Isolation, Purification, and Characterization of Urease From Pigeon Pea *(Cajanus Cajan)*

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Urease was isolated and purified by a series of citrate buffer extractions and chromatography through Sephadex Fine G-200. The molecular weight of the enzyme, obtained through Sephadex G-200 chromatography, was estimated at 540,000 daltons. SDS polyacrylamide gel electrophoresis set the molecular weights of the sub-units at 90,000, 46,000 and 31,000 daltons respectively. The isoelectric point, determined by isoelectric focusing, was about 5.8. Thiosemicarbazide was slightly acted upon, while urea was completely hydrolyzed by this enzyme. Th Km value obtained from the Lineweaver-Burk plot was 9.9 x 10^{-3} mM and V_{max} value of 189 units/mg protein. The Eadie Hofstee diagnostic plot rendered values of 10.4 x 10^{-3} mM and 193 units/mg protein for Km and V_{max} respectively. The low Km value of this enzyme for urea indicates its high affinity for the said substrate. The optimum conditions for the assay, with urea as substrate, were at pH 7.0 and temperature at 40°C. The crude enzyme was stable when suspended in 50% glycerol and stored at 0°C for 6 months. These properties are comparable to the commercially available Sigma jackbean urease.

Key words: pigeon pea, urease purification, urease characterization

Urease (Urea amidohydrolase E.C.3.5.1.5) is an efficient catalyst of urea hydrolysis. It is widely used as part of the reagent kit for Blood Urea Nitrogen (BUN) determination in hospitals and clinical laboratories. Thus the demand for the BUN diagnosis kit using this enzyme is high.

Since commercial preparations of this enzyme is not yet done locally, this study on its isolation and purification from a local source will be of utmost importance. This paper deals with the isolation and purification of urease from pigeon pea *(Cajanus cajan)*. Jackbean seeds *(Canavalia ensiformis)* grown in the institute of Plant breeding (IPB), UP Los Baños were also investigated and used as reference.

MATERIALS AND METHODS

Screening of Legumes for Urease Activity

Five dry legumes were selected for screening on the basis of the following criteria. They: a) belong to the leguminosae family; b) have a high protein content, the value of which is comparable to that of the Philippine jackbean seeds' protein; c) are available; d) are economical; e) have a high urease specific activity.

Extraction

Pigeon pea meal (50g) was mixed with 50 mL of 0.08 M citrate buffer, pH 7.5 in a Waring blendor for 2 min. The slurry was centrifuged in a Sorvall refrigerated centrifuge for 30 min. at 5000 x g. The extract was collected and set aside. The residue was extracted repeatedly until no urease activity was detected. All extracts with urease activity were pooled and labeled as Supernate I.

Supernate I was suspended in celite for 30 mins. with occasional stirring and centrifuged. The resulting supernatant, designated as Supernate II, was purified by gel filtration. Assays for urease and protein were performed on these extracts.

Sephadex Fine G-200 Chromatography

Supernate II, containing o.456 mg protein was loaded onto a column of Sephadex Fine G-200 (0.65 x 45 cm) previously equilibrated with 0.08 M citrate buffer, pH 7.5. Elution was carried out in the same buffer at a flow rate of 0.45 mL per 2 minutes. Fractions of 2.5 mL were collected and assayed for protein and urease. The fraction with urease activity was collected and labeled as Fraction I. This was rechromatographed onto the same column and eluted using the same buffer. This second fraction with enzyme activity was collected and designated as Fraction II. It was then concentrated and stored at 0° - 5°C after adding an equal volume of glycerol. Fractions collected were subjected to gel electrophoresis to determine their purity.

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Polyacrylamide Gel Electrophoresis

All electrophoresis were performed in a horizontal slab gel system following the procedure furnished by Pharmacia (1).

Molecular Weight Determination

Molecular weight of pigeon pea urease was determined using Sephadex Fine G-200 column (0.65 x 45 cm). Standard proteins, namely: thyroglobulin (MW = 669,000), apoferritin (MW = 443,000), catalase (MW = 232,000), bovine serum albumin (MW = 66,000) and pigeon pea urease were applied into the column and eluted with 0.08 M citrate buffer, pH 7.5. Fractions of 2.5 mL were collected. Elution volume (V_e) for each protein was measured. Blue dextran was used to determine the void volume (V_0).

SDS Polyacrylamide Gel Electrophoresis

Analytical sodium dodecyl sulfate polyacrylamide electrophoresis was carried out according to Weber and Osborn system (2.)

Isoelectric Point Determination

A twenty uL aliquot of Fraction II was applied into a 5% polyacrylamide slab gel containing ampholytes ranging from pH 3.5 - 9.5. It was electrofocused for 1.5h at 3°C for 30 minutes at constant power. The pH gradient was determined using a surface glass pH electrode.

Substrate Specificity

To determine its substrate specificity, pigeon pea urease was reacted with equal concentrations (10 mM) of different structure-related substrates namely: urea, thiourea, thiosemicarbazide, guanidine HCL and N,N-dimethylformamide.

Optimum Conditions

Fraction II was subjected to different conditions of temperature and pH to determine its optimum assay condition.

Determination of Km and Vmax

Urease assay was performed at various urea concentrations keeping all other conditions (pH and temperature) constant. Lineweaver-Burk plot were constructed where the Michaelis constant (Km) and maximum reaction velocity (Vmax) were deduced.

Stability Test

The crude extract was mixed with an equal amount of glycerol to make a 50% glycerol solution and stored inside the freezer. Enzyme activity was monitored every month.

Urease Activity Assay

The assay procedure was a modification of the urea test by Roche (3). A 17 mM urea was used as substrate for the reaction.

The reaction mixture consisted of 1 mL of 0.02 M citrate buffer, pH 7, 20 uL of urea and 100 uL urease. The solution was incubated at 40°C for 15 minutes. After cooling, 2.5 mL of salicylate reagent (0.187 M sodium salicylate and 0.002 M sodium nitroprusside) and 2.5 mL of alkaline hypochlorite solution, (0.55 M sodium hypochlorite and 6.25 M sodium hydroxide) were added and mixed. Absorbance was read at 620 nm after incubation at the same temperature for 5 mins. Specific activity was expressed as units of urease activity per mg protein. In this paper, a unit of activity refers to that amount of urease which liberated 1 umol of ammonia from 0.5 umol urea for 15 mins. at 40°C.

Protein Determination

Protein content was determined by Lowry et al as adopted by Concepcion (4), using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Screening of Legumes for Urease Activity

Among the materials screened for urease activity, pigeon pea was not only commercially available and cheap but also gave the highest specific activity (113 units/mg protein) as shown in Table 1. Under the same conditions, pigeon pea surpassed the value obtained for soybean (79 units/mg protein). Soybean has been reported (5) to be a good source of urease together with jackbean seeds, although the latter is said to have 16 times more urease content than soybean. Moreover, the specific activity of pigeon pea urease was found to be 94.17% of the Philippine jackbean (120 units/mg protein) which was used as reference material.

Isolation and purification of Urease from Pigeon Pea

Extraction

A summary of the purification scheme is presented in Table 2. Urease, based on studies by Howell et al (6), exhibits its optimum activity at M/8 citrate buffer, pH 7.5. The resulting supernate I, after several extractions, was found to have a specific activity of 115 units/mg protein. Celite was used to clear the solution, thus making supernate II ready for purification by gel filtration.

Sephadex G-200 Gel Filtration Chromatography

Supernate II containing 0.456 mg protein was

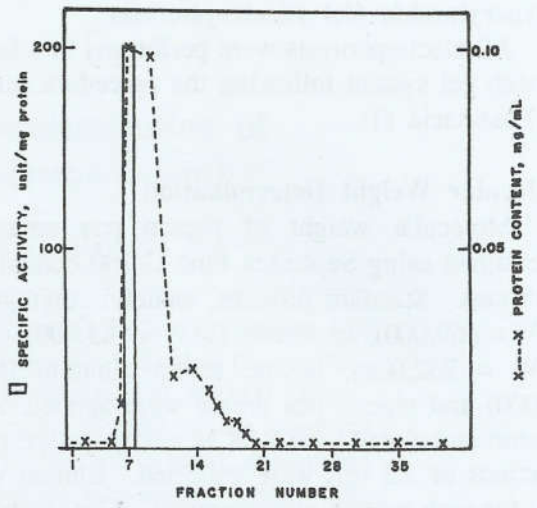
loaded onto the Sephadex G-200 column (0.65 x 45 cm) for purification. Separation of urease from other proteins present in Supernate II is illustrated in Fig. 1. The 7th fraction containing 2.5mL eluate was not only found to have urease activity but it also contains the highest protein concentration. In this step yielding Fraction I, a 1.73-fold purification with 88% recovery was obtained (Table 1). Fraction I which was about 50.71% of the original proteins was rechromatographed on the same column and was eluted with the same buffer.

Figure 2 shows further purification of Fraction I. This step yielded a 46.6% recovery with a specific activity of 436 units/mg protein. Fraction II accounted for 12.30% of the original proteins.

Table 1. Data for the Survey of Urease From Different Legumes

Sample Materials[b]	Total Protein (g/100)	Availability	Price/kg ₱	Urease Sp. Act. (units/mg protein)
Red Kidney bean	19.3	A	₱24.00	14
Cowpea	20.4	A	24.00	24
Mungbean	24.2	A	24.00	50
Soybean	35.8	NRA	32.00	79
Pigeon pea	21.2	A	24.99	113
Jackbean	24.1	NCA	b	120

[a] Value obtained from FNRI Food Composition Table
[b] courtesy of IPB, UPLB
A = available
NRA = not readily available
NCA = not commercially available



FRACTION NUMBER

Fig. 1. Chromatography on Sephadex G-200 of Supernate II. The sample (0.456 mg-protein) was loaded onto the column (0.65 x 45 cm) equilibrated with 0.00 M citrate buffer, pH 7.5 and eluted with the same buffer. The flow rate was 0.45 mL/2 min. Fractions of 2.5 mL were collected.

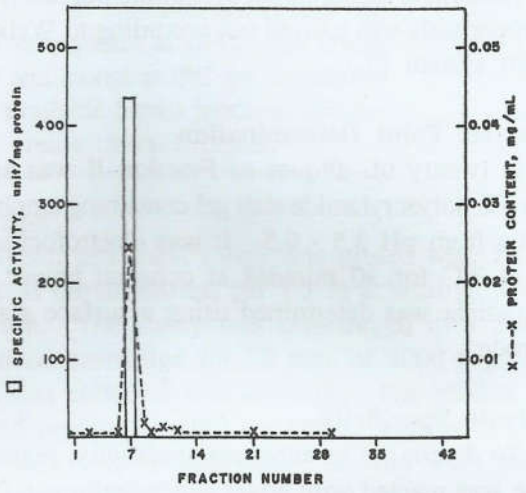


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Fig. 2 Chromatography on Sephadex G-200 of Fraction 1. The sample (0.198 mg protein) was rechromatographed onto the column (0.65 x 45 cm) and eluted with 0.08 M citrate buffer, pH 7.5. The flow rate was 0.45 mL/2 min. Fractions of 7.5 mL were collected.

Table 2. Purification Scheme of Urease from Pigeon Pea

Purification Step	Volume (mL)	Protein		Enzyme Specific Activity			Yield %	Purification Fold
		mg/mL	Total Amt (mg)	units/ mL	units/mg protein	Total units		
Crude Extract (Supernate I)	100	0.488	48.8	56.12	115	5612	"100"	"1.00"
Passing thru the celite (Supernate II)	100	0.456	45.6	53.59	118	5359	95.5	1.03
Gel Filtration Sephadex G-200 (Fraction I)	250	0.099	24.75	19.75	199	4938	88.0	1.73
Gel Filtration Sephadex G-200 (Fraction II)	250	0.024	6.00	10.46	436	2615	46.6	3.78

Determination of Purity by Electrophoresis

Figure 3 shows the bands of proteins produced in every step of the isolation and purification processes. Supernate I (B) revealed 3 bands of protein while Fraction I (C) produced 2 bands. Only one band resulted from Fraction II (D) which indicates an almost 100% purity of the enzyme isolated as determined by densitometry. Sigma jackbean urease (A), on the other hand, was noted to produce 4 bands, which may imply the presence of some impurities (Fig. 3). The single band from a duplicate segment of Fraction II gel (D) was found to have enzyme activity as shown in Fig. 4.

Characterization of Urease From Pigeon Pea

Native Molecular Weight

From the graph shown in Fig. 5, it can be deduced that the estimated molecular weight of pigeon pea urease is 540,000 daltons. This determination was carried out in Sephadex Fine G-200 column. The ratio of elution volume (Ve) to the void volume (Vo) of each protein was plotted against the logarithm of its molecular weight. The MW value obtained for pigeon pea urease was close to the MW of Sigma jackbean urease which is approximately 545,000 daltons (7).

Sub-Unit Molecular Weight

Both pigeon pea (Fraction II) and jackbean urease were subjected to SDS polyacrylamide gel electrophoresis. Using Dalton Mark VI as standard, the estimated molecular weight values obtained for pigeon pea urease were 90,000, 46,000 and 31,000 daltons for each corresponding band (Fig. 6).

While most investigators suggest that only one type of sub-unit exists (8) in urease, the proposed sub-unit molecular weights varied between 30 kD (10), 76 kD (11), 80 kD, 90 kD and 100 kD (12). Dixon et al (12) noted that it is possible the 90,000 - 100,000 dalton sub-unit is composed of two nearly identical 47,000 dalton polypeptides. Contaxis, et al (9), showed that native urease (480,000 daltons) can be dissociated stepwise to 60,000 and 30,000 MW sub-units. Some researches (9, 13) have suggested that sub-units do not contain polypeptide chains linked by disulfide bonds.

Considering the above observations, it may also be possible for pigeon pea to be easily dissociated. Hence, the 46,000 and 31,000 dalton polypeptides could be the result of further dissociation of 90,000 dalton sub-units. If Dixon (14) claimed that urease contains six sub-units, then the pigeon pea urease having a MW of 540,000 daltons could also be a hexamer, having six 90,000 dalton sub-units. The sub-unit MWs obtained for Sigma jackbean urease were 89,000, 52,000 and 28,000 daltons respectively.

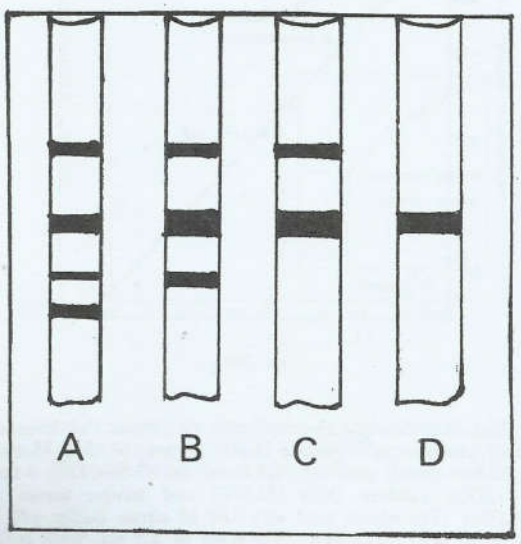

Fig. 3. Conventional Polyacrylamide Gel Electrophoresis of pigeon pea urease. Sigma jackbean urease (A), Supernate 1 (B) Fraction 1 (C,), Fraction 11 (D).

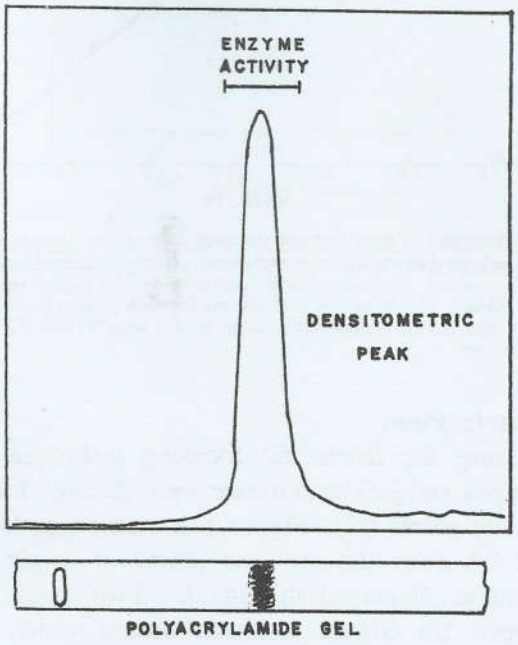

Fig. 4. Polyacrylamide Gel Electrophoresis of Pigeon Pea Urease (Fraction II) showing the densitometric peak which corresponds to the protein band in gel found to possess enzyme activity.

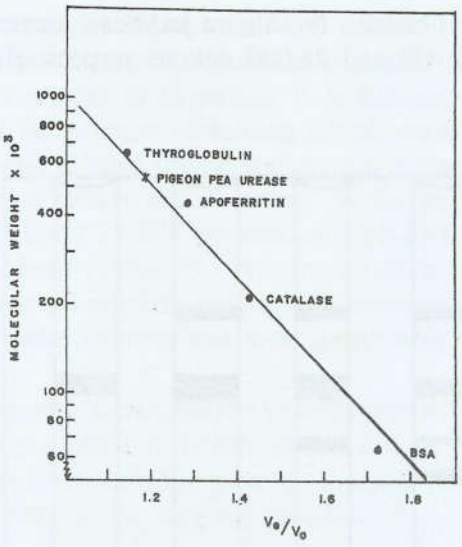

Fig. 5. Molecular Weight Determination of Pigeon Pea Urease by gel filtration of proteins on Sephadex G-200 column (0.65 x 45 cm). The protein markers used were thyroglobulin (MW=669,000): apoferritin (MW=443,000); catalase (MW-232,000) and bovine serum albumin (MW=66,000). The eluent used was 0.08 M citrate buffer, pH 7.5 with a flow rate of 0.45 mL/2 min. Fractions of 2.5 mL were collected.

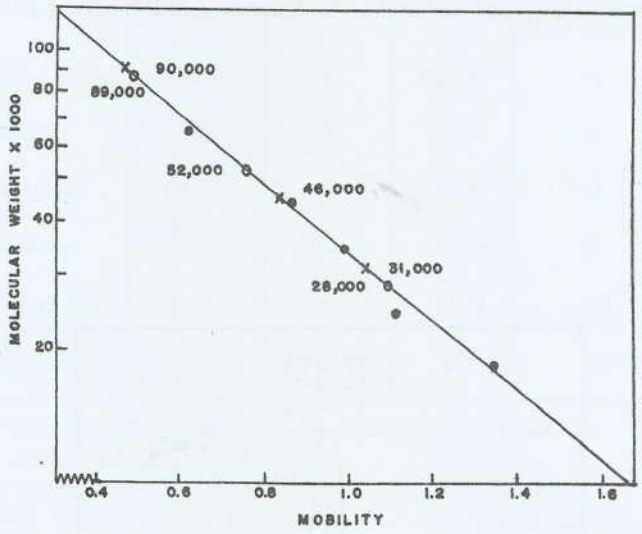

Fig. 6. Molecular Weight Determination of Pigeon Pea Urease sub-units by SDS polyacrylamide gel electrophoresis. Using Dalton Mark VI (•) as protein markers, the molecular weights obtained for pigeon pea urease (x) were 90,000, 46,000 and 31,000 daltons for each protein band whereas the MWs obtained for Sigma jackbean urease (o) were 89,000, 52,000 and 28,000 daltons.

Isoelectric Point

Using the isoelectric focusing technique, both pigeon pea and jackbean urease were checked for their isoelectric points (pI). Pigeon pea urease was focused at pH 5.8 even though the points of origin were different as displayed in Fig. 7. Two bands were developed for Sigma jackbean urease which were focused at pHs 5.28 and 4.25.

Table 3 gives a summary of the characteristics obtained for pigeon pea and jackbean urease. It can be inferred from the table that urease from both sources have similar characteristics.

Kinetics

Substrate specificity. Although urease has been described as absolutely specific for urea (15), other studies (16) proved this wrong. To determine the specificity of pigeon pea urease, different structure-related substances other than urea, namely: thiourea, thiosemicarbazide, guanidine-HCI, N, N-dimethylformamide and nicotinamide were used as substrates. Figure 8 shows this enzyme has high specificity for urea. Other compounds used did not give any reaction. Thiosemicarbazide was only slightly acted upon. Bennet and Wren (17) claimed that thiourea was a substrate of urease having a Km similar to that of urea, but Dixon, et al (18) found no evidence of enzymatic hydrolysis on this substance. Results obtained in this study agree with Dixon's observation that thiourea is not a substrate of urease.

Km and Vmax. The Km of urease varies according to the conditions of measurements. As mentioned in earlier studies, the Km of jackbean urease ranges from 0.003 (15) to 0.017 M (19). The Km of this enzyme was determined by measuring the initial reaction velocities from different urea concentrations, incubated at 40°C for 15 mins. and at pH 7.0. The Lineweaver-Burke plot (Fig. 9) gives an estimated Km value of 9.9 x 10^{-3}M and a Vmax of 189 units/mg protein, while the Eadie-Hofstee diagnostic plot (Fig. 10) shows a Km value of 10.4 x 10^{-3}M and a Vmax of 193 units per mg protein. In contrast, the Michaelis Menten Curve (Fig. 10 inset) shows a Km of 9.8 x 10^{-3}M.

Effect of pH. Figure 11 shows that the activity of pigeon pea urease is optimal at pH 7. Its activity increases from pH 5 to its maximum at pH 7 by 65% and decreases slowly as the pH increases by 12.42%. The optimum pH of Sigma jackbean urease was also determined under the same condition, increasing from pH 5 to its maximal pH 7 by 14.30%. A decrease by only 6.85% is observed at basic pH. Results suggest that the pigeon pea urease is more sensitive at acidic pH than the jackbean urease although both show similar behavior at basic pH.

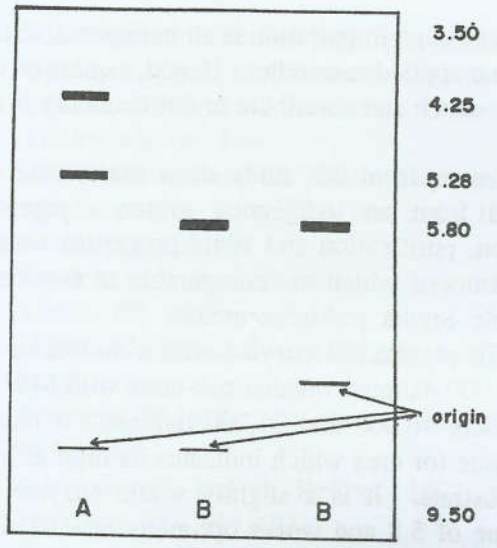

Fig. 7. Isoelectric Focusing of Pigeon Pea Urease. An aliquot from pigeon pea and Sigma jackbean urease were electrofocused on a 5% polyacrylamide gel with a pH gradient of 3.5 - 9.5. The pI for pigeon pea urease (B) was estimated to be at pH 5.0 while that of jackbean urease (A) was at 5.20 and 4.25.

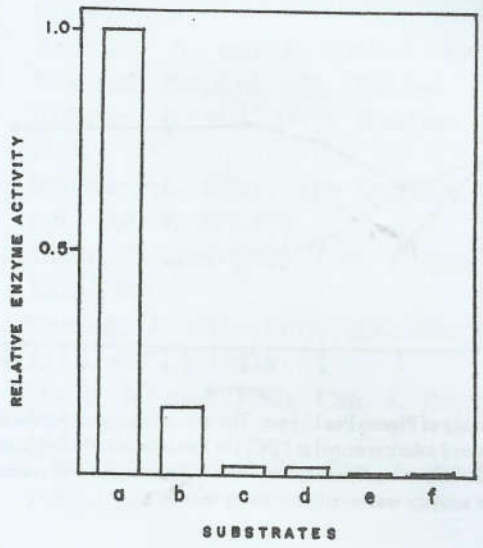

Fig. 8. Substrate Specificity of Pigeon pea Urease. Different related compounds namely: (a) urea, (b) thiosemicarbazide (c) thiourea (d)guanidine HCL (e) nicotinamide, and (f) N,N-dimethylformamide were used as substrates in the assay. Pigeon pea urease showed specificity for urea and a little reaction was exhibited for thiosemicarbazide.

Table 3. Comparison Between Pigeon Pea Urease and Sigma Jackbean Urease

Characteristics	Pigeon Pea Urease	Sigma Jackbean Urease
Molecular Weight	540,000	545,000 (11)
Sub-unit MW	90,000	89,000
	46,000	52,000
	31,000	28,000
Isoelectric Points	5.8	5.28
		4.85

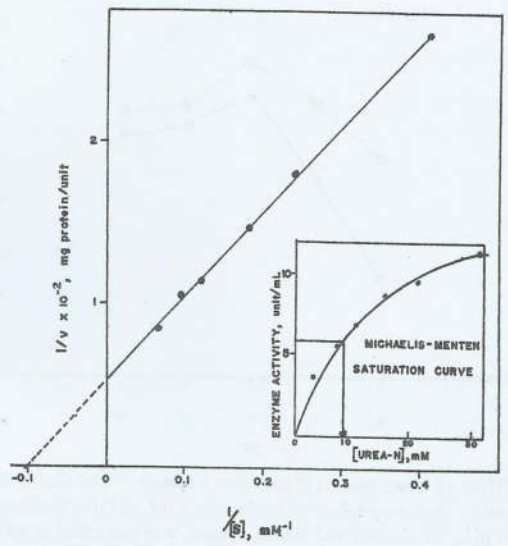

Fig. 9. Lineweaver-Burk plot for Pigeon Pea Urease. Specific activity was measured at varying concentrations of urea. The values were calculated to be 9.9 x 10^{-3} mM for Km and 109 units/mg protein for Vmax.

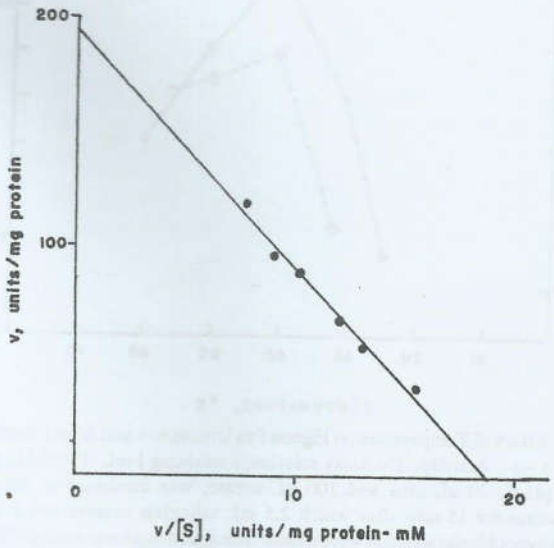

Fig. 10. Eadie Hofstee diagnostic plot for Pigeon Pea Urease. The values were calculated to be 10.4 x 10^{-3} mM for Km and 193 units/mg protein for Vmax.

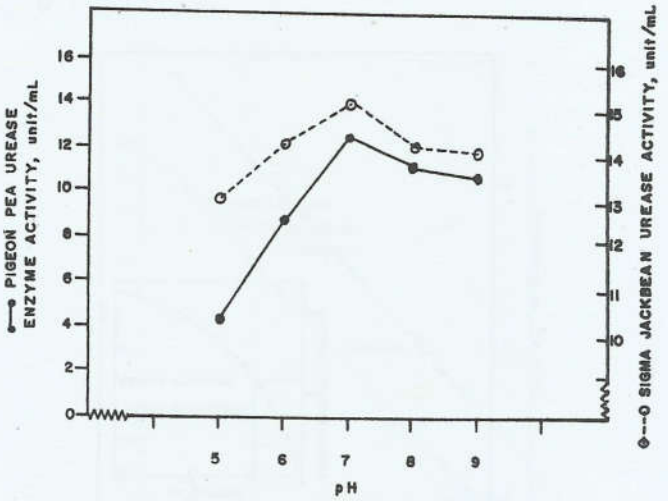

Fig. 11. Effect of assay pH on Pigeon Pea Urease • - • and Sigma Jackbean o - o Activity. The assay solution, containing 1 mL of 0.02 M citrate buffer, at different pHs, 20 uL urea and 100 uL urease, was incubated at 40°C for 15 min after which 7.5 mL salicylate reagent and 2.5 mL alkaline hypochlorite solution were added. The absorbance was read at 620 nm. Each point represents mean value of 5 determinations.

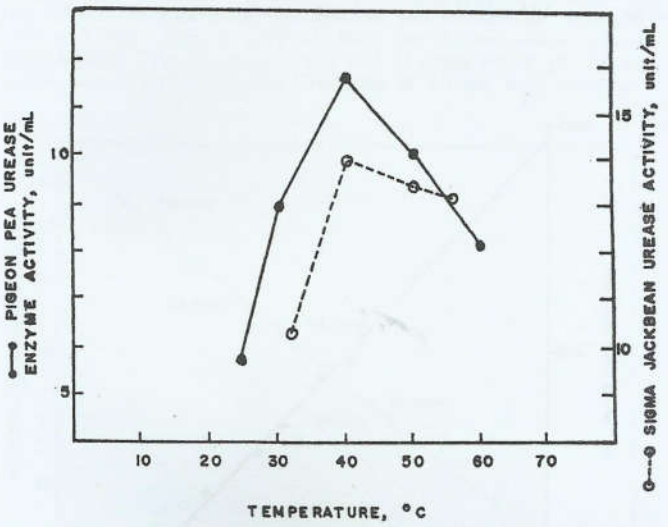

Fig. 12. Effect of Temperature on Pigeon Pea Urease • - • and Sigma Jackbean Urease o --o Activity. The assay solution, containing 1 mL of 0.02 M citrate buffer, pH 7, 20 uL urea and 100 uL urease, was incubated at different temperatures for 15 min after which 2.5 mL salicylate reagent and 2.5 mL alkaline hypochlorite solution were added. The absorbance was read at 620 nm. Each point represents mean value of 4 determinations.

Effect of Temperature. The effect of temperature was determined on both pigeon pea and Sigma jackbean urease. Pigeon pea urease activity at pH 7 is optimal at 40°C as shown in Fig. 12. A 51% increase in activity is observed when the temperature is raised from 25°C to 40°C. Only a 13.44% loss is observed at 50°C but a further increase of 10° gives a 29.46% loss of activity.

Stability. The stability of Fraction II, stored at 0°C, was monitored for two months. There was no loss of activity. The crude enzyme extract in 50% glycerol is apparently stable when stored inside the freezer as shown in Figure 13 since duplicated sample stored inside the refrigerator shows loss of activity.

Urease, an enzyme long introduced in the Philippines through importation is an indispensable tool for basic and applied researches. Hence, a question of how basic research can contribute to our economy is always asked.

Results from this study show that urease can be isolated from an indigenous source - pigeon pea. Isolation, purification and some properties were studied, results of which are comparable to commercially available Sigma jackbean urease.

The pigeon pea enzyme with a molecular weight of 540,000 daltons contains sub-units with MW values of 90,000, 46,000 and 31,000 daltons. It has a low Km value for urea which indicates its high affinity for the substrate. It is a slightly acidic enzyme with a pI value of 5.8 and works optimally at 0.02M citrate buffer, pH 7.0 at 40°C. Suspended in 50% glycerol at pH 7 and stored at 0°C, this enzyme is stable for at least 6 months.

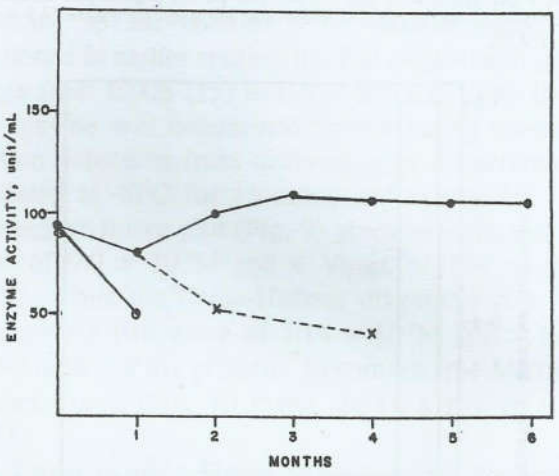

Fig. 13. Stability of Pigeon Pea Urease. The x----x line stands for the Supernate I in 50% glycerol solution stored at 12°C; • - • line indicates the duplicate sample stored at 0°C while o - o stands for the extract without glycerol stored at 12°C. The enzyme activity was monitored every month.

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