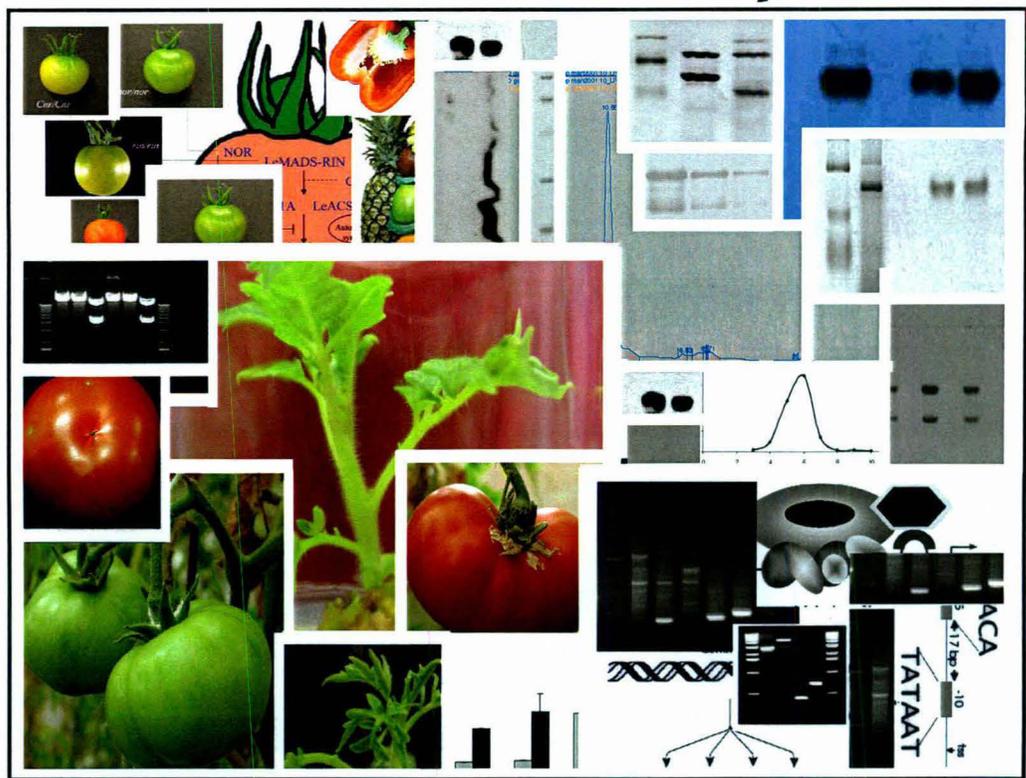


Chapter 5



Summary

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Ripening is a complex process involving major transitions in fruit development and metabolism. Although ripening hallmarks the initiation of fruit senescence, it also initiates number of coordinated changes in number of biochemical pathways. In addition to other biochemical transitions, such as pigment biosynthesis and production of volatiles, softening accompanies the ripening of many fruits. It has been considered axiomatic that these textural changes result primarily from changes in cell wall structure (Huber, 1983). Induction of cell wall hydrolases during ripening interested many physiologists and biochemists who consider them to be the principal cause for programmed changes in cell wall structure. These cell wall hydrolases are known to act on the carbohydrate component of the cell wall which accounts for about 90% of the total dry weight (Cosgrove, 1997). Therefore their activity is directly linked to softening which is of commercial importance.

Glycosyl hydrolases, which are part of the CAZymes (Carbohydrate active enzymes), form a major and diverse group with many individuals unexplored. Till date several hydrolases have been tried and tested to prove their function and regulation during fruit ripening. Although scientists had little success in understanding the role of each hydrolases in cell wall modification and disassembly, each time it was sure step ahead to know their role in the process.

In our study we emphasized on mannosidase, belonging to glycosyl hydrolase family 38 and known to cleave glycosidic bond between the carbohydrates or carbohydrate and protein. The enzyme activity of alpha-mannosidase is known to increase during ripening in many fruits including capsicum and tomato. These two are the most important vegetable fruits for which texture is of utmost importance with respect to consumer acceptance. Therefore we targeted mannosidase enzyme, which is unexploited till date, and investigated its role in ripening associated softening in the above mentioned vegetable fruits. The work is divided into three sections. First being the purification and characterization of α -mannosidase from tomato and capsicum. Second section involving molecular cloning and functional characterization of α -mannosidase from tomato and the third section involved, promoter cloning and *in-silico* analysis.

Purification and characterization of α -mannosidase from tomato and capsicum

- Using PNP- α -D mannopyrinoside as the substrate we identified that breaker stage of tomato has maximum mannosidase activity. In capsicum maximum activity was seen in the stage 8, which represents the last stage of ripening.

- The enzyme was extracted from the crude by 0-70% and 40-60% ammonium sulphate precipitation in tomato and capsicum respectively. Then dialyzed and loaded on DEAE sepharose in tomato and on Q-sepharose in capsicum. Further, fractions showing activity were precipitated and loaded on to sephadex G-100 gel filtration column to purify the protein.
- On non-denaturing (native) PAGE, capsicum protein (CaAMS) and tomato protein (SIAMS) depicted molecular mass of ~ 500 and ~ 360 kD respectively. Separating the same proteins on superdex 200 analytical column depicted a size of 290 kD.
- On SDS-PAGE both the proteins resolved into two subunits, 70 kD was common for both the proteins but the lower subunit was 45 and 50 kD in SIAMS and CaAMS respectively.
- Purity of the enzymes was checked by separating the protein by 2D-Gel electrophoresis and subjecting to LC MS/MS analysis. The lower subunit further separated into three proteins indicating the presence of interacting proteins. Among the possible interacting proteins one was identified to be monosaccharide transporter and other was ATPase.
- Enzyme kinetics revealed that CaAMS has more affinity to the substrate with comparatively lower K_m value of 1.6 mM than the SIAMS which had the K_m of 4.6 mM.
- The optimum pH for both the enzyme was found to be 6.0. CaAMS was found to be more resistant to change in the pH than the SIAMS.
- CaAMS and SIAMS were found to be thermo-stable enzymes and were found to be active up to 60°C. The optimum temperature was 45°C and 55°C for SIAMS and CaAMS respectively.
- Both the proteins were found to be glycoproteins with carbohydrate moiety attached to it and were susceptible to EndoH cleavage.
- Antibodies raised against both the proteins were able to precipitate the protein from the crude and Immunolocalization revealed them to be localized in the cell wall.
- Protein characterization revealed that both the proteins had lot of similarity. Antibodies raised against the proteins were able to cross react with each other.

Cloning and characterization of α -mannosidase from tomato and capsicum

- Polyclonal antibodies were used to screen the expression library. And as an alternative approach, degenerate primers were designed using peptide tags and conserved motifs, obtained by multiple alignments. The amplification was done using cDNA as the template which amplified a fragment of 0.7 Kb corresponding to the

gene. The remaining part of the gene was amplified by rapid amplification of cDNA ends (RACE).

- The full length clone (coding region) was 3090 and 3093 bp long in tomato and capsicum respectively. The deduced amino acid sequence was 1029 and 1030 amino acids for same.
- *In-silico* analysis revealed that the deduced protein from the *SIAMS* and *CaAMS* cDNA sequences were found to have conserved domains characteristic to the family. The N-terminal had "N-terminal glycosyl hydrolase domain", middle region had "glycosyl hydrolase middle" and the C-terminal had "C-terminal glycosyl hydrolase domain". The C-terminal also had a sequence similar to the geraniol responsible factor 15 from *Matricaria chamomilla*. SignalP identified a cleavage site in both the proteins and TargetP designated them to be secretory proteins. TmPred predicted N-terminal to be exposed and identified three transmembrane helices in tomato and two in capsicum protein. 3D JIGSAW server predicted its three dimensional structure using 1HTY (PDB entry) using *Drosophila* golgi mannosidase as the template.
- In order to functionally characterize the gene we transiently suppressed the endogene in tomato by agro-injection. For this, part of the gene was cloned in sense and antisense orientation in the pHANNIBAL vector and then transferred to binary vector pART27. *Agrobacterium* was further transformed with the construct and mobilization was confirmed by colony hybridization.
- The *Agrobacterium* with construct was injected in to the fruit pericarp. After 3-4 days molecular analysis of the agroinjected fruits was done by real time RT-PCR, which revealed that there was suppression of the endogene up to 70% compared to the control. Small interfering RNAs (siRNAs) were detected in the ihp-AMS injected fruit pericarp. Texture analysis of the agroinjected fruits revealed that silencing of SIAMS rendered the fruits 1.6 to 2 times firmer than the vector agroinjected fruits.
- Owing to the success of transient suppression of the gene we used the same vector and silenced the gene to produce stable RNAi lines. This necessitated establishing the regeneration protocol for tomato, which had an efficiency of 7-10%. The putative RNAi lines were identified by the presence of the vector by PCR analysis using the intron specific primers.
- Using real time RT-PCR, reduction in the level of transcript was determined in stable RNAi lines. In the RNAi lines the transcript was suppressed up to 99 %. Then fruits of these lines were analyzed for texture analysis and were found to be 2- 2.5 times firmer than those from the vector transformed lines.

- Due to sequence similar to geraniol responsible factor 15 at the C-terminal of the protein, mannosidase inducibility to geraniol, a monoterpene, was determined. Mannosidase was found to be up regulated in wild type plants.
- In order to study the gene regulation, we determined its expression in ripening impaired mutants. Real time RT-PCR data indicated that the AMS transcript level was suppressed up to 90% in *rin* (*ripening inhibitor*) mutant and 30% in Nr (Never ripe).
- Geraniol treatment in *rin* mutant induced mannosidase to higher extent than in the wild type confirming that geraniol induces mannosidase.
- ACC treatment to *rin* as well as wild type induced mannosidase gene, further revealing its regulation by ethylene.

Promoter cloning and *in-silico* analysis

- The 1.2 Kb upstream sequence of the mannosidase gene was cloned by PCR based genome walking.
- Using PLACE (Plant Cis acting regulatory elements) and PlantCARE (Plant cis acting regulatory elements) database, putative cis-acting regulatory elements were identified.
- Other than the ubiquitous elements like TATA, CAAT boxes, elements like ABRE (Abscisic acid responsive elements), ERE (Ethylene responsive element), GARE (GA-responsive element), ASF-1 binding site which are responsive to auxin and salicylic acid were identified.
- Light responsive elements like, G-Box, GT-1 box and I-Box were also the part of the promoter.
- "CGCG" box was identified which is known to be involved in multiple signal transduction pathway. "GCC" box which is an important component of pathogenesis related (PR) gene was also identified in the *SLAMS* upstream region.

Future line of work

1. To devise experiments which will help to study the oligomerisation of the protein and to elucidate the role of interacting proteins.
2. Crystallize the protein to unravel the structure of mannosidase.
3. To evaluate the T1 generation of RNAi lines for their texture, longevity and some rheological properties of the fruits which are important for the industries.
4. In depth analysis of promoter and its characterization to yield knowledge about the gene regulation.