2. Purification and Testing Homogeneity of the Purified Enzyme

2.1 Introduction
Oxalate oxidase has been purified from a number of sources by applying conventional methods of enzyme purification. Sorghum (var. CSH-5 and CSH-14) oxalate oxidase has been purified by using these methods (Satayapal and Pundir, 1993). We used the same method to purify oxalate oxidase from sorghum (var. Amarnath-2000) due to non availability of var. CSH-5 and CSH-14. Crude extraction of oxalate oxidase was done in a buffer of nearby neutral pH followed by ammonium sulfate fractionation to concentrate the protein. Dialysis of the protein was done to remove excess salts. Ion exchange chromatography was done to separate the protein on the basis of charges. DEAE-Sephacel was used as a matrix. Lastly gel filtration chromatography was done to separate the protein based on its molecular weight. The fraction collected now became quite diluted so lypholization was followed to concentrate the protein.

Homogeneity of the purified enzyme was tested by denatured and non denatured SDS-PAGE. CBB staining was preferred over any other stain due to requirement of the further experiment for sequencing purpose. Polyclonal antibodies were raised against the purified enzyme. ELISA was done to test the specificity of the antibodies in crude as well as purified enzyme from sorghum leaves.

2.2 Chemicals and Instruments Used:

Chemicals
DEAE-Sephacel, oxalic acid, horseradish peroxidase, 4-aminophenozone, and polyvinyl polypyrrolidone (PVPP), were from Sigma Chemicals Co. St. Louis, U.S.A., Sephadex G-200 was from Amershem Pharmacia, Uppsala, Sweden. All others chemicals were of analytical reagent grade.

Instruments and equipments used
Refrigerated centrifuge (Make: Remi 12C, Spectronic-20 (Milton and Roy Co., U.S.A.), Cold room (Blue star, New Delhi), Pesistaltic pump and fraction collector (Pharmacia LKB, Sweden), Water bath shaker and oven (N.S.W., New Delhi), Cyclomixer (Remi equipments, Mumbai), Digital pH meter (335 Sytronics, Ahmedabad), Deep-freezer 40°C (Model–Vestfost, Denmark), Refrigerator (Godrej), Lypholizer (FTS systems, Inc.)
U.S.A., Model # EZ5SOQ. Adjustable volume pipettes (Sigma Chemical CO. St. Louis, U.S.A.) and fixed volume pipettes (Tarson, Calcutta), were used.

2.3 Crude extraction of oxalate oxidase:

Sources of seeds

Sorghum seeds (*Sorghum vulgare* var. Amarnath-2000) were a gift from M/s Nath Seeds Private Limited, Aurangabad, Maharashtra, India.

Collection of plant material

Ten days old seedling plants of sorghum (var. Amarnath-2000) were raised in the laboratory. Sorghum seeds were soaked in distilled water for overnight. Next day, seeds were treated with 0.2% HgCl₂ for one min., washed with distilled water for several times and allowed to germinate in petridishes lined with double layer of moist filter paper at room temperature (33±5)°C and were kept in dark. After 5 days of germination, the seedlings were irrigated daily with Hoagland’s nutrient solution and maintained in a photoperiod of 8-10 h/day. After ten days germination, seedlings were removed from filter paper and their leaves were separated with sharp scissors in dark, washed in chilled distilled water, dried between folds of filter paper, and weighed. These leaves then stored immediately at -20°C until use (Satyapal and Pundir, 1993).

Extraction of oxalate oxidase

Crude oxalate oxidase from 10-days old sorghum leaves was extracted. The frozen leaves (90 g) were homogenized in chilled distilled water in 1:3 ratio (w/v) containing 1% PVPP (polyvinylpolypyrrolidone) in chilled pestle and mortar. The homogenate was squeezed through a double layer of cheese cloth and filtrate was centrifuged in a refrigerated centrifuge at 15,000g for 30 min at 4°C. The pellet was discarded and supernatant was collected and treated as crude enzyme. It was stored at 4°C until use.

2.4 Assay of oxalate oxidase

The assay of oxalate oxidase was carried out (Bais et al., 1980). It was based on the measurement of \( \text{H}_2\text{O}_2 \) generated from oxalate by oxalate oxidase in a colorimetric reaction consisting of 4-aminophenazone, phenol and peroxidase. The assay was carried out in a 15 ml test tube wrapped with black paper. The reaction mixture containing 1.7 ml 0.05M sodium succinate buffer pH 5.5, 0.1 ml CuSO₄(10⁻²M) and 0.1 ml crude enzyme was preincubated at 37°C for 5 min in water bath with constant stirring. The
reaction was started by adding 0.1 ml oxalate (10^{-2}M). After incubation at 37°C for 5 min with constant stirring, 1.0 ml colour reagent was added and tubes were kept at room temperature (30±5)°C for 15 min in dark to develop the colour. A control was prepared by adding 0.1 ml of heated crude enzyme (for 2 min in boiling water bath) in place of crude enzyme in the above assay. A_{520} was read against control in Spectronic-20 and H_{2}O_{2} generated in reaction was determined from standard curve between H_{2}O_{2} concentration and A_{520} prepared in 0.05M sodium succinate buffer pH 5.5.

**Preparation of colour reagent**

Colour reagent consisted of 50 mg 4-aminophenazone, 100 mg solid phenol and 1mg horseradish peroxidase (RZ=1.0) per 100 ml of 0.4M sodium phosphate buffer, pH 7.0. It was stored in amber coloured bottle at 4°C and prepared fresh after one week (Bais et al., 1980).

**Preparation of standard curve of H_{2}O_{2}**

It was prepared by taking various concentrations of H_{2}O_{2} ranging from 10 nmole to 250 nmole in a reaction mixture containing 1.9 ml of 0.05M sodium succinate buffer, pH 5.5. The reaction was started by adding 1.0 ml colour reagent in test tubes. After incubation at
37°C for 10 min. in dark, $A_{520}$ was read and a standard curve was plotted between $H_2O_2$ concentration and $A_{520}$.

**Unit of enzyme**

One unit of enzyme is defined as the amount of enzyme required to catalyze the formation of 1.0 nmole of $H_2O_2$ min under standard conditions of assay.

2.5 **Protein determination**

At each step protein content was determined (Bradford, 1976).

**Preparation of reagents**

**Dye concentrate:** It was prepared by dissolving 100 mg Coomassie brilliant blue (CBB)G-50 in 50 ml of 95% ethanol. Concentrated ortho phosphoric acid (100 ml) was added to it and its final volume was made to 200 ml by mixing distilled water. It was stored in amber colored bottle at 4°C until use. Prior to use, one volume of this concentrated dye was diluted with four volumes of distilled water and filtered through Whatman No.1 filter paper.

**Preparation of standard casein solutions:** Different dilutions of casein solution ranging from 0.2 to 1.0 mg/ml were prepared in 0.1N NaOH.
**Procedure:** To 0.1 ml of casein solution of different concentrations, 5.0 ml of diluted dye was added and $A_{595}$ was read. A standard curve was prepared by plotting casein concentration vs. $A_{595}$. Similarly to 0.1 ml of enzyme preparation, 5.0 ml diluted dye was added and $A_{595}$ was read. The amount of protein was calculated from standard curve of casein between casein concentrations versus $A_{595}$.

### 2.6 Purification of oxalate oxidase

Crude oxalate oxidase was extracted from 10-days old sorghum leaves and purified (Satyapal and Pundir, 1993). All steps of purification were carried out in cold (4-10°C) and dark.

#### 2.6.1 Ammonium sulfate fractionation

Solid ammonium sulfate was added to crude enzyme (15,000g supernatant) slowly with continuous stirring to give a final 0–80% saturation, kept in a refrigerator overnight and centrifuged at 10,000g for 30 min at 4°C in a refrigerated centrifuged. The pellet was dissolved in minimum volume (10-15 ml) of potassium phosphate buffer (0.01M, pH 6.8). The enzyme activity and protein in dissolved pellet were measured.

#### 2.6.2 Dialysis:

The enzyme was dialyzed against 0.01M potassium phosphate buffer, pH 6.8, overnight at 4°C under continuous stirring.

#### 2.6.3 Sephadex G-200 gel filtration:

**Preparation of gel:** 8 g of Sephadex G-200 powder was soaked in 400 ml distilled water and kept at room temperature for 48 h. The gel was deaerated on steam bath for 30 min with continuous stirring and allowed to cool. It was washed several times with freshly prepared distilled water.

**Packing of column:** A glass column of dimension (2.5×25 cm) having sintered plate inside near its lower end was used. The column was washed thoroughly with chromic acid and then distilled water many times, dried and set erect on a clamp stand. The outlet was closed and small amount of potassium phosphate buffer (0.01M, pH 6.8) was added to column. The gel was mixed with a glass rod and poured into column along its sidewall with the help of a glass rod. The gel was allowed to settle down in the column under gravity up to height of 15 cm. The column was washed with potassium phosphate buffer (0.01M, pH 6.8) for 12 h at a flow rate of 0.33 ml/min until pH of outgoing buffer was same as of incoming buffer.
Determination of void volume: One ml of 0.2% blue dextran dissolved in potassium phosphate buffer (0.01M, pH 6.8) was loaded on the top of column and it was allowed to run in the same running buffer at a same flow rate i.e. 0.33 ml/min until blue colour appeared in the effluent. The total volume of buffer passed to start elution of blue dextran was noted.

Bad volume determination: $\Omega r^2h$

Sample loading: The dissolved ammonium sulfate precipitate was applied on the top of Sephadex G-200 column (2.5×25 cm) through its side wall using a pipette slowly without disturbing the upper layer of gel. The column was eluted with eluting buffer (potassium phosphate buffer 0.01M, pH 6.8) at a flow rate of 0.33 ml/min. One void volume of column was allowed to pass. Fractions of 3 ml each were collected until buffer of two more void volumes was passed. Each fraction was monitored for enzyme activity and protein content. Fractions containing maximum specific activity were pooled.

2.6.4 DEAE–Sephacel column chromatography

Preparation of ion exchanger: DEAE–Sephacel (supplied in 20% ethanol by Sigma) was washed with distilled water for several times to remove alcohol. A glass column (2.5×15 cm) having sintered plate at its lower end was fixed erect on a clamp stand and its outlet was closed. The gel was stirred gently and poured along the wall of column with the help of a glass rod and allowed to settle for some time. The outlet of column was opened and allowed the eluting buffer (potassium phosphate, 0.01M, pH 6.8) to pass at a flow rate of 0.33 ml/min until pH of incoming and outgoing buffer was same.

Loading of sample: The pooled Sephadex G-200 fractions were gently applied on the top of the column through its side wall with the help of eppendorf pipette without disturbing the upper layer of the gel. The column was eluted with 0.01M potassium buffer pH 6.8 until no protein was detected in the effluent. The enzyme was eluted with 0.0–0.6M linear gradient of KCl by taking 200 ml of 0.01M potassium phosphate buffer pH 6.8 in a mixer and 200 ml of the same buffer, containing 0.6M KCl in the reservoir. These buffers were taken into the aspirator bottles connected at the bottom and open at the top. The buffer in the mixture was stirred with the help of a magnetic stirrer. The flow rate was maintained at 0.33 ml/min. The fractions of 3 ml each were collected and monitored for enzyme activity and protein. The fractions containing maximum specific
activity were pooled.

2.6.5 **Lyophilization:** The pooled fractions of DEAE-Sephael were lyophilized by lyopholizer to concentrate the enzyme.

2.7 **Testing homogeneity of the purified enzyme**

2.7.1 **Chemicals and instruments used**

**Chemicals**

Ammonium persulfate, and glycerol bromophenol blue, Coomassie brilliant blue, sodium dodecylsulfate (SDS), Tetramethylethylene diamine (TEMED) Acrylamide, bisacrylamide, coomassie blue. N.N. N\(^1\), N\(^1\)-, tris (hydroxymethyl)-aminomethane, glycine, were from Sigma Chemicals Co. St. Louis, U.S.A. All other chemicals were of analytical reagent grade.

**Instruments and equipments used**

Water bath shaker and oven (N.S.W., New Delhi), Cyclomixer (Remi equipments, Mumbai), Digital pH meter (335 Systronics, Ahmedabad). Deep-freezer of -40°C (Model–Vestfost, Denmark), Refrigerator (Godrej), Lyopholizer (FTS systems, Inc. U.S.A., Model # EZ55OQ, Electrophoresis power supply 610 (Systronic instrument Co., Ahmedabad), Electrophoresis apparatus (GENEI), and Adjustable volume pipette range 20–200 \(\mu\)l and 200–1000 \(\mu\)l (Sigma Chemical CO. St. Louis, U.S.A.) and fixed volume pipette of 100 \(\mu\)l and 1000 \(\mu\)l (Tarson, Calcutta), were used.

2.7.2 **SDS-PAGE (Polyacrylamide gel electrophoresis) and CBB Staining:**

**SDS-PAGE (Polyacrylamide gel electrophoresis)**

Polyacrylamide gel electrophoresis (SDS-PAGE) of purified enzyme was carried out in 12% polyacrylamide gel (Sambrook *et al.*, 1989; modified Laemlli, 1970).

**Preparation of reagents and gel for SDS-PAGE:**

1. **Acrylamide and bisacrylamide (30:0.8):** 14.6 g acrylamide and 0.4 g N\(^1\). N\(^1\)-methylen bisacrylamide were dissolved in 20 ml of distilled water and final volume was made up-to 50 ml with distilled water. It was filtered and stored at 4°C in dark.

2. **1.5M Tris-HCl buffer, pH 8.8:** 18.15 g of tris buffer was dissolved in about 40 ml distilled water and pH of solution was adjusted to 8.8 with 1N HCl and final volume was made to 100 ml with distilled water. It was stored at 4°C.

3. **0.5M Tris-HCl buffer pH 6.8:** 6.0 g of tris buffer was dissolved in 40 ml distilled
water; adjusted pH of solution to 6.8 with 1N HCl and final volume was made to 100 ml with distilled water and stored at 4°C.

4. **10% Ammonium per sulfate solution (0.1 g/ml):** It was prepared in distilled water and made fresh before use.

5. **Tris-glycine buffer:** 1.5 g tris buffer and 7.2 g glycine were dissolved in distilled water and its pH was adjusted to 8.5–8.7. Volume was made to 500 ml with distilled water.

6. **Tracking dye:** 0.05% Bromophenol blue was prepared in 1.5M tris-HCl buffer, pH 8.8.

7. **10% SDS (Sodiumdodesylsulphate):** It was prepared in distilled water.

**Separating gel preparation (12%):** It was prepared by mixing distilled water 3.45 ml, solution B 2.5 ml, solution A 4.0 ml, solution D 50 µl, TEMED 5 µl to make 10 ml total volume. For SDS-PAGE 0.1 ml of 10% SDS solution was added in above mixture.

**Spacer gel preparation (4.0%):** It was prepared by mixing 6.14 ml distilled water, 2.5 ml of solution C, 1.3 ml of solution A, 50 µl of solution D and 10 µl of TEMED. In SDS-PAGE 0.1 ml of 10% SDS solution was added in above solution.

**Sample preparation:** 0.02 ml of enzyme sample was mixed with 0.02 ml of 20% glycerol containing 0.05% bromophenol blue. For SDS-PAGE, 0.4 ml of 10% SDS and 0.1 ml of β-mercaptoethanol was added in above solution. The enzyme sample was diluted with sample buffer in 1:1 and heated for 5 min in boiling water bath and cooled.

**Procedure**

Cleared and dried glass slabs with spacer bars adjusted on three sides were fixed and tightly sealed with brown tape. Separating gel was poured up to 2/3 height between the vertical plates. It was allowed to polymerize for approximately 30 min. at room temperature and then stacking gel solution was layered to a height to accommodate the comb and comb was fixed immediately so that wells can be formed for sample application on polymerization. The gel plates were kept undisturbed overnight for polymerization. The spacer fixed on lower side was removed. The upper chamber of the apparatus was filled with electrophoresis buffer and gel plates were kept in lower chamber in such a way that no air bubble was formed between gel and buffer system. The comb was removed. The enzyme sample (40 µl) and solution of standard proteins (40 µl)
were loaded in well with the help of auto pipette. The apparatus was connected to the electrophorethic power supply unit and run at 8 V/cm in the spacer gel while the voltage increased in the resolving gel to 15 V/cm until blue line approached the lower of the resolving gel. The plates were taken out and gel was put into CBB for staining.

**CBB staining:**
Polypeptides separated by SDS-PAGE was fixed with methanol:glacial acetic acid and stained with Coomassie Brilliant Blue R250, a trimethylmethane textile dye also known as acid blue solution of the dye. Excess dye is then allowed to diffuse from the gel during a prolonged period of destaining.

**Making CBB stain:**
Dissolved 0.25 g of CBB R250 in 90 ml of methanol: H₂O (1:1v/v) and 10 ml of glacial acetic acid. The solution filtered through a Whatman No. 1 filter to remove any particulate matter.

**Table 7: CBB stain**

<table>
<thead>
<tr>
<th>CBB Stain</th>
<th>CBB Destain</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coomassie Brilliant Blue (R250)</strong></td>
<td>0.25 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>90 ml</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>10 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>90 ml</td>
</tr>
<tr>
<td><strong>No CBB</strong></td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>90 ml</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>10 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>90 ml</td>
</tr>
</tbody>
</table>

**Procedure:**
1. Gel immersed in 5-6 volumes of staining solution and placed on a slowly rotating shaker for overnight at room temperature.
2. Next day stain was removed by soaking the gel in methanol acetic acid solution without the dye on a shaker, changing the destaining solution time to time until bands appeared.
3. To make a permanent record photograph of the gel was taken.

**2.7.3 Polyclonal Antibody Production:** Polyclonal antibodies were raised against purified enzyme (Sambrook et al., 1989). White rabbit (Plate-1) was injected with 30 mg
purified protein dissolved in .5 ml of potassium phosphate buffer (pH 6.8, .01M) and emulsified in equal volume of Freund’s complete adjuvant.

**Plate-1. Rabbits used for raising polyclonal antibodies:**

This emulsion injected into the rabbit’s limbs immediately. Two booster injections with 10 mg each of purified protein dissolved in .25 ml of potassium phosphate buffer (pH 6.8, .01M) and emulsified in equal volume of Freund’s incomplete adjuvant were given at 15 days intervals. Blood was collected each time from an ear marginal vein, put at room temp for 3-4 h and then centrifuged at 3000g for 20 min. After clotting, the crude serum was prepared by centrifugation supernatant collected and stored at -70°C.

2.7.4 ELISA (Engvall and Perlman, 1971):

**Buffers used in ELISA**

1. **Coating buffer (pH 9.6):** It was prepared by dissolving 1.59 g sodium carbonate (Na₂CO₃), 2.93 g sodium bicarbonate (NaHCO₃) and 0.20 g sodium azide (NaN₃) in 900 ml H₂O, adjusted pH to 9.6 with HCl and made up to 1 L.

2. **PBS (pH 7.4) phosphate buffer Saline:** It was prepared by dissolving 8.0 g sodium chloride (NaCl), 0.2 g monobasic potassium phosphate (KH₂PO₄), 1.15 g dibasic sodium phosphate (Na₂HPO₄), 0.2 g potassium chloride (KCl), 0.2 g sodium azide (NaN₃) in 900 ml H₂O, adjusted pH to 7.4 with NaOH/HCl and made up to 1 L.

3. **PBS-Tween (PBST):** PBS + 0.5 ml Tween 20/L.

4. **Sample extraction buffer (pH 7.4):** PBST + 2% PVP (Sigma PVP-40 polyvinyl pyrrolidone) + 0.02M Sodium sulfite (Na₂SO₃).

5. **Conjugate buffer:** PBST + 2% PVP + 0.2% egg albumin (Sigma A-5253).
6. **Substrate buffer**: 97 ml diethanolamine + 600 ml H₂O + 0.2 g sodium azide (NaN₃) and adjusted to pH 9.8 with HCl and made up to 1 L with H₂O.

**Procedure: DAS-ELISA (Double Antibody Sandwich ELISA)**

1. Coating. Diluted IgG (white cap) 1:1000 in coating buffer and added 200 μl to each well of a microtitre plate.
2. Incubated at 37°C for 2-4 h.
3. Washed plate with PBS-Tween using wash bottle, soaked for a few minutes and repeated washing two times. Blotted plates dry by tapping upside down on tissue paper.
4. Added 200 μl aliquots of the test sample (extracted in sample extraction buffer) to duplicate wells.
5. Incubated overnight at 4°C.
6. Washed three times as in step 3.
7. Diluted antivirus conjugate 1:1000 in conjugate buffer and added 200 μL (dilution depending on antiserum quality) to each well.
8. Incubated at 37°C for 2-4 h.
9. Washed three times as in step 3.
10. Added 200 μl aliquots of freshly prepared substrate (1 mg/ml of p-nitrophenyl phosphate [Sigma 104-105] in substrate buffer) to each well. Incubated at room temperature for 60-120 min or until unambiguous reactions are obtained.
11. Assessed results by visual observation.
2.8 Results and Discuss

Plate: Sorghum seedlings raised in the laboratory and used for enzyme purifications

Extraction & assay of oxalate oxidase: Crude oxalate oxidase was extracted from 90 g leaves cut from the ten days old sorghum seedlings (*Sorghum vulgare* var. Amarnath-2000) raised in the laboratory. Oxalate oxidase assay was done based on the quantification of hydrogen peroxide generated from oxalate by oxalate oxidase using a colour reaction consisting of 4-aminophenazone phenol and peroxidase as chromogenic system (Bais *et al.*, 1980). Fractions which gave positive results in the form of pink colour after the reaction were collected at each step. Activity was calculated from the standard curve plotted between \( \text{H}_2\text{O}_2 \) concentration and \( A_{320} \) (Figure 8). Oxalate oxidase activity increased after ammonium sulfate precipitation and ion exchange chromatography while decreased after gel filtration chromatography due to dilution factor. Specific activity of the enzyme increased with each step of purification (Table 8).

Determination of protein content: Protein content in various enzyme preparations was determined (Bradford, 1976). Protein content decreased with each purification step (table 8).

Purification: Purification of the crude oxalate oxidase (15000g supernatant) was carried out in cold (4°C) which includes:

Ammonium sulfate fractionation (0-80%): Crude enzyme was subjected to 0-80% ammonium sulfate precipitation; it resulted into 9.6 fold purification of enzyme with 60% yield. This step also helped in concentrating the enzyme up to 35 ml to load it onto
Sephadex G-200 gel filtration column. Specific activity of the pooled fractions was 142.8 and the protein was 4.2 mg/ml.

**Sephadex G-200 gel filtration:** The gel filtration of 0-80% ammonium sulfate fraction onto Sephadex G-200 column resulted into a major broad activity peak between fractions 8-14 (Fig. 10). The maximum activity was eluted in fraction 12. The step resulted into 42.2 fold purification of enzyme with an overall 43% recovery of enzyme (Table-8). The fractions containing maximum specific activity were pooled and subjected to DEAE-Sephacel ion exchange chromatography.

![Fig. 10. (G-200) Sephadex Column Chromatography](chart)

**DEAE-Sephacel column chromatography:** The ion exchange chromatography of pooled Sephadex G-200 fractions on DEAE Sphacel column using a linear gradient of KCl (0-0.6M) in 0.01M potassium phosphate buffer, pH 6.8 resulted into one major peak of enzyme activity between fraction 2-6 and a minor peak of enzyme activity between fraction 9-12. The elution profile of major peak of enzyme activity showed that enzyme was eluted near 0.3M KCl concentration (Fig 11). This step gave an overall 108.1 purification fold with 28% yield. The fractions having maximum specific activity were pooled. The pooled fractions showed specific activity of 16.00 units/mg and protein .2 mg/ml (Table-8).
**Lyophilization:** The active fractions of DEAE-Sephacel column were pooled and lyophilized at -40°C to concentrate the enzyme.

**Table: 8 Purification of oxalate oxidase from 10 - days old Sorghum seedlings**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Activity (units/ml)</th>
<th>Specific activity (Units/mg)</th>
<th>Purification (Fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Enzyme</td>
<td>270</td>
<td>5</td>
<td>74</td>
<td>14.4</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>(0-80%)</td>
<td>20</td>
<td>4.2</td>
<td>600</td>
<td>142.8</td>
<td>9.6</td>
<td>60</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>35</td>
<td>.4</td>
<td>250</td>
<td>625</td>
<td>42.2</td>
<td>43</td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td>15</td>
<td>.2</td>
<td>380</td>
<td>1600</td>
<td>108.1</td>
<td>28</td>
</tr>
</tbody>
</table>

The starting material for purification of oxalate oxidase was 90 g leaves of 10-days old sorghum (var. Amarnath-2000) leaves. 15,000g supernatant was treated as crude enzyme. All the steps of purification were carried out at 4-8°C.
**Formula Used:**

Concentration of $\text{H}_2\text{O}_2 \times$ Dilution Factor

**Enzyme Activity**

Enzyme Activity

Time of Incubation to which enzyme act on substrate

**Specific Activity**

$\frac{\text{Enzyme Activity}}{\text{Protein Concentration}}$

**Purification Fold**

$\frac{\text{Specific Activity}}{\text{Crude Specific Activity}}$

**Yield (%)**

$\frac{\text{Fraction Activity} \times 100 \times \text{Total Volume}}{\text{Original Activity}}$
CBB staining:
The number of bands/proteins decreased with increasing purification step. We have the maximum number of proteins in crude extract (CS) while one single prominent band (highly visible) in gel filtration fraction sample (S-1 and S-2). The highly visible band is nearby 60 kDa (Fig. 12) so this band was cut and used for identification and sequencing purpose (As sorghum oxalate oxidase is reported to be a dimer of 60 kDa).

But we did not get the required protein sequence. So the enzyme was purified again by the same method and did SDS-PAGE. We got many bands including the 60 kDa band (Fig. 13).

To know the number of proteins non-denatured SDS-PAGE was done and it showed two bands. One band was nearby 60 kDa while other band much above the marker used in this PAGE (Fig. 14). So to confirm the identity of the enzyme the band was cut and used again for identification and amino acid sequencing by MALDI-LC-MS (Explained in chapter 3).
Fig. 13

Denatured SDS-PAGE (12%) CBB stained of the purified enzyme from sorghum leaves

Marker  Sample

97  66  45  31

Band Used in MLII01-TOB-945

Fig. 14

Non-Denatured SDS-PAGE (12%). CBB stained of the purified oxalate oxidase from sorghum leaves

M  S-1  S-2

M = BSA used as marker S-1 & S-2 = Samples
Polyclonal antibodies were raised against the purified enzyme in white rabbit was tested for its antigenicity in the crude (Fig. 15) as well as purified enzyme (Fig. 16).

**Fig. 15.** DAS-ELISA (Double Antibody Sandwich ELISA) against Crude Enzyme

**Fig. 16.** DAS-ELISA (Double Antibody Sandwich ELISA) against Purified Enzyme
In both cases ELISA results showed positive antigenicity, which was visibly observed with the appearance of the yellow color in samples (2, 3, 4, 5 and 6), while no color was appeared in controls (A, B, C, D, E and F).

Discussion
After purifying the enzyme by conventional methods three times it was noticed that usually it is not possible to purify the enzyme from sorghum up to homogeneity only by the conventional methods as used by earlier researchers for sorghum oxalate oxidase purification (Satayapal and Pundir, 1993). To purify the enzyme up to homogeneity, we need to do further purification steps like affinity chromatography or HPLC or 2D gel electrophoresis followed by MALDI-LC-MS for identification of any specific protein.

In non-denatured SDS-PAGE, we got two clearly visible bands instead of one (Fig. 14) while many bands in denatured SDS-PAGE (7-8), including a band of around 60 kDa (Fig. 13). We got the desired band in SDS-PAGE approximately 60 kDa, raised polyclonal antibodies against it and did ELISA to test the specificity of the enzyme in the crude extract as well against purified enzyme and in both cases got positive results. This 60 kDa band was used for MALDI-LC-MS analysis. The band/peptide (60 kDa) was thought to be oxalate oxidase but identified as beta-amylase by MALDI-LC-MS (As explained in next chapter). But as the sample had oxalate oxidase activity so the other bands should be oxalate oxidase polypeptides. The bands nearby 30 kDa or even below could be oxalate oxidase hexameric subunits while other bands in the gel (Fig. 13) could be dimers, trimers, tetramers or even pentamers of oxalate oxidase which possibly could not be denatured totally and remained intact as subunits. So like barley and wheat; sorghum oxalate oxidase seems to be a hexamer and not dimer as reported earlier.