktikan bahwa virion haNPV dapat menghancurkan struktur histologi midgut.

Kata kunci: SlNPV, *Spodoptera litura*, LD50, tingkat konsumsi, membran peritrophic.

INTRODUCTION

One of the main concepts of IPM is to maintain populations of pests insect so they will not exceed the economic threshold and keep other animals so as not to interfere, so that the balance of ecosystems is maintained (Bonning and Hammock 1996). These organisms are pathogenic naturally against insect larvae with a specific target so as not to interfere with insect species and other non-target species. In addition, these agents are very virulent, easily spread in the population and can be persistent in the long term if environmental conditions allow (Teakle et al. 1994).

Some viruses that attack the plant are able to kill insect pests, such as members of the genus Baculovirus often called Nuclear Polyhedrosis Virus (NPV) (Yamagishi et al. 2003 Maramorosch 2007). Moscardi (1994) states that the application of baculovirus is quite effective as an insect control agent for pest Lepidoptera. NPV can attack several leaf-eating Lepidoptera larvae which destroyed many crops. Until now about 700 viruses have been isolated and identified from insects and other arthropods animals. These arthropod viruses mostly belong to six genera namely virus Baculovirus, Poxivirus, Iridivirus, enterovirus and Rhabdovirus (Cristian 1994). According Barbehenn and Marin (1994), Baculovirus specifically works to and infects several species of insects, usually from the same family.

NPV can suppress the *Spodoptera exigua* caterpillar attack (SeNPV) which attacks the onion leaves for up to 84% (Sutarya 1996). The use of NPV in tomato plants suppresses *Heliothis* sp. caterpillars attack for up to 65% and save the lost crop yields up to 83% (Novizan 2004). This virus is a deadly pathogen because it can damage the peritrophic membrane in the area of middle intestine of

Lepidoptera insect species. Granados and Corsaro (1990) argued that when the virus infects the middle of the insect intestine, the histological structure of the peritrophic membrane which plays very vital role in the digestive process is estimated to be defective, so that the digestive process disrupted and eventually reduce the weight of larvae.

Researches on the presence of peritrophic membrane against pathogen attack in several Lepidoptera larvae have been reported. Among these are the defense mechanisms of *Trichoplusia ni* larvae against viral infection by forming peritrophic membrane (Wang and Granados 1998). *Glossina morsitans morsitans* larvae also form a membrane-peritrophic against *Trypanosoma* infection (Lehane and Msangi 1991). According to Utari (2000), the integrity of peritrophic membrane histological structure on *Helicoverpa armigera* larvae due to HaNPV infection decreases with increasing doses of infection.

Research on NPV infection of some Lepidoptera insect larvae has been reported, but research on the effect of NPV on peritrophic membrane area in the middle intestine of larvae of *S. litura* is rare. This study aims to determine the effect of SlNPV infection on body weight and the damage to peritrophic membrane on the instar-5 larvae of *S. litura*.

MATERIALS AND METHODS

Determination of Lethal Dose 50 (LD_{50})

This study used 5 doses of treatment with 1 type of dosage control. The dose was determined based on the range of LD_{50} with 95% confidence level. This experiment used 6 test larvae for each treatment with 4 replicates; observations were made until the pre-pupa stage. Stages of viral insecticides are not different from the methods performed on the initial study.

Spodoptera litura Nuclear Polyhedrosis Virus (SlNPV) is given to the test larvae through the feeding method with food that has been poured with virus suspension for as much as 10 µL of doses that have been determined. Previously, the larvae are given no food for 24 hours. The next day, all larvae were given fresh food without SlNPV suspension. For control, larvae fed with no virus suspension addition, but distilled water.

Every day for 8 days, the death larvae observed in each treatment is accounted. The time needed by the virus to interact with its host takes several days from the onset of infection until the larvae die.

Observation of larval weight

This observation Is aimed to determine whether there is a larval weight change due to SlNPV infection in the larvae of *Spodoptera litura*. This observation began with separating the last four instars larvae that had stopped eating. If skin had changed, then the body weight of larvae was weighed and then separated individually in each zalp bottle with a piece of food that had been given with virus suspension. The observation in this phase was ended when the mortality observations ended. Furthermore, the final weights of larvae were weighed again.

Histological preparations

The making of an incision across the membrane peritrophic is based on Utari (2000) which has been modified. The middle intestine of *S. litura* instar-5 larvae was taken at the time of 0, 24, 48, 72, 96 hours after infection using an infection dose based on LD50 values of SlNPV delivered from research, with a sharp razor blade. To obtain a good piece, larvae were snared in Bouin's solution on the bearing candles. Part of front intestine and rear colon is pinned with a needle. About five minutes after soaking, the dead larvae were removed from the bearing candles, and their middle colons were taken. Furthermore, the middle intestines were fixed in Bouin's solution for 24 hours. The next day, the dehydration process was made with graded alcohol, namely: 70%, 80%, 90%, 96%, and 100%, respectively, each for three hours.

The Organs purification was done by soaking the object in alcohol 100%, i.e. xilol. Organs that have been clear then infiltrated in paraffin at a temperature of 48°C for 30 minutes, 52°C for 60 minutes, and 56°C for 90 minutes. Furthermore, organs were put into paraffin until frozen, then were sliced crosswise for as thick as 8 um. Incision ribbon was affixed to the glass objects that have been given Mayer's albumin adhesive. Then HE staining was performed by immersing the object for 40 minutes in pure xilol, alcohol 100%, 96%, and 80% respectively for 3 minutes, 70% alcohol for 30 minutes, dye HE for 2 min, water 6 minutes, alcohol 70% + 3 drops of HNO_3 twice dyeing, alcohol 70% and 80% respectively for 3 minutes, eosin Y for 25 minutes, alcohol 96% as much as 3 times dyeing, alcohol 100% as much as two times each for 6 minutes, pure xilol for 10 minutes, then they were given with glue and covered with glass cover.

Peritrophic membrane observation

In observation of this study, data analysis was done descriptively. The result of cross-sectional and longitudinal incision of the intestine was observed under the microscope. Observation of peritrophic membrane structure on the value of LD_{50} was performed at 0, 24, 48, 72 and 96 hours after infected by SlNPV. Determining the level of damage from SlNPV infection was done descriptively based on the presence or absence of peritrophic membrane damage, regenerative cells and basal membrane.

Data analysis

To determine the doses that can cause death on insect by 50% (LD50), the number of individual larvae of *S. litura* who died of infection are recorded every day SlNPV and its repetition until the pre-pupa stage. Furthermore, mortality data were analyzed using Probit analysis of the Polo-PC (Utari 2000). The calculation of the weight of larvae used ANOVA test by meeting the requirements that it should be normal and homogeneous. Homogeneity test used Bartlet test and continued with normality test.

If in the control treatment larval mortality was found, the mortality data corrected using Abbot's formula (Busvhine 1972), namely:

$$Pt = \frac{Po - Pc}{100 - Pc} X100\%$$

Pt : Percentage of dead insects after corrected (%)
Po : Percentage of dead insects for each treatment insects
Pc : Percentage of dead insects in the control

RESULTS AND DISCUSSION

The result of observation on Lethal Dose (LD) of instar-5 *S. litura* larvae is listed in Table 1.

Tabel 1. The value of LD_{10}, LD_{50} and LD_{90} of the instar-5 larvae of S. SlNPV litura at different doses.

LD	LD values	95% Fiducial limit
10	277	156.26-341.63
50	438	365.06-491.06
90	692	595.39-1011.18

Note: The fiducial limits of 95% indicate the range of the upper and lower limit of the SlNPV insecticide obtained by Probit Polo-PC analysis with 95% of confidence level.

From the daily observed mortality data, it is found out that the early symptoms on *S. litura* larvae which were infected by SlNPV appear within 24 hours after treatment (day 1st) with LD 50 values of 438/Inclusion Body (OB) and has a range from 365.06 – 491.06 / OB. As stated by Rohrmann (1994) that in order to create an effect in the host, the amount of inclusion body needed is about 50-thousand per insect. From the number of doses administered during the treatment which was 315 – 615 PIB / mL, it can be seen that the amount has been met the dosage range that can cause the virus to infect its host insect within the body. This is demonstrated by the response of larvae mortality occurred.

In addition to the amount of PIB, mortality of larvae of *S. litura* can also be influenced by the stage of larval development, temperature and insect species (Christian 1994). According Dibyantoro (1996), the optimum temperature for larval development are 23-24°C with relative humidity of 60-65%, while based on the results of measurements during the study at the Laboratory of Animal Structure-UPI Bandung, it is obtained the temperature range between 24-26°C with relative humidity of 59 - 67%. Environmental factors are still within the normal range for larval development so it is unlikely if these factors affect the mortality of larvae of *S. litura*.

In addition to the number of incoming PIB, temperature and insect species can affect the mortality of larvae, the development stage factors are also contributed. According Gothama et al. (1990); Laoh et al. (2003), the organs of young larvae are still weak, especially the middle intestine which is the primary target of pathogen attack, so the NPV is more easily penetrate these organs and damage its vulnerable cells. While in advanced instar larvae, the sensitivity of larvae would be reduced along with the development of body, weight and age of larvae. The organs and tissues of larvae grow and create differentiation. Intestinal wall, peritrophic membrane and integument become thicker and stronger, so they are more difficult to be penetrated by the NPV.

The mechanism of infection to cause death in larvae begins with swallowing polyhedra that enter with food into the body of insects which will then be digested in the middle intestine of the insect. Then the membrane that covers polyhedra will be dissolved in the middle intestine of the insect because of the alkaline conditions in that area. The next step, the virion will do the fusion with the plasma membrane and penetrate the peritrophic membrane or epithelial cells of the middle intestine which is the primary target of NPV infection. In the cytoplasm, virions will release the nucleocapsid. The remaining Nucleocapsids will then enter the nucleus while releasing its DNA and form virogenic stroma. In this condition, these virions replicate or reproduce themselves in the host cell nucleus. Furthermore, these infected cell nucleuses grow, and then lysis and release new virions derived from viral replication.

When the infection occurs continuously, this will damage the entire intestinal tissue and the conditions in the hemolymphs tissues seem turbid because they are full of NPV fluid resulted from replication of newly formed virions within hemosol (body cavity) and other tissues such as epidermis cells, fat cells and trachea (Bedjo et al. 2005). Infected tissue is filled with virions that cause the cells to lysis. Finally, in the advanced stage, larvae will die after the majority of their tissues are infected by NPV.

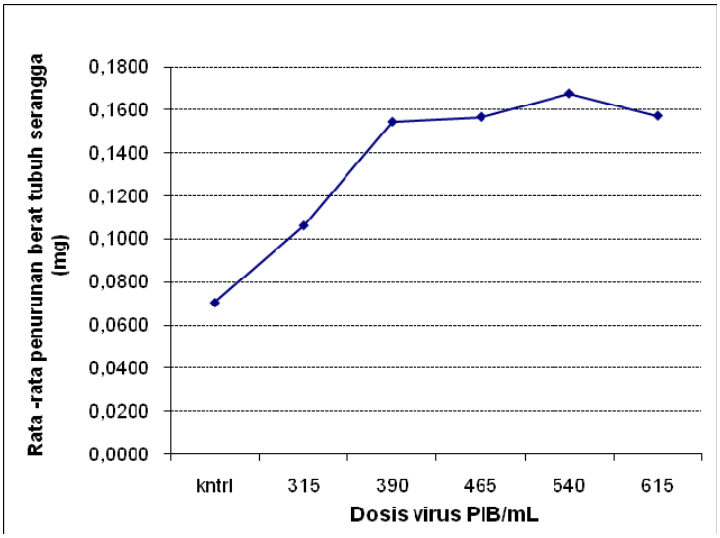
From the observation of symptoms caused by insect larvae after being given the treatment, it appears that there was a change in the body after the insects were infected by SlNPV. The description of changes in the initial symptoms until the larvae died in all treatments is relatively the same, except the controls. At first the larval body was red, especially on the abdomen and shiny, then swell, finally the larvae will be lazy to move and its appetite decreased (Wigglesworth 1984). Feels soft to the touch and it excretes fluid from its body which is turbid and smelly. These symptoms are relatively similar to the symptoms caused on Helicoverpa armigera larvae infected with NPV (Laoh et al. 2003). On the other hand, the deaths in the controls showed no symptoms which are mentioned earlier. Morphological condition of larvae remains the same, but the larvae are reluctant to eat, it appears from the remaining food that is different from the other larvae.

In addition to the ability of the NPV as virus for insect in killing its host as a direct response of its biological activity, it turns out that the impact or consequences arise indirectly to the infected larvae were also seen in larvae that successfully passed to live with the response of "debilitating effect" (Pawana 2000). This impact is the changes of host quality, such as the level of sensitivity to other pathogens, the decrease of reproductive capacity, fertility, sex ratio and a smaller body size.

Insects as the host of baculovirus certainly have the potential to perform self-defense against pathogen attack (Blum 1985). Besides, they can also develop a tolerance level (Sanjaya 2000). In adult insects, adverse effects due to pathogen attacks are treated with the apoptosis response.

Response can prevent the virus to multiply and prevent the spread of infection throughout its body.

In addition to the larvae that die due to viral infection, there are larvae stays alive although they were given the same treatment with SlNPV insecticide. This can occur because of several factors, including the ability to perform self-defense against pathogen attack. According to Blum (1985), a low dose of single cell pathogens such as viruses and bacteria will be responded by the phagocytosis immune, whereas in high doses, the response that occurs is the formation of nodules. Hemocyte that functions as a phagocyte is plasmosit (Wigglesworth 1984). Even in its development, the individual host can release itself from the effect of NPV infection with the mechanism of "maturation resistance" that is in line with the increasing age, the increasing resistance or the decreased sensitivity to the NPV would be obtained (Kurnia et al. 2002).



Gambar 1. Pengaruh dosis *SlNPV* terhadap berat larva instar 5 *Spodoptera litura.*

Observation of larval weight of *S. litura*

Based on the observation of larval weight of *S. litura* that has been done, it seems that there is a correlation between the effects of SlNPV infection and weight larvae. The more and more doses provided to the test larvae, the consumption rate of larvae decreases causing the weight decrease on test larvae (Figure 1).

After the calculation of the weight of *S. litura* larvae, the result is obtained that the SlNPV infected larvae looses its body weight. This decrease can be caused by the transfer of energy, where the energy that would be used for metabolic activity and growth is converted to counter attack pathogens entering the body (Pawana 2000). The results also show that larvae treated with a dose of SlNPV, on next day, when given a new food that is not contaminated, it tends to be quiet and does not eat the food directly. If it is done continuously, the larvae will die. The larvae rejection is presumably because SlNPV consumption through food has led to the change of food quality. Changes in the quality of food, of course, can inhibit the eating activity of larvae and even the larvae can be inert. In low doses, the larvae's feed consumption is still normal so the rejection response is rare. This is because the number of pathogens coming to the body is still below the threshold to be able to cause infection in the larval body. In this condition, the presence of peritrophic membrane as one of the body's defense mechanism still functions optimally in defending the attack of pathogens.

At higher doses, the larvae begin to show the response of inertia to eat the provided food. It is assumed that when the body has been attacked by pathogens, and the body will respond with her body's defense system. But at a certain time, larval body's defense system will decline. It is mentioned before that the protease enzyme secretion from intestinal epithelium has a central role in virus resistance (Bolognesi et al. 2002). Larvae's activity still runs optimally when the body is not susceptible to interference, but when pathogen attacks, the activities will be disrupted resulting in the metabolic process to be not optimum. Thus, if most of the tissues have become infected, then along with the decrease in the body's defense system, the rate of feed consumption will decline; even larvae will stop eating at all that causes a decrease in weight of larvae and the larvae eventually die.

According to Blum (1985) the main target of NPV infection is the area of the middle intestine of insects which are the main digestive organ because it serves as an absorber of nutrients and secretion of digestive enzymes. Therefore, if the intestine is damaged then the activity of digestion will be disrupted. Thus, the metabolism becomes inhibited. Based on this, allegedly baculovirus infection can reduce the weight of larvae.

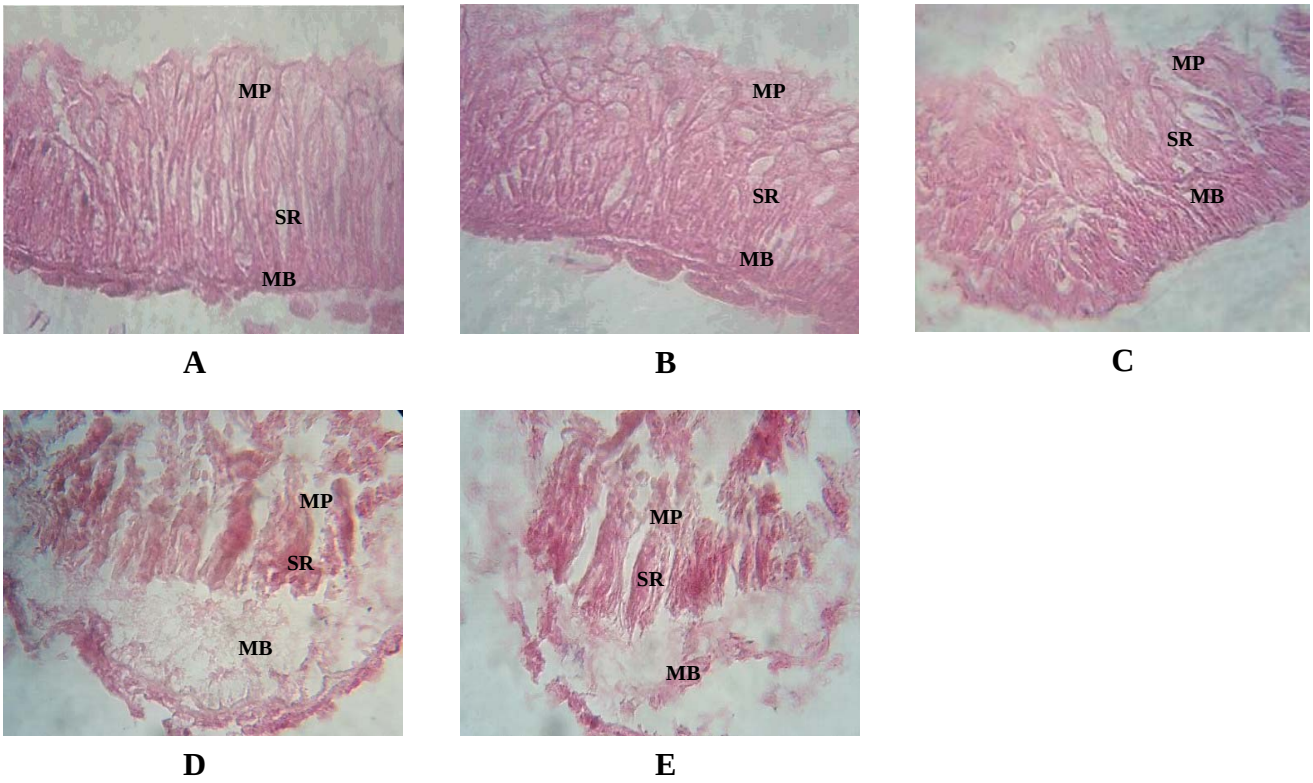
Histology of peritrophic membrane

From the results of earlier studies, it is obtained that doses which are capable of causing 50% death of *S. litura* larvae (LD_{50}) is 465 PIB / mL. At the LD_{50} value, a post-infection incision for 0, 12, 24, 48, 72 and 96 hours is made (Table 2 and Figure 2).

Table 2. The damage level to the intestinal area instar-5 larvae of *S. litura*.

Middle intestinal regions	SlNPV infection time at a dose of 438 PIB / Ml n (%)				
	0	24	48	72	96
Peritrophic membrane	-	3/10 (30)	5/21 (24)	5/9 (55)	3/5 (60)
Regenerative cells	-	-	6/20 (30)	7/15 (47)	7/11 (64)
Alkaline membrane	-	-	-	3/19 (16)	5/15 (33)

Note: from the list, it can be grouped into 4 categories as follows: A. no damage (0%), B. minor damage (10-29%), C. medium damage (30-59%), D. major damage (60-79%).



Gambar 2. Kerusakan struktur membran peritrofik akibat infeksi SlNPV pada selang waktu tertentu. A. 0 juam, B. 24 jam, C. 48 jam, D. 72 jam, E. 96 jam. Keterangan: MP: membran peritrofik, SR : sel regeneratif, MB: Membran basal.

Based on histological observations of the middle intestine of *S. litura* larvae at 0 hour, it turns out that peritrophic membrane structure still appeared intact; constituent tissues of the middle intestine was still in normal condition. Results of histological incision on abdomen of *S. litura* larvae after treatment for 24 hours show the damage to the outermost layer of the middle intestine of larvae which was peritrophic membrane. When compared to the controls, the profile of membrane peritrophic in this treatment begins to disintegrate towards the lumen of middle intestine. Furthermore, after 48 hours of SlNPV infection, the spread of the virus in the body of the insect larvae begins to enter the deeper areas, where the cells making up the middle intestine (regenerative cells) begin to experience degradation so it seems to move toward the intestinal lumen. After 72 hours of treatment, the tissue damage level in the middle intestine began to spread, until reaching the basal membrane. Damage of these cells is estimated by SlNPV infection in the body of the test larvae which occurs due to PIB consuming process. After 96 hours of treatment, the intestine making up tissue was increasingly unclear, making it difficult to find the constituent parts. It is presumably caused by the process of viral infection which has entered the advanced phase. Viruses that have undergone replication in the mid intestine region began to be released into the hemosol and will attack other parts of the body.

Based on the results above, at 0 hours it can not be found any damages caused by SlNPV on the peritrophic membrane structure of *S. litura* instar-5 larvae. Intestinal epithelium was still seemed intact with its constituent cells, which consists of a collection of columnar cells and was arranged densely at their ends and there were regenerative cells at the side of epithelium basal and had a direct boundary with basal membrane (Levy et al. 2004). These conditions clarify that with the integrity of the membrane profile peritrophic the metabolic activity is still going well since the middle intestine to optimize its function as a place of absorption and secretion of enzymes (Kikhno 2002). Mentioned by Wang and Granados (1998) who studied the existence peritrophic on Trichoplusia ni membranes that peritrophic membrane proteins composed of Insect intestinal mucin, which is the largest protein that was conceived by the membrane peritrofik.

On further observation due to infections that contributed SlNPV digested with food, then the membrane structure peritrophic at 24 hours after infection began to experience damage as shown in Table 2 by 10%. This confirms the existence of peritrophic membrane also serves as protection against damage to the intestine was strong by food particles (Day and Waterhouse 1953). The damage increases with the length of time of infection. In early infection, pathogen attack will be responded by the insect's defense system with the existence of morphologically peritrophic membrane that serves as a protection against pathogen attack (Terra 2001). Funakoshi and Aizawa (1989) stated that given the infection process constant, the function of peritrophic membrane untenable.

One of the factors that influence the success of the virus in the histological structure of membrane damage due to viruses peritrophic accelerating factor produce a virus that make virus capable of infecting insect cells and damage membranes peritrophic (Engelhard and Volkman 1995; Lehane 1997). Thus peritrophic membrane will be easier to be penetrated by NPV virions, which in turn will attack the cells next to it. The damage was observed after 48 hours in which the regenerative cells (damaged by 30%) began to disintegrate towards the middle of the gut lumen. With the disruption of the cell, then the middle gut function as a producer of enzymes will be disrupted. Mentioned that one of the enzymes secreted by the gut middle is a protease that acts as an anti-virus (Bolognesi et al. 2002). Therefore, when their activities disrupted the presence of pathogens is the metabolic process will not proceed smoothly.

Based on the observation of the advanced stage of infection at 72 and 96 hours after infection, it is found that peritrophic membranes become less and less intact. As proposed by Rohrmann (1994), that the NPV virions takes several days to express their interactions. Thus, the longer the contact time between NPV virions with host cells, the level of damage caused higher. The damage that occurs in this advanced stage causes the ability of epithelial in shaping peritrophic membrane is disturbed. This is consistent with the statement of Patton (1963) that the peritrophic membrane is the secretion from the epithelium of middle intestine.

CONCLUSION

Number of isolates SlNPV dose having an effect on the death of the instar-5 larvae of *S. litura* by 50% (LD50) is 438 PIB / mL. SlNPV infection was also influential in lowering feed intake of larvae of *S. litura* larvae; causing the weight loss that the largest is in the 540 PIB / mL at 0.1675 mg. Histological Structure of peritrophic membrane after infected by SlNPV appears damaged in line with increasing time of SlNPV infection given, whereas the greatest damage occurs during 96 hours after infection.

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