AMYLASE, PROTEASE, AND LIPASE ACTIVITY OF BUTTERFLY OF *Junonia almana* AND *Junonia atlites*

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ABSTRACT

Major gut digestive enzymes of butterflies are hydrolytic enzymes, i.e. amylase, protease, and lipase. *Junonia* is the most abundant butterfly species in the arboretum area of Universitas Padjadjaran, West Java, Indonesia. This study was aimed to determine the amylase, protease, and lipase activity of butterflies of *Junonia almana* and *Junonia atlites*. All enzyme activity was measured at the maximum wavelength of remaining substrates or products with the UV-visible spectrophotometric method. Both butterflies have higher amylase activity than protease and lipase activity. Amylase activity of *J. atlites* higher than *J. almana* at its optimum temperature and pH, i.e. 37 °C and 6.8. This study has provided the first information about amylase, protease, and lipase activity in *J. atlites* and *J. almana* butterfly.

Keywords: Arboretum, Butterfly, Hydrolytic Enzyme, Indonesia

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INTRODUCTION

Enzymes have specificity in the catalyzed reaction and substrates. Enzyme activity is influenced by substrate concentration, temperature, and pH. Amylase, lipase, and protease enzymes are hydrolytic enzymes, which degraded macromolecules.^{1,2} Enzymes, as biocatalysts for biological reactions, help butterflies to digest their food. The butterfly life cycle is relatively short (2-6 months) and breeds rapidly.³ Butterflies are the highest number in the insect order, around 2500 species exist in Indonesia. There are 600 butterfly species in Java and Bali islands, Indonesia.^{4,5} This condition allows the availability of butterflies for enzyme production on a large scale.⁶

The genus of *Junonia* (Nymphalidae) is an important system model for experimental research in Lepidoptera. This system of development and evolution of this genus can be applied to all other genera. The most abundant genus of *Junonia* in the arboretum area of Universitas Padjadjaran, West Java, Indonesia, is *Junonia almana* (peacock pansies) and *Junonia atlites* (gray pansies).^{7,8} *J. almana* is distributed in an Asian tropical area.⁹ These species consume Acanthaceae, Amaranthaceae, Apocynaceae, Asteraceae, Fabaceae, and Verbenaceae.^{7,10,11} *J. atlites* has similar behavior to other *Junonia*, except when spawning.¹² The major gut digestive enzymes of these two butterflies, the hydrolytic enzymes, have never been studied. In this study, we report the amylase, protease, and lipase activity in *J. atlites* and *J. almana*.

EXPERIMENTAL

Experimental animal

Junonia almana and *Junonia atlites* butterflies were collected from December 2014 to February 2015 from the arboretum area of Universitas Padjadjaran, West Java, Indonesia. Butterflies were identified by the Laboratory of Animal Taxonomy, Department of Biology, Universitas Padjadjaran. The procedures and protocols of the enzyme activity assay were reviewed and approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Padjadjaran No. 732/UN6.C2.1.2/KEPK/PN/2014.

Chemical Materials

All analytical grade of chemicals were purchased from Merck, Germany, i.e. acetonitrile, Coomassie brilliant blue (CBBG), ethanol, hydrochloric acid, iodine, phosphoric acid, potassium iodide, sodium chloride, sodium hydrogen phosphate, sodium hydroxide, sodium phosphate, and soluble starch. Bovine

serum albumin (BSA), *p*-nitrophenol (*p*-NP), *p*-nitrophenyl palmitate (*p*-NPP), and tyrosine were purchased from Sigma-Aldrich, USA.

Protein Extraction

The butterfly wings were cut carefully. The bodies were weighed, then extracted with 0.02 M sodium phosphate buffer (pH 6.8) containing 10 mM NaCl at 4 °C for 2 h. Each extract was centrifuged at 4 °C and 13,000 G for 30 min. The supernatants were collected and stored at -20 °C.¹³

Determination of Total Protein Content

Five concentrations of BSA, extracts, and blank were prepared. The Bradford reagent was added to each solution. The absorbance was measured at 573 nm after 5 min incubation.¹⁴

Amylase Activity Assay

The starch solution (3 mL) and each butterfly extract (250 µL) were incubated for 8 min. Hydrochloric acid (0.5 mL) and iodine (0.5 mL) were added, the absorbance was measured at 627 nm.¹⁵ Amylase activity was calculated by Eqn.-1.

$$U/mg = \frac{A_{std} - A_{sampl}}{A_{std}} \times \frac{(mg) \text{ Starch}}{(mL) \text{ Enzyme in Extract} \times \text{Total Volume} \times \text{Time}} \quad (1)$$

Protease Activity Assay

The casein solution (1 mL) and each butterfly extract (250 µL) were incubated for 30 min. Ethanol (1 mL) was added, placed in a boiling water bath for 5 min, then cooled to the ambient temperature. Each mixture was centrifuged for 10 min at 13,000 G and the absorbance was measured at 277 nm.¹⁶ Protease activity was calculated by Eqn.-2.

$$U/mg = \frac{\mu\text{mol Tyrosin which produce}}{\text{mg Enzyme in Extract} \times \text{Time}} \quad (2)$$

Lipase Activity Assay

The substrate solution (1 mL) and each butterfly extract (250 µL) were incubated for 15 min. The substrate solution was phosphate buffer: ethanol: *p*-NPP solution (95:4:1). Ethanol (1 mL) was added and the absorbance was measured at 405 nm.¹⁷ Lipase activity was calculated by Eqn.-3.

$$U/mg = \frac{\mu\text{mol pNP which produce}}{\text{mg Enzyme in Extract} \times \text{Time}} \quad (3)$$

Statistical analysis

Data were showed as mean and standard deviation (SD), then analyzed by single-factor ANOVA, followed by Duncan Multiple Range Test (DMRT). Data were considered to be significantly different if *p*-value < 0.05.¹⁸

RESULTS AND DISCUSSION

Protein Extraction

The butterflies of *J. almana* and *J. atlites* were collected from December 2014 to February 2015, due to the highest rainfall in arboretum area, which increases butterfly populations associated with the plant fertility level as the butterfly host plant.¹⁹ The rainfall level affected the nectar availability. High water availability causes an increase in plants releasing nectar through the flowers.²⁰ This study collected 72 *J. almana* butterflies (8.67 g) and 53 *J. atlites* butterflies (5.22 g). Butterfly's body produces a crude extract with a concentration was 14.29 mg/mL for *J. almana* and 14.28 mg/mL for *J. atlites*. All crude extract was stored at -20 °C, to stop all enzymatic reactions that cause protein degradation or produce adverse substances.²¹

Determination of Total Protein Content

Bradford's method¹⁴ with BSA as a standard was used to determine the total protein content. In the Bradford method, the amount of protein-CBBG complex is proportional to the positive charge on the protein.²² The

maximum wavelength of the protein-CBBG complex was 573 nm, which corresponds to its complementary or transmitted color, i.e. orange wavelength (570-620 nm).²³

The correlation coefficient of the BSA calibration curve was 0.995, which met the ICH criteria.²⁴ It showed that instrument response is equal to the concentration. The absorbance of crude extract of *J. atlites* and *J. almana* was 0.78 ± 0.01 and 0.85 ± 0.01 , respectively. The calculated total protein content of *J. atlites* and *J. almana* with a linear regression equation was 83.30 ± 0.10 and 90.21 ± 0.09 $\mu\text{g/mL}$, respectively. The total protein content of *J. atlites* was lower than *J. almana*, which significantly different ($p = 1.76 \times 10^{-7}$). These values were higher than the total protein content of *Catopsilia pomona* butterfly (71.98 ± 0.18 $\mu\text{g/mL}$).¹³ Crude extract of *J. almana* and *J. atlites* contain 58.30% and 60.63% of protein, respectively. This result showed that the crude extracts contain non-protein compounds which soluble in phosphate buffer.

In this study, the optimum pH and temperature of amylase, protease and lipase were determined. At optimum pH, the enzyme three-dimensional structure is the most convenient to binding to the substrate. When the hydrogen ion concentration change, the enzyme activity is progressively lost until the enzyme became denatured.²⁵ pH alteration occurs in amino acid residues to maintain the tertiary and the quaternary structure of the active enzyme.²⁶ Increased temperature causes increased kinetic energy, thereby increased the collision intensity between the substrate and the enzyme. Further increased temperature will decrease enzyme activity, due to denaturation. Enzymes encounter conformational changes at high temperatures, causes the substrate inhibition to enter the active site of the enzyme.²⁵

Amylase Activity Assay

The sensitivity of amylase activity which determines with iodimetry by the Fuwa method (mg of starch equivalents consumed/min) was five times higher than the activity units (mg of glucose equivalents produced/min) measured with the dinitrosalysilic acid method.²⁷ The starch type with the glucose number difference affects the formed starch-iodine complex.¹⁵

Butterflies need monosaccharides as an energy source of nectar. When a butterfly flies, the needed energy increase, so there will be increased amylase activity. The butterflies are dependent on the amylase endurance,²⁸ cause when fly, the butterfly's temperature is more than 30 °C or 5-10 °C higher than normal body temperature. In insects, most amylase is active at neutral or slightly acidic pH.^{29,30}

Table-1: Amylase Activity in Various Temperature and pH

Parameter		Amylase activity (U/mg)		p-value
		<i>J. atlites</i>	<i>J. almana</i>	
Temperature (°C)	27	6.41 ± 0.01	6.28 ± 0.01	5.46×10^{-5}
	37	8.08 ± 0.01	7.61 ± 0.01	8.36×10^{-7}
	47	6.60 ± 0.06	6.82 ± 0.01	4.42×10^{-5}
pH	5.8	3.16 ± 0.01	2.93 ± 0.01	4.42×10^{-5}
	6.8	6.41 ± 0.01	6.28 ± 0.01	5.46×10^{-5}
	7.8	1.98 ± 0.02	0.78 ± 0.01	3.64×10^{-8}

The optimum temperature and pH of *J. atlites* and *J. almana* amylase were 37 °C and 6.8. Amylase activity of *J. atlites* was better than *J. almana* at optimum temperature and pH. The amylase activity of both butterflies was significantly different (Table-1). The optimum temperature and pH of *J. atlites* and *J. almana* were similar to the stomach amylase of hemiptera insects, i.e. 25-40 °C³¹ and 6.5,³² respectively. The butterflies are active at 16-42 °C and 28-30 °C is requiring for flight.¹⁹ The decreased amylase activity at 47 °C caused by exposure of the hydrophobic residues to the enzyme surface which important to stabilize the amylase structure. Amylase activity at 47 °C was higher than 27 °C, which indicates amylase has a high resistance to extreme environments. The charge of amino acid residues at the optimum pH is in an appropriate state, so the enzyme activity is efficient.³³

The amylase activity of *J. atlites* and *J. almana* was higher than *C. pomona* butterfly at 27 °C, i.e. 5.85 ± 0.01 U/mg, but lower than *C. pomona* butterfly, at 37 and 47 °C, i.e. 8.21 ± 0.01 and 7.74 ± 0.11 U/mg,

respectively.¹³ These results showed that amylase of *C. pomona* more stable than *J. atlites* and *J. almana* in different temperatures. The amylase activity of *J. atlites* and *J. almana* was higher than *C. pomona* butterfly at the studied pH. These results showed that amylase of *J. atlites* and *J. almana* more active than *C. pomona* in different pH.

Protease Activity Assay

Insects need protein for structural proteins, enzymes, receptors, transporters, and storage.³⁴ The protease activity was determined based on the amount of producing tyrosine from casein hydrolysis, then measured at 277 nm, i.e. the maximum wavelength of the aromatic amino acids.³⁵

Table-2: Protease Activity in Various Temperature and pH

Parameter		Protease activity (U/mg)		p-value
		<i>J. atlites</i>	<i>J. almana</i>	
Temperature (°C)	27	0.76 ± 0.02	1.27 ± 0.01	1.42 x 10 ⁻⁶
	37	0.29 ± 0.01	1.11 ± 0.01	5.34 x 10 ⁻⁷
	47	0.20 ± 0.01	0.45 ± 0.01	7.88 x 10 ⁻⁸
pH	5.8	0.39 ± 0.01	0.64 ± 0.01	4.62 x 10 ⁻⁵
	6.8	0.76 ± 0.02	1.27 ± 0.01	1.42 x 10 ⁻⁶
	7.8	0.43 ± 0.01	0.65 ± 0.01	4.45 x 10 ⁻⁸

The optimum temperature and pH of *J. atlites* and *J. almana* protease were 27 °C and 6.8. Protease activity of *J. almana* was better than *J. atlites* at optimum temperature and pH. The amylase activity of both butterflies was significantly different (Table-2). The optimum temperature of *J. atlites* and *J. almana* were similar to the stomach protease of *Ectomyelois ceratoniae*, i.e. 30 °C with casein as the substrate.³⁶ The optimum pH of *J. atlites* and *J. almana* were classified as neutral protease, correspond to the general body pH of the insect³⁷ and in the range of protease, i.e. 7.0 to 9.0, due to serine protease in the extract.³⁸ Both protease activities decreased with increasing temperature. This result showed that amylase work more dominant than protease, when a butterfly flies, due to nectar degradation as an energy source. At the optimum temperature and pH, protease activity of both butterflies was higher than *C. pomona*, i.e. 0.59 ± 0.01 U/mg.¹³ These results showed that protease of *J. atlites* and *J. almana* more active than *C. pomona* in studied temperature and pH.

Lipase Activity Assay

Lipase activity was determined based on hydrolysis ester bonds in the oil as the substrate into glycerol and its fatty acids.³⁹

Table-3: Lipase Activity in Various Temperature and pH

Parameter		Lipase activity (U/mg)		p-value
		<i>J. atlites</i>	<i>J. almana</i>	
Temperature (°C)	27	2.46 ± 0.06 x 10 ⁻⁴	21.43 ± 0.02 x 10 ⁻⁴	1.12 x 10 ⁻¹²
	37	1.79 ± 0.04 x 10 ⁻⁴	4.38 ± 0.03 x 10 ⁻⁴	1.11 x 10 ⁻⁸
	47	2.15 ± 0.03 x 10 ⁻⁴	5.68 ± 0.02 x 10 ⁻⁴	1.85 x 10 ⁻⁸
pH	5.8	3.75 ± 0.04 x 10 ⁻⁴	5.88 ± 0.02 x 10 ⁻⁴	1.14 x 10 ⁻⁵
	6.8	2.46 ± 0.06 x 10 ⁻⁴	21.43 ± 0.02 x 10 ⁻⁴	1.12 x 10 ⁻¹²
	7.8	1.22 ± 0.02 x 10 ⁻⁴	9.71 ± 0.02 x 10 ⁻⁴	2.05 x 10 ⁻¹¹

The optimum temperature of *J. atlites* and *J. almana* lipase activity was 27 °C, while pH optimum was 5.8 for *J. atlites* and 6.8 for *J. almana*. Lipase activity of *J. almana* was higher than *J. atlites* at optimum temperature and pH. The lipase activity of both butterflies was significantly different (Table-3). The optimum temperature of *J. atlites* and *J. almana* were similar to lipase activity in *Ectomyelois ceratoniae*, i.e. 30 °C.³⁶ The optimum pH of *J. atlites* and *J. almana* were in the range of lipase activity in insects, i.e. 5.5-7.5.^{40,41} Both lipase activities decreased with increasing temperature. When flying, the dominant enzyme was amylase, due to energy production from nectar degradation. At the optimum temperature and pH, lipase activity of both butterflies was higher than *C. pomona*, i.e. 0.59 ± 0.01 U/mg.¹³ These results showed that lipase of *J. atlites* and *J. almana* more active than *C. pomona* in studied temperature and pH.

CONCLUSION

Junonia atlites and *Junonia almana* have higher amylase activity than protease and lipase activity at optimum temperature and pH.

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