5. Summary, Conclusion and Future Prospects

Oxalate oxidase has been reported from bacteria, fungi, mosses and plants but this enzyme is not found in animals. In man, oxalic acid or oxalate is a metabolic end product of glycine metabolism and there is no enzyme present to act on oxalate. Oxalate oxidase is employed in diagnosis and treatment of various diseases like the formation of stones (crystals of calcium oxalate) in the urinary tract, chronic renal failure and calcium oxalate nephrolithiasis, hyperoxaluria, hereditary intolerance to the normal levels of oxalate in the diet, consumption of ascorbate, ethylene glycol, diethylene glycol, or xylitol; inhalation of the anaesthetic methoxyflurane and infection with Aspergillus, known as aspergillosis. In cystic fibrosis patients as a consequence of antibiotic treatment that eliminates Oxalobacter formigenes from the gut flora and in premature babies fed with infant formula rather than breast milk, in cardiomyopathy and cardiac conductance disorders, pyridoxine deficiency etc.

The treatment of almost all such diseases, which is caused by higher concentration of oxalate in human body, has been suggested either in vivo or in vitro. Recently, human gene therapy has been suggested for the treatment of fatal genetic disorder primary hyperoxaluria, which leads to deposition of calcium oxalate throughout the body. Commercial dimensions of oxalate oxidase range widely from healthcare, agriculture, environment and industry. Though oxalate oxidase available in the market (SIGMA) but it has drawbacks due to its sensitivesness towards Cl⁻ and NO₃⁻ ions, which interfere in the detection of oxalate in biological fluids (that is in its use for diagnostic purpose). We have chosen oxalate oxidase from sorghum leaves due to its reported insensitivesness towards Cl⁻ and NO₃⁻ ions. The aim of our investigation was molecular characterization of oxalate oxidase purified from sorghum leaves.

To achieve this aim, oxalate oxidase has been purified from 10-days old sorghum leaves (Sorghum vulgare var. Amarnath-2000). The enzyme was isolated by the conventional methods of enzyme purification including ammonium sulfate precipitation, ion exchange
chromatography, gel filtration chromatography, dialysis and then this purified enzyme was lyophilized. Protein concentration as well as assay of ox-ox was done at every step during enzyme purification. The leaves were homogenized in chilled distilled water containing 1% (w/v) polyvinylpolypyrrolidone in 1:3 ratio (w/v) in a pestle and mortar and homogenate was centrifuged at 15,000 g for 30 min at 4°C. The supernatant was collected and treated as crude enzyme. The crude enzyme was purified by 0-80% ammonium sulfate precipitation, dialysis. Gel filtration was done on Sephadex G-200 using 0.01M potassium phosphate buffer pH 6.8 and ion exchange chromatography on DEAE-Sephacel column using a linear gradient of KCl (0-0.6M) in 0.01M potassium phosphate buffer pH 6.8 for elution of enzyme. The active fractions were pooled and lyophilized to concentrate the enzyme. An over all purification of 108.1 fold with 28% yield was achieved. The purified enzyme had a specific activity of 1600 U/mg. One unit of enzyme is defined as the amount of enzyme, which generates 1 μm of H₂O₂ per min under standard assay conditions.

To see the homogeneity of the purified enzyme, non denatured SDS-PAGE was done. Polyclonal antibodies were raised from the purified enzyme in white rabbits. ELISA was done by using these primary antibodies in crude extract as well as with purified enzyme which showed positive response in both cases. The 60 kDa band was cut and given for MALDI-LC-MS analysis. But surprisingly, instead of oxalate oxidase the band was identified as β-amylase which had very close resemblance with maize β-amylase. It seems that oxalate oxidase had been degraded most probably either during lyophilization or SDS-PAGE. So the whole process repeated again to purify the enzyme except lyophilization. In non-denaturated SDS-PAGE we got two clearly visible bands instead of one, while in denaturated SDS-PAGE we got 7-8 bands including a band around 60 kDa. Protein concentration as well as assay for oxalate oxidase was done at every step of enzyme purification. The assay results were positive for oxalate oxidase enzyme. Again 60 kDa band was given for MALDI-LC-MS analysis and got the same results i.e. β-amylase.

To confirm the results the β-amylase assay of the crude as well as purified enzyme was
done. It showed positive results in both cases. To know the glycoprotein nature, the enzyme was heated in orcinol-H$_2$SO$_4$ reagent; it gave brownish colour indicating its glycoprotein nature. The carbohydrate content of the enzyme was calculated from the standard graph of glucose between glucose concentration vs $A_{420}$ (Fig. 18) and was found to be 0.40 µg confirming the glycoprotein nature of sorghum leaves β-amylase.

We didn’t get any other 60 kDa band, though few low molecular weight bands are clearly visible (Fig. 13). As the sample showed positive oxalate oxidase and 60 kDa band did not come out to be oxalate oxidase. It seems that the sorghum oxalate oxidase is not a dimer and its subunits are not of 60 kDa (as reported earlier) instead it should be hexamer in nature like wheat and barley oxalate oxidase as there are few clearly visible low molecular weight bands in the gel along-with 60 kDa band which was identified as β-amylase (Fig. 13). The other bands in the gel having different molecular weight (Fig. 13) could be dimers, trimers, tetramers or even pentamers of oxalate oxidase which could not be denatured totally and remained intact as subunits while the band nearby 30 kDa or even below could be oxalate oxidase hexameric subunits.

Primers were designed by DNASTAR from conserved sequence data already available from other species of Gramineae family and genomic DNA was extracted from green young sorghum leaves. Attempts were made to amplify the gene by using these primers but these attempts failed. It may be due to many factors including mistakes in primer designing or synthesis. Interestingly, when we analyzed the data for fresh primer designing then came across putative sorghum oxalate oxidase clone sequence data itself. Sorghum oxalate oxidase resembles highly to barley, rice and maize oxalate oxidase and orthologous in nature. As the oxalate oxidase gene sequence already came out in public domain so another attempt to design primers for its amplification stopped.

The doubt raised by our findings about sorghum oxalate oxidase number of subunits to some extent also confirmed by oxalate oxidase orthologous nature and that sorghum oxalate oxidase highly resemble to barley, wheat and rice. From these reports and our studies it could well be concluded that sorghum oxalate oxidase could not be a dimer but should be hexamer in nature. With these findings, the doubt naturally comes to mind if...
sorghum oxalate oxidase is not a 60 kDa dimer then is it really Cl⁻ and NO₃⁻ insensitive as reported earlier (the factor that we chose sorghum oxalate oxidase for our studies due to its commercial importance). As the sample purified by earlier researchers/cited reports said to be homogenous in nature, having single band of 60 kDa. If oxalate oxidase is not 60 kDa then we have reasons to doubt if it had even oxalate oxidase? As earlier reports said that oxalate oxidase is a dimer having single band of 1,20,000 daltons by native PAGE and 60,000 by SDS PAGE revealing that enzyme is a homodimer. Hence we did chloride and nitrate insensitivity test and our doubt proved to be true. Our sample had positive assay results for oxalate oxidase activity but it is chloride and nitrate sensitive! So the idea to express sorghum oxalate oxidase in E. coli dropped.

**Future Prospectus**

The present investigation was undertaken with the objectives of molecular characterization of sorghum oxalate oxidase by purifying the enzyme by conventional methods to sequence its amino acid sequence to amplify the gene by PCR and then its expression in E. coli due to its insensitiveness towards chloride and nitrate ions. We did our sincere efforts with truly scientific spirit. Though results came out to be unexpected than what has been hypothesized. Instead of losing enthusiasm we went ahead to confirm our doubts even beyond the confined lines mentioned in our synopsis. In view of our findings sorghum oxalate oxidase lost its reported advantage over barley at the commercial front as it is not chloride and nitrate insensitive as reported earlier.

But oxalate oxidase at broad spectrum is an important multifunctional enzyme having its uses in healthcare, agriculture, environment and industry. Its studies at molecular level and 3D structure determination from different species and their comparison at gene level could be helpful in tracking evolutionary history of these related enzymes that is oxalate oxidase, superoxide dismutase and oxalate decarboxylase. In turn these studies could pave the way for designing new molecules/drugs specific for different diseases related to higher concentration of oxalate oxidase in healthcare and other industries which use oxalate oxidase or oxalate decarboxylase.