PURIFICATION, IMMOBILIZATION AND APPLICATION OF UREASE ENZYME FROM PIGEONPEA SEEDS (*Cajanus cajan* L.)

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Abstract

Urease (E.C 3.5.1.5) was isolated and purified from dehusked pigeonpea seeds (*Cajanus cajan* L.). The purification method involved ammonium sulphate precipitation, Sephacryl S-200 gel filtration and DEAE Sepharose ion-exchange chromatography. The specific activity, the relative purity of the enzyme urease was increased by 398 fold over that of crude extract. The purified urease enzyme was found to have the specific activity of 306.67 μmol $min^{-1}mg^{-1}$ of protein. The purity of the enzyme was confirmed by non SDS-PAGE as single band. The molecular weight for purified urease was found to be 478 kDa (kilodalton). The immobilization of the purified urease was carried out by gel entrapment technique using sodium alginate. Determination of urea content in dried skate fish before and after removal by pigeonpea urease was carried out by using immobilized urease. After 66 h, 75.94 % of urea was removed from dried skate fish.

Keywords: Urease, Pigeonpea, non SDS-PAGE, immobilization, sodium alginate, gel entrapment technique

Introduction

Urease (urea amidohydrolase, EC 3.5.1.5) is a nickel-containing enzyme that catalyzes the hydrolysis of urea to yield ammonia and carbamate, the latter compound decomposes spontaneously to generate a second molecule of ammonia and carbon dioxide (Andrew *et al.*, 1986; Hausinger, 1993; Mobely*et al.,* 1995; Park and Hausinger, 1995).

$$H_2N–CO–NH_2 + H_2O \xrightarrow{\text{Urease}} NH_3 + NH_2–COOH \xrightarrow{\text{Spontaneous}} 2NH_3 + CO_2$$

Urease has many industrial applications, e.g., in diagnostic kits for measuring urea, in alcoholic beverages as a urea reducing agent (Fujinawa and Dela, 1990; Fumuyiwa and Ouch, 1991), and in biosensors of haemodialysis systems for determining blood urea (Smith *et al.,* 1993). Urease is also

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important in medical field because it may help human pathogens survive in the stomach lining and cause peptic ulcers.

Pigeonpea(Figure 1) is widely cultivated in Upper Myanmar. Thus, urease can be obtained from a rather inexpensive and readily available source like Pigeonpea. In this research work, urease was isolated and purified from Pigeonpea seeds collected from Natogyi Township, Mandalay Region.

Botanical description of Pigeonpea as follows.

Family	-	Fabaceae
Genus	-	*Cajanus*
Species	-	*cajan*
Scientific Name	-	*Cajanuscajan* L.
English Name	-	Pigeonpea
Myanmar Name	-	Pe-sin-ngone

(a) (b) (c)

Figure 1: (a) Flower, (b) pods and (c) seeds of Pigeonpea

Materials and Methods

Common laboratory tools were used throughout the experiment at the Biochemistry Research Division, Department of Medical Research (Lower Myanmar). The advanced instruments which are used in the characterization of samples and purification of pure compound are shown below.

1. Dialysis bag, digital balance (A-200S, Sartorius Service Hot Lines, Japan)
2. Lyophilizer (Freeze-dryer machine) (J.T/C, 4451F, LABCONCO,–150ºC)

3. Refrigerated centrifuge (Model CD-70 SR, maximum speed 5,000 rpm, Tomy Seiko Co., Ltd, Tokyo, Japan)
4. UV visible spectrophotometer (Bausch and Lomb Spectronic – 21)
5. Perista mini-pump (SJ-1220)
6. Biomini UV-monitor (AC-5200 L), mini-recorder (SJ-3462)
7. Mini-collector (SJ-1400NC) (Atto Corporation, Tokyo, Japan)
8. Fraction collector (SF-200A, Toyo Co., Ltd., Japan)

Sample Collection

Pigeonpea seeds were collected from Natogyi Township, Mandalay Region.

Dehusked pigeonpea seeds were ground into fine powder by a blender. Then the raw materials were stored under 4ºC in the well-stoppered glass bottle and used throughout the experiment.

Extraction of Urease from Dehusked Pigeonpea Seeds

Dehusked pigeonpea seeds were ground into fine powder by a blender. Pigeonpea seed powders (227 g) were thoroughly mixed with 800mL of phosphate buffer of pH 7.0 containing 1mM EDTA and 1mM β-mercaptoethanol and left for overnight at 4ºC. The mixture was filtered through two layers of muslin and the filtrate was collected.

Solid ammonium sulphate (50.00 g) was added slowly to the resulting filtrate to make 20% saturation, stirred slowly for 20 min and centrifuged at 3200 rpm for 30 min. The pellet (0-20 portion) was discarded and the supernatant fraction brought to 60% saturation by slow addition of 101.40 g of solid ammonium sulphate. Precipitated protein urease was sedimented as above. The protein suspension was transferred to a dialysis tubing and dialyzed against in a 10-fold excess of the same buffer with three changes after allowing 2h for equilibration.

After removal of ammonium sulphate by dialysis, the dialyzed sample was concentrated with acetone and then the concentrated sample was kept in a lyophilizer for 20 min. Then it was centrifuged at 3500 rpm for 10 min. After that, the enzyme precipitate was collected yield %. Urease activity was

determined by Nessler's method at 480 nm and protein content was determined using Bovine Serum Albumin from Sigma at 550 nm.

Purification of Urease Enzyme by gel filtration Chromatography

Gel filtration was carried out in a column (2 x 39 cm) packed with pre-swollen Sephacryl S-200 using 0.1M phosphate buffer (pH 7.0) and equilibrated with the same buffer (500 mL).

Crude urease (1.0 g) was dissolved in 2 mL of pH 7.0 phosphate buffer. This solution was applied to a Sephacryl S-200 gel filtration column (2 x 39 cm) previously equilibrated with the same buffer. The flow rate was adjusted to 12 mL hr^{-1} by a mini-pump and 1.5 mL fractions were collected per tube using a fraction collector. After collection, the protein content of each tube was checked by measuring the absorbance at 280 nm wavelength using a UV-visible spectrophotometer.Each tube was also measured for urease activity in the same manner.The fractions with highest urease activities were pooled and concentrated with acetone (1:9 ratio).

Purification by Ion Exchange Chromatography

The ion exchange chromatography was packed in a column (1 × 25 cm) with DEAE Sepharose using 0.1M sodium phosphate buffer (pH 7.0) and equilibrated with the same buffer.

The concentrated urease fraction obtained by Sephacryl S-200 gel filtration column was applied to a DEAE Sepharose. The flow rate was adjusted to 12 mL/h using a mini-pump and 1.5mL fraction was collected per tube using a mini-collector. During collection, the protein content of each tube was continuously monitored at 280 nm wavelength by a bio-mini UV-monitor. After collection, the protein content of each tube was confirmed by measuring at 280 nm wavelength using a UV-visible spectrophotometer.

The active protein fractions of fraction numbers 10 to 16 and 31 to42 were pooled. The pooled fraction of highest urease activity was concentrated with acetone (1:9 ratio) and kept in a lyophilzer for 10 min. Then it was centrifuged at 5000 rpm for 5 min, and the enzyme precipitate was taken out.

Electrophoresis and molecular weight determination

Electrophoresis was begun when the polymerized gel slab was placed in the electrophoresis apparatus containing electrophoresis buffer (pH 8.3). The 20 µL sample contained 100 µg of protein. Both sample (20 µL) containing 100 µg of protein and standard molecular weight marker proteins (20 µL) containing Urease-tetramer, Urease dimer, Albumin, bovine dimer and Albumin, chicken egg were loaded in the sample well and standard well respectively.

Initially, the current was maintained at 15 mA (100 V) for 15 min until the samples had travelled through the stacking gel. Then the current was increased to 30 mA (200 V) until the bromophenol blue dye had reached near the bottom of the gel slab. It was required for 1.5 h.

At the end of running, the gel was washed with distilled water for five times, and fixed with fixing solution. Then the gel was placed in Petri dish with staining solution for 2 h, and subsequently destained with destaining solution until a clear gel was obtained.

Immobilization of Urease Enzyme by Gel Entrapment

Sodium alginate powder (3.0 g) was added to the distilled water (100 mL) in a 500 mL beaker and stirred for 30 min. After sodium alginate was completely dissolved, the solution was left undisturbed for 30 min to eliminate air bubbles that could later be entrapped and cause the beads to float. It was boiled until sodium alginate had hydrated and left to cool. Next, 1 mL urease enzyme solution was mixed with 10 mL of 3% sodium alginate solution. The enzyme alginate mixture was obtained.

With a 5 mL disposable syringe, enzyme alginate mixture was dipped into 1% calcium chloride solution from a height of approximately 10 cm. The beads formed were hardened in calcium chloride solution for 30 min and then washed with distilled water before use. The alginate beads were stored at 4°C in 0.1 M phosphate buffer (pH 7.0)

Comparison of the Activities of Soluble Urease and Immobilized Urease

The procedure for urease activity determination was the same as mentioned above except that the final purified free enzyme solution (0.1 mL) and 13 immobilized enzyme beads (0.1 mL) were used in each test tube.

For comparative study of urease activities between soluble and immobilized urease enzyme, the absorbance of the orange brown colour of each reaction mixture was measured at 480 nm.

Determination of Optimum pH for Soluble and Immobilized Urease Catalyzed Reaction of Urea

For soluble urease, an aliquot (0.1 mL) of phosphate buffer (pH 7) and 0.1 mL of 0.5 M urea solution were brought to incubate at 37°C for 5 min. The reaction was started by adding 0.1 mL of final purified enzyme solution. After 5 min, 0.1 mL of 0.5 M hydrochloric acid was added to terminate the reaction. After that, 4 ml of distilled water and 0.1 mL of Nessler's reagent were added. The amount of ammonia liberated was measured at 480 nm in a UV-visible spectrophotometer against the reagent blank.

For immobilized urease, the whole of the above procedure was carried out using 13 beads (0.1 mL) of immobilized urease in place of 0.1 mL of enzyme solution.For blank solution, 0.1 mL of distilled water was used in place of 0.1 mL of enzyme solution.Similarly phosphate buffer solution of different pH values (5.0, 5.5, 6.0, 6.5, 7.5 and 8.0) were employed in the above experiment instead of phosphate buffer (pH 7.0) for each experimental run. The absorbance values were measured at 480 nm and the optimum pH was found from the enzyme activity *vs* pH plot.

Application of Immobilized Urease for Determination of Urea in Dried Skate Fish

Immobilized urease was used for determination of urea in dried skate fish (Figure 2 a). Dried skate flesh (Figure 2 b) (without bone) (20 g) was ground using 20 mL of distilled water and the liquid was squeezed and this process was repeated for another 2 times. Then residual precipitate was

ground with 10 mL of distilled water and the liquid was squeezed and process was repeated for another 3 times. The total liquid was centrifuged at 3200 rpm for 30 min and the clear supernatant fluid was made up to 100 mL with distilled water in a volumetric flask to obtain the sample solution.

(a) (b)

Figure 2: (a) Skate fish and (b) Skate flesh

Sample solution (0.1 mL) was added to a test tube and 0.1 mL of phosphate buffer (pH 7.0) were also added. After that, this mixture was incubated at 37°C for 5 min. The reaction was started by adding 13 beads of immobilized enzyme (0.1 mL of enzyme solution). After that, this mixture was incubated at 37°C for 5 min. A0.1 mL of 0.5 M HCl was added to the reaction mixture in order to terminate the reaction. After that, 4 mL of distilled water and 0.1 mL of Nessler's reagent were added. The absorbance of the orange brown colour of the reaction mixture was measured at 480 nm.

Urea content in dried skate flesh was removed by prepared urease and the remaining urea content in dried skate flesh was determined by prepared immobilized urease. Dried skate flesh (200 g) was thoroughly mixed with 10 mL of 1% of urease enzyme solution and dried in sunlight.

After 12 h, 20 g of dried skate flesh was taken out and ground using 20 mL of distilled water and urea content was determined using immobilized urease. Determinations of urea content were also carried after 18 h, 36 h, 42 h and 66 h after mixing with prepared urease and dried in sunlight.

Results and Discussion

Urease was isolated from dehusked pigeonpea seed powder by ammonium sulphate precipitation method. Ammonium sulphate precipitation method was chosen for salt fractionation because of its high solubility in water, lack of toxicity, cheapness and lack of harmful effects on enzyme activity.

Urease Purification

Urease was purified by gel filtration chromatography employing Sephacryl S-200 followed by anion exchange chromatography using DEAE-Sepharose (Figure 3).

Purification of pigeonpea urease is summarized in Table1. The enzyme was enriched 398 fold compared to its crude extract. The purified enzyme displayed a specific activity of 306.67 µmol min^{-1} mg^{-1} protein.

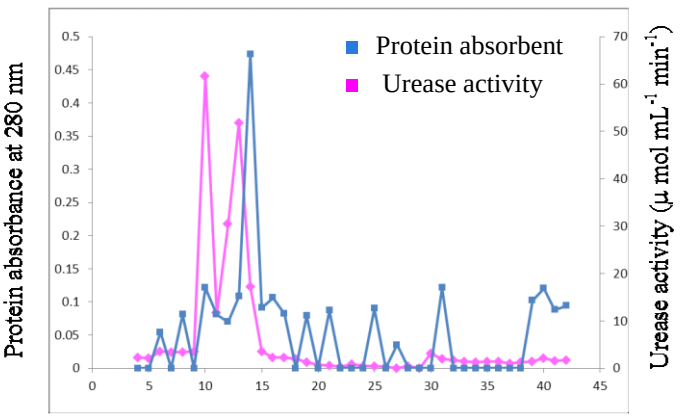


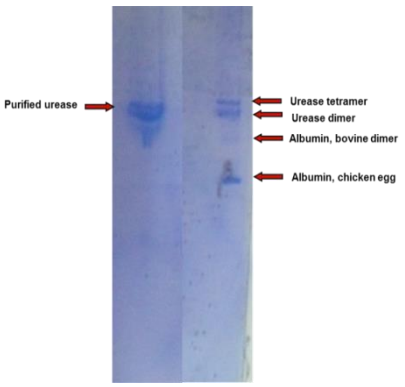
Figure 3: Chromatogram of urease activity on DEAE sepharose after sephacryl S-200 gel filtration chromatography

Table 1: Purification of Pigeonpea Urease

Purification step	Total Activity (µmol min^{-1})	Total protein (mg)	Specific activity (µmol min^{-1} mg^{-1})	Purification (fold)
Crude extract	51962.65	67195.00	0.77	1.00
20%$(NH_4)_2SO_4$ Precipitation	49504.66	5926.25	8.35	10.84
Dialyzed sample after 60 % $(NH_4)_2$ SO_4 precipitation	812.30	38.80	20.93	27.18
Sephacryl S-200	653.64	23.28	28.10	36.49
DEAE-Sepharose	263.74	0.86	306.67	398.00

Molecular Weight Determination by Non SDS-PAGE

In this study, proteins from the Pharmacia high molecular weight (HMW) calibration kit: urease tetramer (480,000), urease dimer (240,000), albumin, bovine dimer (132,000) and albumin, chicken egg (45,000) were used for molecular weight determination by non SDS-PAGE. Proteins migrated in polyacrylamide gels of the correct porosity according to protein size. Figure 4 depicts the electrophoretic examination of the urease from final purification step.



(a) (b)

Figure 4: Photograph of sodium dodecylsulphate polyacrylamide gel electrophoresis
Lane (a)Purifiedurease
Lane(b)Standardhigh molecular weight marker proteins

The purified urease enzyme showed a single band on non SDS-PAGE.

The distance migrated by each protein was used to construct a standard curve from which the molecular mass of urease can be calculated based on the mobility under the same electrophoretic condition (Table 2).

Table 2: Relationship between molecular weight of standard high protein markers (HMW) and relative mobility (R_f) values obtained from non SDS-PAGE

No.	HMW marker protein	Molecular weight (Dalton)	Log molecular weight	R_f
1.	Urease tetramer	480,000	5.681	0.486
2.	Urease dimer	240,000	5.380	0.527
3.	Albumin, bovine dimer	132,000	5.121	0.625
4.	Albumin, chicken egg	45,000	4.653	0.777

The R_f value of purified urease was found to be 0.49. From Figure 5 the standard curve of log of known HMW marker proteins *vs*R_f values, the molecular weight of urease from dehusked Pigeonpea seedswas determinedas 478 kDa.

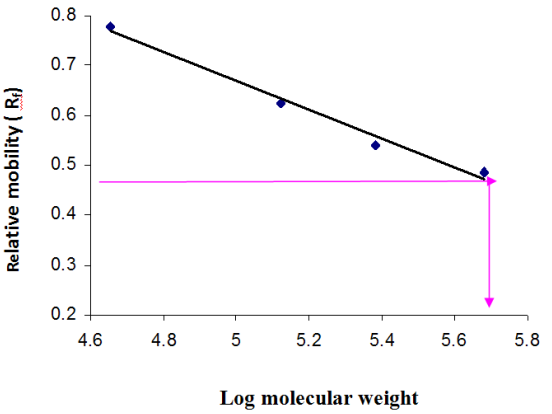


Figure 5: Log molecular weight of standard protein markers as afunction of $R_f$values

Immobilization of Urease by Entrapment in Calcium Alginate Beads

Figure 6 shows the immobilized urease enzyme beads by gel entrapment method. The total amount of urease immobilized within calcium alginate bead was 7.4 µLprotein per bead.

Figure 6 : Photograph of immobilized urease in calcium alginate beads

Comparison of the Activities of Soluble Urease and Immobilized Urease

The activities of both soluble urease and immobilized urease were examined in 0.1 M phosphate buffer (pH 7) at 37°C. The enhancement in the activity of the entrapped enzyme in comparison to that in the usual aqueous medium was noted. The data recorded in Table 3 showing about 1.6 fold increase in the activity.

Table 3: Comparison of the Activities of Soluble Urease and Immobilized Urease

No.	Urease Activity (µmol min^{-1} ml^{-1} of ammonia)		Ratio = $\frac{B}{A}$
	In aqueous medium(A)	Entrapped medium (B)	
1	309.74	493.72	1.59
2	291.65	482.45	1.65
3	282.73	474.61	1.67

Effect of pH on the Activities of Soluble and Immobilized Urease

The pH at which a certain enzyme will cause a reaction to progress most rapidly is called the optimum pH. At optimum pH, enzyme activity is maximum and above and below this pH, the activity is low (Lehningher, 1975).

In the present work the activities of soluble urease and immobilized urease were investigated at different pH values (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) with urea substrate. Effect of varying pH of the assay buffer (0.1M phosphate) is shown in Table 4 and Figure 7.

The results indicated that soluble urease had an optimum at pH.7 for urea and that enzyme activity decreased rapidly on either side of this value particularly towards more acidic pH values. The decrease in activity was due to the ionic states of the amino acid residues of the urease enzyme and substrate urea molecules, causing varying efficiency in the binding of substrate.

Table 4: Effect of pH on the Activities of Soluble and Immobilized Urease Catalyzed Reactions

pH	Soluble urease ($\mu mol\ ml^{-1}min^{-1}$)	Immobilized urease ($\mu mol\ ml^{-1}min^{-1}$)
5.0	112.03	223.90
5.5	117.58	229.28
6.0	162.95	284.00
6.5	227.76	315.70
7.0	287.95	495.64
7.5	162.95	612.09
8.0	129.61	199.27

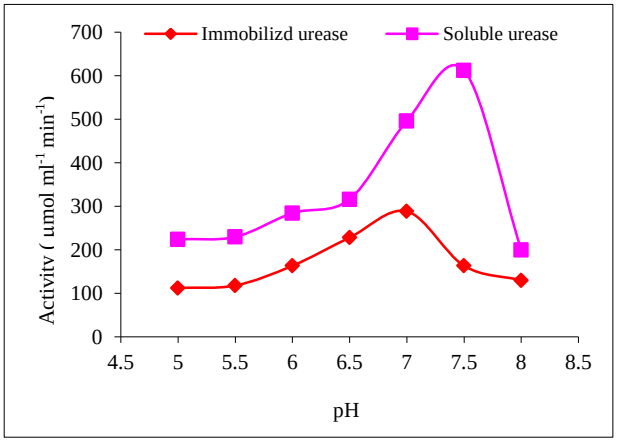


Figure 7: Plot of soluble and immobilized urease activities as a functionof pH

Application of Immobilized Urease for the Determination of Urea

Skate, fish of the family Rajidae contains urea and trimethylamine in large amounts making it unpopular as daily dishes due to its ammonicalodour. In this investigation, urea content in dried skate (*Raja hollandi*) flesh was determined by urease immobilized in calcium alginate beads. It was observed that urea content in dried skate flesh was 4.53 %. In the present work urea content in dried skate fish was removed by urease and the residual urea content was determined by prepared immobilized urease. Table 5 and Figure 8 show that the urea content decreased gradually with increased in time. After 66 h, 75.94 % of urea was removed.

Table 5: Removal of Urea Content as a Function of Time

Time (h)	Urea (%)	Removal percent (%)
0	4.53	-
12	3.32	26.71
18	2.89	36.20
36	2.25	50.33
42	1.85	59.16
66	1.09	75.94

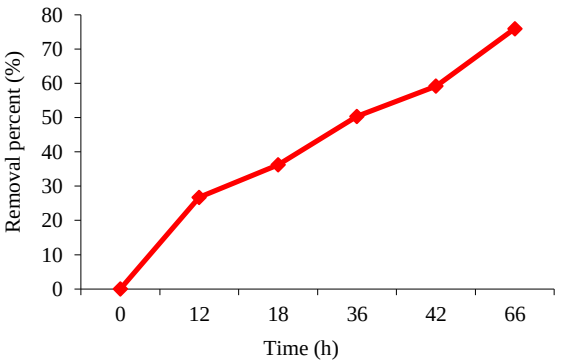


Figure 8: Plot of removal of urea content as a function of time

Conclusion

Urease was isolated and purified from Pigeonpea seeds collected from Natogyi Township, Mandalay Region. Ammonium sulphate precipitation method, gel filtration chromatographic method (SephacrylS-200) and ion-exchange chromatographic method (DEAE Sepharose) were used successively to get better purity. The purity of urease enzyme was confirmed by non SDS-PAGE technique as single band and the molecular weight of purified urease was determined to be 478 kDa. The immobilization of the purified urease was carried out by using gel entrapment technique using sodium alginate. Comparative studies of optimum pH between soluble and immobilized enzymes were carried out.

Immobilized urease showed a shift in optimum pH from 7 (soluble enzyme) to 7.5 in phosphate buffer. Immobilized urease was used for the determination of urea content in dried skate fish sample. Urea contents were found to be 4.53% in these samples. Moreover, removal of urea in dried skate fish was carried out by mixing the flesh of dried skate fish with purified urease. The residual urea was determined by Nessler method employing immobilized urease. After 66h, 75.94 % of urea was removed from dried skate fish due to the loss of ammonia the dried skate fish were more palatable to the consumers.

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