The Characteristics of Urease Enzyme of Green Bean Seeds (Vigna radiata L.) and Its Activity as An Antifungal Against Candida albicans

Niken Istikhari Muslih 1,*, Zusfahair 1, Dian Riana Ningsih 1, Fajar Nuradha 1

1 Department of Chemistry, Mathematics and Natural Science Faculty, Jenderal Soedirman University, Purwokerto 53122, Indonesia

* Corresponding author: niken.istikhari@unsoed.ac.id

https://doi.org/10.14710/jksa.27.3.145-150

Abstract

Urease is an enzyme responsible for catalyzing the hydrolysis reaction of urea into CO2 and NH3. The urease isolated in this study came from green bean seeds. The urease enzyme was then tested for its antifungal activity against Candida albicans. The research aims to extract and characterize the urease enzyme from green bean seeds and explore its potential use as an antifungal agent. Green bean seeds were smoothed with a mortar and pestle, followed by homogenization using a stirrer and cold centrifugation. The crude extract of the urease enzyme was assessed for its activity through the Nessler method and measured by employing a UV–Vis spectrophotometer at a wavelength of 500 nm. The well–diffusion method was conducted to determine the antifungal activity of rough enzyme extracts against C. albicans. Positive controls were 100% ketoconazole, and negative controls used a pH buffer of 7. The characterization of the urease enzyme from green bean seeds revealed the optimum urease activity at a concentration of 0.25 M, pH level of 7, and an incubation temperature of 35°C with a value of 32.115 U/mL. Maximum reaction rate (Vmax) and Michaelis–Menten constant value (Km) were obtained at 56.497 U/mL and 0.215 M, respectively. Antifungal tests of C. albicans resulted in strong inhibitory activity at a concentration of 100% crude urease extract of 12 mm inhibition zone. The inhibitory concentration value grows at least 0.5% by 0.25 mm and positive control of 19.802 mm.

1. Introduction

Infection is a condition when microorganisms such as bacteria, viruses, or fungi enter the body and begin to reproduce. Infections can happen to anyone and anywhere, including in tropical areas like Indonesia, where humidity levels are high enough for the microorganisms to grow well. The most common cause of infection is a fungal infection. Candida albicans is a common pathogenic fungus that causes human infections [1]. The infectious condition caused by the fungus C. albicans is known as candidiasis, a fungal disease of the skin, nails, respiratory tract, and gastrointestinal tract of an acute and subacute disease of the skin, nails, respiratory tract, and gastrointestinal tract. Therefore, there is a need for exploration of natural antifungal drugs and the discovery that canatoxins are ureases that are capable of inhibiting the radial growth of filament fungus [3]. The fungitoxic activity of urease occurs at submicromolar doses, making this protein 2–3 times more potent than other plant–derived antifungal proteins. The fungitoxic activity of these ureases results in damage to cell walls and cell membranes and plasmolysis. Therefore, it can be concluded that the urease enzyme has potent antifungal activity.

Urease enzymes can be isolated from bacteria, fungi, and plants that have an essential function within the nitrogen cycle in nature [4, 5, 6]. Beans such as peanuts and long beans are often used as sources of enzyme producers of urease [7]. Green bean seeds belong to the family Fabaceae, which produces seeds. The protein...
content of 24% in green bean seeds indicates a high level of enzymes. Therefore, the urease enzyme from green bean seeds can be used as an antifungal.

One way to test antifungal activity is by using a series dilution technique to determine the minimum amount of antifungal substances needed to inhibit the growth of microorganisms in vitro, which is called Minimum Inhibitory Concentration (MIC). MIC is determined by variations in the sample concentration that can inhibit the growth of C. albicans. Good antifungal activity is when MIC occurs at low sample concentrations, but the impedance is high [8]. In this study, urease enzyme extraction was performed using the maceration method of green bean seeds with distilled water. The crude extract resulted from an antifungal test against C. albicans, characterized by variations of concentration, pH, temperature, and determination of MIC.

2. Experimental

This research started with sampling green bean seeds from Wage Market, Purwokerto. The seeds were macerated with distilled water, smoothed with a mortar and pestle, and homogenized using a stirrer and cold centrifugation. Crude urease extract was obtained for further characterization. The activity of the urease enzyme was calculated with a linear regression equation from the standard curve of ammonium sulfate.

2.1. Materials and Equipment

The materials used in this study included green bean seeds, distilled water, ammonium sulfate (Merck), sodium dihydrogen phosphate dihydrate (Merck), sodium hydrogen phosphate dihydrate (Merck), urea (Merck), sulfuric acid (Merck), Na–Wolframite (Merck), Nessler reagent (Merck), buffer citrate (Merck), buffer tris–hydrogen chloride (Merck), Sabauraud dextrose agar (SDA) (Merck), Sabauraud dextrose agar (SDA) (Merck) and Candida albicans fungi (ATCC 10231) from Biological Laboratory, Bogor Agricultural Institute.

The equipment used in this research included glasses commonly used in biochemical laboratory, analytical balance (ACIS), filler (Glasmir), autoclave (Hirayama), pH meter (Lutron), vernier caliper (Krisbow), UV–Vis spectrophotometer (Shimadzu 1800), incubator thermometer (Memmert), centrifuge (O’haus), refrigerator (Panasonic), crock bor, Drigalski spatula, and petri dish.

2.2. Extractions

About 10 g of green beans were measured and soaked in distilled water for 6 hours. The seeds were subsequently smoothed with mortar and pestle. The green bean seeds were then soaked in 40 mL of phosphate buffer pH 7 at 4°C and mixed with a stirrer for 3 hours, resulting in two distinct layers. The first layer was a filtrate, and the second was a suspension. The filtrate was separated using a muslin fabric. The resulting filtrate was centrifugated at 12,000 rpm at 4°C for 15 minutes. The crude extract was tested for its activity and characterized by substrate concentration, pH, and temperature variations.

2.3. Standard Curve Determination

The standard curve was determined using a standard ammonium sulfate solution with 10, 11, 12, 13, and 14 ppm concentrations. The standard solution was taken as much as 1.5 mL plus 250 μL of the Nessler reagent. The absorption value was measured at 500 nm wavelength.

2.4. Urease Activity Test of Crude Extract

A total of 1 mL of 0.25 M urea was inserted into the sample tube plus 1.9 mL pH 7 phosphate buffer solution and 0.1 mL enzyme crude extract. One mL of 0.25 M urea was inserted into the sample tube and added with 2 mL pH 7 phosphate buffer solution as a blank tube. Both tubes were incubated for 15 minutes at 35°C for further cooling with ice. The solution in the sample tube and the blank tube, respectively, was added 1 mL 2/3 N H_{2}SO_{4} to stop the activity of the enzyme urease and with 1 mL Na– Wolframite to complete the reaction of H_{2}SO_{4}. Each tube went through 15 minutes centrifugation process. The sample tubes and blanko solutions were taken in 1.5 mL, respectively, followed by 250 μL of Nessler reagent. The solution then measured its absorption using a UV–Vis spectrophotometer at 500 nm wavelength. The urease enzyme activity of green bean seeds was determined using the standard ammonium sulfate curve [9].

2.5. Determination of Optimum Substrate Concentration

The determination of urease activity on the variation of the substrate concentration was done using a procedure similar to the activity test. However, the substrate concentrations were varied at 0.15, 0.2, 0.25, 0.3, and 0.35 M and then incubated for 15 minutes using the specified substrate concentration variation. The data was used to determine the maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_{m}).

2.6. Determination of pH Optimum

The urease activity under different pH conditions followed the same procedure as the urease enzyme activity test. However, the pHs of the substrate were varied at pH levels of 6, 6.5, 7, 7.5, and 8 in 0.2 M buffer solution at optimal substrate concentrations. Their activities were tested at pH 6, 6.5, 7, and 7.5 with phosphate buffer, whereas pH 8 with tris–hydrogen chloride buffer.

2.7. Determination of Optimum Temperature

The same procedure was used to determine urease activity at different temperatures as for the enzyme activity test. However, it used a range of incubation temperatures of 25, 30, 35, 40, and 45°C. The urease enzyme was controlled at the ideal substrate concentration and pH. The reaction time was 15 minutes, with the determined temperature variation, and its activity was measured.

2.8. Antifungal Activity Test

The initial phase involved regenerating C. albicans by inoculating a fungal culture into 15 mL of SDA medium. This was achieved by transferring one loopful of C.
The antifungal enzyme in the phosphate buffer pH 7 at 4°C had been smoothly hydrolyzed with phosphate soaked with distilled water for 6 hours. Green bean seeds, sensitivity, is a low approach is used because it is simple, has great activity test to ensure that ammonia is not produced. The urease enzyme activity test of crude extract was measured by the amount of the formed ammonia. Urease enzyme activity was expressed in units per milliliter (U/mL). One unit of activity was expressed as the quantity of ammonia formed in micrograms per milliliter per minute from the hydrolysis of urea catalyzed by the urease enzyme in the sample.

In this research, the standard curve used ammonium sulfate because the ammonium sulfate in the water would produce the same ammonia ion as the hydrolysis result of urea [10]. The reaction of ammonium sulfate in water can be seen in Reaction (1).

\[
(\text{NH}_4)\text{SO}_4 + \text{H}_2\text{O} \rightarrow 2\text{NH}_3^+ + \text{SO}_4^{2-} + \text{OH}^- + \text{H}^+
\] (1)

Hydrolisis reaction of urea by urease is shown in Reaction (2).

\[
\text{CO}((\text{NH}_2)_2 + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{NH}_3
\] (2)

The ammonium ion produced during the reaction was quantified using the Nessler method and UV-Vis spectrophotometer at 500 nm wavelength. The concentration curve of ammonium sulfate against absorption produces a linear curve, as seen in Figure 1.

The relation between concentration and absorption values was either straight or linear. This was in line with Lambert–Beer’s law; as the concentration of a solution was higher, the absorption value of the solution was also higher [12]. Ammonium sulfate concentrations ranging from 10 to 14 ppm were utilized to generate standard curves. These concentrations were selected to achieve wavelengths with high and consistent absorption values within the range of 0.2 to 0.8, as the Lambert–Beer law is applicable within this range. From Figure 1, a linear equation was derived: \( y = 0.105x - 0.924 \). With an \( R^2 \) value of 0.998 for this regression equation, it can be concluded that the standard curve of ammonium sulfate exhibited a correlation coefficient close to 1. This indicates a strong correlation between the variables involved [13].

![Figure 1. Standard curve of ammonium sulfate](image-url)
The enzyme activity of urease was evaluated using Nessler’s reaction, resulting in the formation of a complex yellow-brown-colored compound. The color absorption was measured with a UV-Vis spectrophotometer. Based on the results obtained, the value of the activity of the urease enzyme produced was 32.086 U/mL.

### 3.2. Characterization of Urease Enzyme

Urease enzyme activity was influenced by several factors, including substrate concentration, pH, and temperature. The optimal substrate concentration was 0.25 M (Figure 2a), with the highest urease activity value of 32.115 U/mL. Substrate was able to bind to the active side of the enzyme so that it could produce a product. Urease activity increases at a concentration of 0.15–0.25 M and then decreases at a concentration of 0.3–0.35 M. At low substrate concentrations, the enzyme has many active sites available for binding to the substrates. As a result, the activity of enzymes increases as the substrate concentration increases. However, after reaching the optimal concentration, all the active sites may already be filled with the substrates, and the enzyme has reached a saturation point. Therefore, adding more substrates will not increase the enzyme activity further or even inhibit enzymes [14].

Urease enzymes from green bean seeds had optimal activity at pH 7 (Figure 2b) with urease enzyme activity values of 32.065 U/mL. Under optimum pH conditions, enzymes had the active side conformation that corresponds to the substrate so that the collision between the enzyme and the substrates occurs very effectively and facilitates the formation of complex enzyme substrates, as a result of which more and more products were formed and produce high values of enzymatic activity [15]. At a pH of 6–7, urease activity increases because, in this stage, many of the functional groups of amino acids in the enzymes (such as carboxylic and amino acid groups) were in ionic form. This allowed for the formation of ionic bonds between these groups and the ionic groups on the substrate, enhancing the interaction between the enzyme and the substrate. At a pH of 7.5–8, urease enzyme activity decreased because of the increased alkalinity [16]. This happened because, under non-optimal pH conditions, the enzyme undergoes conformational changes, leading to alterations in its structure and subsequent loss of activity.

The determination of enzymatic kinetics could be seen through the relationship between 1/V and 1/[S] based on the Lineweaver–Burk equation (Figure 3). The maximum rate of reaction (Vmax) of the urease enzyme from the green bean seed to hydrolyze its substrate was 56,497 U/mL. Substrate concentration at the catalytic concentration for the Lineweaver–Burk equation (Figure 3). The relationship between 1/[S] and 1/V

![Figure 3](image.png)

### 3.3. Minimum Inhibitory Concentration (MIC)

MIC was conducted to determine the minimum concentration of the sample that could inhibit the growth of C. albicans. Antifungal activity was considered high when MIC occurred at low sample concentrations but had
a large barrier force. MIC was determined by testing several concentrations of samples produced by dilution. The concentrations used in this study were 100, 50, 25, 12.5, 6.5, 3, 1.5, 1, 0.5, and 0.25% (v/v). The results of the MIC test can be seen in Figure 4.

The barrier zone for crude urease enzyme extraction from green bean seeds grew as concentrations rose. Figure 4 shows that the higher the extract concentration, the higher the active substance, such as hydroxyl groups. It disrupts organic components and nutrition transport, leading to toxic effects and inhibiting harmful spores in fungi, resulting in enhanced antifungal activity [20, 21]. The antifungal activity continues to be classified according to the diameter of the barrier zone from the weak to the strong. The diameter of the barrier zone of antifungal activity was classified as follows [22]: The barrier zone diameter > 20 mm has a very strong fungal growth barrier response; the barrier zone diameter 11–20 mm has a strong fungal growth barrier response; the barrier zone diameter 5–10 mm has a moderate fungal growth barrier response; the barrier zone diameter < 5 mm, weak fungal growth barrier response.

A positive control (ketoconazole) and a 100% urease enzyme crude extract with a sequential barrier zone of 19.802 and 12 mm. MIC was formed on a 0.5% urease enzyme crude extract with a sequential barrier zone of 0.205 mm. Crude urease enzyme extracts from at an incubation temperature of 35°C with an activity at a substrate concentration of 0.25 M, pH 7, and 4.

Ketoconazole exerts its antifungal effect against C. albicans by disrupting cell membranes. This disruption occurs through the inhibition of ergosterol synthesis, a process facilitated by interacting with C-14 alpha demethylase—an enzyme reliant on the P-450 cytochrome system. This inhibition prevents the conversion of ergosterol to its thinner form, rendering the fungi structurally unstable. Consequently, the fungus may form pseudohyphae or hyphae, leading to cell death [24].

4. Conclusion

Urease enzymes from green bean seeds had optimum activity at a substrate concentration of 0.25 M, pH 7, and at an incubation temperature of 35°C with an activity value of 32.115 U/mL. Crude urease enzyme extracts from green bean seeds could inhibit C. albicans antifungal activity at concentrations of 100% with a barrier zone of 12 mm and a growing barrier concentration at a minimum of 0.5% with a diameter of a barrier zone of 0.205 mm.

Acknowledgment

The authors gratefully acknowledge the Biochemistry Team, Mathematics and Natural Science Faculty, and Jenderal Soedirman University for supporting our work by providing necessary lab facilities during research.

References


[14] Nisha Kumari, Veena Jain, Sarla Malhotra, Purification and characterization of extracellular acidophilic α-amylase from *Bacillus cereus* MTCC
https://doi.org/10.5897/AJMR12.1371


https://doi.org/10.14710/jksa.12.1.7-13


https://doi.org/10.18860/al.v0i0.2297


https://doi.org/10.1016/j.toxicon.2007.07.008