Isolation and Characterization of α-Amylase Enzyme on Brown Planthopper (*Nilaparvata lugens* Stal) On Rice

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Abstract

Brown leafhopper (*Nilaparvata lugens* Stal) is an important pest in Indonesia that causes heavy losses. The rice was damaged by *N. Lugens* sucking plant liquid in the form of carbohydrates in the form of starch. Environmentally friendly control methods are needed to reduce the increasingly high use of inorganic pesticides. Control of *N. lugens* with biotechnology can be done by inhibiting the metabolic cycle in these insects. This method utilizes the proteins available in rice seeds to stop the performance of enzymes α-amylase in the digestive system of *N. lugens*. α-amylase is an enzyme that plays a role in the process of starch degradation so that it becomes a simpler form both in microorganisms. The characterization of α-amylase enzymes in insects is an important first step to the determination of appropriate protein inhibitors so that they can be used to produce pest-resistant Genetically Modified Organisms crops. The research was conducted in the Agrotechnology laboratory, University of Jember. The research began with isolation and purification of α enzymes from brown stems and then continued with testing of α-amylase activity. Test parameters include the effect of temperature, pH, and substrate concentration on the activity of the α-amylase enzyme. The results showed that supernatant extracted from brown planthoppers produces a clear zone in the agar medium, which means the activity of enzymes α-amylase in the hydrolysis of starch. The pH value of 6 provides the most optimum conditions for the activity of α enzymes. The α-amylase enzyme is able to work optimally in the temperature range of 30°C - 45°C, and experiences a decrease in activity when the temperature reaches 50°C. The α-amylase enzyme shows the ability to hydrolyze the amylase substrate to a concentration of 0.8 $\mu g.\mu L^{-1}$.

Keywords: α-amylase, *Nilaparvata lugens* Stal, Rice.

INTRODUCTION

Brown planthopper (*Nilaparvata lugens* Stal) is a significant pest occurred in rice cultivation in Indonesia. Brown planthopper attacks cause heavy losses and pose a threat to rice farmers in Indonesia. Data shows that brown stem attacks can cause up to 50% damage to vulnerable varieties such as Cisadane [1]. A fairly high degree of damage can lead to a significant decrease in yield. Baehaki and Mejaya [2] said that brown stem attacks can cause an average loss of 1-2 $tons.ha^{-1}$ in severe attack conditions. It causes dependence on inorganic pesticides to be higher for pest control [3].

Control of brown planthopper utilizing biotechnology can be done by inhibiting the metabolic cycle in these insects. This control method is carried out by stopping the performance of α enzymes using protein inhibitors available in rice seeds. α-amylase is an enzyme that plays a role in the degradation process of starch into a simpler form, both in microorganisms, plants, and humans [4]. α-amylase is included in the enzyme endoamylase, which catalyzes the hydrolysis of starch into shorter oligosaccharides through the division of bonds α-1,4-glycosidic [5]. Bahagiawati in her research explained that enzyme inhibitors α-amylase can suppress the growth of *Callosobruchus maculatus* warehouse pest insects, by extending the larval period in such insects and increasing the percentage of larval mortality [6].

Brown planthopper sucked carbohydrates in the form of starch from rice plants. This process can be inhibited with protein inhibitors α amylase that can be obtained from plants, humans, and microorganisms. This protein inhibitor can be found in several types of food crops such as wheat (*Triticumaestivum*), barley (*Hordeum vulgareum*), sorghum (Sorghum bicolor),rice (*Oryza sativa*), and some legume plants such as gude beans (*Cajanuscajan*), red delinquent beans (*Vignaunguiculata*), and beans (*Phaseolus vulgaris*) [4]. Characterization of α enzymes in insects is an important first step and must be taken to go to the stage of determining appropriate protein inhibitors so that they can be used to produce pest-resistant Genetically Modified Organisms (GMO) crops. Previous research has discussed the influence of various factors applied to the process of characterization of α enzymes in insects. Research by Abdolmaleki

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et al. [7] showed that the activity of the enzyme α-amylase in species *Andralus spinidens* can be influenced by temperature, pH, and concentration conditions of certain elements. Other results were shown in Ravan's study [8], where the enzyme α-amylase in the species *Eurygaster maura* is active at pH 6, and the temperature is 35-40°C. It shows the characteristics of different α enzymes in each insect species, so characterization is needed to support the process of finding suitable inhibitor proteins.

MATERIAL AND METHOD

Isolation and purification of α-amylase

The study was conducted at the Agrotechnology Laboratory, Faculty of Agriculture, University of Jember. Isolation and purification of α-amylase from *N. lugens* Stal are carried out by preparing 2 grams of *N. lugens* Stal stored at -20°C for 30 minutes. The frozen *N. lugens* Stal is crushed using a mortar with 8 mL of sodium phosphate buffer of 20 mM pH 7 and then centrifuged at 10,000 rpm for 20 minutes with a temperature of 4°C. Clean supernatant is used as an α-amylase material [9].

Detection of α-amylase enzyme activity

Detection of alpha-amylase enzyme activity is carried out by the method of Silaban and Simamora [10]. The supernatant obtained through the isolation process is tested to determine the activity of enzymes α-amylase. Testing is carried out by the starch method to plate. Agar medium containing 2% starch is used in this method. The medium is to be hollowed out at 4 points and placed with an enzyme solution at 4 points. Medium to be incubated for 30 minutes at a temperature of 37°C and then added Iodine. Indications of the activity of the enzyme α-amylase are indicated by the formation of a clear zone around the point added to the enzyme of the brown stem leafhopper.

Testing of enzyme activity α-amylase

The activity of α-amylase was measured using Bernfeld's method [11]. A total of 25 µL of enzyme α-amylase from the isolation of brown planthopper was added to 475 µL buffer of 1% amylase reaction. The reaction mixture is then vortex until homogeneous and then incubated at 30°C for 10, 20, and 30 minutes. The reaction ends by adding 500 µL of 3.5-dinitrosalicylic acid reagents, then heated to boiling water for 10 minutes. The reaction mixture is cooled, and subsequently, absorbance is measured using a spectrophotometer at a wavelength of 560 nm.

Testing the effect of pH on α-amylase enzymes

The effect of pH on α-amylase was measured at different pH values. pH was adjusted using a mixture of K-Phospate and aqua dest buffers of 50 mL. The addition of HCl and NaOH was done to set the pH value at 5, 6, 7, and 8. The mixture of 20µL α-amylase and 475 µL buffer of 1% amylase reaction was incubated at 30°C for 10 minutes. The reaction was stopped to add 500 µL regent 3.5-dinitrosalicyliclic. Enzyme activity was measured on a spectrophotometer at a wavelength of 560 nm.

Testing the effect of temperature on enzymes α amylase

The effect of temperature on α-amylase activity is determined by the incubation of 20 µL α-amylase and 475 µL buffers of 1% amylase reaction at different temperatures, namely 30, 35, 40, 45, and 45°C. The pH conditions are adjusted to the optimum results obtained in the previous test. Temperature treatment is carried out with two different incubation intervals, 10 and 20 minutes. The reaction is stopped to add 500 µL regent 3.5-dinitrosalicyliclic. Further enzyme activity is measured on the spectrophotometer at a wavelength of 560 nm.

Testing the effect of substrate concentration on α-amylase enzymes

The effect of substrate concentrations on α-amylase enzymes was measured in several samples with amylum stock solution concentration of 5 $\mu g.\mu L^{-1}$ with volumes of 0, 10, 20, 40, 80, 120, 160 µL. The starch solution, according to the specified amount, is added 20µL extract of the enzyme α-amylase and 475 µL buffer of 1% amylase reaction and aqua dest until it reaches 1 mL resulting in a concentration of 0, 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 $\mu g.\mu L^{-1}$. The mixture is incubated for 10 minutes. The pH and temperature conditions are adjusted to the optimum conditions obtained in the previous test. The reaction is stopped to add 500 µL reagent 3.5-dinitrosalicylic. Furthermore, enzyme activity is measured on the spectrophotometer at a wavelength of 560 nm.

Measurement of protein molecular weight

Measurement of the molecular weight of proteins in the sample was done using the Bradford method [12]. Measurements are carried out in several stages, namely the manufacture of BSA (Bovine Serum Albumin) with a concentration of $1\mu g.\mu L^{-1}$ as a standard protein, the manufacture of Bradford reagents with Coomassie Brilliant Blue (CBB) G-250 staining, the

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manufacture of standard curves, and the measurement of protein levels obtained from the calculation of absorbance values entered into linear equations. The measurement of protein levels was carried out with six samples of protein counts of 0, 5, 10, 15, 20, and 30 µL using 5 µL of enzyme extracts in each sample.

RESULT AND DISCUSSION

Enzyme Activity α-amylase

The supernatant obtained through the isolation process of brown planthopper is tested on a medium agar that has been added iodine to detect the presence of enzymes α-amylase. The results of testing on the medium agar, it appears the emergence of clear zones that show the activity of enzymes α-amylase in hydrolysis starch compounds (Fig. 1).

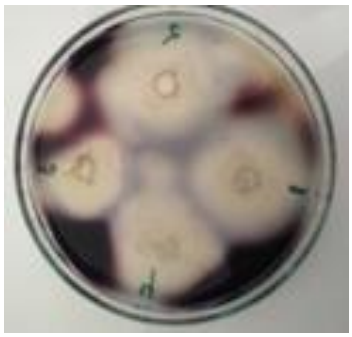

Figure 1. The results of testing the activity of the enzyme a-amylase in agar media

The results of isolation and testing in this study showed the presence of the enzyme α-amylase in the digestive system of brown leafhoppers, which played a role in the starch hydrolysis process. This enzyme has an important role in the digestion of insects, to break down starch in plant tissues into oligosaccharides before being hydrolyzed into glucose by glucosidase [13]. The formation of clear zones in the agar medium that has been added with iodine proves the activity of the enzyme α-amylase in hydrolyzing starch.

The results of reaction testing showed that the enzyme α-amylase had good rection in different incubation intervals. At intervals of 10 minutes, the absorbance value shows a result of 1.267. The reaction also showed an increase directly proportional to the incubation interval at 20 and 30 minutes (Table 1).

Table 1. Test of enzyme reaction α-amylase

Incubation Interval (Minutes)	Absorbance Value
10	1.267
20	1.689
30	1.821

Effect of pH on α Enzymes

Different pH values indicate an influence on the activity of α-amylase enzymes. This is reviewed from the absorbance value and enzyme activity obtained in Table 2.

Table 2. Effect of pH on the activity of α-amylase enzymes

Buffer pH	Incubation Interval (minute)	Enzyme Activity ($U.mL^{-1}$)	Absorbance Value
5	10	14.138	0.044
6	10	77.472	0.234
7	10	77.138	0.215

A pH value of 6 indicates the most optimal result against the activity of the α-amylase enzyme based on testing. Based on this data, it can be concluded that an increase in pH above the optimum value tends to cause a decrease in enzyme activity α-amylase.

The pH condition plays an important role in ensuring the function of the α-amylase enzyme can work. Changes in pH values can affect the total load of enzyme proteins α-amylase, both through changes in structure and changes in charge in amino acid residues that function in binding substrates [14]. Changes in pH conditions are what can cause an increase or decrease in the activity of α-amylase enzymes. Ravan's research [8] states that the enzyme α-amylase in insects generally tends to be most active under neutral to slightly acidic pH conditions. It is by the data obtained in this study. The enzyme α-amylase from the brown stem leafhopper showed the best activity under pH 6 conditions, and its activity value did not decrease significantly until the neutral pH 7 condition. The more acidic pH condition of 5 and pH 8, which is more alkaline, affect the activity of the enzyme α-amylase, seen from the decrease in the value of its activity.

Data on the influence of pH conditions and optimum values in this study is supported by the results of previous studies that show similar pH ranges provide optimum conditions for enzyme activity α-amylase. Research on *Callosobruchus maculatus* [15] and *Andralus spinidens* [7] mentioned the optimum pH 6 for enzyme activity α-amylase, while research in the species *Oryctes owariensis* mentioned pH 7 [16].

The enzyme α extracted from insects has a different optimum pH range depending on the species. The enzyme α-amylase extraction from the order Hemiptera generally has an optimum pH range ranging from 6 to 7 as found in *Podisus maculiventris, Graphosoma lineatum, Eurygaster maura, Eurygaster integricep*s, and *Aphis fabae*.

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This optimum pH value is a picture of the pH conditions in the lumen of the insect midgut and becomes part of the insect adaptation process to digest nutrients sourced from its host plant [17].

Effect of Temperature on α-amylase Enzymes

The temperature effect on the activity of the enzyme α-amylase was tested under a pH of 6 conditions and showed optimum conditions based on Table 3 data. Temperature exerts a different influence on the activity of enzymes α-amylase extracted from brown planthoppers. It is indicated in the absorbance value and enzyme activity obtained in Table 3.

Table 3. Effect of temperature on enzyme activity α-amylase

Mouth (°C)	Incubation Interval (minute)	Enzyme Activity ($U.mL^{-1}$)	Absorbance Value
30	10	157.138	0.473
35	10	154.805	0.466
40	10	149.805	0.451
45	10	144.472	0.435
50	10	89.138	0.269

A temperature of 30°C produces optimum conditions for the activity of the enzyme α-amylase. Increasing the temperature to the optimum point can accelerate the rate of enzyme catalysis reaction due to the higher kinetic energy and frequency of collisions between the molecules involved [18]. Data from this study showed a temperature of 30°C provided optimum conditions for the activity of α-amylase enzymes isolated from brown planthopper. The temperature rises to 45°C do not significantly affect the activity of α-amylase enzymes, so it can be concluded that the temperature range of 30°C - 45°C supports the activity of enzymes α-amylase. These results are by research related to the influence of temperature on the activity of α enzymes in other insect species.

Previous research has shown optimum temperature conditions for α-amylase enzyme activity in Eurygaster maura range from 30°C - 40°C [8], 35°C - 40°C in *Aeolesthes holosericea* [19], 35°C in *Plagiodera versicolora* [20], 37°C in *Leptinotarsa decemlineata* [21], and 45°C in *Andralus spinidens* [7]. Insects such as brown leafhoppers are poikilotherm organisms. Their body temperature and physiological processes are influenced by environmental conditions [22]. The optimum temperature range for the activity of this α-amylase enzyme is vital, as an overview of the appropriate environmental conditions for brown planthoppers to attack their hosts.

The temperature of 50°C is the point where the activity of the enzyme α-amylase decreases significantly. High temperature, as a trigger for decreased activity, was also mentioned in the study of Patil *et al.* [19]. There was a significant decrease in the activity of the enzyme α-amylase when the temperature was raised to 50°C. Damage to the constituent components of enzymes due to high temperatures is suspected to be the cause of the decreased activity of enzyme α-amylase. High-temperature conditions cause the kinetic energy of enzyme molecules to b too high, thereby breaking the bonds that maintain the shape of the enzyme and causing the denaturation of hydrophobic residues on the surface of the enzyme [14,23]. These hydrophobic residues include tryptophan and phenylalanine, which play a role in stabilizing the enzyme α-amylase. Based on the data obtained, it can be concluded that the enzyme α-amylase from brown stem leafhoppers can work optimally in the temperature range of 30°C - 45°C and decreases when the temperature reaches 50°C.

Effect of Substrate Concentration on α-amylase Enzymes

The concentration of the added amylase substrate affects the activity of the enzyme α-amylase based on the tests performed. It is reviewed from the absorbance value and the results of the calculation obtained in table 4.

Table 4. Effect of substrate concentration on enzyme activity α-amylase

Substrate Concentration ($\mu g.\mu L^{-1}$)	Number of Enzymes (μL)	Enzyme Activity ($U.mL^{-1}$)	Absorbance Value
0.00	20	47.472	0.144
0.05	20	63.138	0.191
0.10	20	54.138	0.164
0.20	20	57.805	0.175
0.40	20	68.805	0.208
0.60	20	91.138	0.275
0.80	20	109.805	0.331

The addition of substrate concentrations is related to the increased activity of enzyme α-amylase resulting from isolation from brown planthopper. In general, enzyme activity tends to increase with the addition of the concentration of the amylase substrate.

The Lineweaver-Burk analysis is carried out to find out the kinetic parameters of the enzyme α-amylase at different concentrations of amylase substrates (Fig. 2). The maximal velocity (Vmax) of the enzyme α-amylase brown planthopper s was recorded at 43.47 $U.mg^{-1}$ protein and Km 1%.

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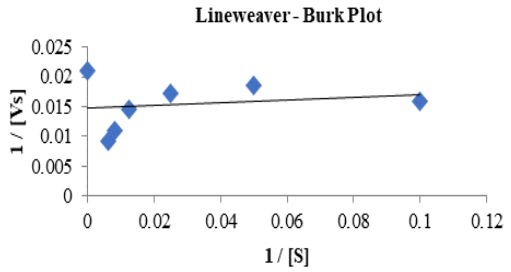

Figure 2. Lineweaver – Burk plot of alpha-amylase enzyme activity brown planthopper at substrate concentrations different.

Dissolved protein levels of the enzyme α-amylase of brown planthopper

The absorbance results of the BSA standard protein solution are used to create the standard curve of the protein (Fig. 3). The protein levels of the enzyme α-amylase brown planthopper are obtained through the substitution of the absorbance value of the enzyme protein α-amylase in the regression equation of the protein standard curve. As a result, the protein levels of the enzyme α-amylase showed a value of 16.478 $\mu g.\mu L^{-1}$, which can be seen in Table 5.

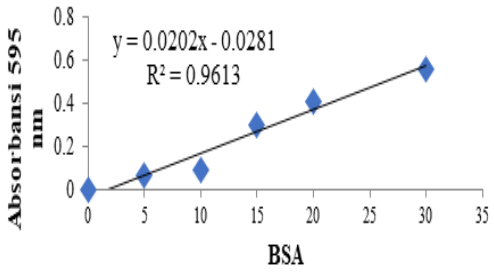

Figure 3. Protein Standard Curve

The enzyme α-amylase extracted from the brown stem leafhopper showed the ability to hydrolyze the substrate well to the highest concentration on the test. Enzymes show increased activity with the addition of the concentration of the amylase substrate until it reaches the highest activity value at the substrate concentration of 160 µL. The number of enzymes added to each substrate concentration influence treatment is 20 µL or equivalent to 330 µg, referring to the dissolved protein content.

Table 5. Dissolved protein levels of brown planthoppers

Sample Protein	Absorbance Value 595 nm	Protein up to ($\mu g.\mu L^{-1}$)
Brown planthopper	0.329	16.478

The pH conditions and optimum temperature obtained previously were used as standard in this test. Thus, the optimal enzyme performance to even the highest substrate concentration. The activity of the enzyme α-amylase increased under conditions of high substrate concentration [24]. Research on the enzyme α of the *Mythimna* separate species showed an increase in enzyme activity as the concentration of the amylase substrate increased. The increase or decrease in enzyme activity at various concentrations depends on the active site of the available enzymes. The decrease in enzyme activity can be caused by the active site of the enzyme is full. This research shows that under optimum conditions, brown planthoppers can hydrolyze the substrate at several concentrations by utilizing the enzyme α on its own [25].

Lineweaver-Burk analysis showed maximal velocity (Vmax) of the enzyme α-amylase brown planthopper was recorded at 43.47 $U.mg^{-1}$ protein and Km 1%. The Km value has an inverse relationship with the substrate concentration needed to unify the active site of the enzyme α-amylase. So that the lower the value of Km then indicates a strong bond [26]. The value of Km enzyme α-amylase use of this amylase substrate is not much different from the results obtained by Zibaee *et al.* [27]. The enzyme α-amylase of the species Andrallus spinidens has Vmax 7.14 $U.mg^{-1}$ protein and Km 1.04% in the starch substrate.

CONCLUSION

Supernatant extracted from brown planthoppers produces a clear zone on the medium to which iodine is added. It indicates the activity of enzyme α-amylase in the hydrolysis of starch.

A pH value of 6 provides the most optimum conditions for the activity of enzymes α-amylase extracted from brown planthoppers. An increase in pH value of more than 6 leads to a gradual decrease in enzyme activity.

The enzyme α extracted from brown planthoppers can work optimally in the temperature range of 30°C - 45°C and decreases in activity when the temperature reaches 50°C. The enzyme α-amylase from brown planthoppers showed the ability to hydrolyze amylase substrates to a concentration of 0.8 $\mu g.\mu L^{-1}$. Enzyme activity tends to increase with the addition of substrate concentrations.

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