

## Determination of Urease Biochemical Properties of Asparagus Bean (*Vigna unguiculata ssp sesquipedalis* L.)

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**Abstract.** Urease is enzyme that plays a role in nitrogen metabolism during plant germination. Plants that produce a lot of urease are grains. This study used asparagus bean as source of urease. The purpose of this research is to learn the effect of germination time on the activity of urease enzyme from asparagus bean and its biochemical properties. The research was started by germination of asparagus bean on day 2, 4, 6, 8, 10 and 12. Asparagus bean sprouts were extracted using acetone and separated by centrifugation to obtain the crude extract of urease. The biochemical properties of the crude extract of urease was further determined including: the effect of temperature, pH, substrate concentration, and metal addition to urease activity. The urease activity is determined by the Nessler method. The germination time of asparagus bean in yielding urease enzyme reached the optimum activity on the 8th day with activity value of 593.7 U/mL. The biochemical properties of urease from asparagus bean have optimum activity at 35 °C, pH 7.0 and substrate concentration 0.125% with activity value of 600 U/mL. Addition of CaCl<sub>2</sub>, SnCl<sub>2</sub> and ZnCl<sub>2</sub> metals decrease the activity of urease.

### Introduction

Urease has been isolated and characterized from several types of bacteria, fungi and plants [1]. Urease is commonly found in seeds such as chickpeas (*Cicer arietinum* L.) [2], *Pisum sativum* L. [3], *Cajanus cajan* [4] and cotton (*Gossypium hirsutum*) seeds [5]. Urease is one form of enzyme that plays a role in nitrogen metabolism at plant germination [3]. The urease enzyme can catalyze the pathogenic reaction of urea in plant cells. In the germination stage it will produce growth hormones followed by activation of hydrolytic enzymes such as  $\alpha$ -amylase lipase, peptidase and urease until optimum growth is reached when the beans have perfectly formed the roots, stems and leaves.

Urease is also important in human bodies due to the fact that many urinary tract and gastroduodenal diseases [6], including in cancer [7]; are related in some ways to this enzyme. Along with the increased use of urease enzymes, it is necessary to find the source of urease enzyme from raw material that is easily obtained. One ingredient that has the potential to be explored as a source of urease enzymes is the asparagus bean. Asparagus bean are abundant and easy to cultivate in Indonesia. So far, the use and utilization of asparagus bean is minimal and mostly as vegetables. In this study asparagus bean will be used as a source of urease. The use of asparagus bean as a source of enzymes is expected to increase the economic value of asparagus bean.



In this research urease enzyme isolation process was conducted from asparagus bean seeds which has been germinated by urease then extracted using acetone solvent and separated by centrifugation method so that the crude extract of urease is produced. The crude extract of the urease is determined by its biochemical properties including: the influence of various temperature variations, pH, substrate concentration, and metal addition to urease enzyme activity.

## Methods

### Tools and materials

The tools that used in this study are glassware, micropipette (Soccorex), tip (Biologix), digital scales (Ohaus), pH meters (Hanna Instrument), cuvet, UV-Vis spectrophotometer (Shimadzu UV-1800) Incubator (Mettler), centrifuge (Quantum), bath, refrigerator, mortar and pestle, plastic wrapping, stirrer, cotton, and muslin fabric, stirrer, funnel and filter paper. The materials that used in this study were asparagus bean (*Vigna unguiculata ssp sesquipedalis* L.) seeds obtained from Purwokerto Wage Market,  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ,  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ,  $\text{C}_6\text{H}_8\text{O}_7 \cdot 2\text{H}_2\text{O}$ ,  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ ,  $\text{C}_4\text{H}_{11}\text{O}_3\text{N}$ , HCl,  $\text{CuSO}_4$ ,  $\text{CaCl}_2$ ,  $\text{SnCl}_2$ ,  $\text{ZnCl}_2$ ,  $\text{CO}(\text{NH}_2)_2$ , acetone,  $\text{H}_2\text{SO}_4$ , Na-Wolframat, Nessler reagents and aquadest.

## Procedures

### Isolation of urease from asparagus bean [3]

Isolation was done on the seeds of asparagus bean, where as much as 200 grams of seeds were soaked in water for 6 hours, then drained and put into a plastic container filled with wet cotton, then covered with wrapping and left in the dark room on room temperature and germination was done with variation of time 0, 2, 4, 6, 8, 10 and 12 days. The next step was urease extraction.

The urease extraction was carried out as follows: As much as 20 grams of spouts was taken and mashed with mortar and pestle then soaked in 80 mL of acetone 20% in cold temperature (4 °C) and stirred with stirrer for 3 hours to produce 2 layers of filtrate and suspension. The filtrate was separated by means of a muslin cloth. The filtrate obtained was centrifuged at 7,000 rpm for 15 minutes at 4 °C. The resulting supernatant is used as a crude urease extraction.

### Urease activity test of crude extract [8]

The urease activity test was performed by 1 mL urea concentration of 0.1% plus 1,95 mL of buffer phosphate solution of pH 7 inserted into sample tube. A total of 0.05 mL of a crude extract solution of the urease enzyme was added. The tube was incubated at 35 °C for 15 minutes. The tube was cooled with ice. As much as 1 mL 2/3N of  $\text{H}_2\text{SO}_4$  solution was added to the sample tube to stop urease enzyme activity and 1 mL Na-Wolframat was added to perfect  $\text{H}_2\text{SO}_4$  work. The blank tube was filled with 3 mL of distilled water. Both tubes were centrifuged for 15 minutes and supernatant was taken by filtration process. Deuteronomy was done 3 times. A total of 1.5 mL of solution of each sample tube and blank was taken. Each sample solution and blanks were then added with 250  $\mu\text{L}$  of Nessler reagent. The solution uptake was then measured by using UV-Vis spectrophotometry at  $\lambda$  500 nm.

Determination of urease activity was done by using the following formula:

$$\text{Activity unit} = \frac{[S] - [B] \times 5 \text{ mL}}{0,05 \text{ mL} \times 1,5 \text{ mL} \times 15}$$

Explanation:

[S]	= concentration of sample
[K]	= concentration of control
5	= volume of solution (mL)
0.05	= volume of enzyme (mL)
1.5	= test volume (mL)
15	= incubation time (menit)

The urease estimation was performed using the standard ammonium sulfate curve. One unit of activity is defined as "the amount of ammonia formed  $\mu\text{mol}$  per mL of hydrolysis of urea by urease enzyme within 15 minutes".

### Determination of urease biochemical properties of asparagus bean seeds

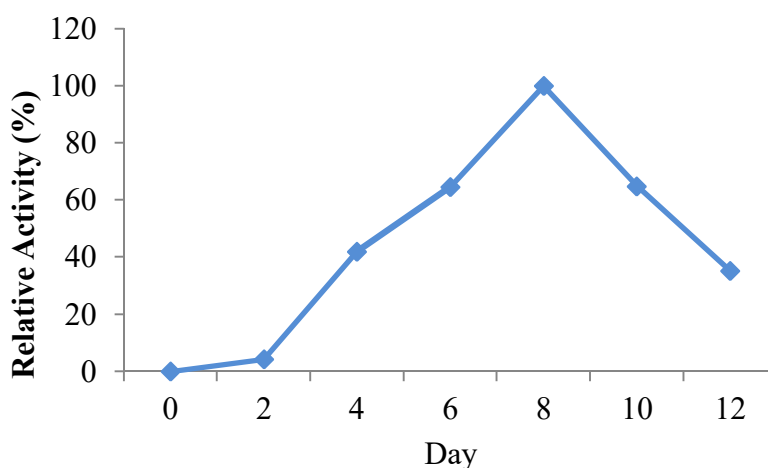
Determination of the biochemical properties of urease was begun by examining the effect of incubation temperature variations on urease activity. Determination of urease activity was performed in similar way as in activity test but conducted at variation of incubation temperature of 25, 30, 35, 40 dan 45 °C. The enzyme reaction mixture was adjusted to 0.2 M phosphate buffer solution pH 7, with 15 minutes of reaction time. At the optimum temperature, the effect of substrate pH variation on urease activity was further tested. Variations of urea pH substrates used were pH 5.0; 6.0; 7.0; 8.0 and 9.0 in 0.2 M buffer solution. The effect of substrate concentration on urease activity was tested on concentration variation; 0.05; 0.075; 0.1; 0.125 and 0.15%.

The effect of metal addition to urease activity was done by the addition of CaCl<sub>2</sub>, SnCl<sub>2</sub>, and ZnCl<sub>2</sub> with varying concentrations of 10<sup>-3</sup>-10<sup>-8</sup> M. The enzyme activity was tested by adding 1 mL of urea 0.1% and enzyme solution (0.05 mL Enzyme + 0.1 mL metal + 1.85 mL 0.2 M bufer). The enzyme reaction mixture is adjusted under optimum conditions using the addition of the specified metal.

## Results and Discussion

### Effect of Asparagus Bean Growing Period on Urease Activity

Isolation of urease in this study was carried out through the stage of germination of asparagus bean seeds. Long dried seeds were soaked in water. The process of immersion makde the cells in the plant tissue become actively growing due to imbibisi process, which is the absorption of water into plant tissue cavities. Seeds were grown using wet cotton and in dark conditions for 0, 2, 4, 6, 8, 10 and 12 days. Asparagus bean sprouts were then extracted and centrifuged at cold temperatures to prevent the occurrence of denaturation due to hot temperatures. The supernatant obtained was a crude extract of urease enzyme which then tested for its activity. The effect of germination time on urease activity can be seen in **Figure 1**.



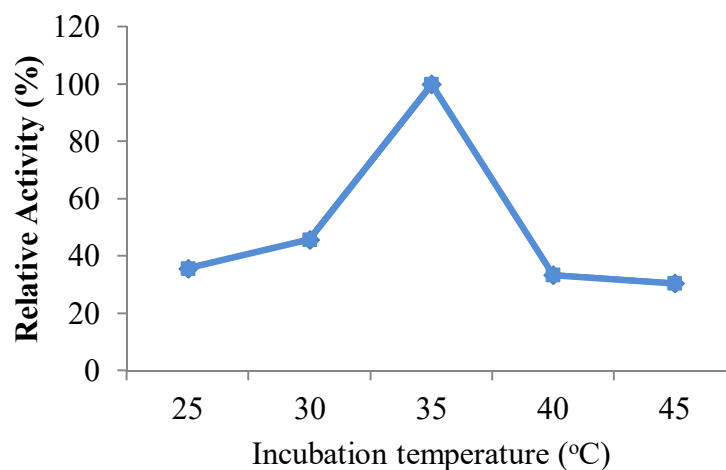
**Figure 1.** Curve of asparagus bean germination time on relative urease activity

**Figure 1** shows the urease activity getting significantly increased after the 2nd day until the 8th day. In this situation, the germination process began, where the embryo in the seeds grew into new plants, thus triggering the growth of shoots from within the seed. The process of plant cell division was done with the help of growth hormone that also activated hydrolytic enzymes such as amylase, phosphatase, lipase, peptidase and urease. The optimum urease activity took place on the 8th day of germination with activity value of 593.7 U/mL. In these circumstances urease had high activity in catalyzing the chemical reaction and most urea was hydrolyzed by urease to produce the nitrogen used as plant growth [9]. The germination time for 8 days was used for further research.

### Determination of Urease Biochemical Properties of Asparagus Bean Seeds

### ***Effect of incubation temperature variation on urease activity of asparagus bean***

Activity of enzyme is influenced by several factors one of them is temperature. In this study the variation of incubation temperature used was 25, 30, 35, 40 and 45 °C. The effect of temperature on urease activity can be seen in **Figure 2**.



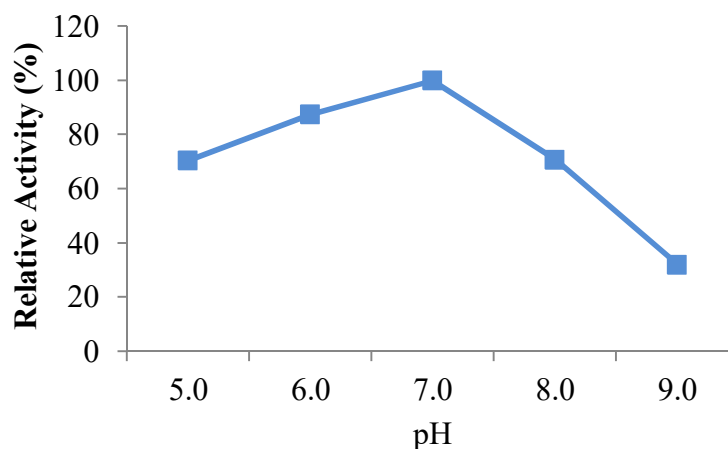
**Figure 2.** Curve of relation of the incubation temperature to the relative urease activity of the asparagus bean

**Figure 2** shows the activity of urease enzyme at 25 °C to 35 °C has increased significantly. In these circumstances there is an increase in collisions between enzyme molecules and substrate. The resulting collision is small or rare due to the small kinetic energy resulting in low activity. The activity of urease reaches optimum condition at 35 °C with activity value equal to 612.4 U/mL. Activity can be increased due to increased kinetic energy which increased the chance of collision between enzyme molecules with substrate and forming complex of enzymes, so that the resulting product increases. The activity decreased significantly after reaching the optimum. According to [10], temperature which rises above optimum temperature results in decreased enzyme activity. Decrease in activity is caused by the enzyme denaturation process by high heat. This is because high temperatures will break secondary bonds such as hydrogen bonds that retain enzymes at their natural temperature, so that the secondary and tertiary structures of the enzyme will be partially damaged followed by a decrease in activity.

Research by [3] states that the optimum temperature of urease isolated from *Pisum sativum* L. is at 40 °C. Pervin et al., (2013) state that the optimum temperature of urease isolated from the chickpea seed (*Cicer arietinum* L.) is at 48 °C. Most enzymes have optimum activity in the 20 - 50 °C temperature range. Enzymes that actively work at that temperature range belong to the mesozyme group [11].

### ***Effect of pH variation on urease activity of asparagus bean***

In this study, the variation of pH being used was 5.0; 6.0; 7.0; 8.0 and 9.0. Data on the effect of pH on urease activity can be seen in **Figure 3**.



**Figure 3.** Curve of the relation of pH of the substrate to the relative activity of urease from asparagus bean

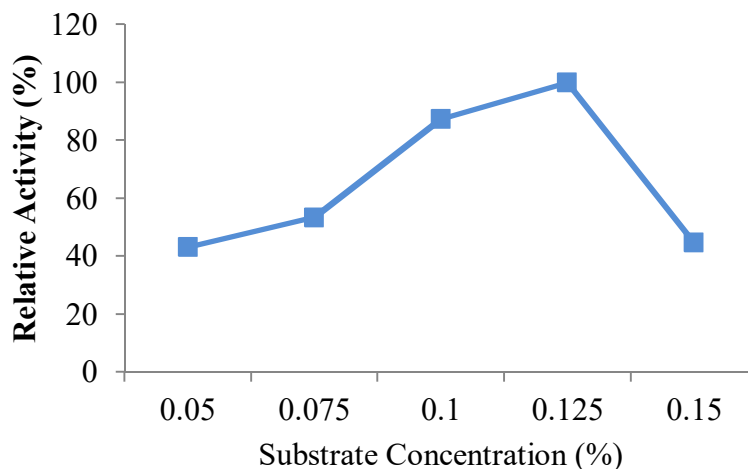
The data in **Figure 3** shows the urease activity rose sharply at pH 5.0 to 7.0. In this state, the interaction between the enzyme and the substrate was still low so that the resulting activity was small. This is because the conformation of the enzyme was not yet ideal. The pH value of the environment affects the effectiveness of the active side of the enzyme in forming the enzyme-substrate complex. Changes in pH lead to changes in ionization levels in enzymes or substrates that affect activity. Activity increased until it reaches the optimum condition produced at pH 7.0 with activity value of 613 U/mL, when the optimum pH of enzyme conformation was in ideal condition. This causes the interaction between enzyme and substrate to be maximal in urea degradation process and formed the product. The enzyme has an active site with certain groups acting as a catalyst in the formation of the enzyme-substrate complex. Enzyme activity is closely related to its structure, structural changes will cause changes in enzyme activity.

Enzyme activity decreased sharply after reaching optimum condition. This is due to the change in acidity which causes decreased activity. In an atmosphere that is too acidic or alkaline, the conformation of the enzyme will change and cause disruption of the activity of the enzyme. Conformation of altered enzymes can lead to decreased enzyme activity due to enzyme conformation different from substrate conformation [12].

The optimum pH value of urease activity isolated from chickpeas (*Cicer arietinum L.*) was at pH 7.2 [13], *Cajanus cajan* was pH 7.5 [4]. Based on the results of this study it can be seen that the optimum pH for some urease enzymes generally lies in neutral pH.

#### ***Effect of substrate concentration variation on urease activity of asparagus bean***

In this research, the variation of substrate concentration used were 0,05; 0.075; 0.1; 0.125; and 0.15%. Data of effect of substrate concentration on urease activity can be seen in **Figure 4**.



**Figure 4.** Curve of relation of substrate concentration to relative urease of asparagus bean

The data in **Figure 4** shows the urease activity rose sharply with increasing substrate concentration and reached optimum at 0.125% concentration. When the substrate concentration was enlarged, substrate binding by the enzyme will increase. Thus, the concentration of the enzyme-substrate complexes was greater and the activity of the enzyme will also increase. Relative activity decreased at 0.15% concentration. According to [14], the more substrate concentration, the more the active side of the enzyme in contact with the substrate also increases, resulting in more substrates being hydrolyzed into the product. But too much product causes inhibition because the product will stick to the enzyme's allosteric side so that the enzyme's active side can no longer be occupied by the substrate. This resulted in decreased enzyme activity when substrate addition with greater concentration after reaching optimum condition.

#### ***Effect of metal addition to urease activity of asparagus bean***

This study used a variety of metals such as metal  $\text{CaCl}_2$ ,  $\text{SnCl}_2$  and  $\text{ZnCl}_2$  with variation of concentration  $10^{-3}$ -  $10^{-8}$  M. The data of effect of metal addition to urease enzyme activity can be seen in **Table 1**.

**Table 1** The effect of metal concentration on relative activity (%) and inhibition (%)

Type and Metal Concentration (M)	Relative Activity (%)	Inhibition (%)
No metal	100	0
CaCl <sub>2</sub> 10 <sup>-3</sup>	37	63
CaCl <sub>2</sub> 10 <sup>-4</sup>	46	54
CaCl <sub>2</sub> 10 <sup>-5</sup>	56	44
CaCl <sub>2</sub> 10 <sup>-6</sup>	64	36
CaCl <sub>2</sub> 10 <sup>-7</sup>	77	23
CaCl <sub>2</sub> 10 <sup>-8</sup>	83	17
SnCl <sub>2</sub> 10 <sup>-3</sup>	48	52
SnCl <sub>2</sub> 10 <sup>-4</sup>	58	42
SnCl <sub>2</sub> 10 <sup>-5</sup>	67	33
SnCl <sub>2</sub> 10 <sup>-6</sup>	78	22
SnCl <sub>2</sub> 10 <sup>-7</sup>	85	15
SnCl <sub>2</sub> 10 <sup>-8</sup>	96	4
ZnCl <sub>2</sub> 10 <sup>-3</sup>	50	50
ZnCl <sub>2</sub> 10 <sup>-4</sup>	57	43
ZnCl <sub>2</sub> 10 <sup>-5</sup>	69	31
ZnCl <sub>2</sub> 10 <sup>-6</sup>	78	22
ZnCl <sub>2</sub> 10 <sup>-7</sup>	88	12
ZnCl <sub>2</sub> 10 <sup>-8</sup>	91	9

The data in **Table 1** shows that the higher metal concentrations added, the greater the inhibition to urease activity. The greatest inhibition occurred in the addition of CaCl<sub>2</sub> metal at a concentration of 10<sup>-3</sup>M. The results of this study were comparable to that of the [15] on the urease enzyme isolation from *Rhizopus oryzae* which states that the addition of CaCl<sub>2</sub> metal with increasing concentration (10<sup>-3</sup>-10<sup>-5</sup> M) decreases the activity of urease enzymes.

Addition of ZnCl<sub>2</sub> concentration increased caused the activity of seed urease *Cicer arietinum* L. also decreased. This is because the ZnCl<sub>2</sub> metal will inhibit the enzyme binding to the substrate so that the resulting product becomes less and less. Addition of some heavy metals one of which is Zn<sup>2+</sup> produces an effect in decreasing the activity of urease. The inhibition that occurred was resulted from the reaction between metal molecules and the active part of the enzyme ie thiol (-SH) [13]. The inhibitor binds the enzyme to cause the enzyme not to bind to the substrate on its active side (competitive inhibitor). The competition that occurs depends on the concentration of substrate and inhibitor. Inhibitors did not only work to bind the active side of the enzyme, but also to the enzyme section so as to alter the three-dimensional conformation of enzyme-building proteins. This condition does not produce substrate-enzyme complexes so that the urease activity decreased.

## Conclusion

Based on the result of the research, it can be concluded that: time of germination of asparagus bean in yielding of enzyme urease reach optimum activity on day 8 with activity value equal to 593,7 U/mL. The biochemical properties of urease from asparagus bean seeds have optimum activity at 35 °C, pH 7.0 and 0.125% substrate concentration with activity value of 600 U/mL. The addition of CaCl<sub>2</sub>, SnCl<sub>2</sub> and ZnCl<sub>2</sub> metals decrease the activity of urease.

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