Chapter II
CHAPTER II
EXTRACTION AND PARTIAL PURIFICATION OF UREASE
FROM DOLICHOS BIFLORUS

2.1. INTRODUCTION

A. Ureases: General outline

Urease [urea amidohydrolase, E. C. 3. 5. 1. 5] a nickel containing metalloenzyme catalyzes the hydrolysis of urea to ammonia and carbon dioxide. [1, 2, 3, 4]. Urease was the first enzyme crystallized by Sumner from jack beans (Canavalia ensiformis) in 1926 [5]. It exhibits very high substrate specificity and catalyzes urea hydrolysis at a rate $10^{14}$ times faster than uncatalysed reaction. This enzyme is ubiquitous in nature and present in many organisms including plants, bacteria, fungi and invertebrates.

Bacterial ureases play important role in pathogenesis of a number of bacterial species including Proteus mirabilis, Staphylococcus saprophilicus, Yersinis enterocolitic, Ureaplasma urealiticus and others [6]. Urease activity in bacteria is required to use urea as a sole nitrogen source [7]. Therefore presence of urease was used as a screening test for isolating pathogens from the bacterial strains [8]. One of the most frequently mentioned examples in recent literature is the urease from Helicobactor pylori because of its essential role in pathogenesis of this microorganism [9].

Urease activity was also found in several species of fungi, however, nucleotide sequence of genes encoding urease were reported for only few of them including a fungal respiratory pathogen of human Coccidiodes immitis [10] and Schizosaccharomyces pombe [11]. While in invertebrate Aplysia californica urease was present with carbonic anhydrase and involved in formation and homeostasis of calcium carbonate inclusions in
the lumen of gravity-sensing organ, the statocyst [12, 13]. Urease is a cytosolic enzyme and in most of the cases studied, the activity is mainly associated with the soluble fractions of the cells [5, 13].

B. Plant ureases

a. Occurrence and sub cellular location of plant urease

Urease is widely distributed in plant kingdom. Sumner for the first time crystallized urease from jack bean in 1926 and demonstrated the proteinaceous nature of enzyme with the help of urease. This enzyme is widely spread in all plants albeit at lower level but abundant in legume seeds. It has been purified from several leguminous plant seeds and studied extensively [14, 15]. Recently urease has been extracted and purified from plant leaves of *Morus alba* [16] and soya bean [17].

Urease has been isolated and extensively studied from the seeds of various leguminaceae, cucurbitaceae, asteraceae and pinaceae members while in other higher plants the low levels were observed. The enzyme has been found in leaves, roots, and bark of plants with actively growing tissues possessing greater activity [14, 18]. Urease has also been involved in the assimilation of urea normally formed in the plant as a result of hydrolysis of arginine.

b. Physiological role

Urease is the key enzyme involved in urea metabolism in plants. The primary role of urease is to allow the organism to use external or internally generated urea as a nitrogen source [6, 7]. Significant amount of plant nitrogen comes through urea and it is generated from arginine and possibly from degradation of purines and ureides [19]. Urea cannot be used as nitrogen source by plants as it is. Urease hydrolyzes urea to form
ammonia, which is further incorporated into organic compound by glutamine synthetase and is made available to plant. The ubiquitous urease is responsible for recycling of metabolically derived urea. The metabolism of urea in plants is shown in Scheme 2.1.

### Scheme 2.1. Significance of urease enzyme in physiology of plants. [From Ref No. 21]

The importance of urease enzyme was demonstrated by the observation that plants with reduced growth show accumulated urea [20]. Genetic and chemical blocking of enzyme activity caused the necrotic leaf tip [21, 22, 23]. The physiological role of urease has been generally proposed to function in assimilation of urea derived from ureides or arginine [24]. Arginine is the predominant amino acid in the stem of mulberry tree and considered as major nitrogen source but urease is required to make it available for the plant. In case of silk worms grown on mulberry leaves, it has been shown that mulberry leaf urease is directly involved in silk protein. Urease activity was found in digestive track of these insects and urea secreted by midgut epithelium was decomposed into ammonia by the enzyme. The ammonia produced is found to be useful in the
synthesis of silk protein in insects [24]. This reveals that urease plays important role in urea metabolism.

c. Enzyme assays

Urease is highly specific towards the substrate urea and catalyses its hydrolysis to form ammonia and carbon dioxide.

\[
\text{H}_2\text{N} - \text{C} - \text{NH}_2 \xrightarrow{\text{Urease}} 2 \text{NH}_3 + \text{CO}_2
\]

The reaction products exist in both gaseous and ionized form \( \text{viz.} \, \text{CO}_2/\text{HCO}_3^- \, \text{and} \, \text{NH}_3/\text{NH}_4^+ \) depending upon the pH of the reaction mixture. During the hydrolysis of urea the first reaction product is carbamate, which further decomposes to \( \text{CO}_2 \) and \( \text{NH}_3 \). Hence as far as the enzymatic reaction is concerned, possible strategies to measure urea concentration are to measure pH change, \( \text{CO}_2/\text{HCO}_3^- \) production or \( \text{NH}_3/\text{NH}_4^+ \) production (Scheme 2.2).

**Scheme 2.2.** Various strategies to determine urease activity. * indicates the measurable moiety.
Most of the methods described for determination of urease activity measure liberated ammonia. Sumner's classic method, utilizes Nessler's reagent for the estimation of urea [25]. In Stutts and Fridouch method, the ammonia produced is coupled to horse radish peroxidase [26]. Urease activity was also measured by coupling ammonia production to NADH oxidation brought by glutamate dehydrogenase. NADH oxidation was measured as decrease in absorbance at 366 nm [27, 28]. Another indirect titration method was employed in which measured amount of HCl was added to reaction mixture and the excess unneutralised acid was titrated against NaOH [29]. Urease activity was also measured by radio assay method where the enzyme was incubated with $^{14}$C urea, $^{14}$CO$_2$ was eliminated by boiling the sample and the radioactivity of residual urea was then measured [30]. Recent methods involve direct potentiometric determination of ammonia with cationic sensitive glass electrode [31, 32].

The second type of activity measurement is in terms of the amount of HCO$_3^-$/CO$_2$ produced. The carbon dioxide formed is aerated into the solution of lime to check the evolution of CO$_2$ after enzymatic hydrolysis of urea.

Among the various methods available, the spectrophotometric assays for NH$_3$/NH$_4^+$ moiety are the most popular one such as Nesslerization method and hypochlorite method.

**i) Phenol hypochlorite method**

This method is reported by Berthelot where urease acts on urea to liberate ammonia, which reacts with phenol in presence of hypochlorite to form an indophenol. The indophenol gives blue colored compound with alkali. Nitroprusside acts as a catalyst. The color obtained is measured at 630 nm [33, 34].
ii) Nesselerisation method:

Breakdown of urea by urease enzyme gives ammonium hydroxide that reacts with Nesseler's reagent and gives a brown colored complex. The intensity of the complex is measured at 520 nm, which is proportional to the concentration of urea in the solution. [35, 36]. The reactions involved in both the methods are given in chapter I.

d. Protein structure

i) Structure of subunit

Jack bean urease is reported to have six equivalent subunits. Urease exists enzymically as a trimeric or hexameric polymer of six similar polypeptide chains. Urease from this source has been extensively studied and the sequence of amino acids is deduced as single N-terminal methionine residue and single C-terminal sequence Tyr-Leu-Phe was found. It has five unique tryptophan sequences Trp-Ala, Trp-Glu, Trp-Gly, Trp-Met and Trp-Arg.

The smallest active unit found has a molecular weight of about 240,000 Daltons suggesting that this unit has only one active site. Formation of urease polymers is not a nonspecific process based on necessity of protecting hydrophobic residues; it is likely that association is a specific function of enzyme.

One fundamental difficulty of trimeric model is that it is theoretically impossible for three identical units to react with urea, a substrate of two fold symmetry. The possibility of some form of ultramicroheterogenety needs further consideration. In this connection it is notable that urease from Neurospora crassa has been shown to have two genetic loci [37]. Undialyzed preparation of urease contained 1-2% carbohydrate,
measured as glucose equivalents. Further work is necessary to establish unequivocally whether or not urease is a glycoprotein.

ii) Molecular weight

The determination of molecular weight (Mr.) for urease has been often carried out using gel filtration or gel electrophoresis. The molecular weights of many plant ureases have been reported on the basis of SDS-PAGE where a single protein staining band was observed which explained the presence of subunits of identical molecular mass [17, 38, 39].

Initially, Sumner reported the molecular weight of jack bean urease by using ultracentrifugation as 483 kDa. Later on, this value was confirmed by SDS-PAGE as 488 kDa [40]. SDS-PAGE of jack bean urease indicated that it is composed of a single polypeptide subunit with apparent molecular mass of 90.5 kDa [41]. Legume seed ureases have same subunit size (90-93.5 kDa) [2, 42] while bacterial ureases have one large and two small subunits. These results revealed that a single type of urease subunit appears to be conserved among all plants. Several studies on plant ureases have shown that enzyme exists as a trimer or hexamer in its native form [42]. The molecular mass of urease from soya bean has been reported to be 480 kDa [17] and the molecular mass from watermelon seeds has been reported to be slightly lower, as 470 kDa [43]. A recent report from mulberry leaf urease shows the molecular mass to be 175 kDa and was found to be a homodimer [16].

e. Purification of urease

The details of various methods used in extraction and purification of urease from jack bean seeds are available. Urease was first purified and crystallized from jack bean
(Canavalia ensiformis) by Sumner [5]. Later on, many researchers have purified urease from jack beans to apparent homogeneity using standard procedures such as solvent precipitation, salt fractionation, ion exchange chromatography, gel filtration and affinity chromatography techniques.

Extraction of urease has been carried out by employing different buffer systems such as tris-HCl, tris-maleate and tris-acetate at different pH, while Sumner has reported extraction of urease in 31% acetone. In majority of the reports EDTA and mercaptoethanol have been added during extraction, whereas extraction of urease from mulberry leaves was carried out in presence of polyvinylpyrrolidone.

Recently urease was extracted and purified from various other plant seeds such as soya bean, pigeon pea, watermelon etc. Urease purified from soya bean shows 500 fold purity and electrophoretic homogeneity. The purification was achieved by using column chromatographies such as DEAE-cellulose, hydroxylapatite and Agarose A-15m.

Urease from pigeonpea was first subjected to three step solvent extraction procedure followed by DEAE-cellulose chromatography. The enzyme shows high fold purification with 8% recovery [44]. In another report the urease from pigeonpea was extensively studied, where the purification was achieved upto homogeneity using Sephadex G-200 column chromatography in combination with ion exchange chromatography. The purified urease has shown 200 fold purification with 12 % recovery [45].

Urease was also extracted and partially purified from the seeds of watermelon of ‘Ogonek’ variety. This enzyme has shown only 4 fold purification after ammonium sulphate fractionation, which is very low as compared to other reports. The watermelon
urease has shown molecular weight as 483000 Daltons, which was comparable with that of jack bean [43].

Recently, urease was purified from leaves of mulberry by several chromatographic techniques including HPLC and affinity. The enzyme was obtained with very high purity (5700 fold) to apparent electrophoretic homogeneity, though the yield is very less (3.6%) [16].

f. Present work

In this part of the thesis we present an account of our studies on extraction and partial purification of enzyme carried out from a novel plant source, *Dolichos biflorus*, which is cultivated in India for edible purposes.

![Figure 2.1. A) Plant of *Dolichos biflorus* showing dry pods. B) Dry seeds obtained from the pods of *Dolichos biflorus*](image)

The enzyme is partially purified by using ion exchange and size exclusion column chromatography which is further characterized by Native PAGE. The enzyme activity is measured by hypochlorite assay and the kinetic parameters such Km, Vmax and optimum pH and optimum temperature are determined.
2.2. EXPERIMENTAL ASPECTS

A. Materials and methods

Chemicals

Various pulses and seeds of leguminoceae and cucurbitaceae family were purchased from local markets. The chromatographic matrices DEAE-Cellulose, sephadex G-200 and Blue dextran were obtained from Pharmacia Fine chemicals. The chemicals sodium nitroprusside, sodium hypochlorite, urea (extra pure), bovine serum albumin, Bromo Phenol Blue and Cresol Red were purchased from SRL India. The reagents for electrophoresis were procured from BioRad (India). All other chemicals were of analytical grade. All the solutions were prepared in double distilled ammonia free water.

B. Solutions and reagents

a. Extraction of urease

Physiological saline: 0.85% NaCl in distilled water.

Assay of urease: All the reagents were prepared in ammonia free water.

Assay Buffer: Sodium phosphate buffer, 0.1 M pH 7.0

Assay reagents

Phenol nitroprusside: 1.25 gm phenol + 6 mg sodium nitroprusside in 100 ml water

Sodium hypochlorite: 600 mg of sodium hydroxide + 0.25 ml sodium hypochlorite in 100 ml water.

Substrate: Urea 4 mM in ammonia free water

b. Lowry’s protein estimation reagents

Protein standard solution: 1mg/ml BSA in distilled water

Working standard: 100 μg /ml BSA
Folin phenol reagent: Folin phenol solution, prepared in our laboratory was used with proper dilutions.

Lowry reagents i) Lowry A: 2% Sodium carbonate in 0.1 N NaOH
ii) Lowry B₁: 1% Sodium potassium tartarate in water
iii) Lowry B₂: 1% Copper sulfate in water

Lowry C was prepared freshly by mixing 100ml of Lowry A + 1 ml of Lowry B₁ + 1 ml of Lowry B₂ reagent.

c. Purification of urease

Ion exchange chromatography

Matrix: DEAE-Cellulose

Washing buffer: Sodium phosphate buffer (0.1 M, pH 7.5)

Elution buffer: Sodium phosphate buffer (0.1 M, pH 7.5) with KCl linear gradient (0.1 M-0.3 M)

Gel Filtration chromatography

Matrix: Sephadex G-200

Elution buffer: Sodium phosphate buffer (0.1 M, pH 7.5)

d. Characterization of urease

Electrophoresis (Native PAGE)

Acrylamide solution: 30 gm of acrylamide +0.8 gm of N, N’ methylene bis-acrylamide

Stacking gel buffer: Tris –HCl buffer pH 6.8

Ammonium per sulphate: 10%

TEMED

Tracking dye: Bromo phenol blue (BPB) 0.01% in 60 % sucrose solution.
Protein staining reagents

Staining: 0.1 % coomassie brilliant blue R 250 solution in Methanol: Acetic acid: Water (5:1:4 v/v)


Activity staining reagents

Cresol Red: 50mg/100ml

Sodium salt of EDTA (Na₂EDTA): 100mg/100ml

Urea: 1.5 gm/100ml

C. Extraction of Urease

a. Assay of urease activity

Urease catalyses hydrolysis of urea to form ammonia and carbon dioxide. The measurement of ammonia released during reaction was measured for quantifying the urease activity. The standard assay system contained 100µl of urease enzyme; 0.1 ml standard urea (0.160 mM) and 0.8 ml of sodium phosphate buffer (0.1M, pH 7.0) in a final volume of 1 ml. The substrate was replaced by distilled water for the blank. The reaction mixture was incubated at 37 °C for 10 minutes and ammonia formed was determined by adding 1 ml of phenol nitroprusside and 1 ml of sodium hypochlorite followed by incubation of 10 minutes at 37 °C. The ammonia formed was measured using a standard curve of ammonium sulphate. The blue colored complex was formed for which the absorbance was recorded in a wavelength range of 400 nm to 800 nm to find absorption maxima (λmax). One unit of urease activity was defined as µmoles of ammonia formed /min/ml of enzyme. Activity of urease was determined at each stage of purification.
b. Protein estimation:

Protein content of urease extract was estimated by the method of Lowry *et al.* [46] with bovine serum albumin (0.1 mg/ml) as a standard. The absorbance was measured at \( \lambda_{\text{max}} 660 \text{ nm} \).

The calibration curve was plotted for the concentration of standard protein (20 – 100 \( \mu \text{g} \)) against absorbance. The protein concentration of the enzyme extract was estimated at all stages of purification to determine specific activity.

c. Preparation of crude enzyme extract:

Dried seeds were used for the extraction of urease enzyme. Seeds along with husk were crushed in a blender to make the fine powdered meal and stored at 4 °C under dry conditions for the extraction of enzyme. 10 ml of chilled physiological saline was added to 1 gm of powdered meal and stirred slowly for 10 minutes in ice.

The slurry was then subjected to centrifugation at different speed and time intervals. When it was centrifuged at 10,000 rpm for 15 minutes, the supernatant obtained was clear milky white solution, showing maximum urease activity, while the pellet showed trace activity. The extraction was also carried out in sodium phosphate buffer (0.1 M, pH 7.0) and sodium phosphate buffer saline (PBS).

d. Screening of various seeds for urease activity

The crude extract was prepared from the seeds of various plants as mentioned in Table 2.1 under identical conditions.
Table 2.1. Various plant seeds screened for urease activity.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Name of the plant</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Canavilia ensiformis</em></td>
<td>Jack bean</td>
</tr>
<tr>
<td>2</td>
<td><em>Glycine max</em></td>
<td>Soya bean</td>
</tr>
<tr>
<td>3</td>
<td><em>Cajanus cajan</em></td>
<td>Pigeon pea</td>
</tr>
<tr>
<td>4</td>
<td><em>Cajanus indicus</em></td>
<td>Tur</td>
</tr>
<tr>
<td>5</td>
<td><em>Dolichos biflorus</em></td>
<td>Kultih</td>
</tr>
<tr>
<td>6</td>
<td><em>Lens esculenta</em></td>
<td>Mung bean</td>
</tr>
<tr>
<td>7</td>
<td><em>Citrullus vulgaris</em></td>
<td>Watermelon</td>
</tr>
<tr>
<td>8</td>
<td><em>Luffa aurantia</em></td>
<td>Dodka</td>
</tr>
<tr>
<td>9</td>
<td><em>Cucumis sativus</em></td>
<td>Cucumber</td>
</tr>
<tr>
<td>10</td>
<td><em>Momardica charantia</em></td>
<td>Karle</td>
</tr>
<tr>
<td>11</td>
<td><em>Cucurbita maxima</em></td>
<td>Pumpkin</td>
</tr>
</tbody>
</table>

The enzyme activity and protein was checked for the extract from each source to determine specific activity. The enzyme extracted from a new source *Dolichos biflorus* has shown high specific activity, which is comparable with that of crude jack bean urease and hence chosen as a source of urease.

D. Purification of urease

a. Removal of miscellaneous matter

The organic solvent precipitation is generally carried out to separate the desired protein from miscellaneous matter.

In the present studies chilled acetone (30 %) was gradually added to crude enzyme extract to solubilise the contaminating proteins. The precipitate was centrifuged at 10,000 rpm for 10 minutes at 4°C to separate out from supernatant. Urease activity and protein content were checked for precipitate as well as for supernatant. The supernatant showed almost 90% activity considering activity of crude extract as 100%, while the pellet showed very less activity. The supernatant was subjected further for salt precipitation.
b. Salt fractionation

Ammonium sulphate is the most commonly used salt for the precipitation of proteins, as it is highly soluble in water, cheap and has no detectable undesirable effects on the protein conformation.

In case of urease the acetone precipitated supernatant was subjected to ammonium sulphate fractionation in the range of 20%-70% saturation. The precipitate obtained after every step was collected after centrifugation, dissolved in a sodium phosphate buffer (0.1 M, pH 7.0) and dialyzed against 0.01 M phosphate buffer (pH 7.0) to remove excess salt. The supernatant and dialyzed residue after each step of precipitation was checked for activity and protein. The maximum urease activity was obtained at 60 % saturation.

c. Purification of urease by chromatographic techniques

i) Ion exchange chromatography

DEAE-cellulose was used as ion exchange matrix during the purification of urease. The matrix was repeatedly washed with ammonia free and kept in glass distilled water for 2 hours for swelling. It was then suspended in 0.5 M HCl for 30 minutes. After this activation it was washed continuously with water till the pH increased up to 4.0. Thus the matrix, activated with chloride ions was further suspended in NaOH (0.5 M) for 30 minutes and rinsed with water to attain pH 7.0. The anion exchanger thus formed was packed in a glass column (diameter 2 cm height 25 cm). Before loading the sample, the column was equilibrated with sodium phosphate buffer (0.1M, pH 7.5). The 30 ml of dialyzed fraction (2.1 mg/ml) was loaded on the column. After loading the dialyzed fraction the column was washed with sodium phosphate buffer to remove the excess or
unbound protein from the column. Initially fractions of 10 ml were collected during washing until they were free from protein. The enzyme, which has been retained on the column, was eluted by increasing the ionic strength of the buffer. Sodium phosphate buffer with KCl was used for elution and 5 ml fractions were collected. A linear KCl gradient (0.1 M - 0.3 M) was applied till the complete elution of protein was achieved. Activity of each fraction was determined by hypochlorite assay while protein content by checking absorbance at 280 nm.

ii) Size exclusion chromatography

Sephadex G-200 was suspended in phosphate buffer (0.1 M, pH 7.5) for swelling and equilibration for 8 hours. The equilibrated matrix was packed in a glass column with dimensions 1.5 cm x 70 cm. Initially Blue dextran was loaded on Sephadex G-200 column to determine void volume of column. The peak fractions from ion exchange chromatography were pooled (15 ml, 1mg/ml) and loaded on Sephadex G-200 column. The sodium phosphate buffer (0.1M, pH 7.5) was passed through column and fractions of 3 ml were collected until the loaded protein gets eluted completely from the column. Each fraction was assayed for urease activity as well as for protein.

E. Physicochemical characterization of the enzyme.

a. Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out under nondenaturing conditions to ascertain the homogeneity of the purified preparation. Native PAGE of partially purified and commercial urease was carried out by the method described by Ornstein and Davis [42] using 7.0 % polyacrylamide gel in the tris-glycine buffer at pH 8.3.
Preparation of gel

The polymerizing solutions were brought to room temperature and degassed before mixing. The solutions were mixed in proportions to form a 7% gel as given in standard electrophoresis chart and the mixture was poured in between two sealed glass plates.

A comb was inserted in the solution properly to form wells and a small volume of n-butanol saturated with water was layered on the top of this solution to prevent atmospheric oxidation. The gels were then allowed to polymerize. Residual ammonium persulphate was removed by carrying out pre-electrophoresis for 15 minutes using a current 3mA per well.

ii) Preparation of sample

100-150 μg of protein was mixed with BPB in sucrose (60%) and 250-500 μl aliquots of the sample were prepared.

iii) Electrophoresis

After pre-electrophoresis the protein sample, 50 μl-75μl was loaded on the gel without removing the reservoir buffer. The samples were loaded in the wells and a current of 3 mA per well was applied till the tracking dye reached the bottom of the gel.

iv) Protein Staining

Coomassie brilliant blue (CBB R250) was used as a protein stain. 1.25 gm of CBB was dissolved in 1 liter mixture of methanol, acetic acid and water and filtered through Whatman filter paper No.1 before use. The gel was kept in a staining solution overnight and then in a mixture of methanol, acetic acid, and water (5:1:4) for destaining.
v) Activity staining

For activity staining, the gels were equilibrated with Cresol Red and Na₂EDTA followed by incubation for 4 hours with the same solution containing 0.25 M urea [45].

b. Kinetic studies

The partially purified urease was further characterized for kinetic constants such as Km, Vmax, optimum pH and optimum temperature.

i) Effect of enzyme concentration

The rate of an enzyme-catalyzed reaction is directly proportional to the concentration of the enzyme at optimum assay conditions provided the substrate concentration is present in excess.

To study the effect of enzyme concentration on hydrolysis of urea, the concentration of partially purified urease (1:2 diluted) was varied from 25 μl - 150 μl. The reaction was carried out as described earlier in assay methods.

ii) Effect of substrate concentration

The enzyme kinetics at varying substrate concentration explains the nature of the enzyme-catalyzed reaction, affinity of the substrate towards the enzyme and also the maximum velocity of the reaction. Lineweaver-Burk plot is preferentially applied to determine the kinetic constants, Km and Vmax.

The effect of substrate concentration on the rate of product formation of urease activity of the enzyme extract was studied spectrophotometrically (λmax 633nm) by varying urea concentration (0.04 mM to 0.32 mM) in presence of assay buffer, keeping the enzyme concentration constant in a total volume of 3 ml, as described in assay method. The Km
and Vmax were calculated from double reciprocal plot for molar substrate concentrations against velocity.

iii) Effect of pH

Enzyme is catalytically active within a limited range of pH. It is well established that the change in pH cause changes in ionic characters of amino acids and carboxylic groups of the enzyme thereby, affecting the catalytic activity of the enzyme.

The optimum pH for urease activity was determined in buffers between pH 4.0 – 8.0 (Acetate buffer pH 4.0-5.5, Sodium phosphate buffer 6.0-7.5, Tris-HCl buffer pH 8.0) under the assay conditions mentioned earlier.

iv) Effect of temperature

In case of enzymes the relationship between reaction rate and temperature is exponential. At higher temperatures above 50°C enzymes generally lose their activity, while at lower temperature the rate of enzyme catalysis slows down. Therefore, optimum temperature, at which the enzyme appears to have maximum activity, is normally chosen for the study of enzyme activity. Enzyme assays are carried out routinely in the range 30°C to 37°C.

The optimum temperature of the partially purified enzyme extract was studied for its urease activity using urea as substrate. The assay mixture was incubated at varying temperatures ranging between 15 °C to 70 °C for 10 minutes prior to addition of colour forming reagents.
2.3. RESULTS AND DISCUSSION:

A. Enzyme extraction

The urease extracted from the seeds of *Dolichos biflorus* was in its active form, when extracted in physiological saline. Physiological saline was preferred for extraction of urease to sodium phosphate buffer (0.1 M, pH 7.0) or phosphate buffer saline (PBS) since maximum activity was observed in the extract. The conditions for urease extraction were critically optimized, as a slight change in pH, temperature, extraction procedure or time of extraction process affects the efficiency of extraction of an enzyme. The extract was clear and remained active up to 8 days on storage at 4 °C.

a. Determination of urease activity

The hypochlorite assay was optimized for the determination of urease activity. A spectra was obtained for the blue colored complex formed in the range of 400-800 nm. (Figure 2.2)
The blue color developed after addition of phenol nitroprusside and sodium hypochlorite to the reaction mixture was showing maximum absorption at 633 nm and hence this wavelength was selected for further measurements.

The activity of extracted urease was assayed using urea as a standard substrate. The graphical profile of time versus absorbance for different concentrations of urea is shown in Figure 2.3.

The reaction has shown a gradual increase in the intensity of the color of the complex formed, after addition of color developing reagents to the reaction mixture containing urease, urea and phosphate buffer (0.1 M, pH 7.0). The response increases gradually up to 10 minutes after which it stabilizes. The graphical profile of time Vs absorbance for different concentration of urea is shown in Figure 2.3. The recorded response was showing a linear profile when urea concentration was plotted versus absorbance, recorded after 10 minutes (Figure 2.4). Therefore all the measurements were recorded at 10 minutes time interval.

![Figure 2.3. To determine response time at different urea concentrations from 0.04 mM to 0.24 mM.](image-url)
Figure 2.4. A linear response of urease enzyme for different urea concentrations (0.04mM- 0.240mM) After 10 minute.

The protein concentration of the extract was estimated from the calibration plot by using bovine serum albumin as a standard protein by Lowry’s method [46] (Figure 2.5).

Figure 2.5. Calibration plot for protein estimation using BSA as a standard protein by Lowry’s method.
B. Screening of different plant seeds for urease activity

The crude extract was prepared from the powdered meal of various seeds mentioned earlier in Table 2.1 under identical conditions. The urease activity and protein was checked for each extract and the specific activities were determined, as given in Table 2.2.

Table 2.2. Specific activities of various seeds screened for urease activity.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Name of the plant</th>
<th>Specific activity Units/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Leguminosae family</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><em>Canavalia ensiformis</em></td>
<td>40</td>
</tr>
<tr>
<td>2.</td>
<td><em>Glycine max.1</em></td>
<td>32</td>
</tr>
<tr>
<td>3.</td>
<td><em>Cajanus cajan</em></td>
<td>32</td>
</tr>
<tr>
<td>4.</td>
<td><em>Cajanus indicus</em></td>
<td>24</td>
</tr>
<tr>
<td>5.</td>
<td><em>Dolichos biflorus</em></td>
<td>36</td>
</tr>
<tr>
<td>6.</td>
<td><em>Lens esculenta</em></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><strong>Cucurbitaceae family</strong></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td><em>Citrullus vulgaris</em></td>
<td>28</td>
</tr>
<tr>
<td>8.</td>
<td><em>Luffa autangula</em></td>
<td>—</td>
</tr>
<tr>
<td>9.</td>
<td><em>Cucumis sativus</em></td>
<td>—</td>
</tr>
<tr>
<td>10.</td>
<td><em>Momardica charantia</em></td>
<td>—</td>
</tr>
<tr>
<td>11.</td>
<td><em>Cucurbita maxima</em></td>
<td>26</td>
</tr>
</tbody>
</table>

The crude extract of jack bean urease has shown the highest specific activity, but jack beans cannot be used as a source of enzyme because the plant is not cultivated in India on a large scale and importing purified jack bean urease is quite expensive. Therefore *Dolichos biflorus* was chosen as a source of enzyme because the crude extract of it shows fairly high activity comparable with that of jack bean crude extract.

In the present studies, we wish to employ the urease enzyme in its immobilized form for the fabrication of clinically significant urea biosensors. Therefore there is a need to have purified urease preparation for the construction of biosensors to increase the specificity of the measurements, to reduce the quantity of enzyme required and to avoid
the interferences. However, the highly purified enzyme may not be suitable for this purpose due to instability and high cost. Hence we have carried out partial purification of urease enzyme to obtain a contamination free form of enzyme with high specific activity.

C. Purification of enzyme

The crude extract was first subjected to 30% acetone precipitation to remove the mucilaginous matter from the extract. Organic solvent fractionation is a routinely carried out step in enzyme purification prior to or after the fractionation with salts. It is based upon differences in the solubility of proteins in aqueous solution of polar organic solvents such as ethanol, acetone and n-butanol. These organic solvents lower the dielectric constant of the medium, in turn decreasing the protein solubility. The protein contamination with comparatively more hydrophilic groups is removed using this method by adjusting the ratio of the organic solvent to aqueous medium.

Here 30% acetone precipitation has enhanced the specific activity of the extract almost four times. The supernatant, showing urease activity was further subjected to ammonium sulphate fractionation. During fractional precipitation a measurable amount of urease activity was observed in all fractions although the supernatant was rich in the activity till 50% concentration of ammonium sulphate. After 60% precipitation, there was no urease activity in supernatant indicating that at this concentration, urease in the solution was precipitated completely.

The enzyme activity and protein was determined at each step, the values given in Table 2.3. The dialyzed fraction free from ammonium sulphate was further purified by ion exchange and size exclusion chromatography. The purification was attempted by
using these two chromatographic techniques in two different sequences. First one was size exclusion chromatography and then ion exchange chromatography, while in second case ion exchange chromatography was carried out first followed by size exclusion. The second one has shown more increase in the specific activity than the first one. Therefore the second strategy has been followed for urease purification.

The elution profile of DEAE-cellulose column is given in Figure 2.6. A linear gradient of KCl was applied to the column in the range (0.1M-0.3M) and 5 ml fractions were collected. Urease activity was eluted as a single peak with 0.2 M KCl elution in the elution volume between $325 \text{ - } 360$ ml. The peak fractions showing maximum activity were pooled and loaded on the size exclusion Sephadex G-200 column. The void volume was determined by using blue dextran, which was found to be 38 ml. The urease was eluted in the fractions just after void volume (48-60 ml), indicating that urease from *Dolichos biflorus* has a fairly high molecular weight. The elution profile of sephadex G-200 column was given in Figure 2.7.

The pooled peak fractions from this column have shown high urease activity with very low protein contents, thus indicating 189 times increase in specific activity with fairly good yield (18%). These results are quite comparable with the reports on purification of urease from *Cajanus cajan* [42] by using both the chromatographies ion exchange and size exclusion, where 200 fold purification has been achieved with relatively less yield (12%).
Figure 2.6. Elution profile of *Dolichos biflorus* urease on DEAE-Cellulose chromatography for enzyme activity and protein (absorbance at 280 nm). The enzyme was eluted from column with sodium phosphate buffer (0.1M, pH 7.5) containing 0.2 M KCl.
Figure 2.7. Elution profile of *Dolichos biflorus* urease on sephadex G-200 Column. Elution was carried out by using sodium phosphate buffer (0.1 M, pH 7.0).
Table 2.3. Purification of urease from *Dolichos biflorus* seeds.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Activity (U/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Fold Purification</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>420</td>
<td>20.58</td>
<td>700</td>
<td>34.01</td>
<td>-----</td>
<td>100</td>
</tr>
<tr>
<td>Acetone Precipitation (Supernatant)</td>
<td>590</td>
<td>3.5</td>
<td>428.54</td>
<td>122.44</td>
<td>3.6</td>
<td>86</td>
</tr>
<tr>
<td>60% ammonium sulphate fractionation</td>
<td>30</td>
<td>2.1</td>
<td>4410</td>
<td>2100</td>
<td>61.74</td>
<td>45</td>
</tr>
<tr>
<td>DEAE-Cellulose (Peak Fractions)</td>
<td>15</td>
<td>1.0</td>
<td>4512</td>
<td>4512</td>
<td>132.70</td>
<td>23</td>
</tr>
<tr>
<td>Sephadex G-200 column (Peak Fractions)</td>
<td>6</td>
<td>0.71</td>
<td>4600</td>
<td>6424</td>
<td>189</td>
<td>18.7</td>
</tr>
</tbody>
</table>
D. Physicochemical characterization:

a. Electrophoresis

A 7% Native PAGE of partially purified urease from *Dolichos biflorus* showed three bands, one major and two minor bands while crude extract has shown 5-6 bands on protein staining with coomassie blue. The major protein band corresponds to the commercial jack bean urease band (Figure 2.8) indicating the similarity between the urease from *Dolichos biflorus* with that of jack bean urease with respect to charge and molecular weight. The major protein band coincides with the band obtained by activity staining. (Figure 2.9). For activity staining the native PAGE was incubated with cresol red, which distinctively stained the urease on Native PAGE. Thus the presence of urease was confirmed by activity staining.

![Figure 2.8. The Native PAGE stained with Coomassie blue to locate urease enzyme represents a) Crude extract b) partially purified enzyme c) Commercial jack bean urease.](image)
b. Kinetics Studies

Efficiency of the enzyme catalysis is determined by evaluating the rates of reaction. Enzyme kinetics deals with factors affecting the rate of enzyme-catalyzed reactions. The most important factors are enzyme concentration, substrate concentration, pH, temperature and inhibitors.

The parameters affecting the rate of reaction such as enzyme concentration, substrate concentration, optimum pH and optimum temperature were optimized.

c. Enzyme concentration:

Figure 2.10. shows the graph of activity against the increasing enzyme concentration which gives the formation of ammonia in terms of intensity of blue color is directly proportional to the enzyme concentration up to 100μl of enzyme under specified
conditions. Therefore the enzyme concentration was selected in the linear range of the assay.

![Graph](image)

**Figure 2.10. Optimization of urease concentration required for hydrolysis of urea.**

d. Substrate concentration:

The rate of urea hydrolysis was monitored as a function of urea concentration from 0.04 mM to 0.32 mM. The plot of substrate concentration Vs initial velocity of the catalytic reaction gave a hyperbolic profile (Figure 2.11). At low concentrations, the initial rate was proportional to the substrate concentration, while at concentration higher than 0.24 mM the velocity of the reaction was independent of substrate concentration. The Line-Weaver Burk plot of reciprocal initial velocity against reciprocal substrate concentration is depicted in Figure 2.12. The Km and Vmax values calculated from this plot are found to be 1.85 mM and 10 units respectively, which were comparable with the reported values for jack bean urease.
Figure 2.11. Initial velocity of the reaction versus urea concentration (0.04 mM - 0.320 mM).

Figure 2.12. Line-Weaver Burk Plot of urease activity with urea as a substrate. Km was calculated from the x-intercept value fitting in the equation \( V = \frac{1}{K_m} \).
e. Optimum pH of the assay system

The pH optimum of most of the plant ureases studied, lies between 6.0- 8.0. Generally the pH curve of the enzyme activity shows bell shape with a single peak. The effect of pH on the urease activity is shown in Figure 2.13. The optimum pH for the urease activity was observed to be 7.0, which is in good agreement with that of the reported values [42, 17].

![Graph](image)

**Figure 2.13.** Effect of pH on urease activity determined in the pH range 4.0-8.0.

f. Temperature of the assay system

The urease enzyme was found to be stable in a wider temperature range from 30-60°C. The effect of temperature on the activity of enzyme was depicted in the figure 2.9. The urease enzyme from *Dolichos biflorus* has shown maximum activity at 37 °C. Therefore, in all urease assays, the reaction mixture was incubated at 37 °C.
2.4. CONCLUSIONS:

The crude extract of urease enzyme from *Dolichos biflorus* seeds has shown comparable specific activity comparable with that of crude jack bean urease, hence selected as a new source of enzyme for further studies. The crude extract was clear, yellowish-milky white in color and retained its activity for 8 days when stored at 4 °C. It was purified by solvent precipitation, salt fractionation followed by ion exchange chromatography and size exclusion chromatography. The purified fraction showed 189 times fold purification in specific activity and obtained in fairly good yield (18 %). A 7% native PAGE showed one major and two minor bands on protein staining where the major protein band corresponds to the commercial jack bean urease band. Activity staining of native gel with cresol red also showed a single band corresponding to the major protein band which ascertains the presence of urease enzyme.
The hypochlorite assay was employed for the determination of urease activity. The kinetic parameters such as $K_m$, $V_{\text{max}}$, optimum pH and optimum temperature were found to be comparable with the reported values for other plant ureases and commercial jack bean urease as well. The partially purified urease from *Dolichos biflorus* with high specific activity may be suitable for sensing purposes.
References


(1975) 4130.


2470.

387.


