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The Role of Acetylcholine Esterase in Resistance Mechanism of *Plutella xylostella* to Emamektin Benzoate

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Abstract

One of the resistance mechanism of *P. xylostella*to emamektin benzoate is target insensitivity which is acetylcholine esterase that responsible for resistance occurrence. The objective of this study was to determine the role of acetylcholinesterase in the resistance mechanism of *P. xylostella* population to emamektin benzoate. For enzyme activity analysis, larvae homogenate of the third instar of *P. xylostella* was prepared. The number of insects required for each scour is 1 for each field population. The protein content in *P. xylostella* homogenate was measured by the Folin-Ciocalteu test. Non-specific esterase activity with an absorption rate was read using ELISA reader tool with λ = 450 nm.The inhibition level of acetylcholinesterase activity by emamectin benzoate in the tested population was 36.84%. The highest inhibition occurs in Puasan (Ngablak) population. The result shows that a α-naphthyl acetate substrate was used so that it was recorded as non-specific esterase activity and did not exhibit esterase activity which specifically describes emamectin benzoate. Non-specific esterase enzyme activity of either α or β-naphthyl acetate substances to benzoic emamectin in the tested population most of the population was still susceptible. On α-naphthyl acetate substrate, the highest absorbance value found in susceptible population to benzoate emamectin (0.773), while the lowest found in Babrik (Ngablak) population (0.083).

How to Cite

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INTRODUCTION

Insecticide resistance occurs through an accelerated natural selection process, resulting in a new population with resistant genes (Abdullah *et al.,* 2004). This acceleration is due to the strong selection pressure due to the increasing frequency of insecticide use. A highly intensive insecticide application will kill susceptible individuals in the population, whereas more resistant individuals will survive and multiply. Eventually, this population will be dominated by resistant individuals (Brengues *et al.*, 2003).The mechanism of resistance in *P. xylostella* is very dependent on the action of emamectin benzoate insecticide. The mechanism of non-metabolic resistance caused by decreased sensitivity of acetylcholinesterase more quickly captures acetylcholine than inhibitors (insecticides) so that the action of acetylcholinesterase is not inhibited and acetylcholine will decompose into acetic acid and choline. In case of inhibition, acetylcholine does not decompose, but it is accumulated in the form of acetylcholine so that excitement does not stop and will cause death (Walsh *et al.*, 2001).

The objective of this study was to determine the role of acetylcholinesterase in the mechanism of resistance *P. xylostella* to emamectin benzoate with the assumption that resistant insects have acetylcholinesterase that is less sensitive to the insecticide, so it does not affect *P. xylostella.* The benefit of this study is that strong selection pressure due to increased frequency of insecticide use will kill susceptible individuals, whereas resistant individuals will survive, ultimately to be dominated by resistant populations.

METHODS

Bioassay of emamectin benzoate on *P. xylostella*

The bioassay of emamectin benzoate on the third instar of *P. xylostella* larvae (F2) was performed by feeding method. It was conducted using six series of different concentrations for each population, and the treatment was replicated three times. Mortality observations were performed 24 to 72 hours after treatment. The magnitude of insect population resistance was indicated by calculating the value of the resistance factor (RF). Insect population was categorized as resistant if RF $\geq$ 4. Data were analyzed using probit analysis. Biochemical analyses were performed on insect populations that had been resistant to emamectin benzoate. Analysis of acetylcholinesterase activity and non-specific esterase was measured using ELISA with the wavelength of (λ) 450 nm. The bioassay data of insensitivity of acetylcholinesterase in the form of color intensity of enzymatic reaction result was qualitatively determined (Wang & Tang, 2005).

Analysis of Enzyme Activity

For enzyme activity analysis, larvae homogenate of the third instar of *P. xylostella* was prepared. The number of insects required for each scour is 1 for each field population. Insect body was crushed by using glass homogenizer (1 ml capacity) with 0.25 M KPO4 buffer. Scouring of larvae was done on the ice plate. The homogenate was then centrifuged at a rate of 10.000 rpm for 30 minutes at a room temperature of 40C. The supernatant was used as the source of the enzyme. In the test tube (10 ml), 200 µL homogenate of third instar larvae of *P. xylostella* was poured and add 100 µl KPO4 0.25 M at negative control + 100µL ATCH (dissolve 75 mg of acetylthiocholine Iodide (ATCH) and 21 mg propoxur in 10 ml acetone and + 90 mL 0.25 M KPO4 Buffer). Each solution was mixed and + 100 µL DTNB (dissolved 13 mg 5.5-dithio-bis 2-nitrobenzoic acid into 100 ml 0.25 M KPO4 Buffer) and 100 µL test insecticide solution at each well and mix. Read with the microplate reader at 405 nm.

The blank mixture contains the same components as the tested solution, except the homogenate of the enzyme source, was replaced by phosphate buffer. The light absorbance of the test solution measured at a wavelength of 405 nm against the blank with the ELISA reader AChE activity (v) expressed as a hydrolyzed substrate molar per minute of protein was calculated by the formula based on Ellman's *et al.*, 1961 as modified by Rhee *et al.*, 2001.The inhibition percentage of AChE enzyme activity by certain insecticide was calculated using Lowry's formula (Lowry *et al.*, 1951).

The protein content in the homogenate of *P. xylostella* was determined by the Folin-Ciocalteu test. The standardized curve was made based on the absorbance value of BSA solution dose with the specified level (Lowry *et al.* 1951). The biochemical test through the increase of non-specific esterase enzyme by reading the absorption value using ELISA reader tool with λ = 450 nm, then analyzed by descriptive observational by determining the cut-off positive. Determination of cut-off positive for spectrophotometer reading based on a calculation of an average value of vulnerable uptake value + 2 SD (Sasim *et al.* 2014). The results interpreted to be susceptible if absorption value <mean negative control + 2SD, tolerant if absorption value> negative control avera-

ge + 2SD, but still below average negative control + 4SD, and resistant if absorption value> average negative + 4SD control. Quantitative biochemical test results through the reading of absorption value showed the difference of absorption value.

Analysis of non-specific esterase enzyme activity

Non-specific esterase enzyme activity was analyzed based on Lee's method (Lee, 1991; Smirle *et al.*, 2010). One-third instar larvae of each field population were crushed by using a homogenizer glass of temperature 40C to make homogenate, then dissolved in 0.5 ml PBS (Phosphate Buffer Sulfate). Homogenate was taken 50 µl with the micropipette and inserted into the microplate, add 50 µl of substrate (containing 3 mg α naphthyl acetate dissolved in 0.5 ml acetone) then taken 100 µl inserted into PBS to 10 ml volume, let stand 60 minutes. Each microplate containing 50 µl homogenate and 50 µl substrate (coupling reagent consisting of 30 mg of Fast blue + 70 ml SDS 5% + 3 ml aquades), let stand 10 min, and from red color gradually become blue. The reaction was discontinued by the addition of 50 µl of 10% acetic acid, the result readable absorbance value with Elisa reader with λ 450 nm.

RESULTS AND DISCUSSION

The findings showed that enzyme activity varies from one population to another population. This shows that each population develops a different detoxification system. This occurs because test populations exposed to different insecticidal compounds. Hence, the detoxification system developed is also different. The high activity of detoxification enzyme occurs because of enzyme mutation or because of the increase of expression of the enzyme. Higher enzyme activity will increase resistance to insecticidal compounds because of the ability of insects to convert toxic insecticidal compounds into other less toxic compounds increases. To confirm whether the high activity of the tested enzyme occurred due to enzyme mutations or increased expression of the enzyme, further research is needed at the molecular level by studying the structure and level of expression of the enzyme compared with the laboratory standard.

The difference in susceptibility status in each population of *P. xylostella* may occur due to differences in the increase in non-specific esterase activity of each population, resulting in differences in the activity of the enzyme. Molecularly the increase of esterase enzymes is due to the amplification of the gene encoding (coding) esterase (esterase α-2 and esterase β-2), thus causing an increase in the percentage of gene expression (Young *et al*. 2004).

Figure 1. The stages of eggs, third instar larvae, and pupae of *P. xylostella* (L.)

According to Benyamin & Alphey (2017), the difference in vulnerability status in which resistant genes are dominant leads to increased insect populations. Decreased susceptibility status occurs because insects have an enzyme system that neutralizes toxic doses. Gene complexity affects the decrease in susceptibility status; the more genes regulate insect resistance, the slower the occurrence of resistance, and vice versa. The results of enzyme analysis in this study showed that enzyme activity varies from one population to another population. This shows that each population develops a different detoxification system. This can happen because test populations exposed to different insecticidal compounds, so the detoxification system developed is also different. The high activity of detoxification enzyme occurs because of enzyme mutation or because of the increase of expression of the enzyme (Li *et al.*, 2017) Higher enzyme activity will increase resistance to insecticidal compounds because of the ability of insects to convert toxic insecticidal compounds into other less toxic compounds increases.

The difference in susceptibility status in each population of *P. xylostella* may occur due to differences in the increase in non-specific esterase activity of each population, resulting in differences in the activity of the enzyme. Molecularly the increase of esterase enzymes is due to the amplification of the gene encoding (coding) esterase (esterase α-2 and esterase β-2), thus causing an increase in the percentage of gene expression. According to Richard & Constant (2013), the difference in vulnerability status in which resistant genes are dominant leads to increased insect populations. Decreased susceptibility status occurs because insects have an enzyme system that neutralizes toxic doses. Gene complexity affects the decrease in susceptibility status, the more genes that regulate insect resistance, the slower the occurrence of resistance, and vice versa (Nuessly *et al.*, 2007; Roush, 2005). According to Heming-

way *et al*. (2004), the difference in susceptibility status is influenced by the duration and frequency of exposure to insecticides, the frequency of the genes present, the interactions between the carrier genes, and the presence of previous insecticidal selection.

The result of the inhibition test of acetylcholinesterase enzyme activity by emamectin benzoate in eleven population showed that the resistance level of *P. xylostella* to the emamectin benzoate correlated with the level of acetylcholinesterase susceptibility. The rate of inhibition of acetylcholinesterase activity by the emamectin benzoate insecticide in the tested population ranged from 0-36.84%. The highest inhibition occurs in the population coming from Puasan (Ngablak), the percentage of inhibition is 36,84%, while the population of Kejajar, Gondosuli, Kenteng, Gedongsongo, and Selo do not occur inhibition because the population is not exposed by emamektin benzoate. The higher level of resistance compared with the level of enzyme acetylcholinesterase to the emamectin benzoate insecticide suggests the existence of other mechanisms involved in the resistance of *P. xylostella* to the insecticide, i.e., the increased activity of detoxification metabolism (Li *et al*., 2017)

Oxidation metabolism is the main pathway of emamectin benzoate detoxification in *P. xylostella*. (Khan *et al*., 2016). Insecticides absorbed and entered the intestine, all or some of the insecticides undergo an enzymatic chemical change and their (metabolite) changes become inactive or less active, called the detoxification process or bioinactivation (first pass effect), there is also a result of the change actually strengthened (bioactivation) or similar activity (Smagghe, 2004).

In contrast to the insensitivity of the AChE enzyme to the emamectin benzoate insecticide among the population of *P. xylostella*, the activity of the esterase enzyme is not to explain the difference in the level of resistance of *P. xylostella* population to the insecticide. It is possible that in this study the α-naphthyl acetate substrate is used so that the recorded activity is non-specific esterase and does not show any esterase activity that specifically describes emamectin benzoate (Table 2).

The results showed that the non-esterase enzyme activity of either α or β-naphthyl acetate substances against the emamectin benzoate insecticide in eleven tested populations for the majority population is still susceptible, i.e., Kaponan, Kertek, Keteb, Babrik, Plalar, Puasan, Gondosuli. While others are resistant, i.e., the population of Kejajar, Kenteng, and Selo.In the α-naphthyl acetate substrate, the highest susceptible population was 0.773 for benzoic emamectin, and found in population Gondosuli (Tawangmangu), whereas the lowest was found in the Babrik (Ngablak) population of 0.083. The highest tolerant population was found in Gedongsongo at 1.504, and the lowest in the Selo population was 1.281. On the β-naphthyl acetate substrate, the highest susceptible population was found in the Gondosuli population with the absorbance value of 0.488 and the lowest in the Babrik population of 0.081. The highest tolerant population in the population Gedongsongo absorbance value of 0.967 and the lowest in the population Kenteng (Sumowono) of 0.671.

Differences in susceptibility status based

Table 1. Percentages of acetylcholinesterase activity by emamectin benzoate insecticide

Population	AChEspecific activity (M substrate / minute / µg protein)		Inhibiton of AChE activity (%)
	With Insecticide	Without Insecticide	
Kaponan	0.023	0.024	4.17
Kertek	0.020	0.021	4.76
Keteb	0.014	0.015	6.67
Babrik	0.010	0.014	28.57
Puasan	0.012	0.019	36.84
Plalar	0.012	0.014	14,29
Kejajar	0.015	0.015	0.00
Gondosuli	0.014	0.014	0.00
Kenteng	0.016	0.012	0.00
Gedongsongo	0.015	0.014	0.00
Selo.	0.016	0.015	0.00

Table 2. Non-specific esterase enzyme activity in the homogenate of third instar larvae of *P. xylostella* on α-naphthyl acetate

No. Population	Absorbance value of non spesific esterase (α)			Absorbance value of non spesificesterase (β)			Means of Absorbance value of non-specific esterase on		Enzyme status in α-naftil acetate	Enzyme status in β-naftil acetate
	Replication						α-naftil acetate	β-naftil acetate		
	I	II	III	I	II	III				
1. Kaponan	0.244	0.277	0.225	0.178	0.174	0.184	0.232	0.179	Susceptible	Susceptible
2. Kertek	0.357	0.298	0.318	0.206	0.217	0.217	0.323	0.213	Susceptible	Susceptible
3. Keteb	0.222	0.228	0.207	0.156	0.158	0.152	0.219	0.155	Susceptible	Susceptible
4. Babrik	0.088	0.085	0.075	0.088	0.085	0.075	0.083	0.081	Susceptible	Susceptible
5. Plalar	0.276	0.270	0.245	0.276	0.270	0.245	0.264	0.193	Susceptible	Susceptible
6. Puasan	0.209	0.211	0.201	0.209	0.211	0.201	0.207	0.177	Susceptible	Susceptible
7. Kejajar	0.950	1.604	1.644	1.276	0.833	0.792	1.399	0.967	Tolerant	Tolerant
8. Gondosuli	0.733	0.654	0.933	0.804	0.357	0.307	0.773	0.488	Susceptible	Susceptible
9. Kenteng	0.996	0.357	0.337	0.713	0.719	0.580	0.563	0.671	Susceptible	Tolerant
10. Gedngsongo	1.489	1.529	1.493	0.744	0.708	0.778	1.504	0.743	Tolerant	Tolerant
11. Selo	1.284	1.178	1.382	0.462	0.482	0.510	1.281	0.485	Tolerant	Susceptible

Note:
Biochemical data of color intensity of non-specific esterase enzyme activity measured by reading absorbance value (AV) using ELISA reader at λ = 450 nm. Susceptible if AV value <negative control level + 2SD = (α = 1.149 and β = 0.669), tolerant if AV> negative control rate + 2SD, but still below negative control mean + 4SD, and resistant if AV value> negative control + 4SD = (α = 2.215.d and β = 1.257)

on non-specific esterase enzyme activity in *P. xylostella* populations occur because the gene differences in each study population result in differences in the activity of individual non-specific esterase enzymes. Molecularly the increase in esterase enzyme in strains is caused by the amplification of the gene encoding (coding) esterase enzymes (esterase α-2 and esterase β-2), thus causing an increase in the percentage of gene expression (Hemingway *et al.*, 2004).

The significance of this study is the finding that the occurrence of enzyme-related resistance is closely related to enzyme mutations. These mutations may accelerate the activity of catalytic enzymes or increase the amount of enzyme expressed. Increased enzymes activity leads to the conversion of insecticide toxicity to non-toxic substances.

The benefit of this research is the confirmation that intensive insecticide use is indeed the cause of insect resistance. Intensive insecticide applications include kind of insecticide, high insecticide dose, and the short interval of spraying.

CONCLUSION

The resistance mechanism of *P. xylostella* population to benzoic emamectin is determined primarily by an increase in the rate of detoxification by acetylcholinesterase enzyme, the activity of esterase enzymes cannot be used to explain the difference in resistance level of *P. xylostella* population to emamectin benzoate. Differences in susceptibility status based on non-specific esterase enzyme activity in *P. xylostella* populations can occur because the gene differences in each population result in differences in the activity of individual non-specific esterase enzymes.The molecular increase in esterase enzyme in the strain is caused by the amplification of the gene encoding (coding) esterase enzymes (esterase α-2 and β-2), thus causing an increase in the percentage of gene expression

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