4.0 SUMMARY
SUMMARY

Oxalic acid accumulates in a number of biological systems as a nonmetabolic pool in which the turnover is not occurring. This along with its strong acidic nature and powerful chelating capacity indicates its role as a stress factor in various cases. The oxalic acid levels increase in conditions of primary and secondary hyperoxaluria in humans and this results in impaired renal functions, disturbances in glycine metabolism, reduced blood coagulability (Luque de Castro, 1988; Conyers et al, 1990). Oxalic acid also serves as a mobile toxin which allows the spread of pathogenic fungus, Sclerotinia sclerotium in Helianthus spp. The oxalic acid produced by the fungus from oxaloacetate or from glyoxylate is secreted into host tissues. This lowers the pH to enhance the activity of extracellular enzymes secreted by fungus (Maxwell, 1973). Oxalic acid moves up the xylem elements from stem base to leaves etc. and allows for fungal spread. Oxalate production by other fungi has also been linked to their pathogenicity e.g. oxalic acid production occurs in the virulent strains of plant pathogen, Endothia parasitica (Havir and Anagnostakis, 1983). Oxalic acid produced by Endothia parasitica might chelate calcium from cell wall components in the host bark enabling the advance of mycelium in cankers. Oxalic acid is an important substrate which is involved in neurotoxin biosynthesis in the protein rich legume, Lathyrus sativus. The neurotoxin, ODAP or β-N-oxalyl, L-α,β-diaminopropionic acid synthesized from oxalic acid is the metabolic antagonist of glutamic acid which is involved in transmission of nerve impulses in the brain (Mickel-
Oxalic acid is the end product of various metabolic pathways in a large number of organisms. Oxaloacetate, ascorbate and glycolate/glyoxylate are the precursors of oxalic acid in many cases (Jakoby and Bhat, 1958; Houck and Inamine, 1987; Yanagawa et al, 1990). The enzyme systems exist which degrade oxalic acid by oxidation (Sugiura et al, 1979) or decarboxylation (Shimazono and Hayashi, 1957) or by activation followed by decarboxylation (Baetz and Allison, 1989). Thus there is a need to study the oxalic acid degradation enzymes in detail since such studies may have potential role in alleviating the stress conditions generated by oxalic acid in the cases mentioned above. There is also the need for developing a good assay system to monitor urinary and serum oxalate levels.

In this work the enzyme oxalic acid decarboxylase from the basidiomyceteous fungus *Collybia velutipes* was studied in detail. A partial purification of the enzyme had been reported by Shimazono and Hayashi (1957). Some of the properties had been determined by them using crude enzyme extracts or 1st or 2nd acetone fractions. The enzyme was shown to be active at pH 4.5 and was very substrate specific as it did not catalyze degradation of any other mono/di/tri carboxylic acids. The enzyme did not require ATP, coenzyme A, acetate or Mg$^{2+}$ for its activity. Since the enzyme catalyzed a single step breakdown of oxalic acid in absence of any cofactor requirement, it was chosen for further study. The major objective of this work was purification of
enzyme to homogeneity, biochemical characterization of its properties, construction of cDNA library and immunoscreening with anti-oxalate decarboxylase antibody, isolation and characterization of the cDNA clones.

The following results were obtained in the study of oxalate decarboxylase.

A. Purification and biochemical characterization of oxalate decarboxylase.

1. The protein, oxalate decarboxylase was purified from cultures which were grown for 25 days and then induced by addition of 12.5 mM oxalic acid. The enzyme activity was detected 12 hours after the addition of oxalic acid and maximum activity was reached 2 or 3 days after induction. The mycelium from three days after induction was homogenized in buffer at acidic pH and the homogenate subjected to multiple acetone fractionation steps and chromatofocusing.

2. Chromatofocusing resolved oxalate decarboxylase into two isoenzymic forms. Peak A eluted at the pH 3.30 and peak B eluted at pH 2.5. The peak A was purified to 1666 fold with a 2.9% recovery while peak B purified to 614 fold with 16% recovery. Though the yield of peak A was much less than that of peak B, it was considered for further study because of the purified form obtained on a single passage through a column. Some contaminants that copurified with peak B were eliminated by a passage through gel filtration column (Sepharose 4B).
3. That both isozymes, peak A and peak B of oxalate decarboxylase were related was proved by their amino acid composition and V8 peptide map. The two proteins showed a very high content of acidic amino acid residues (22%) which could account for their low pI. The two peaks had very similar 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 seen in the peptide maps of the two peaks digested with V8 protease. Thus the two forms must have arisen as allelomorphs at a single locus because of slight differences seen in the content of methionine, cysteine; the percentage of acidic residues amidated in vivo was not determined.

4. The peak A was shown to homogeneous by various criteria.

(a) It eluted as a single protein peak in FPLC superose 12 chromatography; no other proteins were detected by absorbance at 280 nm.

(b) The protein was diluted serially starting from 3 µg and the purity determined on 7%-15% polyacrylamide gel. Purity of the protein was calculated to be >96%.

(c) 10 µg of the protein was resolved on two dimensional gel electrophoresis. The separation in first direction was by isoelectric focusing and in the 2nd direction by 12% SDS-PAGE gel. A single species of protein was detected by this method.

5. The activity-band correlation was done on non-denaturing polyacrylamide gel. The migratory distance of enzyme activity
co-related with the single stained band on the gel.

6. The molecular mass of 560 kDa for the native enzyme was estimated by gel filtration through FPLC Superose 12 column. Electrophoresis on 7%-15% concentration gradient SDS-PAGE showed a single protein of 64 kDa. The enzyme appeared to occur as an aggregate under non-denaturing conditions. The size obtained from deglycosylation is 55 kDa.

7. The apparent Michaelis constant of 4.5 mM and $V_{\text{max}}$ of 0.133 mmol/l$^{-1}$/min$^{-1}$ was obtained for peak A, under standard assay conditions. The enzyme was shown to be highly specific for oxalic acid as a substrate as it did not degrade any of the other carboxylic acids used. The enzyme was inhibited competitively by phosphate which increased the $K_i$ to 9.2 mM.

8. The enzyme had a pH optimum of 3.0 and lost 50% of its activity on either side of the optimum. The enzyme did not give irreversibly inactivated when incubated with buffers of different pH values.

9. The thermal instability studies showed that the enzyme retained 78% of its activity after 20 min incubation at 80°C. There was a negligible loss of oxalate decarboxylase activity on incubation at 60°C; almost all the activity was lost within 5 min of heating at 96°C. The enzyme was moderately thermostable.

10. The enzyme was not inhibited by any of the sulphydryl group inhibiting substances. It retained 45% of its activity after pre-incubation with 10% SDS for 30 min at room temperature. It
lost almost all of the activity when heated to 66°C in presence of 10% SDS. It was largely stable in various reagents.

11. The enzyme stained positive with Periodic acid-Schiff's base reagent; it bound to ConA-Sepharose and was eluted with 0.5M α-methylmannoside. The enzyme was acted upon by Endo H which caused its size to reduce to 55 kDa. The enzyme had a total carbohydrate content of 15%.

B. Immunological characterization.

12. Antibodies were raised against the enzyme by injecting heat denatured oxalate decarboxylase into New Zealand white rabbit. The antibodies were able to precipitate enzyme activity from solution and the 1:5000 dilution crude antiserum could detect 1 ng of peak A protein. The affinity purified antiserum cross reacted with peak B of oxalate decarboxylase and with oxalyl CoA decarboxylase (Oxalobacter, strain OxB). The serum also cross-reacted with all the peptides obtained by digestion of peak A and peak B protein with protease from S. aureus V8. The affinity purified antiserum at 1:1000 dilution cross-reacted with deglycosylated forms of peak A and peak B proteins.

13. The purified protein bound to protein A-Sepharose to which the IgG from anti-oxalate decarboxylase antiserum was bound. It did not bind to pre-immune IgG immobilized on protein A-Sepharose.

C. cDNA synthesis and cloning.
The enzyme was studied at molecular level by isolating the mRNA at 12 hr of oxalate induction and preparing cDNA library from this. The cDNA library was made in the lambda expression vector gt11 and the specific antibodies were used to screen the library.

14. The in vitro translated mRNA from 12 hr oxalate-induced stage when immunoprecipitated gave 55kDa band. No protein was immunoprecipitated from translated 0 hr mRNA. The intensity of 55 kDa band decreased when purified oxalate decarboxylase was used for competition experiments. Thus it appeared that the translatable mRNA was present only at 12 hr after induction.

15. The mRNA was isolated from 12 hr of induction with oxalic acid and cDNA synthesized ranged from 0.5 kb to less than 2 kb in size. The flush ended cDNA was methylated, ligated to Eco RI linkers and then to Eco RI arms of lambda gt11.

16. Approximately 246,000 recombinant plaques were obtained from 4 independent ligations. Twelve plaques were randomly picked and phage DNA isolated from these. The plaques were analyzed for insert size. The insert sizes ranged from 0.1 kb to 2.1 kb and an average size of 0.5 kb was obtained.

17. About 47,000 recombinants were screened with antibody pre-treated with E. coli lysate. Sixteen immunopositive plaques were
obtained and plaque purified. The plaques gave white color with X-gal when induced with IPTG indicating that these are recombi­nants. The signal intensity was found to vary with the plaques and is shown in the decreasing order of intensity: clone nos. 3 and 15 > 4 & 9 > 1, 6, 7, 10, 11, 12, 13, 14, 16 > 8 > 2.

18. The phage DNA from 15 immunopositive clones was immobilized onto Gene Screen Plus membrane in duplicate and probed with oxalate-induced and uninduced cDNA probes. Differential hybridization of 16 immunopositive clones showed that 15 hybridized to the cDNA probe from oxalate plus RNA and gave no signal with uninduced cDNA. Clone no.15, which had given a very strong signal on immunoscreening also hybridized to uninduced cDNA probe. Clone 8 and non-recombinant lambda gt11 didnot give any signals with either probe.

19. Phage DNA was isolated from all of the immunopositive plaques and was digested with Eco RI, end-labeled and resolved on 2% agarose and 6% acrylamide gel. Almost all the clones showed presence of multiple inserts. Clone nos. 1, 3, 8 & 15 had inserts >1kb size along with small sized inserts. No inserts were seen for clones 6 and 14.

20. Relatedness of these inserts was studied by using 1.2 kb insert from clone no.3 as a probe. This was subcloned into pT218U; the Eco RI digest of the subclone was resolved on LMP agarose and the 1.2 kb insert incised and labeled by random primer labeling method. This 1.2 kb probe hybridized to most of the larger inserts of immunopositive clones except clone no. 8.
but did not hybridize to the very small-sized inserts obtained in all the cases. To study further if all the smaller inserts are related, one of these viz. 0.3 kb insert was used as a probe. This however, did not cross hybridize to any of the other inserts and hybridized only to itself. This indicated that probably random ligation of unrelated cDNA inserts had occurred during cloning.

21. Almost all of the immunopositive plaques synthesized fusion proteins, the sizes of which were comparable to insert sizes. Clone no.8 however did not show any crossreactivity to anti-oxalate decarboxylase antibody. Clone no.1 encoded a fusion protein of 54 kDa size which was picked up by specific antiserum; with anti β-galactosidase antibody, a 110 kDa protein was seen. This indicated presence of an operon fusion. However, this fusion protein was encoded by the nucleotide sequences from relevant 1.2kb insert plus those from unrelated 0.3 kb insert, both of which had been randomly ligated in clone no.1.

22. The 1.2 kb probe was used to probe the genomic blot. Single bands were obtained with the digests of Bam HI, Eco RI, Hind III, Pvu II, SspI, Xba I and Xho I indicating the presence of single copy gene. The two bands of unequal intensities obtained with Kpn I and Pst I were due to the presence of internal sites (single sites for each enzyme) in 1.2 kb cDNA insert for these enzymes.

23. The 1.2 kb probe hybridized to a single species of mRNA of 1.5 kb size from 12 hr oxalate induced poly(A⁺) RNA and no hybri-
dization to RNA from uninduced lane was seen. From the same batch of culture, samples were collected from different stages of induction and analyzed for RNA levels and enzyme activity. The Northern of total RNA showed that 1.5 kb band was absent at 0 hr and peaked at 12 hrs, after which there was a decline in its levels. On 2nd day no RNA was detected. The enzyme activity was detected 12hr after addition of oxalic acid and peak activity was obtained 2nd day after induction. Thus a temporal relationship was observed between mRNA levels and enzyme activity.

24. The unrelatedness of 0.3 kb insert was indicated by the different patterns of genomic Southern and Northern analysis. The 1.7 kb mRNA was identified at 0 hr as well as 12 hr and the induction pattern was found to be very different from that obtained with 1.2 kb insert as probe; there appeared to be a constitutive expression of its mRNA. For convenience, the same argument was extended for other unrelated inserts which did not crosshybridize to the other larger 1.2 kb probe or to the 0.3 kb probe.

25. The clone no. 15 hybridized to the uninduced cDNA probe in differential hybridization experiment because of its 0.1 kb insert which had randomly ligated to the 1.1 kb cDNA. When the 1.1 kb insert was used to probe the blots, it gave hybridization pattern identical to that of 1.2 kb from clone no.3 on genomic Southern and Northern blots.
26. Hybrid selection was done using only one of the clones because (i) it cross hybridized to all other clones (2) when 1.1 kb insert from clone no.15 was used as a probe for Northern analysis, it picked up 1.5 kb mRNA at 12 hr and did not hybridize to 0 hr mRNA. The induction pattern was identical to that of 1.2 kb insert from clone no.3.

27. The subclone of 1.2 kb cDNA insert was used to hybrid select the mRNA. The hybrid-selected mRNA on in vitro translation and immunoprecipitation gave a band of 55 kDa, which was similar to the size obtained with total poly(A⁺) mRNA and corresponded to the size of deglycosylated protein. This 55 kDa protein was not obtained with uninduced mRNA. In an independent experiment, no translated product was seen when vector sequences (nonrecombinant) were used to hybrid select the RNA.

Thus 1.2 kb insert from lambda clone 3, subcloned into pT218U was the putative oxalate decarboxylase clone based on 3 lines of evidence:

a. It hybridized to cDNA probe from oxalate induced mRNA and not with uninduced cDNA viz. it is differentially expressed.

b. The 1.5 kb mRNA to which it is hybridized was induced by oxalate.

c. It hybridized to mRNA which on in vitro translation and immunoprecipitation gave a 55 kDa product. This protein band was obtained with 12 hr induced mRNA and not with 0 hr uninduced mRNA and corresponded to the size of deglycosylated protein.

Since the cDNA insert obtained above was not of full length, its
expression could not be studied.

Further work will be done using this cDNA clone as a probe to isolate genomic clones from a genomic library. Further confirmation of the clone would be done by sequencing the genomic clone and comparison of the derived amino acid sequence with the N-terminal sequence of the purified protein. The structure of the genomic clones and the poly (A+) RNA will be studied in detail and attempts will be made to express this in some other system.