EXTRACTION, PARTIAL PURIFICATION, AND CHARACTERIZATION OF UREASE FROM CHICKPEA SEEDS (*Phaseolus vulgaris* L.)

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Abstract. Urease is an enzyme which as an essential role as a catalyst in the hydrolysis reaction of urea into ammonia and CO2. Urease has been extracted from legumes, and chickpea seeds standing out among the legume varieties with the potential as a urease source. This research aims to delineate the attributes of urease derived from chickpea seeds. The urease enzyme was isolated from the chickpea seeds, resulting in a crude enzyme extract. Subsequently, this crude extract underwent purification utilizing ammonium sulfate salt through the precipitation principle. Fractions exhibiting the highest activity were subject to characterization encompassing pH, temperature, substrate concentration, and the addition of metallic elements. The urease activity assay employed the Nessler method, with the absorbance measurements conducted using a spectrophotometer at the wavelength of 500 nm. Urease originating from chickpea seeds demonstrated an optimal activity at the pH of 7, incubation temperature of 35 °C, and substrate concentration of 0.2 M, resulting in the F30 activity of 17.720 U/mL.

1. Introduction

Enzymes are biocatalysts that can accelerate a chemical reaction by reducing the activation energy of that chemical reaction [1]. Some beneficial properties of enzymes, when used, include their high efficiency, ability to operate under mild conditions, safety, ease of control, the ability to replace hazardous chemicals, and biological degradability. Enzymes find extensive use in the industrial sector due to these properties, and they are also highly specific compared to inorganic catalysts. One enzyme widely employed in industry is urease, a metalloenzyme that catalyzes the hydrolysis of urea into ammonia and carbon dioxide. Urease plays a significant role in clinical treatment analysis, blood and urine analysis, artificial fertilization, kidney failure, and drug analysis. Urease enzymes are also utilized in wastewater treatment and food processing [2].

Urease activity is widely found in almost all plant tissues. In previous research, urease has been isolated from *Vicia faba* L [3], *Pisum sativum* [4], and *Vigna unguiculata* subsp *sesquipedalis* L. [5], and other legume groups. This research isolated urease from the legume group known as chickpea seeds. Chickpea seeds are readily available and cost-effective plants. In Indonesia, the production of chickpea seeds in 2021 reached 320.774 tons, with 27.560 tons being produced in Central Java [6]. The enzyme obtained from isolation is in the form of crude extract. Purification of the crude extract is necessary to enhance its activity.

The crude urease extract can undergo partial purification through ammonium sulfate fractionation. Protein purification using ammonium sulfate is one of the initial purification methods for enzymes. The selection of ammonium sulfate is based on its high solubility level, which makes it easily interact with water molecules. Precipitation with salt is done following the principle of salting out. The solubility of proteins decreases at high salt concentrations. Increased salt concentration causes water to separate from the proteins, leading to the formation of hydrophobic interactions between proteins, resulting in precipitation [7].

The fractions obtained from purification are further subjected to dialysis. Dialysis is a method that can be applied to enhance the purity of the enzyme after fractionation with salt. In the dialysis process, a semi-permeable membrane is used to separate large molecules from small ones [8]. To make these fractions suitable for industrial use, they are then characterized, including assessing the impact of temperature, pH, substrate concentration, and the addition of metals on the urease activity derived from chickpea seeds.

2. Methods

2.1.Materials and Instruments

The instruments used in this research included common laboratory glassware, incubator, refrigerator (LG), analytical balance (O'Haus), centrifuge (O'Haus), and UV-Vis spectrophotometer (Shimadzu UV-1800). The materials used in this research consisted of chickpea seeds obtained from Pasar Wage Purwokerto, ammonium sulfate, Nessler's reagent, urea, casein, Na_2HPO_4, NaH_2PO_4, Na_2CO_3, NaOH, $CuSO_4$, Na(K)-Tartrate, Folin-Ciocalteau reagent, acetic acid, cellophane bags, and distilled water. All chemicals used were purchased from Merck, Germany.

2.2. Determining Chickpea Seeds Plant

The determination of chickpea seeds plants was conducted at the Laboratory of the Faculty of Biology, Universitas Jenderal Soedirman.

2.3. Germinating Chickpea Seeds

The germination of chickpea seeds was carried out by soaking chickpea seeds in distilled water for 6 hours, after which the seeds were planted on cotton media for 0, 2, 4, 6, and 8 days.

2.4. Sample Preparation [5]

A total of 10 g of chickpea seeds were soaked in distilled water for 6 hours at 4 °C. After which, these chickpea seeds were planted for 4 days on cotton as the media. After 4 days, they were finely ground using a mortar and pestle that had been cooled by placing them in a refrigerator for approximately 24 hours. The finely ground chickpea seeds sprouts were transferred to a beaker glass and mixed with 30 mL of pH 7, 0.2 M phosphate buffer. The mixture was stirred for 30 minutes at a cold temperature. After 30 minutes, the suspension was filtered using muslin cloth. The obtained filtrate was centrifuged for 15 minutes at a speed of 7000 rpm at 4 °C, and the supernatant was tested for activity and subjected to fractionation using ammonium sulfate.

2.5. Fractionating Crude Urease Extract [8]

Fractionation was carried out by gradually adding ammonium sulfate at 15, 30, 45, and 60% saturation-concentrations. The fractionation process at 15% began with the preparation of 8.4 g of ammonium sulfate, which was then slowly added to 100 mL of crude urease extract while stirring with a magnetic stirrer. This mixture was subsequently centrifuged at

a speed of 7,000 rpm for 15 minutes at 4 °C. The obtained precipitate was dissolved in 10 mL of pH 7, 0.2M phosphate buffer and stored at 4 °C, referred to as the 15% fraction (F15). The supernatant from the first fractionation was supplemented with 30% ammonium sulfate, and the same procedure was repeated for 30% fraction (F30). The same treatment was carried out for 45% fraction (F45) and 60% fraction (F60). All four fractions obtained were subsequently dialyzed. The activity of each dialyzed fraction was tested. The fraction exhibiting the highest activity underwent characterization, including assessments of temperature, pH, substrate concentration, and the addition of metals using the same method as the urease activity test.

2.6.Determining Urease Activity [9]

A total of 1 mL of a 12,000 ppm urea solution was introduced into a reaction tube, along with 0.9 mL of pH 7 phosphate buffer (0.2 M) and 0.1 mL of urease solution. The solution was then incubated for 15 minutes at 35 °C. After the 15-minute incubation, the solution was placed into a cooling cabinet for 5 minutes. Subsequently, 0.1 M HCl was added to halt the hydrolysis reaction. The solution was centrifuged at a speed of 500 rpm for 15 minutes, and 1.5 mL of the supernatant from the centrifugation was collected and placed into a reaction tube. Then, 0.25 mL of Nessler's reagent was added, and the solution was thoroughly mixed until it was homogeneous. The absorbance of the solution was measured using a UV-Vis spectrophotometer at a wavelength of 500 nm. A blank solution was prepared in the same manner, but without the enzyme solution.

The urease activity was estimated using a standard curve of ammonium sulfate. One unit of activity was defined as the "amount of ammonia formed (µmol) per mL per minute from the hydrolysis of urea by urease.".

2.7. Characterizing Urease [9]

The urease enzyme was characterized aiming at determining its optimal conditions and behavior. This involved investigation of temperature variations (25, 30, 35, 40, and 45 °C), pH variations (ranging from 5 to 9, with specific buffers for each pH), and substrate concentration variations (ranging from 0.1 to 0.35 M). Additionally, the influence of added metals (Ni^{2+} and Hg^{2+}) on urease activity was assessed. Prior to testing, each enzyme extract was supplemented with 0.1 ppm of Ni^{2+} and Hg^{2+} at an enzyme-to-metal ratio of 9:1. After a 10-minute incubation, the enzyme's activity was determined following the same procedure as in enzyme activity determination.

3. Results And Discussion

3.1. Determining Chickpea Seeds Plant

Based on the determination results, chickpea seeds plants can be classified as follows:

Family : Fabaceae
Genus : Phaseolus
Species : *Phaseolus vulgaris* L.

3.2. The Effect of Chickpea Seeds Germination Time on Urease Enzyme Activity

The data from the determination are presented in **Figure 1**. Based on Figure 1, it is evident that the highest urease activity occurs during a 4-day germination period, with a urease activity value of 14.600 U/mL. During the germination process from day 0 to 2, carbohydrates and fats as an energy source begins degrading, while protein degradation starts around day 4. Proteins are broken down into amino acids, and these amino acids are subsequently oxidized, resulting

in the production of urea. The formation of urea triggers the sprout to produce urease enzyme to hydrolyze urea into ammonia. Urease activity decreases after day 4 due to a decrease in urease production as the stored food reserves are depleted [10].

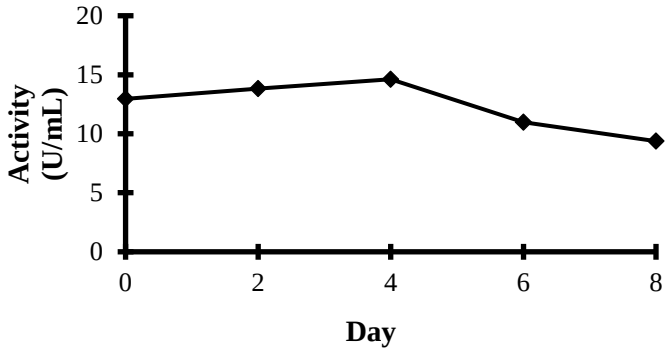

Figure 1. Relationship of germination duration with urease activity

3.3. Urease Fractionation

The data from the enzyme fractionation process are presented in **Table 1**.

Table 1 Data from Enzyme Fractionation

Phase	Activity (U/mL)		Protein (mg/mL)		Specific activity (U/mg)		Purity
Crude Extract	14.620 0.058	±	4.635 0.049	±	3.161 0.044	±	1
F15	15.861 0.030	±	2.431 0.044	±	6.548 0.128	±	2.072
F30	17.720 0.025	±	1.746 0.013	±	10.208 0.062	±	3.229
F45	15.813 0.008	±	3.171 0.033	±	5.001 0.049	±	1.582
F60	15.556 0.198	±	3.978 0.002	±	3.943 0.052	±	1.247

Based on Table 1, it is evident that each fraction exhibits higher activity and specific activity compared to the crude extract. Higher activity indicates that the enzyme obtained is purer. F30 has the highest specific activity at 10.149 U/mg with a purity of 3.229. This fraction is presumed to have relatively lower solubility compared to F45 and F60. Proteins with lower solubility tend to precipitate first when ammonium sulfate is added. The protein content of the fractionated enzyme is lower than that of the crude extract since the fractionation process separates proteins in the solution based on their solubility [10]. F30 is further characterized by determining the optimal conditions, including variations in temperature, pH, substrate concentration, and the influence of metal additions on urease activity derived from green bean.

3.4. Characterization of Urease F30 from Chickpea Seeds

3.4.1 Effect of pH

The relationship between pH variations and urease F30 activity can be observed in **Figure 2**.

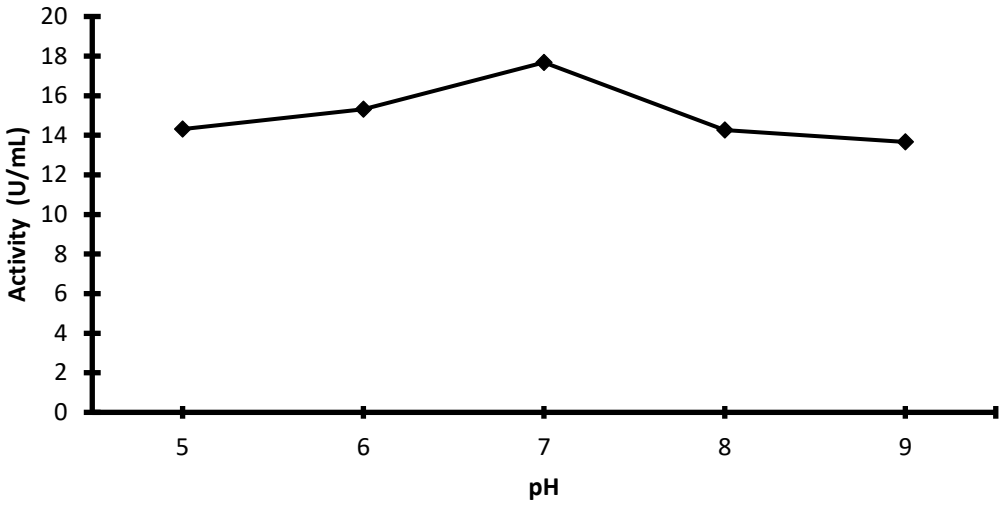

Figure 2. Effect of pH variations on urease F30 activity

As shown in Figure 2, the optimum activity of the urease enzyme occurs at pH 7, with an F30 activity value of 17.711 U/mL. At the optimum pH condition, the enzyme adopts an active site conformation that is suitable for the substrate. This leads to highly effective interactions between the enzyme and the substrate, facilitating the formation of enzyme-substrate complexes and resulting in a higher enzyme activity [11]. At pH 5, 6, 8, and 9, enzyme activities are lower than those at pH 7. This can be attributed to the fact that at non-optimal pH levels, the enzyme undergoes a conformational change in its active site, leading to structural alterations that can disrupt enzyme activity [11]. Previous studies have also reported that urease enzymes from legume-type plants such as yardlong beans and cowpeas have optimum activity at pH 7 [9].

3.4.2 The Effect of Incubation Temperature

The relationship between temperature variations and urease enzyme activity can be observed in **Figure 3**.

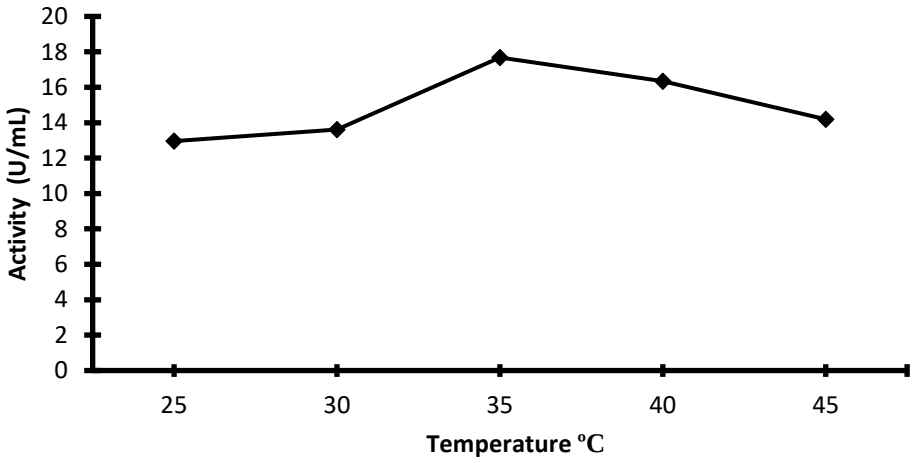

Figure 3. The Effect of Incubation Temperature on Urease Enzyme Activity

Based on Figure 3, urease enzyme activity increases from 25 to 35 °C and reaches its highest activity at an incubation temperature of 35 °C, with an F30 activity value of 17.711 U/mL. At lower temperatures, enzyme activity decreases as the kinetic energy of molecules is generally lower, leading to slower enzyme-substrate interactions. Temperatures that are too high can cause enzyme denaturation, resulting in the loss of catalytic activity [12].

3.4.3 Effect of Substrate Concentration

The relationship between substrate concentration and urease enzyme activity can be observed in **Figure 4**.

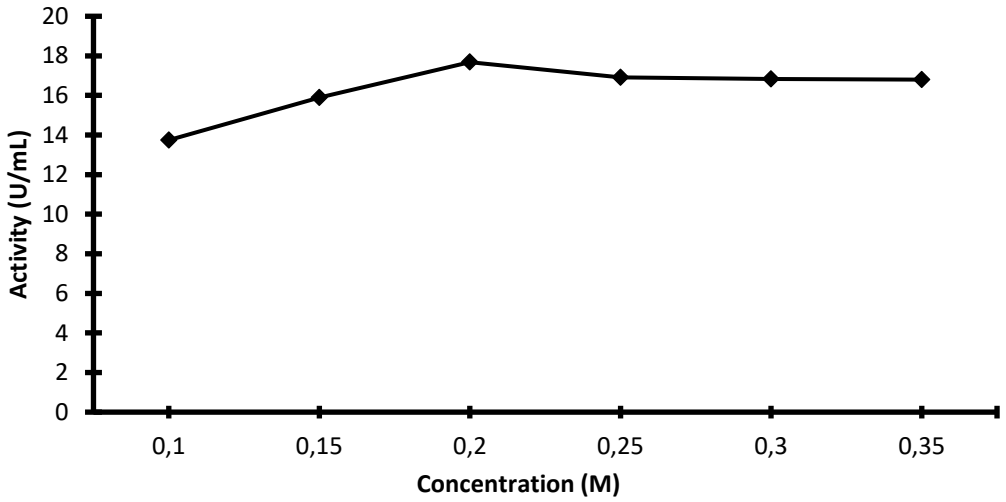

Figure 4. Relationship of substrate concentration and urease enzyme activity

Based on **Figure 4**, as the substrate concentration increases, the urease enzyme activity also increases and reaches its optimum point at a substrate concentration of 0.20 M, with an F30 activity of 17.720 U/mL. At substrate concentrations of 0.25-0.35 M, enzyme activity decreases. This occurs as the products formed inhibits enzyme activity, resulting in a decrease in enzyme activity [13].

The data obtained from the variation in substrate concentration were used to determine Vmax and KM. The curves showing the relationship between 1/V and 1/[S] for the crude enzyme extract and Fraction 30 can be observed in **Figure 5** and **Figure 6**, respectively.

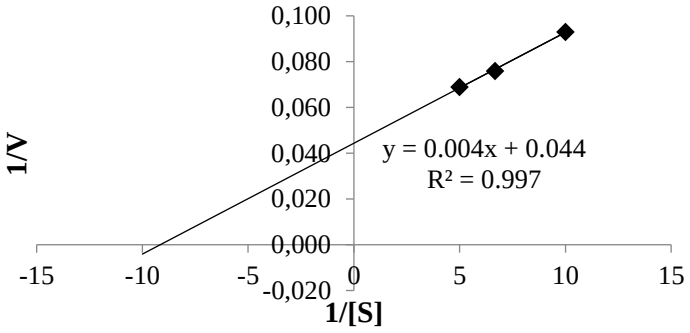

Figure 5. Curve showing the relationship between 1/V and 1/[S] for the crude extract

Based on **Figure 5**, the regression equation obtained is y = 0.004x + 0.044. The calculated Vmax value is 22.727 U/mL, and the K_M value is 5.460 ppm (0.091 M).

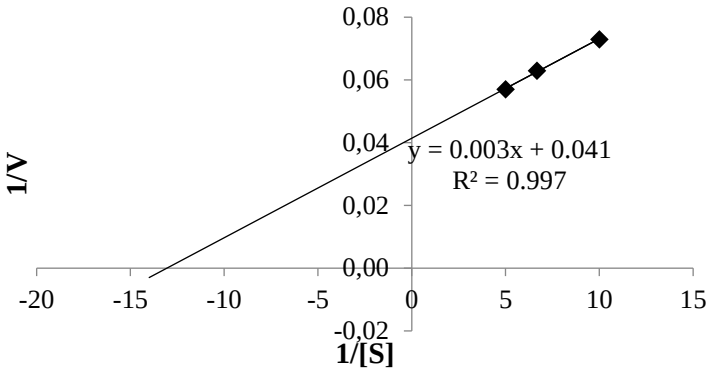

Figure 6. Curve showing the relationship between 1/V and 1/[S] for Urease F30

Based on **Figure 6**, the regression equation obtained is y = 0.003x + 0.041. The calculated Vmax value is 24.390 U/mL, and the KM value is 4.380 ppm (0.073 M). The KM value obtained for F30 is smaller than that of the crude extract, indicating that the F30 enzyme has a higher affinity for the substrate. High affinity indicates that the enzyme has a high ability to bind to the substrate, resulting in higher efficiency in product formation. High enzyme affinity also indicates a higher reaction rate under optimum conditions.

3.4.4 The Effect of Metal Addition

The relationship between the addition of heavy metals and urease enzyme activity can be observed in **Table 2**.

Table 2. Relationship between the addition of heavy metals and urease F30 activity.

Reagen	**Activity (U/mL)**	**Relative activity (%)**
Control	17.720	100
Ni^{2+}	17.025	96
Hg^{2+}	15.637	88

Based on **Table 2**, the addition of metals can decrease the activity of the urease enzyme. The activity of the sample without metals in F30 is 17.720 U/mL. The addition of metal ions Ni2+ and Hg^{2+} to F30 decreases urease activity to 17.025 (96%) and 15.637 U/mL (88%), respectively. Ions such as Na^{+}, Ca^{2+}, Ni^{2+}, and Cu^{2+} act as inhibitors for urease enzyme isolated from long bean seeds [9]. Additionally, ions like Cu^{2+}, Cd^{2+}, Hg^{2+}, and Co^{2+} are inhibitors for urease enzyme isolated from pumpkin seeds [14].

Metal ions can inhibit enzyme activity by either replacing the metal co-factor at the enzyme's active site, leading to a decrease in enzyme activity, or by directly binding to the enzyme at sites other than the active site, altering the enzyme's conformation and affecting its activity [15].

4. Conclusion

Urease from chickpea seeds, purified with ammonium sulfate in the 30% fraction (F30), exhibits optimal activity at pH 7, incubation temperature of 35°C, and substrate concentration

of 0.2 M, with an activity value of 17.720 U/mL. Metal ions Ni^{2+} and Hg^{2+} act as inhibitors for the urease enzyme.

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