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RESEARCH ARTICLE

Partial purification and characterization of urease from black-eyed pea (*Vigna unguiculata ssp unguiculata L.*)

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Abstract

Urease is one of the most important enzymes in the industry. The aim of this research was to isolate and to partial-purity urease from black-eyed pea including urease characterization. Research begins by germinating the black-eyed pea. Germinated black-eyed peas were extracted using phosphate buffer pH 7 and separated by centrifugation to obtain a crude extract of urease. The crude extract of urease was further fractionated using acetone at concentrations of 20, 40, 60, and 80%. The fraction which has highest specific activity then determined by molecular weight using SDS PAGE method and characterized including the influence of temperature, pH, substrate concentration, and metal addition to urease activity. The urease activity is determined by the Nessler method. The specific activity increased during the fractionation phase and specific activity is obtained amount 428.59 mU/mg with a purity level of 2.2 times in FA 80. The results of electrophoresis analysis showed that FA 80 estimated to have four polypeptides with a molecular weight of about 15, 17, 35 and 55 kDa. The result of characterization was obtained the optimum FA 80 urease activity at temperature 30 ^{o}C, pH 7, substrate concentration 0.125% (w/v) with K_M value 17.8 mM. Urease FA 80 from black-eyed pea was classified as a metaloenzyme. The addition of $CaCl_2$, NaCl, $NiCl_2$ and $CuCl_2$ metals at various concentrations decreased the urea activity of FA 80. The higher metal concentration was added then the FA 80 urease activity decreased further.

***Keywords*:** Characterization, partial purification, urease, black-eyed pea

INTRODUCTION

Urease is an enzyme that hydrolyzes urea into ammonia and carbon dioxide. The main role of urease is to provide internal and external energy for the organism to use urea or hydroxyurea as a source of nitrogen. There are three enzymes that play a role in urea metabolism: arginase, urease and glutamine synthetase. In plants, only urease can capture nitrogen from urea.

Urease is found abundantly in some plant tissues, especially in the seeds of some family members of Fabaceae (legumes) and Cucurbitaceae (cucurbits). The urease enzyme plays a role in the seed germination process by degrading the urea formed from the activity of the arginase enzyme. Urease enzyme was isolated and purified from cotton *(Gossypium hirsutum)* seed (Menegassi et al., 2008), jack bean (*Canavalia ensiformis*) (Balasubramanian & Ponnuraj, 2009), *Momordica charantia* seed (Krishna, Singh, Patra, & Dubey, 2011), *Cajanus cajan* (Banerjee & Aggarwal, 2012). In this study urease has been isolated from black-eyed pea is a plant that has been known and cultivated by the community. How to consume a black-eyed pea is very limited thus black-eyed pea is not as popular as soybeans. Currently, black-eyed pea is only used as vegetables. Increased economic value black-eyed pea can be done by making it the source of the urease enzyme. So far, urease enzymes are still imported. So with the exploration of black-eyed pea as a source of urease can overcome adversity to get urease, reduce imports of such materials, and increase the income of farmers.

Some urease applications include industrial waste treatment, the alcoholic beverage industry, used in hemodialysis and its potential use in space missions as life support (EL-Hefnawy, Sakran, Ismail, & Aboelfetoh, 2014). Urease can also be used in medical diagnostics and is used for measurement of urea content in human blood. Urea is one of the protein metabolism products. Urease hydrolyzes urea into ammonia and carbon dioxide. The activity of urease can be determined by measuring the amount of ammonia produced. Ammonia levels can be detected using the Nessler method. The principle of the Nessler method is the reaction between Nessler reagent ($K_2H_4I_4$) with ammonium in an alkaline solution then form a brown yellow colloidal dispersion. The intensity of the color that will occur is directly proportional to the ammonium concentration. The color formed is measured by the spectrophotometer at a wavelength of 500 nm.

The activity of enzymes is influenced by environmental factors that alter the form of enzymes or inhibit substrate access to the active site of the enzyme. These environmental factors include temperature, pH, substrate concentration, incubation time, activators, and inhibitors. The aim of this research is isolation and partial purification of urease from black-eyed pea and to determine its characteristic.

EXPERIMENTAL

Materials

The tools that used in this study are glass tools, supporting tools such micropipette (DragonLAB), tip (Biologix), syringe (Terumo), digital scale (Ohaus), magnetic stirrer (Cimarec), refrigerator (LG), pH meter (Hanna Instrument), cuvet, UV-Vis spectrophotometer (Shimadzu UV-1800), SEM, centrifuge, blender, electrophoresis. The

ingredients that used in this study are black-eyed pea (*Vigna unguiculata ssp unguiculata* L.), urea (Merck, Germany), Nessler reagents (Potassium iodide, $MgCl_2$, KOH), acetic acid (Merck, Germany), sodium acetate, sodium phosphate (Merck, Germany), sodium Hydrogen phosphate (Merck, Germany), $CuCl_2$, $CaCl_2$, $NaCl_2$, $NiCl_2$, EDTA, Tris (hydroxymethyl) aminomethane (Merck, Germany), HCl (Merck, Germany), acetone (Merck, Germany).

Isolation of urease from black-eyed pea

a. Germination (EL-Hefnawy et al., 2014)

Two hundred grams of black-eyed pea soaked in water for 6 hours, then drained and put into a plastic container filled with wet cotton. The plastic container covered with plastic wrap and germinated in dark at room temperature. Observed germination with variation time 0, 2, 4, 6, 8, 10 and 12 days

b. Extraction (Banerjee & Aggarwal, 2012)

Two hundred grams of germinated black-eyed pea mashed using mortar and pestle then suspended in 800 mL phosphate buffer (pH=7) which has temperature 4 ºC. Occasional stirring for 3 hours was required then two layers of filtrate and suspension produced. The filtrate is separated by muslin cloth. The filtrate was centrifuged at 7.000 rpm for 15 minutes at 4 ºC. The supernatant was isolated and used as a crude contract.

Acetone fractionation

Crude extracts are gradually fractionated using acetone at cold temperatures (-12 ºC) with acetone concentration of 20, 40, 60, 80%. The protein precipitate was separated by centrifugation at 7000 rpm for 30 minutes. All the fractional precipitate were dissolved with 30 mL 0.2 M phosphate buffer (pH = 7). The dialysate solution was centrifuged for 10 minutes, the obtained filtrate was called 20% acetic fraction (FA 30), FA 40, FA 60, and FA 80. The acetone fraction then measured in activity. The crude extract and the acetone fraction with the highest specific activity will have further characterization include: temperature, pH, substrate and metal concentration. Molecular weight determination of fraction with the highest specific activity is done by the method of SDS PAGE.

Urease assay

The urease assay (Jayaraman & Jayaraman, 2004) was carried out as follows: Enzyme extract (0.05 mL) was added to 1 mL of 0.1 % urea concentration and 1 ml of phosphate buffer solution (pH = 7). The tube test was incubated at 35 °C for 15 minutes. The tube test is cooled with ice. One milliliter of 0.67 N H_2SO_4 was added to tube test immediately to stop urease enzyme activity. One mL Na-Wolframat was added to complete H_2SO_4 work. One tube test prepared for a blank solution, then it's filled with 3 mL of distilled water. Both tubes (blank and sample tube test) were centrifuged for 15 min and taken supernatant by filtration. The repetition is done 3 times. A total of 1.5 mL of a solution of each sample and the blank tube is taken. Each sample and blank solution were added with 250 µL of Nessler reagent. The solution measured using UV-Vis spectrophotometry at λ 500 nm. The urease estimation was performed using the curve of standard ammonium sulfate. One unit of activity is defined as "the amount of ammonia formed (µmol) per mL per minute from urea that hydrolyzes by urease".

Enzyme characterization

The first characterization performed on temperature variations. Determination of urease activity on temperature variation was done as same as enzyme assay but has a variation of incubation temperature which is 25, 30, 35, 40 and 45 ºC. The solution was incubated 15 minutes at phosphate buffer (pH 7 of 0.2 M) then the enzyme activity measured. At the optimum temperature conditions, the determination of urease activity on pH variation was performed on urea variation of 0.1% (w/v) pH 5 (citrate buffer), 6, 7 (phosphate buffer), 8 and 9 (Tris-HCl buffer) in 0.2 M buffer solution. To study the effect of incubation time on urease activity, the assay used time 10, 15, 20, 25 and 30 minutes under optimum temperature and pH conditions. To study the effect of substrate concentration on urease activity, the assay used urea concentration of 0.05; 0.075; 0.1; 0.125; 0.15% (w/v) dissolved at optimum pH then incubated at optimum temperature and incubation time. To study the effect of metal and EDTA the assay used $CaCl_2$, $NiCl_2$, NaCl, $CuCl_2$ and EDTA at concentrations 10^{-3}, 10^{-4} and 10^{-5} M. Enzyme activity was assayed by adding 1 mL urea 0.1% (w/v) and enzyme solution (0.05 mL enzyme + 0.1 mL metal + 1.85 mL 0.2 M buffer). The assay done under optimum conditions using the addition of the specified metal.

SDS PAGE analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed at reduced condition using 12% polyacrylamide as running gel and 5% polyacrylamide as stacking gel. Five and ten microliters of each protein fraction were analyzed by SDS-PAGE (LaemmLi., 1970). Protein band was detected using Coomassie brilliant blue dye. Molecular marker kit (Bio-Rad) was used for the reference of protein molecular weight.

Data analysis

Data were analyzed by ANOVA method to differentiate repetition variations and the differences between treatments. The fixed variables are pH, temperature, substrate concentration and the effect of metal. Each variable was analyzed by Anova one factor separately and performed gradually. The independent variable is the activity of urease. ANOVA results showed a significant difference followed by a Tukey test with 95% preciseness degree.

RESULTS AND DISCUSSION

Isolation of urease from black-eyed pea

Urease is enzyme that plays a role in the germination process. This enzyme can catalyze the pathogenic reaction of pathogenic urea in plant cells into ammonia and CO_2. So the first step that was done in urease isolation is germination process of black-eyed pea. Germination was done for 0, 2, 4, 6, 8, and 10 days. The germinated black-eyed pea was weighed and mashed with mortal in a cold temperature and subsequently dissolved in a 0.2 M phosphate buffer (pH = 7). The solution was filtered using a muslin cloth and then stirred for 3 hours at a cold temperature. The solution was centrifuged at 7000 rpm for 30 minutes at 4 °C. The supernatant was isolated and used as a crude extract. The crude extract then tested to measure enzyme activity. The highest enzyme activity was found on day eight (Data is not shown). Crude extract from day eight then used for further research to be fractionated using acetone solution.

Acetone fractionation

The urease fractionation from black-eyed pea using acetone is done to increase the purity of crude extracts. Acetone is an organic compound that can damage enzymes. Therefore before it's used acetone should be stored in the refrigerator. The superiority of acetone fractionation compared with ammonium sulfate fractionation is that acetone fractionation does not need a dialysis treatment process to remove any salts which remaining. Acetone fractionation is carried out at cold temperatures (to prevent enzyme damage) by using the ice placed around it. The results of fractionation of acetone can be seen in **Table 1**.

Table 1. Data of urease fractionation from black-eyed pea using acetone.

Phase	Activity (U/mL)	Protein (mg/mL)	Specific Activity (U/mg)	Purity
Crude Extract	211.26 ± 5.14	1.06 ± 0.00	198.74 ± 4.37	1
FA 20	175.06 ± 1.65	1.00 ± 0.00	175.52 ± 1.9	0.9
FA 40	230.12 ± 9.97	1.19 ± 0.01	193.37 ± 9.55	1
FA 60	257.11 ± 6.67	0.77 ± 0.00	335.74 ± 6.93	1.7
FA 80	152.45 ± 3.21	0.36 ± 0. 00	428.59 ± 8.30	2.2

An important parameter in the enzyme purification step is the specific activity of the extracted enzyme. A specific activity represents the true activity of the active protein only (EL-Hefnawy et al., 2014). The data in Table 1 show that specific activity was increased during the fractionation phase and in FA 80 a specific activity was obtained 428.59 U/mg with a purity level of 2.2 times.

Acetone is an effective organic solvent in the precipitation process. Organic solvents (eg acetone) will reduce the water dielectric constant, thereby reducing the solubility of proteins because the interactions between protein molecules are preferred over the molecules of proteins to water. Acetone fractionation was done to precipitate enzyme solution to produce enzyme precipitate in each fraction. The precipitation of the enzymes at these fractions occurs due to differences in solubility in the acetone solvent. Urease from black-eyed pea on FA 80 is thought as the one that have high solubility in acetone solvents.

Urease from black-eyed pea have been fractionated using acetone and followed by a freeze dryer then determined by molecular weight using SDS-PAGE. The results (**Figure 1**) show that urease from black-eyed pea is thought to have four polypeptides with a molecular weight of about 15, 17, 35 and 55 kDa. The Urease of the *Proteus mirabilis* strain comprises four polypeptides of molecular weight of about 15, 29, 45 and 66 kDa (Mohammed, Elshahaby, & Hafez, 2014). Urease *Lactobacillus reuteri* consists of three polypeptides with molecular weight of about 68; 16.1; and 8.8 kDa (Kakimoto, Sumino, ichi Akiyama, & Nakao, 1989).

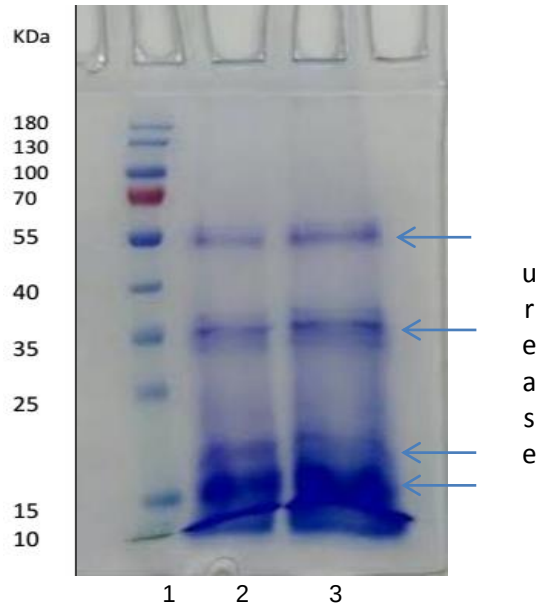

Figure 1. SDS page profile of protein marker (1) Urease from black-eyed pea has been fractionated using acetone and followed by a freeze dryer (2) (3).

Characterization of urease FA 80 from black-eyed pea

The effect of temperature on FA 80 urease activity

To determine the temperature that produces the optimum activity, a temperature variation was performed in the FA 80 urease from black-eyed pea. The temperature variations used were 25, 30, 35, 40 and 45 ºC (**Figure 2**).

The data in **Figure 2** shows that the urease activity at 25 °C is still low. This is because not all substrates bind to the active site of the enzyme, so the resulting product is still small. At a temperature of 30 °C the activity of the urease enzyme is increasing and stable to 35 °C. At this temperature the kinetic energy increases so as to increase the probability of substrate enzyme interaction to produce the product. The rise of temperature further decreases the activity of the urease enzyme significantly. The decrease in activity caused enzyme molecules beyond the energy barrier. This causes damage to the hydrogen and hydrophobic bonds that responsible for maintaining the three-dimensional enzyme structure (EL-Hefnawy et al., 2014). The data in **Figure 2** summed the FA 80 urease activity have optimum activity at a temperature of 30 - 35 ºC. These results are closely related to those reported (El-Shora, 2001).

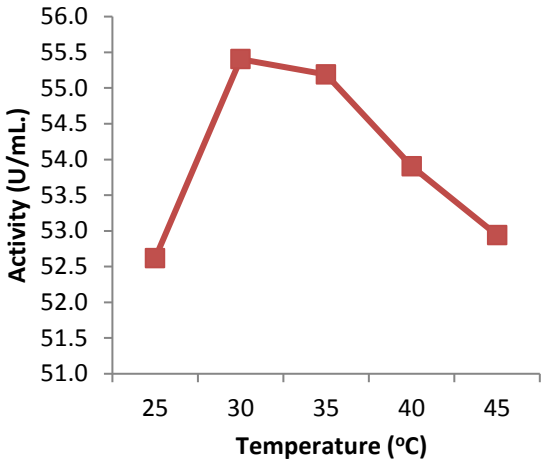

Figure 2. The effect of temperature variation on FA 80 urease activity at pH constant.

The effect of pH on FA 80 urease activity

To see the effect of pH on FA 80 urease activity, the assay used pH substrate variations of 5, 6, 7, 8 and 9. The results of pH effect on enzyme activity can be seen in **Figure 3**.

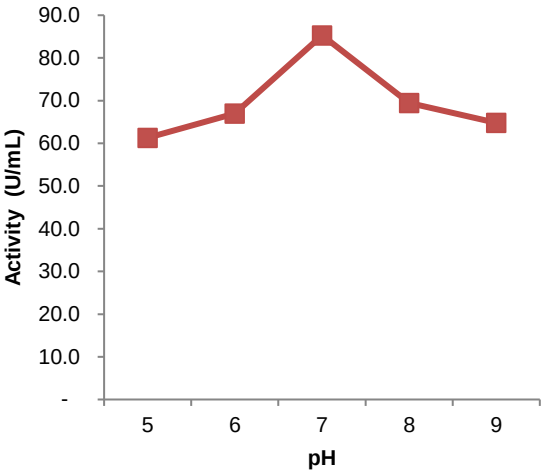

Figure 3. The effect of substrate pH variation on FA 80 urease activity at constant temperature

Based on the data in **Figure 3**, the FA 80 urease from black-eyed pea has the optimum activity at pH 7. From that result it may be concluded that FA 80 urease from black-eyed pea is categorized as urease with neutral pH value. The optimum pH of urease from *rhyzopus oryzae* is pH 7 (Geweely, 2006), from *Pisum Sativum* L seed is 7.5 (EL-Hefnawy et al., 2014), and from chickpea (*Cicer arietinum* L.) seed is 7.2 (Shaela, Sana, Habibur, & Shaha, 2013). pH substrate plays an important role in enzyme activity. The presence of active sites in amino acids will be influenced by changes in pH that may alter the ionization of these amino acids (Amin, Bhatti, & Asgher, 2010).

The effect of substrate concentration on FA 80 urease activity

To study the effect of substrate concentration on FA 80 urease activity, the assays used variation of substrate concentration of 0.05; 0.075; 0.1; 0.125; and 0.15% (**Figure 4**).

The data in **Figure 4** shows that the addition of substrate concentration increase the FA 80 activity and optimum urease activity obtained at 0.125% substrate concentration, furthermore at 0.15% substrate concentration, the data shows enzyme activity no longer

depend on substrate concentration. This is because the enzyme has been saturated with the substrate at that concentration (Kumari, Jain, & Malhotra, 2013).

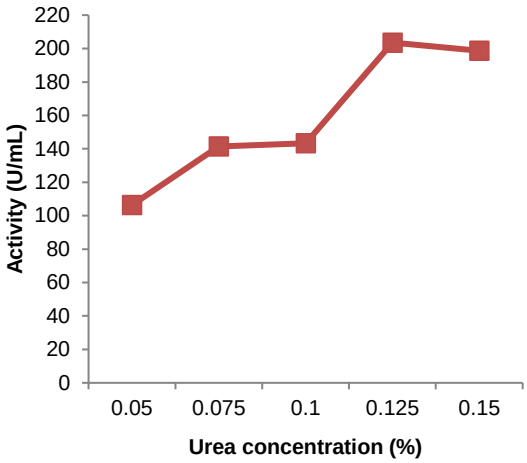

Figure 4. The effect of substrate concentration variation on FA 80 urease activity at constant pH and temperature.

The kinetics constants (K_M)

K_M value of FA 80 urease from black-eyed pea determined by incubating enzyme solution in urea solution at various concentrations under pH 7 and temperature 30 ºC. The K_M value is calculated using the Lineweaver-Burk equation (**Figure 5**).

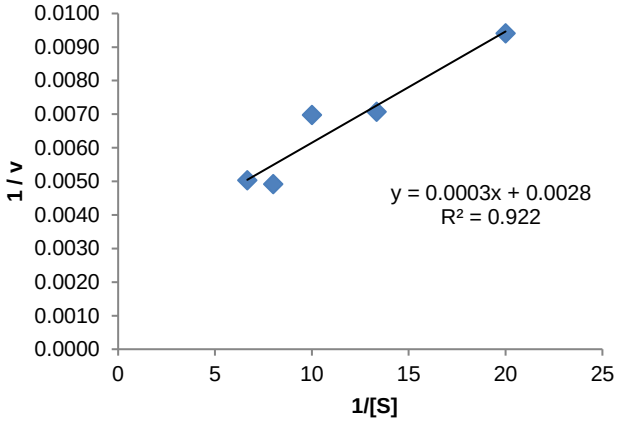

Figure 5. The relation curve between 1/V with 1/[S] based on the Lineweaver-Burk urease equation FA 80 from black-eyed pea.

The data in **Figure 5** obtained the value of K_M which is 17.8 mM. The value of K_M from *Momordica Charantia* was 34 mM (Krishna et al., 2011), urease from soybean seeds ranging from 19-476 mM depending on the buffer system that used. Research that conducted (EL-Hefnawy et al., 2014) obtained KM urease value from Pisum Sativum L is 500 mM, it shows low enzyme affinity to the substrate (Das, Kayastha, & Srivastava, 2002). Smaller K_M values indicate the strong bond between enzyme and substrate, substrate-enzyme complexes also form faster so that activity is higher.

The effect of EDTA and metal ions added to the FA 80 urease activity

The data of the effect EDTA and metal ions addition ($CaCl_2$, NaCl, $NiCl_2$ and $CuCl_2$) addition respectively at concentrations of 10^{-3}, 10^{-4}, and 10^{-5} M to FA 80 urease activity from black-eyed pea are shown in **Table 2**.

The data in **Table 2** shows that the addition of EDTA at concentrations of 10^{-3}.10^{-4}, and 10^{-5} M decreased the activity of FA 80. EDTA can reduce the activity of enzymes by chelates the metal that became enzyme activator. EDTA will bind metals that should bind to the active site of the enzyme and produce a complex. The active side is supposed to be active by the activator metal, but since it has been bound by EDTA it prevents substrate binding on the enzyme's active side then the enzyme activity decrease. The urease activity from black-eyed pea decreased with the addition of EDTA so it can be concluded that the urease from black-eyed pea is classified as metalloenzymes. Metalloenzymes are enzymes that bind strongly to certain metal ions that can activate enzymes (Kumari et al., 2013).

Table 2. Effect of EDTA and metal ions addition on FA 80 urease activity.

Reagents (M)	Relative Activity (%)	Inhibition (%)
Control	100	0
$CaCl_2$ (10^{-3})	74	26
$CaCl_2$(10^{-4})	89	11
$CaCl_2$(10^{-5})	93	7
NaCl (10^{-3})	72	28
NaCl (10^{-4})	91	9
NaCl (10^{-5})	91	9
EDTA (10^{-3})	73	27
EDTA (10^{-4})	83	17
EDTA (10^{-5})	85	15
$NiCl_2$ (10^{-3})	31	69
$NiCl_2$ (10^{-4})	35	65
$NiCl_2$ (10^{-5})	37	63
$CuCl_2$ (10^{-3})	35	65
$CuCl_2$ (10^{-4})	41	59
$CuCl_2$ (10^{-5})	50	50

Urease activity of FA 80 from black-eyed pea decreased with increasing concentrations of $CaCl_2$, NaCl, $NiCl_2$ and $CuCl_2$, and the lowest activity was found in the addition of $NiCl_2$. The research conducted by (Smith, King Jr, & Goodman, 1993) also found that addition of $NiCl_2$ metal ions decrease the activity of *Aspergillus niger* urease. The addition of $CuCl_2$ as heavy metals also decreases the activity of urease. The deflation of activity by 50% occurred in the addition of Cu^{+2} ions 10^{-5} M. Similar results were also found in urease from *Cajanus cajan* seed (Banerjee & Aggarwal, 2012). Inhibition of heavy metals such as Cu^{+2} in enzyme activity shows the presence of thiol (-SH) groups on the active site of the enzyme. These results are related to urease studies of *Cicer arietinum L.* (Shaela et al., 2013) and urease from jack bean (Krajewska, Zaborska, & Chudy, 2004).

CONCLUSION

Urease from black-eyed pea has been fractionated using acetone at a concentration level of 20, 40, 60, 80%. The specific activity increased during the fractionation phase and specific activity is obtained amount 428.59 U/mg with a purity level of 2.2 times in FA 80. The results of the electrophoretic analysis showed that FA 80 from black-eyed pea urease estimated to have four polypeptides with a molecular weight of about 15, 17, 35 and 55 kDa. The result of characterization was obtained the optimum FA 80 urease activity at temperature 30 ºC, pH 7, substrate concentration 0.125% with K_M value 17.8 mM. Urease FA 80 from black-eyed pea are classified as

metalloenzymes. The addition of $CaCl_2$, NaCl, $NiCl_2$ and $CuCl_2$ metals at various concentrations decreased the urea activity of FA 80. The higher metal concentration was added then the FA 80 urease activity decreased further.

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