2.0 PURIFICATION AND CHARACTERIZATION OF OXALATE DECARBOXYLASE
The enzyme oxalate decarboxylase was purified from the basidiomyceteous fungus, *Collybia velutipes*. This enzyme catalyzes the decarboxylation of oxalic acid with formation of stoichiometric quantities of formic acid and carbon dioxide. The partial purification and characterization of the enzyme had been studied by Shimazono and Hayashi (1957). It was shown to be highly specific for oxalic acid as substrate in absence of any cofactor requirement. Instead it was shown that a small amount of oxygen was required although the overall reaction did not stoichiometrically utilize oxygen.

Of the other enzymatic systems utilizing oxalate that had been described in detail, one involved formation of oxalyl CoA (*Pseudomonas oxalaticus*) and was implicated as participating in *Neurospora*. After repeated transfers the pseudomonad deteriorated and was not available for study; the *Neurospora* enzymes were not studied in detail (Jakoby, 1962).

### 2.1 MATERIALS AND METHODS

#### 2.1.1 Organism and growth conditions

*C. velutipes* (strain S, A.T.C.C 13547) was grown on the surface of medium containing 5% dextrose, 1% peptone, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O and 1% Difco malt extract at pH 5.2. The organism was grown from mycelial inoculation at 25°C in stationary cultures in a volume of medium, one-fifth the volume of culture flasks. About 25 days after inoculation, the enzyme oxalate decarboxylase was induced by addition of oxalic acid to 12.5 mM each culture flask.
The mycelium was harvested 2 to 3 days after the addition of oxalic acid and the mycelial pad was washed with cold distilled water and stored at -20°C. *C. velutipes* was maintained on slants of the same medium (Jakoby, 1962).

2.1.2 Purification

**Step 1: Preparation of crude extract**

The frozen mycelium was ground in a Waring blender for 10 min with either dry ice or liquid nitrogen. The powder was extracted with three volumes of 0.1M potassium citrate buffer, pH 3.0 for 10 min at 4°C with constant mechanical shaking and the suspension was centrifuged at 10,000 Xg for 30 min at 4°C. The supernatant was filtered through a double layer of cheese cloth.

**Step 2: Precipitation with acetone**

The acetone cuts were adopted from Shimazono and Hayaishi (1957). The percentages quoted are on vol/vol basis assuming additive volumes.

(a) Low temperature for acetone precipitation was maintained by an ice-salt bath at -10°C. The sample was chilled to 0°C and the first acetone cut of 0-33.3% was given by dropwise addition of chilled acetone to supernatant with constant mechanical stirring (Acetone-1). The mixture was equilibrated for 15 min and the precipitate formed was removed by centrifugation in a precooled rotor at 10,000 Xg, 20 min, 2°C. The precipitate obtained from 33.3%-50% cut was dis-
solved in one-fifth the starting volume of cold 0.1M potassium acetate buffer at pH 4.5. The enzyme solution was dialyzed for 16 hours, 4°C against two changes of 0.02M potassium acetate buffer, pH 4.5 and a small precipitate formed during dialysis was removed by centrifugation (Acetone-II).

(b) Supernatant was brought to 40% acetone (Acetone-III) and the precipitate obtained was discarded. The precipitate from further 50% cut was dissolved in a small volume of 0.02 M potassium acetate, pH 4.5 (Acetone-IV).

Step 3. Chromatofocusing. DEAE-Sepharose CL-6B (Pharmacia) was equilibrated in 0.02 M potassium acetate, pH 4.5 and used to pack a 10 ml column (1 x 13 cms bed). The resolubilized precipitate from the last acetone cut was loaded at a flow rate of 10 ml/hr. The column was washed with two column volumes of 0.02 M potassium acetate, pH 4.5 and the elution effected by developing an internal pH gradient using 4 mM acidic buffer mix (4 mM each of DL-aspartic acid, L-glutamic acid and glycine in water, pH 2.4). The elution was done at a flow rate of 10 ml/hr and 2 ml fractions collected; proteins were monitored by absorbance at 280 nm; the fractions were assayed for enzyme activity and the pH of each fraction determined. The fractions containing enzyme activity were pooled and dialyzed against water and concentrated in Amicon microconcentrator (30,000 M.W cut off). The enzyme was stored at 4°C.
2.1.3 Enzyme assay.

The oxalate decarboxylase activity was determined by measuring the liberation of $^{14}$CO$_2$ from [$^{14}$C]-oxalic acid (Amersham, 4.1 mCi/mmol). The enzyme assay was carried out in small glass vials which contained 1 ml of the following reaction mixture: 0.2 M potassium citrate pH 3.0, 0.005M potassium oxalate, pH 3.0, 5.5 nmoles (0.0227 μCi) of [$^{14}$C]-oxalic acid and 0.2 ml of enzyme solution. The tubes were preincubated for 5 min before the addition of enzyme. The tubes were sealed with rubber stoppers (Suba-seal) and incubated at 37°C for 30 min in a shaking water bath. The reaction was terminated by injection of 0.2 ml of 50% v/v trichloroacetic acid through the rubber caps and the tubes were shaken for additional 60 min to trap all the $^{14}$CO$_2$ evolved in the 0.2 ml methylbenzethonium hydroxide (Sigma) placed in a plastic vial inside the glass tube. The plastic wells were withdrawn and the contents transferred to 5 ml of toluene based scintillation fluid and counts determined in a liquid scintillation counter. Blank tubes were set up in which the 0.2 ml of 50% TCA was added before the enzyme or the enzyme was omitted. In kinetics experiments, the counts obtained from the activity of the boiled denatured enzyme were also considered.

Definition of a unit. One unit was defined as the amount of enzyme releasing 1 μmole of $^{14}$CO$_2$ per min at 37°C under standard assay conditions.
2.1.4 Protein estimation

Protein was estimated by Lowry microassay method (Peterson, 1977) using Bovine gamma globulin as the standard, or by Coomassie blue dye binding method (Bradford, 1976).

2.1.5 Activity-band correlation

The homogeneous oxalate decarboxylase (0.5 μg or 0.2 Units) was run in two lanes of 6% non-denaturing polyacrylamide gel at room temperature. After electrophoresis, one lane was stained with Coomassie blue R-250 and the other lane was cut into twelve 4 mm sections. The sections were placed in microfuge tubes with 200 μl of 0.1M potassium acetate, pH 4.5. The acrylamide was mashed and soaked overnight at 4°C. Enzyme activity was determined and correlated to the band in stained lane.

2.1.6 Gel electrophoresis of proteins

(a) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The discontinuous buffer system described by Laemmli (1970) was employed for electrophoresis in Mighty small apparatus (Hoefer) at 120 V constant voltage. Required volume of acrylamide was added from a 30% acrylamide plus 0.8% bis-acrylamide stock to 0.375 M Tris.Cl pH 8.8, 0.04% TEMED, and 0.5% ammonium per sulfate (APS) for the preparation of a resolving gel. Stacking gel had 4% acrylamide and 0.125 M Tris.Cl pH 6.8, 0.04% TEMED, and 0.5% APS. Reservoir buffer consisted of 0.025 M Tris base, 0.192 M glycine and
0.1% SDS, pH 8.3. Samples were prepared in 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue.

(b) Non-denaturing (native) PAGE. Native PAGE was done using Tris-glycine (pH 8.3-9.5) buffer of Ornstein-Davis high pH system (Davis, 1964). The protein was stacked at pH 8.3 in stacking gel containing 4% acrylamide, 0.125 M Tris-Cl pH 6.8, 0.01% TEMED and 0.2% APS. The resolving gel consisting of 6% acrylamide, 0.375 M Tris-Cl pH 8.8, 0.01% TEMED and 0.2% APS, separated the proteins at pH 9.5. Approximately 0.5 μg of purified oxalate decarboxylase was prepared in 0.625 M Tris pH 6.8, 10% v/v glycerol and 0.02% w/v bromophenol blue and loaded onto the gel. The protein was electrophoresed in reservoir buffer containing 0.3% Tris base and 1.44% glycine, pH 8.3.

(c) SDS-PAGE gradient gel. A 7-15% gradient gel was prepared for molecular size estimation of oxalate decarboxylase and to establish the purity of enzyme. The linear gradient slab gel was made by mixing 7% gel solution in mixing chamber with 15% gel solution in the reservoir chamber of a gradient maker. 7% gel mixture contained 7% acrylamide-bisacrylamide (30:0.8), 0.375 M Tris-Cl pH 8.8, 0.1% APS, 0.125% TEMED in 17 ml total volume. The 15% gel mixture contained 15% acrylamide:bisacrylamide (30:0.8), 0.375 M Tris-HCl pH 8.8, 25% glycerol, 0.05% APS, 0.15% TEMED in a total volume of 17.0 ml. A 4% stacking gel was poured.
Sample preparation and gel run was as described above.

(d) Two-dimensional gel electrophoresis. The separation in first dimension was by isoelectric focusing and in the second dimension by SDS-PAGE (O'Farrell, 1975).

First dimension: Isoelectric focusing

(i) Glass tubes (3mm internal diameter) were rinsed with the following in the given order: chromic acid, alcoholic KOH (37.6% KOH in ethanol) and distilled water. Tubes were dried and one end sealed with parafilm and marked at 12 cms. to ensure equal gel lengths.

(ii) Gel solution (9.5 M urea, 4% acrylamide, 2% NP-40, 2% pharmalytes pH 2.5-5 and 0.1% APS, degassed) was poured with Pasteur pippete and overlayered with water. After polymerization tubes were inserted into the apparatus.

(iii) Water from the gel tops was replaced by 25 µl of IEF sample buffer (9.5 M urea, 2% NP-40, 2% Pharmalytes 2.5-5, 5% 2-ME), which was overlayered with 20 µl sample overlay solution (9.5 M urea, 1% Pharmalytes pH 2.5-5) and 20 mM NaOH to fill the tubes. 20mM NaOH was poured in the upper tank and the lower tank had 10 mM phosphoric acid as the anolyte. Gels were prerun at 200 V for 15 min, 300 V for 30 min, and 400 V for 30 min. After the prerun the purified lyophilised oxalate decarboxylase (10 µg) was loaded in 25 µl IEF sample buffer at the basic end and the gel was electrophoresed for 14 h at 350 Volts.
(iv) After the run, the gels were carefully removed from the tubes by rimming with water filled syringe and the acidic end of the gel was marked by making a small cut. Gels were kept in SDS-PAGE sample buffer for 30 min at room temperature, and then stored at -70°C until used for 2nd dimension.

One of the gels was used for measuring the pH gradient. One cms slices in 4 ml degassed water kept for 12 h was used to measure pH.

Second dimension. 12% SDS-PAGE was done as described above. Standard molecular weight markers (Sigma, SDS-7) were prepared in 1% agarose blocks and placed on one side of the rod gel.

2.1.7 Amino acid composition

Samples were hydrolyzed in 6M HCl in evacuated and sealed tubes at 110°C for 22hr. The hydrolyzates were analyzed with in amino acid analyzer (LKB 4151 Alpha Plus). Cysteine and cystine were determined as cysteic acid after performic acid oxidation. Tryptophan was not determined. The amino acid composition was determined by Dr. Nagraj, CCMB.

2.1.8 Molecular size determination.

The molecular size of oxalate decarboxylase was determined by gel filtration chromatography. Purified enzyme (100 µg in 100 µl) was loaded on a FPLC gel permeation column.
(Superose 12; 10 x 300 nm) at a flow rate of 0.5 ml/min, using 0.02 M potassium acetate buffer, 0.1 M KCl pH 4.5 at room temperature. Proteins were detected at 280 nm. Standard proteins used were thyroglobulin (660 kDa, Pharmacia), ferritin (440 kDa, Pharmacia), catalase (210 kDa, Pharmacia), aldolase (158 kDa, Pharmacia), alcohol dehydrogenase (150 kDa, Sigma) and carbonic anhydrase (29 kDa, Sigma). The subunit composition was determined by sodium dodecyl sulfate – polyacrylamide slab gel electrophoresis in 7% to 15% gradient gels with Hoeffer-Minigel apparatus using Laemmli discontinuous dissociating buffer system. Proteins were stained with Coomassie brilliant blue R-250.

2.1.9 Biochemical properties

2.1.9.1 Kinetic properties

(a) The Michaelis constant $K_m$ of the enzyme (800 ng/ml) was determined using relative substrate concentrations of 0.1, 1.0, 1.11, 1.25, 1.43, 1.67, 2.0, 2.5, 3.33, 5.0 and 10 mM. Data was plotted according to Lineweaver-Burk reciprocal plot (Lineweaver and Burk, 1934).

(b) Inhibition of oxalate decarboxylase by $\text{PO}_4^{2-}$ was studied by including 90 mM KH$_2$PO$_4$ in the enzyme assays at different concentrations of oxalic acid.

(c) Substrate specificity of the enzyme was studied with 10 mM concentrations of pyruvic acid, acetic acid, formic acid, succinic acid, oxaloacetic acid and citric acid.
2.1.9.2 Effect of pH

(a) The stability of enzyme at different pH values was studied by precipitating oxalate decarboxylase with 80% (v/v) acetone and resolubilizing the enzyme at a concentration of 4 µg/ml in buffers of different pH values, at 25°C for 3h. Enzyme activity was determined at pH 3.0 using standard assay system.

(b) Enzyme was preincubated at 4 µg/ml in buffers at different pH values and enzyme activity determined at that pH. The following buffers were used, each at 0.05 mM: HCl-KCl at pH 1 & 2, potassium citrate at pH 3 & 6, potassium acetate at pH 4 & 5, Tris.Cl at pH 7,8 & 9.

2.1.9.3 Thermostability

All thermal inactivation studies were performed in 0.02M potassium acetate buffer pH 3.0. Purified enzyme was diluted at a concentration of 4 µg/ml; 200 ul aliquots were placed in tubes and incubated in water bath at desired temperature. Duplicate samples were removed at 10 min intervals and placed in an ice bath. Oxalate decarboxylase activity assayed immediately and compared to a control incubated at 0°C. Percent activity remaining over control was obtained.

2.1.9.4 Digestion with protease from S.aureus V8 (Cleveland, 1983).
(a) 10 μg of purified proteins were heat denatured for 10 min and incubated with 100 μg/ml of protease at 37 °C for 2 hr. The peptides were resolved on 15% SDS-PAGE and stained with Coomassie blue.

(b) Ten μg of purified protein was resolved on 12% SDS-PAGE and the bands excised and equilibrated in V8 protease buffer (Cleveland, 1978) for at least 2 hr at room temperature. Digestion with V8 was done in the 4.5% stacking gel of a 15% SDS-PAGE at room temperature by switching off the current for 30 min when the dye reached the stacking gel boundary. After electrophoresis the peptides were transferred to a nitrocellulose membrane and immunodetected as described in sections 2.1.9.3

2.1.9.5 Glycoproteic nature

(a) Glycoprotein staining (Polyacrylamide gel electrophoresis: laboratory techniques, Pharmacia)
   (i) The gel was fixed in 5 volumes of 7% acetic acid for 1 hr at 4 °C.
   (ii) After briefly rinsing with water, the gel was incubated in 5 volumes of 1% periodic acid in cold 7% acetic acid for 1 hr, at 4 °C in the dark.
   (iii) The gel was washed in several changes of 7% acetic acid for 1 hr at 4 °C in dark.
   (iv) The color was developed by incubating the gel in cold Schiff's reagent.
   (v) Excess reagent was washed off with acidic 0.5% meta-
bisulfite solution.

(b) Concanavalin A reactivity (Reithman, 1988; Clegg, 1982)

The Con-A reactivity of the glycoprotein was detected after separation of 10 µg of protein on 10% SDS-PAGE and transfer to nitrocellulose by the method of Towbin et al (1979). The filter containing transferred proteins was treated as follows:

(i) It was blocked for 2 hr in blocking solution (10 mM Tris.Cl, pH 7.4, 0.9% NaCl, 0.5% Tween 20 containing 10 µm each of Mg²⁺, Ca²⁺, Mn ²⁺).

(ii) The filter was transferred to a solution of 16 µg/ml Con-A (Sigma) in Tween-TTS plus cations for 1hr, washed 5 times for 5 min each with Tween-TTS plus cations, and then incubated for 1hr in a solution of HRP (horse radish peroxidase, Sigma: Type 1,50 µg/ml in TTS plus cations).

(iii) The HRP-active band was stained with DAB (0.025%) plus 0.01% H₂O₂. The binding of the enzyme to Con A-Sepharose was done as described earlier (Brattain et al, 1977).

(c) Phenol-sulfuric acid method

The total neutral sugar content of the enzyme was determined by the method of Mckelevy and Lee (1969). To 0.5 ml of sample solution, 0.3 ml of 5% aqueous phenol and 1.8 ml of conc. H₂SO₄ were added. The contents were vortexed vigorously and the tubes were cooled to room temperature. The absorbance was read at 480 nm. Glucose was used as a standard.
Deglycosylation (Trimble and Maley, 1984)

1μg of purified protein was deglycosylated using Endoglycosidase H (Boehringer Manneheim). The lyophilised protein was solubilised in 50 mM sodium acetate pH 5.5, and denatured at 100 °C for 5 min in presence of SDS such that the w/w ratio of SDS to protein was 1.2. After the solution was cooled to room temperature, PMSF was added to 1 mM and 10 μU of Endo H (40 mU/mg) was added. The reaction was incubated at 37°C for 22 h. The protein was precipitated with 10 % v/v TCA at 0°C, for 1 hr. The precipitate was washed with 1:1 volume of ether:ethanol and redissolved in Laemmli sample buffer and electrophoresed on 10% SDS-PAGE.

Immunological characterization

Preparation of antiserum

The oxalate decarboxylase (1 mg/ml) was heat denatured by boiling for 10 min in PBS presence of 0.5% SDS. The protein antigen (150 μg) in PBS was emulsified with Freund's complete adjuvant and the thick emulsion injected subcutaneously in New Zealand White rabbit. Subsequent boosters were given in Freund's incomplete adjuvant subcutaneously after a period of three weeks. Fourth injection was given intravenously with the enzyme that was dialyzed against PBS and heat denatured for 10 min in absence of SDS. Test bleeds were done from the ear vein to check for the production of
specific antibodies 10 days after injection (Harlow and Lane, 1988). Antibody titer was monitored using Ouchterlony immunodiffusion technique (Garvey, 1977). Once good titers developed serum samples of 20 ml were collected and processed according to Garvey (1977) and stored in presence of azide at -20 °C.

2.1.10.2 Western blotting

Proteins were transferred to nitrocellulose membrane (Schleicher & Schuell) according to the procedure of Towbin et al (1979) in a transfer buffer containing 25 mM Tris base, 192 mM glycine, and 20% methanol. Transfer was carried out in electrotransfer apparatus (Hoefer) at 150 mA constant current for 3 hr, 15°C. After the transfer the blot was stained in 0.5% Ponceau S in 1% acetic acid, for 5 min and destained in 1% acetic acid (Salinovich and Montelaro, 1986). Standard proteins were marked and blot was completely destained in water.

2.1.10.3 Immunodetection of proteins (Superscreen immunoscreening system, Amersham).

(i) The blots were rinsed in TBS (10 mM Tris.Cl pH 7.5, 150 mM NaCl), and blocked in 5% non-fat dry milk (Amersham) in TBST plus 0.1% sodium azide for 2 hr.

(ii) Filters were washed thrice, for 10 min each in TBST (TBS + 0.05% Tween 20) and incubated for 2 hr at room temperature with primary antibody at 1:5000 (crude antiserum) or 1:1000 (affinity purified) dilutions in TBST.
(iii) After 3 X 10 min rinses in TBST, secondary antibody viz. anti-rabbit IgG labeled with alkaline phosphatase (Promega) or HRP (Amersham) was used at a dilution of 1:7500 (AP) or 1:1000 (HRP) as recommended by the suppliers. Incubation was at room temperature for 2 hr.

(iv) Filters were washed again in TBST. Antigen-antibody complexes were detected using NBT/BCIP in alkaline phosphatase buffer (100mM Tris.Cl pH 9.5, 100 mM NaCl, 5 mM MgCl₂ or DAB in TBS plus 30% H₂O₂). Reaction was stopped in water.

2.1.10.4 Affinity purification of antibodies (Iwaki, 1989)

50 μg of purified peak A protein was transferred to nitrocellulose membrane and the protein band visualized by Ponceau S staining. The band was incised and cut into small strips in deionized water. The strip was blocked in 5% non-fat dry milk in TBS (50 mM Tris.Cl pH 7.6, and 0.15 M NaCl) for 1 hr at room temperature. The blot was washed in TBST (TBS + 0.05% Tween 20) and incubated overnight with 1 ml of diluted antiserum (25 % v/v in TBS). The blot was washed 4 times with TBS and the bound antibodies were eluted in 0.4 ml of 0.2 M glycine-HCl pH 2.5 for 2 min in presence of 0.2% BSA. The eluted antibody was immediately neutralized by adding equal volume of 1M potassium phosphate, pH 9.0 plus 0.2% BSA. The volume was made to 1 ml in TBS and affinity purified antibody was stored at 4°C in 0.05% azide.
2.1.10.5 Immunotitration of enzyme activity.

Purified oxalate decarboxylase (1.5μg) in 0.02 M potassium acetate buffer pH 4.5 was incubated with different volumes of preimmune and immune sera for 2hr at room temperature. The enzyme-antibody complexes were spun down at 12,000 xg, 10 min and enzyme activity in supernatant determined.

2.1.10.6 Immunoprecipitation (Anderson and Blobel, 1978)

(A) Preparation of Protein-A Sepharose beads

(i) Protein-A Sepharose beads (5mg/reaction, Pharmacia) were swollen in 0.5 M sodium phosphate buffer, pH 7.0 for 30 min. Beads were washed twice in 0.1 M sodium phosphate buffer pH 7.0.

(ii) The beads were divided in two parts and a part was incubated with preimmune serum and the other half with the immune serum and rotated for 2hr at room temperature.

(iii) Beads were spun down and washed twice with wash buffer and resuspended in a small volume of wash buffer.

(B) Immunoprecipitation.

(i) Purified protein (3μg) was directly incubated with preimmune IgG bound to Protein A-Sepharose. The in vitro translated products were solubilized by heating in boiling water bath for 4 min in presence of 4 % SDS. This was diluted by first adding equal volume of sterile water and then with 4 volumes of dilution buffer (2.5% Triton-X 100, 90 mM NaCl, 6 mM EDTA, 50 mM Tris.Cl, pH 7.4). It was spun at room
temperature for 5 min.

(ii) The supernatant was clarified by rotation for 2 hr with Protein-A Sepharose beads to which preimmune IgG was bound. The beads were removed by centrifugation and the supernatant incubated with anti-oxalate decarboxylase antibodies bound Protein A-sepharose for 2 hr, room temperature or for 4°C overnight.

(iii) Beads were washed four times in wash buffer (0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA) and twice in sterile water. The immunoprecipitated antigen was eluted from the beads by boiling for 4 min in 1X Laemmli buffer. The supernatant was loaded onto 10% SDS-PAGE gel. The gel was dried, fluoroographed (Chamberlain, 1979) and exposed to a preflashed X-ray film (Kodak-XAR-5). The purified protein was visualized directly in the CBB stained gel.

2.2 RESULTS

2.2.1 Purification

The oxalate decarboxylase was purified from the oxalate induced crude extract of *C. velutipes*. Maximal activity was obtained 2 or 3 days after the addition of oxalic acid. Specific activity of the enzyme varied between different batches of culture. The enzyme was purified by extraction at acidic pH. The acetone extraction steps were taken from Shimazono and Hayashi (1957) except that the last 2 acetone cuts were not repeated. The protein precipitation is effect-
ed by reduction in the solvating power of water for the charged hydrophilic enzyme as the concentration of acetone is increased gradually (Scopes, 1987). Very low temperatures are needed to be maintained to avoid denaturation due to heat generation on organic solvent precipitation. At this step the protein was purified 320 fold. The precipitate from the last acetone step was redissolved in 0.02 M potassium acetate buffer, pH 4.5 and subjected to chromatofocusing. The enzyme bound to the ion exchanger at pH 4.5 at low ionic strength and was eluted by developing an internal pH gradient using an acidic buffer mix. Most of the contaminating proteins were lost in the initial wash and in the last salt wash.

The results of a typical purification procedure are given in Table 2.1. The enzyme resolved into two peaks on the chromatofocusing column: a minor peak called A eluted at pH 3.3 and peak B eluted at pH 2.5 as a major peak (Fig.2.1). Peak A was purified 1666-fold with about 3% recovery while peak B coeluted with two minor contaminants and was purified 614-fold with 16% recovery (Table 2.1). However, these contaminants disappeared after a passage through Sepharose 4B gel filtration column. The peak A protein was used for further work despite its low yield because of the ease of obtaining a highly purified form through use of a single column.
FIG. 2.1 Elution profile of oxalate decarboxylase from chromatofocusing column.

The protein from Acetone-IV step was loaded onto DEAE Sepharose CL-6B (1 x 13 cms) equilibrated in 0.02 M potassium acetate buffer pH 4.5. Bound proteins were eluted with a decreasing pH gradient. The activity was associated with peak A eluting at pH 3.3 and with peak B eluting at pH 2.5. Inset shows the protein bands corresponding to the two peaks. Peak A (lane A) and peak B (lane B) were resolved on 11% SDS-PAGE and stained with Coomassie blue. Molecular weight markers to the left are from Sigma (SDS-7).
Table 2.1

Purification table from a typical experiment

<table>
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<tr>
<th>Purification step</th>
<th>Total Protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification fold</th>
<th>Yield %</th>
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<td>1. Crude extract</td>
<td>4480.0</td>
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<td>2. Acetone II</td>
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<td>3. Acetone-IV</td>
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<td>260</td>
<td>68.8</td>
<td>328</td>
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<td>4. Chromatofocusing</td>
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<td></td>
</tr>
<tr>
<td>a. Peak A</td>
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<td>350.0</td>
<td>1666</td>
<td>2.9</td>
</tr>
<tr>
<td>b. Peak B</td>
<td>1.24</td>
<td>160</td>
<td>129.0</td>
<td>614</td>
<td>16.8</td>
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* 150g of liquid nitrogen ground powder used
2.2.2 Criteria of purity

The serial two-fold dilutions of the enzyme showed that at least 45 ng of protein can be detected by Coomassie blue staining (Fig.2.2A). A single band was detected in all the lanes indicating absence of any other contaminant. About 10 μg of protein gave a single spot in two dimensional gel electrophoresis (Fig.2.2B) involving IEF in the first dimension and a 12% SDS-PAGE in the second. When electrophoresed on non-denaturing gel, the enzyme migrated as a slightly diffuse protein band as seen by CBB staining. The migration distance of enzyme activity correlated with that of the single stained band in non-denaturing PAGE (Fig.2.3). No protein bands or enzyme activity was found in any other part of the gel. Thus the protein band corresponding to peak A had the oxalate decarboxylating activity. The peak A eluted as a single peak in FPLC gel permeation chromatography (data not shown).

2.2.3 Amino acid composition.

A very high content of acidic amino acids (22%) characterized both the protein peaks (Table 2.2). This could account for their low pl values, although the proportion amidated in native protein was not determined. The two peaks had very similar amino acid composition but for a two-fold higher methionine and tyrosine content in peak B and a two-fold higher cysteic acid in peak A. Further relatedness was indicated by the peptide map of the two peaks using S.
FIG. 2.2 Criteria of purity.

(A) Two-fold serial dilutions (lanes 1 to 7) of oxalate decarboxylase starting with 3 μg (lane 1) protein were resolved on 7%-15% gradient SDS-PAGE.

(B) 10 μg of oxalate decarboxylase (peak A) was resolved by isoelectric focusing (pH 2.5-5.0, ampholytes, Pharmacia) in first dimension and by 12% SDS-PAGE in the second dimension. Lane S shows SDS-7 (Sigma) calibration standards for molecular weight estimation. In (A) & (B) the gels were stained with Coomassie Blue.
FIG. 2.3 Activity-band correlation.

500 ng protein was electrophoresed in two lanes of 6% non-denaturing polyacrylamide gel; one lane was stained with Coomassie blue and the other was cut into twelve 4 mm sections and enzyme activity was assayed and correlated to the band in stained lane. The migration distance (R_f of 0.35, gel slice no.4) of enzyme activity (0.2 units) correlated with that of the single stained band (500 ng). No protein band or enzyme activity was found in any other part of the gel. The stained gel slice containing the protein is shown at the top.
Table 2.2

Amino acid Composition

<table>
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<th></th>
<th>Peak A</th>
<th></th>
<th>Peak B</th>
<th></th>
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</thead>
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<td>Asx</td>
<td>10.57</td>
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<td>10.31</td>
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<td>His</td>
<td>2.68</td>
<td></td>
<td>2.74</td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>8.44</td>
<td></td>
<td>9.44</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>7.23</td>
<td></td>
<td>7.19</td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>8.56</td>
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<td>8.48</td>
<td></td>
</tr>
<tr>
<td>Cys$^c$</td>
<td>0.66</td>
<td></td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>0.84</td>
<td></td>
<td>1.85</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>11.03</td>
<td></td>
<td>10.92</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>6.31</td>
<td></td>
<td>6.52</td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>7.79</td>
<td></td>
<td>7.19</td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>4.00</td>
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<td>3.66</td>
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<td>Pro</td>
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<td>8.46</td>
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<td>Phe</td>
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<td>4.68</td>
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</tr>
<tr>
<td>Met</td>
<td>0.34</td>
<td></td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>ND$^b$</td>
<td></td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Ammonia</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

a: Mole percent
b: Not determined
c: Determined as cysteic acid
 aureus V8 protease (Fig. 2.4). Thus the two forms of the enzyme had related though not identical aminoacid composition as well as peptide maps. Slight differences in the peptide maps could be due to the presence of carbohydrates at the cleavage sites of the protease.

2.2.4 \( K_m \)

From Lineweaver-Burk plot (Fig. 2.5A), the apparent \( K_m \) value of 4.5 mM was calculated for potassium oxalate as the substrate. This gave a \( V_{\text{max}} \) of 0.133 mmol 1\(^{-1} \) min\(^{-1} \). The enzyme was competitively inhibited by phosphate ions; a \( K_i \) of 9 mM was calculated when 90 mM \( \text{PO}_4^{2-} \) was added to the reaction (data not shown). The enzyme was specific for oxalate as the substrate as citric acid, acetic acid, oxaloacetic acid, succinic acid, formic acid were not acted upon by the enzyme.

2.2.5 Effect of pH

Curve A in Fig. 2.5B shows that the preincubation of enzyme at different pH values had no effect on the activity measured at pH 3.0. Thus the enzyme was not irreversibly inactivated over a wide range of pH values. Curve B, the activity obtained when the enzymatic reaction was performed at different pH values, indicated that the pH optimum is 3.0. Hence decrease in activity on either side of the optimum is probably due to the effect of pH on the ionizable groups of the active site or substrate.
FIG. 2.4 Comparison of Cleveland digest patterns from the two forms of oxalate decarboxylase.

(A) 10 μg of peak A and peak B protein in V8 protease buffer was heat-denatured and treated with 100 μg/ml of protease V8 for 1h at 37°C. The peptides were analyzed on 15% SDS-PAGE and stained with Coomassie blue. Lanes: 1, peak A and 2, peak B protein.

(B) Peptide maps were generated directly in 4.5% stacking gel of a 15% resolving gel. 10 μg of peak A and peak B polypeptides were cut from 11% SDS-polyacrylamide gel, and digested with 100 μg/ml V8 protease (S. aureus, Sigma). The peptides were electroblotted to a nitrocellulose membrane and immunodetected with 1:5000 dilution of anti-oxalate decarboxylase antibody. Lanes: 1 (peak A protein) and 2 (peak B protein).
FIG. 2.5 (A) Substrate saturation kinetics

$K_m$ of purified oxalate decarboxylase (0.8μg) was determined with different concentrations of oxalic acid. Data from direct plot was analyzed by Lineweaver-Burk plot (inset).

(B) Effect of pH on activity and stability of oxalate decarboxylase.

Curve A shows the effect of pH on enzyme stability i.e. the percent activity recovered at pH 3.0 after preincubating enzyme (4 μg/ml) at 25°C for 3h at indicated pH values. Curve B shows the percent activity obtained when the assay was done at different pH values.

(C) Heat sensitivity of oxalate decarboxylase

The enzyme (4μg/ml) in 0.1M potassium acetate pH 4.5 was preincubated at 60°C, 80°C or 96°C and aliquots were removed at 10 min intervals and placed in an ice bath. The enzyme was assayed as described in 'Materials and Methods' and compared to a control incubated at 0 °C at all times.
2.2.6 Effect of temperature

The effect of temperature on the stability of purified oxalate decarboxylase is shown in Fig. 2.5C. The enzyme retained 78% of the initial activity after 20 min of incubation at 80°C but almost complete inactivation occurred within 10 min at 96°C.

2.2.7 Molecular mass determination.

The molecular mass of the native enzyme estimated by gel filtration was 560 kDa (data not shown). Electrophoresis on 7%-15% concentration gradient SDS-PAGE showed the presence of a single polypeptide of 64 kDa (Fig. 2.2A). When the enzyme was treated with Endoglycosidase H, the size of the deglycosylated protein was 55 kDa (Fig. 2.7). The protein was deglycosylated using Endo β-N acetylglucosaminidase H which is specific for high mannose and certain hybrid Asn-linked type oligosaccharides. The substrate was heat denatured to expose all the glycan units prior to Endo H treatment. The deglycosylated enzyme migrated faster (lanes 3 & 4) than the untreated one (lanes 1&2). The molecular size of the deglycosylated enzyme was 55 kDa. A faint band lower down (28 kDa) was due to Endo-H protein as judged by increasing intensity in samples digested with higher units of Endo-H.

2.2.8 Other properties.

The enzyme was unaffected by sulfhydryl group reagents as the enzyme retained almost complete activity in presence of
Table 2.3

Properties of Peak A of oxalate decarboxylase

<table>
<thead>
<tr>
<th>1. Km</th>
<th>4.5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Vmax</td>
<td>0.133 nmol L⁻¹ min⁻¹</td>
</tr>
<tr>
<td>3. Ki(PO₄⁻)</td>
<td>9.0 mM</td>
</tr>
<tr>
<td>4. pI [Chromatofocusing] [IEF]</td>
<td>3.3</td>
</tr>
<tr>
<td>5. Molecular size:</td>
<td></td>
</tr>
<tr>
<td>a. Superose 12 (FPLC)</td>
<td>560 kDa</td>
</tr>
<tr>
<td>b. SDS-PAGE (7%-15%)</td>
<td>64 kDa</td>
</tr>
<tr>
<td>c. Deglycosylated</td>
<td>55 kDa</td>
</tr>
<tr>
<td>6. pH optimum</td>
<td>3.0</td>
</tr>
<tr>
<td>pH stability range</td>
<td>2.0 - 9.0</td>
</tr>
<tr>
<td>7. Temperature optimum</td>
<td>37°C</td>
</tr>
<tr>
<td>8. Thermal inactivation</td>
<td>10% decrease in activity at 80°C per 10 min</td>
</tr>
<tr>
<td>9. PAS staining</td>
<td>Positive</td>
</tr>
<tr>
<td>10. Hexose content</td>
<td>15%</td>
</tr>
<tr>
<td>11. Effect of: Remaining activity</td>
<td></td>
</tr>
<tr>
<td>a. 8ME (25 mM) (100 mM)</td>
<td>42%</td>
</tr>
<tr>
<td>b. Cysteine (0.1mM-1.0 mM)</td>
<td>100%</td>
</tr>
<tr>
<td>c. pCMBS (50 mM)</td>
<td>95%</td>
</tr>
<tr>
<td>d. Iodoacetate (50 mM)</td>
<td>84%</td>
</tr>
<tr>
<td>e. Mg²⁺ (1mM)</td>
<td>90%</td>
</tr>
<tr>
<td>f. SDS: 10% (25°C) 10% (60°C)</td>
<td>45%</td>
</tr>
<tr>
<td>g. Triton X-100 (5%)</td>
<td>100%</td>
</tr>
<tr>
<td>h. Ethylene glycol (50%)</td>
<td>50%</td>
</tr>
<tr>
<td>i. Butanol (20%)</td>
<td>85%</td>
</tr>
<tr>
<td>j. KCl (3M)</td>
<td>90%</td>
</tr>
</tbody>
</table>
50 mM pCMBS or 50 mM iodoacetate. It retained 45% of its activity after incubation with 10% SDS for 30 min, room temperature. When heated to 60 °C in presence of 10% SDS, the enzyme lost all the activity (Table 2.3).

2.2.9 Enzyme stability

Enzyme preparations were stable at both 4°C and -20°C and more than 70% of the initial activity could be measured after 4 months of storage at 4°C at 1 mg/ml concentration in 0.02 M potassium acetate pH 4.5.

2.2.10 Glycoproteic nature

The protein was stained positive with Periodate-Schiffs base reagent (Fig.2.6-1). The uninduced crude extract (lane U) did not show have a distinct band corresponding to purified oxalate decarboxylase (lane O). But the crude extract prepared from oxalate induced culture (lane I) had a 64 kDa band. Some other proteins were also found to be glycosylated in the crude extract. The enzyme showed reactivity to Con A (Fig.2.6-2). The enzyme was bound to Concanavalin A-Sepharose and eluted with 0.5 mM α-methyl mannoside (data not shown). The neutral sugar content was estimated to be 15% by phenol-sulfuric acid method.

2.2.11 Immunological characterization

The antibodies against the enzyme were raised in rabbits and the initial titer was checked by immunoprecipitin lines
FIG. 2.6. (1) Periodic acid Schiff's staining. Crude extracts (100 μg) from uninduced (lane U) and oxalate induced (lane I) cultures and 4 μg purified oxalate decarboxylase (lane O) were resolved on 11% SDS-PAGE. Crude extract from uninduced cultures did not contain the band corresponding to purified protein.

(2) Con-A reactivity of oxalate decarboxylase. 16 μg of peak A protein was resolved on 12% SDS-PAGE and electroblotted to nitrocellulose membrane and its ConA reactivity was studied as described in 'Methods'.
FIG. 2.7 Deglycosylation of oxalate decarboxylase. 1 µg of the peak A enzyme was treated with 1 mU (lane 3) and 10 mU (lane 4) of Endo H for 22 h and resolved in 11% SDS-PAGE. Lanes 1 & 2 contain untreated controls for peak A. Peak B treated with 10 mU of Endo H (lane 5) and lane 6 with glycosylated peak B protein (1 µg) are also shown. Lane S (right) shows the molecular weight markers from Pharmacia and the lane S (left) are the high molecular weight, pre-stained markers (BRL).
on Ouchterlony plates (data not shown). The immunotitration of enzyme with 8 μl of the crude oxalate decarboxylase antiserum precipitated more than 60% of the initial activity (Fig. 2.8A). In Western blots, oxalate decarboxylase antiserum, at a dilution of 1:5000, could detect about 1 ng of peak A protein (lane D, Fig. 2.8B). The antiserum which was affinity purified (Section 2.1.10.4) against peak A protein cross-reacted with the peak B protein (lane C), and oxalyl CoA decarboxylase from Oxalobacter formigenes (lane B, Fig. 2.8B). It did not cross-react with oxalate oxidase from H. vulgare (Lane A, Fig. 2.8B). The crude antiserum cross-reacted with all the peptides obtained from V8 protease digest of protein peaks A and B (Fig. 2.4B) and with the deglycosylated protein (data not shown).

The purified protein bound to anti-oxalate decarboxylase antibody IgG immobilized on protein-A Sepharose (Fig. 2.9, lane B) but it did not cross-react with preimmune IgG-protein A sepharose (lane A).

2.3 DISCUSSION

Oxalate decarboxylase was isolated from the fungus Collybia velutipes by the acetone fractionation steps and further purification effected by chromatofocusing. Shimazono and Hayaishi (1957) had employed acetone fractionation steps to obtain partially purified enzyme. The properties were determined using crude extract or the first and second acetone precipitates and the enzyme was assayed manometrically or
FIG. 2.8 Immunological characterization.

(A) 1.5 μg of enzyme in 0.02 M potassium acetate buffer, pH 4.5 was incubated with different volumes of serum (1) at 25°C for 2 h. Immunocomplexes were spun down at 12,000 xg for 10 min and residual activity determined by standard assay. Control incubation was carried out with preimmune serum (PI).

(B) Immunoreactivity of affinity purified antiserum against oxalate decarboxylase to other oxalate degrading proteins.

Lane A, Oxalate oxidase (H. vulgare, 10 μg, Sigma). Lane B, Oxalyl CoA decarboxylase (Oxalobacter formigenes, strain, OxB, 10 μg). Lane C, peak B oxalate decarboxylase (1 μg). Peak A of oxalate decarboxylase was loaded in lane D (1 ng), lane E (10 ng), lane F (100 ng) and lane G (1 μg).
FIG. 2.9  Immunoprecipitation of oxalate decarboxylase. 5 μg of the peak A protein was incubated with IgG from preimmune or immune sera bound to protein A-sepharose beads. The bound protein was eluted from the beads and resolved on 10% SDS-PAGE and the gel stained with Coomassie blue. Lane A shows that no protein bound to preimmune beads. In lane B, oxalate decarboxylase band was obtained above the heavy chain IgG polypeptide.
spectrophotometrically. In this work the enzyme was purified to apparent homogeneity and its purity was checked in various ways. The enzyme was found to be glycosylated and the high apparent molecular size obtained by gel filtration could be due to the tendency of certain glycoproteins to interact noncovalently in solution (Kleinman, 1986; Farasch-Carson, 1989). The apparent molecular size of 64 kDa obtained consistently at all gel percentages by SDS-PAGE using Laemmli buffer system should be considered an approximation since the enzyme has carbohydrate and is of acidic nature. Glycoproteins may (i) bind SDS in a ratio different from that of other proteins, or (ii) form asymmetric SDS-protein complexes thus resulting in anomalous migration when compared to other molecular size standards (Cozens and Reithmeier, 1984). However, the size of the deglycosylated form was 55 kDa. The two forms of the enzyme resolved by chromatofocusing showed a few differences in their aminoacid composition; thus the two forms although related are not identical and these two may have arisen from multiple alleles at a single locus. The differences in the pI could also arise from different degrees of amidation of acidic aminoacids as well as from the compositional, structural or site-specific heterogeneity which contributes to the microheterogeneity in the constituent oligosaccharide chains of the glycoproteins (Lamport, 1980).

The peak A which could be purified to homogeneity after a passage through single column was studied in detail. The kinetic properties of the purified enzyme were found to be
different from those reported earlier; the \( K_m \) was reported to be 2mM and the maximal activity of decarboxylation was observed at a pH range of 2.5-4.0. The differences could arise from the different methods of enzyme assays employed and the use of partially clarified extracts. The enzyme was found to be stable over a wide range of pH and was moderately thermostable. These properties could be related to the glycoproteinic nature of the enzyme. Protein conformation could be altered by presence of oligosaccharides as these may direct folding of nascent proteins since N-linked glycosylation is an early event in protein translation in rough endoplasmic reticulum (RER). Chu (1978) found that the presence of carbohydrate in yeast invertase stabilized the enzyme to mild acid, heat, or repeated cycles of freezing and thawing. Oligosaccharides also decreased susceptibility of proteins to proteases (Olden, 1982). Thus the stability of the oxalate decarboxylase to pH and temperature could also be due to the presence of glycan units. The enzyme retained 45% of its activity in presence of 10% SDS at room temperature (Table 23). Since glycoproteins bind less SDS per gram weight of the protein, this glycoprotein may not be completely denatured. However, when heated to 66°C in presence of 10% SDS almost all of the activity was lost. The enzyme did not require any kind of cofactor for its activity and was shown to be highly specific for oxalic acid as a substrate. The immune serum could detect a minimum of 1 ng of protein. The antiserum against peak A of oxalate
decarboxylase could precipitate the enzyme activity from solution. The antiserum crossreacted to peak B protein and to an unrelated oxalyl-Co A decarboxylase from *Oxalobacter formigenes*, an anaerobic bacterium. Further studies required cloning of the gene encoding oxalate decarboxylase.