Chapter 2

Purification and Characterization Of Mannosidase from Capsicum and Tomato
CHAPTER 2
Purification and Characterization of α-Mannosidase from Tomato and Capsicum

2.1 Introduction
The apparent changes in molecular size of cell wall polymers accompanying fruit ripening imply the role of enzymes capable of degrading specific cell wall components. Consequently, the activity of ripening-induced cell wall hydrolases had been subject of intense investigation. Studies till date have focused relatively on the few enzymes that accumulate to high levels in a particular fruit. Most notable are polygalacturonase and carboxymethylcellulases which accumulate to higher level, but not in all fruits. Molecular genetic approaches have helped to identify more cell wall hydrolases from different fruits which show peak during ripening. Among them was alpha-mannosidase, which showed maximum activity during ripening of many fruits. The enzyme Alpha-mannosidase belonging to glycosyl hydrolase family 38 was purified from capsicum and tomato. Although both the vegetable fruits belong to the same family Solanaceae, but they differ in their respiratory behavior and ethylene production. Tomato, called as the “centre piece” of the family, is a climacteric fruit and on the other side capsicum is a non-climacteric type of fruit. Interestingly, mannosidase exhibited increased activity peak during ripening/softening of tomato (Suvamalatha and Prabha, 1999) as well as capsicum (Priya Sethu and Prabha, 1997). This enzyme is known to cleave short chain oligomannose residues (~8-10) present in oligosaccharides and glycoproteins.

The activity of the mannosidase varies with the stage of development/ripening in bell-pepper, tomato, grape, muskmelon, olive and pear (Pharr et al., 1976; Watkins et al., 1988; Ahmed and labavitch, 1980; Fils Lycaon and Buret, 1991; Heredia et al., 1993; Burns and Baldwin, 1994; Priya Sethu and Prabha, 1997). Although Watkins et al. (1988) showed quantitative differences in the activity but no direct association between the activity of this enzyme and other glycosidases was observed. Many scientists used this enzyme as a vacuolar marker (Boller and Kende, 1979; Glund et al., 1984) and many reports suggest that it may be involved in the turnover of endoplasmic reticulum glycoproteins and glycolipids (Murray, 1983). Mannosidase was also found to be associated with cotyledon tissue and showed significant changes during different physiological stages (Neely and Beevers, 1980; Plant and Moore, 1982). Many reports are available wherein the mannosidase has been investigated for different functions in plants like biotic stress, seed germination, N-glycans modification in the plant system and plant development. Moreover, it may also attack specific cell wall structures of plant pathogens (Boller, 1987). Above all very few reports are available where mannosidase has been examined for its role in fruit ripening and/or softening, although other family members have already been targeted for the above said process.
Consistent increase in the mannosidase activity during fruit ripening and distinguishing itself as the most active enzyme amongst the glycosidases in many fruits including tomato and capsicum, prompted us to purify, characterize and elucidate its function in both the systems. We refer the protein of tomato as SIAMS (*Solanum lycopersicum* Alpha-Mannosidase) and Capsicum as CaAMS (*Capsicum annum* Alpha-Mannosidase).

### 2.2 Materials

All the chemicals used were of highest purity and the solutions were made in double distilled water. All the solutions were sterilized, either by autoclaving at 15 pounds/sq inch for 20 min or by filter sterilization.

#### 2.2.1 Solutions and Chemicals

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| **Dilution Buffer**                    | 100 mM Tris-HCl (pH 8.5)  
20% (v/v) Glycerol  
4% (w/v) SDS  
20 mM DTT  
1 mM PMSF                                  |                 |
| **Rehydration Buffer**                 | 8 M Urea  
2 M Thiourea  
0.5% (v/v) Triton X-100  
4% (w/v) CHAPS  
20 mM DTT  
0.5% (v/v) phenylmethylxylate (pH 4-7)  
0.05% (w/v) bromophenol blue              |                 |
| **12.5% polyacrylamide gel** (Resolving gel: 40 ml) | Acrylamide (30:0.8)  
4X buffer (pH 8.8)  
MilliQ  
SDS (20%)  
APS (10%)  
TEMED                                  | 16.68 ml  
10 ml  
12.92 ml  
200 μl  
200 μl  
15 μl                          |
| **4X Stacking Buffer**                 | 1.5 M Tris HCl (pH 6.8)                                                   |                 |
| **8X Resolving Buffer:**               | 3 M Tris HCl (pH 8.8)                                                     |                 |
| **TEMED**                              | Used as supplied and stored at 4 °C                                       |                 |
| **Towbin’s buffer**                    | 25 mM Tris  
190 mM Glycine  
20% Methanol                                 |                 |
| **Reservoir Buffer**                   | 0.25 M Tris (pH 8.3)  
1.92 M Glycine  
1% SDS                                         |                 |
| **Blocking solution:**                 | TBS + Fat free dry milk                                                  |                 |
| **Laemmli loading Buffer**             | 50 mM Tris-Cl pH 6.8  
2% SDS  
10% Glycerol  
0.1% Bromophenol Blue (BPB)  
100 mM DTT or 0.05 % BME to be added fresh. |                 |
| **AP Buffer**                          | 100 mM Tris-Cl pH 9.5  
100 mM NaCl  
50 mM MgCl₂                                  |                 |
| **AP Colour development solution**     | 10 ml AP Buffer  
66 ul NBT (50mg/ml,70%1mM DMF)  
33 μl BCIP (50mg/ml DMF)                    |                 |
| **PNP-α-D-Mannopyranoside**            | 20mM prepared in dissolving it in small amount of DMSO and making up the volume with water |                 |
| **PMSF**                               | 100 mM stock in 100% isopropanol  
Final concentration  5 mM                       |                 |
Destain Solution
50% Methanol
10% Acetic Acid
40% Water
and Fast Destain is 50% methanol

Comassie Staining solution
50% Methanol
10% Acetic Acid
40% Water
0.2% BPB (Bromo-phenol Blue)

APS (Ammonium per Sulphate)
10% APS solution prepared in water

Para formaldehyde
4% para-formaldehyde solution prepared in phosphate buffer.

TCA
20% (v/v) Prepared in water

Extraction Buffer Tomato
100 mM Tris-Cl pH 7.8
0.25M NaCl
5mM PMSF

Extraction Buffer Capsicum
100 mM Tris-Cl pH 7.0
0.25M NaCl
5mM PMSF

Native loading Buffer
50 mM Tris-Cl pH 6.8
10% Glycerol
0.1% Bromophenol Blue (BPB)

10X PBS (Litre)
2.0 g KCl
80 g NaCl
17.8 g Na₂HPO₄
2.4 g of KH₂PO₄

Bradford Reagent
Bradford reagent from Bio-Rad was diluted five times with autoclaved MQ and used.

2.2.2 Plant Materials and growth conditions
Tomato Plants (cv. Pusa ruby) was used for all the experiments. The seeds were germinated in small pots and after three weeks the seedlings were transplanted to big pots in the greenhouse. The greenhouse conditions were 25/23°C, 70% humidity under 14/10 h light/dark regime. Mutants used were procured from TGRC (Tomato Genetic Resource Centre) and were in Ailsa Craig background.

For harvesting the fruits, the flowers were tagged at anthesis and fruits after 35 days of anthesis were considered mature green (MG), MG+2 days as breaker stage (BR), BR+3 days as pink (PK) and PK+3 days as red stage.

In case of capsicum we used a locally available variety for our study. The variety we use changed its colour from green to red during last stage of ripening like tomato. And it did not differ in the activity from the other available varieties which remained green throughout the ripening. The flowers were tagged at anthesis and fruits were harvested at different stages of ripening. We identified eight different stages named S1 to S8 in which first four stages were regarded as developmental stages and last four were designated as ripening stages. S1 is 7 DAF (Days After Fruit set), S2 is 14 DAF, S3 is 21 DAF, and S4 28 DAF. In ripening
stages S5 is 4 DAH (Days After Harvest), S6 8 DAH, S7 15 DAH and S8 is 21 DAH. For the purification we used S8 stage fruits which showed maximum enzyme activity.

2.3 Methods

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2.3.1 Purification of Alpha-mannosidase from tomato

1. Pusa Ruby variety was used for isolation of protein. Fruits were harvested at breaker and the pericarp of the fruit was separated from the seeds.

2. The frozen fruit pericarp was powdered in liquid nitrogen and suspended in one fourth volume of 100mM Tris-Cl pH7.8 buffer with 0.25 M NaCl and 5mM PMSF (Extraction buffer) and extracted overnight at 4°C.

3. Then the extract was filtered through four layers of cheese cloth. The filtrate was centrifuged at 11,000 rpm to settle down all the debris. The supernatant thus obtained was subjected to 0-70% of ammonium sulphate precipitation. Ammonium sulphate precipitation is taken in order to obtain a higher degree of purification. But care has to be taken while precipitating the protein, because increase in temperature during the process will increase the risk of inactivation of the enzyme and frothing caused by vigorous stirring may also result in protein denaturation. Precipitation using neutral salts, like ammonium sulphate is most common method as precipitated protein is not denatured and activity is recovered upon redissolving the pellet. These salts are known to stabilize against denaturation, proteolysis or bacterial contamination.
Salting out, as it is commonly called, depends on hydrophobic nature of the surface of the protein. When salts are added to the system, water solvates the salt ions and as salt concentration increases water is removed, exposing the hydrophobic patches. These hydrophobic patches can interact with each other, resulting in aggregation. The amount of ammonium sulphate required to give % saturation can be calculated by the formula
\[ g = \frac{533 (S_2-S_1)}{100-0.3 S_2} \]
Where g is number of grams of ammonium sulphate, S₁ is the final concentration and S₂ is the initial concentration. The thumb rule in protein precipitation is that the first cut or precipitation should be the maximum % saturation that does not precipitate the protein of interest and the second should be minimum % saturation which precipitates all the protein of interest.

4. Then centrifuged at 10,000 rpm for 10 min. The pellet obtained after the precipitation was reconstituted in minimum amount of buffer and dialyzed overnight against 25mM Tris-Cl pH 7.8 with one change.

5. The sample was then chromatographed on DEAE Sepharose pH 7.8, and eluted with increasing linear salt gradient.

6. DEAE Sepharose is shipped in 20% ethanol which has to be washed several times with autoclaved water until the ethanol smell disappears and further it has to be saturated with the equilibration buffer. The fractions were checked for the activity using PNP- nitrophenyl manno pyranoside as the substrate.

7. The enzyme got eluted at 100 mM salt concentration which was indicated by enzyme assay recorded at absorbance of 405nm. The samples with the activity were analyzed on 12.5% SDS-PAGE and then pooled. The pooled sample was subjected to 0-80% ammonium sulphate precipitation and centrifuged at 10,000 rpm for 10 min.

8. The pellet thus obtained was dissolved in minimum quantity of extraction buffer and directly loaded on the gel filtration column. Fractions were collected after the void volume, analyzed for the presence of enzyme and checked on SDS-PAGE. The enzyme rich fractions, as indicated by absorbance at 405nm, were concentrated using centicon (YM-10) and stored at 4°C. One important point to be kept in mind is that the volume loaded on the gel filtration column should be 1-5% of the total column volume.

2.3.2 Purification of α-mannosidase from Capsicum
The enzyme α-D-mannosidase was purified to electrophoretic homogeneity from pericarp of the capsicum.

1. The capsicum pericarp was separated from the seeds and homogenized in ¼th volume of extraction buffer (100 mM Tris-Cl pH 7.0 with 0.25 mM NaCl and 5 mM PMSF). Protein was extracted overnight at 4 °C in cold room with continuous shaking.
Purification and characterization of α-Mannosidase

2. Next day the slurry was filtered through 4 layers of cheese cloth and spun at 10000 rpm to remove the debris. The remaining protein solution was fractionated by 40-60% ammonium sulphate precipitation. The pellet obtained was dissolved in minimum quantity of buffer and dialyzed overnight in the buffer devoid of salt with one change.

3. Then the protein was loaded on to Q-sepharose ion exchange column and the flow through was collected as the protein elutes in it and never binds to the matrix.

4. The flow through was precipitated again with 0-80% ammonium sulphate and directly loaded on to the Sephadex G-100 gel filtration column. Fractions containing the activity were checked on 12.5% SDS-PAGE and concentrated using centricon.

2.3.3 Preparation of standard curve and protein estimation

Standard curves are used for quantitative analysis of unknown substances by comparison to a "standard". Protein was estimated by commassie blue dye binding method (Bradford, 1976). To quantitate the protein, standard curve method was used. To prepare the curve known quantity of BSA (Bovine Serum Albumin, stock 10mg/ml) was used.

1. BSA was serially diluted to make dilutions from 1-15 μg and added to Bradford reagent solution.
2. Change in the OD with respect to blank (Bradford reagent only) was recorded at A_{595} using spectrophotometer.
3. The absorbance was plotted (on Y-axis) against the concentration (on X-axis) in a scatter plot and a trend line drawn passing through the origin. Further the protein concentration of the unknown was calculated using the equation of the straight line.

2.3.4 Preparation of standard curve for para-nitrophenol

Para nitrophenol (sigma) (PNP) is a yellow coloured chemical substance which was used to plot the standard curve to quantify the breakdown of substrate in the enzyme assay.

1. PNP was dissolved in water to make a stock solution of 100 μM and further serially diluted to form a series of concentrations ranging from 1 to 50 μM in 1 ml volume.
2. OD values were recorded for all the concentrations at 405 nm (Absorption Maximum of PNP) and a trend line was drawn passing through the origin, plotting concentration (in μM) on X-axis and OD values on Y-axis in scatter plot.
3. Further all the enzyme assay calculations were done using the formula y = mx, where m is slope of straight line passing through the origin and x and y represent the concentration and OD values respectively.
2.3.5 Enzyme assay
The enzyme assay was done using PNP-alpha-D-mannopyranoside as substrate.
1. In 940 µl of water 20 µl of 20 mM substrate was used and aliquot of enzyme was added to the mixture.
2. Blank reaction had only MQ and substrate. The reaction was incubated at 37 °C for 15 min and was stopped by adding of 40 µl of 0.5M Na₂CO₃. The colour developed was recorded at 405 nm.
3. To quantitate the amount of PNP released, a standard curve of PNP was used. One unit of the enzyme is defined as 1 µM of PNP released /minute at 37°C under standard assay conditions. The reaction involves the conversion of p-nitrophenol to p-nitrophenolate (a yellow coloured substance) on addition of NaOH or Na₂CO₃.

2.3.6 Oligomerization
Protein oligomerization was estimated by SDS-PAGE using sample loading buffer (Native buffer) which contains either SDS or beta mercaptoethanol, or in combination.
1. One µg of the purified protein was subjected to reducing and non-reducing conditions and resolved on 12.5% SDS-PAGE.
2. To create the reducing environment, 0.05% 2-Mercaptoethanol was used with native buffer in presence or absence of denaturing agent Sodium-dodecylsulphate (SDS).
3. After boiling the protein in presence of SDS and or 2-mercaptoethanol the disulphide bonds between the polypeptides in the protein will break and number of subunits or the polypeptide chains will be easily identified. In case of samples which are not boiled in presence of SDS and 2-mercaptoethanol the protein tends to break incompletely into the individual polypeptides, hence maintaining both the species viz. native as well as the individual subunits.

2.3.7 Activity band correlation
1. One µg of homogeneous purified Alpha-mannosidase protein from capsicum and tomato was run in two lanes of 6% nondenaturing polyacrylamide gel (Native) in the cold room.
2. After electrophoresis, one lane was stained with comassie blue R-250 or silver stain. A gel slice from the unstained lane was cut corresponding to the band position in the stained lane. Then the gel band was mashed and soaked in the protein extraction buffer and left overnight at 4°C. Similarly, gel pieces were cut throughout the lane at equal interval in order to check any trace activity other than the native band.
3. Next day the enzyme activity was determined by taking the supernatant after centrifuging it at 13,000 rpm for 10 min.
2.3.8 Gel electrophoresis of proteins
The discontinuous buffer system described by Laemmli (1970) was employed for electrophoresis in Mighty small / Mighty tall apparatus (Hoefer) at constant voltage of 70V.

1. A 30% acrylamide containing 0.8% bis-acrylamide stock was treated with mixed bed ion exchange resin (Bio-rad) for 2-4 hrs with shaking to remove ionic impurities.
2. The resolving gel consisted of 12.5% acrylamide, 0.375M Tris-Cl, pH 8.8, 0.01% TEMED and 0.2% ammonium persulphate (APS).
3. Stacking gel had 4% acrylamide and 0.125M Tris-Cl, pH 6.8, with same amounts of TEMED and APS.
4. Protein samples were mixed with sample buffer (Laemmli, 1970) and denatured in boiling water bath for 5 min prior to loading it on the gel. Along with protein samples, molecular weight markers (Bio-rad) containing mixture of proteins viz. Phosphorylase b (97.4 kD), Serum Albumin (66.2 kD), Ovalbumin (45.0 kD), Carbonic anhydrase (31.0 kD), Trypsin inhibitor (21.5 kD) and Lysosome (14.4 kD) was run.

2.3.9 Non-denaturing (native) PAGE
1. To separate the proteins in native condition 6% PAGE was used. It contained only the resolving gel with 6% acrylamide, 0.375M Tris-Cl, pH 8.8, 0.01% TEMED and 0.2% ammonium persulphate (APS).
2. The protein was mixed with the loading dye in 1:1 ratio. The loading dye consisted of only Tris-Cl pH 6.8, 15% glycerol and 0.02% of BPB and devoid of any denaturing and reducing agents.
3. The gel was run in the cold (4 °C) at 50 V constant using the running buffer with 0.3% Tris base and 1.44% glycine, pH 8.3. Further the gel was either silver stained or stained with coomassie R-250.

2.3.10 Staining and destaining of the gel
1. Gels were stained using 0.2% commassie Blue R-250 stain in 50% methanol, 10% acetic acid and 40% water. After staining the gel was destained in 50% methanol or in the staining solution without the commassie stain.
2. For silver staining, silver stain plus kit (BioRad) was used and stained according to the manufacturer’s protocol.

2.3.11 Molecular size determination
Native molecular weight of alpha-mannosidase was determined on superdex G-200 analytical column.
1. The column was equilibrated with 20 mM Tris-Cl pH 7.8 and 20 mM Tris-Cl pH 7.0 for tomato and capsicum, respectively.

2. Further the column was calibrated with standard proteins of known molecular weight proteins like; Thyroglobulin (669 kD), Ferritin (440 kD), Catalase (232 kD), Lactate Dehydrogenase (140 kD) and Bovine serum Albumin (67 kD).

3. Void volume of the column was determined by passing the blue dextran through the column. Then 100 μl of unknown protein was loaded on the column and run with same flow rate as that of the standards. Each protein standard as well as the sample was run in duplicate and average value was taken for the calculation.

4. Calibration curve for molecular weight was drawn by plotting Kav values against log molecular weights. Kav was calculated using the formula

\[
\text{Kav} = \frac{V_e - V_o}{V_t - V_o}
\]

Where

- \( V_e \) = Elution volume of the protein
- \( V_o \) = Void volume (Elution volume of the Blue Dextran)
- \( V_t \) = Total Bed volume

2.3.12 Kinetic properties

The Michaelis Menten constant (Km) of the enzyme (1μg/ml) was determined using relative substrate concentrations of 1mM, 2mM, 5mM, 10mM, 15mM and 20 mM. The data was plotted according to Lineweaver-Burk reciprocal plot (Lineweaver and Burk, 1934). Km is roughly an inverse measure of the affinity or strength of binding between the enzyme and its substrate. The lower the Km value, greater is the affinity.

1. The study of the rate at which an enzyme works is called enzyme kinetics. For the study of kinetics we set up a series of tubes containing graded concentrations of substrate.

2. At time interval of zero, a fixed amount of the enzyme was added and over the next few minutes (15 min in our experiment), reaction was stopped every minute by adding 0.5 M Na₂CO₃.

3. Then the activity was measured spectrophotometrically and average change in OD₄₀₅ per minute was recorded. Since the OD is directly proportional to the concentration of the products, it can be used as a measure of the rate or velocity of the reaction.

4. This exercise is repeated with different concentration of substrate, keeping the amount of enzyme constant. Further the values are plotted on double-reciprocal or Lineweaver-burk plot. The reciprocal of velocity (1/v) was plotted in Y-axis and the reciprocal of substrate was plotted on X-axis.
2.3.13 Effect of pH on enzyme activity and stability
The pH is known to influence the rate of an enzyme catalyzed reaction. To determine the optimum pH for enzyme activity and its stability at different pH following method was followed.

1. Different buffers ranging from pH 4 to 10 were prepared and the enzyme assay was performed at 37°C for 30 min followed by adding 0.5 M Na₂CO₃ to stop the reaction.
2. The OD values were recorded and activity of the enzyme was calculated. Further activity was plotted against the pH values. The Peak of the curve represented the optimum pH required for the enzyme activity.
3. To know the enzyme stability, known amount of enzyme was incubated at different pH ranging from 4-10 and then activity assay was performed at the optimum pH.

2.3.14 Effect of temperature on enzyme activity
All the thermal inactivation studies were performed in milli Q (pH 5.8 - 6.0).

1. Purified enzyme was diluted to 5μg/ml and 200 μl aliquot was incubated at desired temperature. In our study we used temperature ranging from 4 °C to 75 °C.
2. After incubating the enzyme at a particular temperature, fixed amount of enzyme, in duplicate, was removed at 10 min interval and mannosidase enzyme assay was performed at 37 °C. This procedure was repeated at different temperatures viz. 10 °C, 20 °C, 37 °C, 42 °C, 55 °C, 60 °C, 65 °C, 70 °C and 75 °C.
3. Reduction in (%) activity was calculated in comparison to control incubated at 0 °C and plotted on the graph taking temperature on X-axis and % activity on Y-axis.

2.3.15 Glycoprotein staining and Endo H digestion
Glycoprotein staining was performed according to instruction manual of GelCode® Glycoprotein staining kit (PIERCE biotechnology).

1. After electrophoresis, the gel was fixed in 50% methanol for 30 min. Then the gel was washed twice with 3% acetic acid for 10 min.
2. The gel was then transferred to 25 ml of oxidizing solution and gently agitated for 15 min. Further the gel was washed for 5 min with 3% acetic acid for 4 times.
3. Further the gel was transferred to 25 ml Gel Code® Glycoprotein stain for 15 min. Then the gel was washed extensively with 3% acetic acid and further with ultra pure water. Glycoproteins appear as magenta bands.
4. Endo H (NEB) was used to remove carbohydrate residues from proteins. Reaction was done according to the manufacturer's protocol with minor modifications.
5. 500 ng of the protein was denatured by boiling it for 5 min in 1X denaturing buffer in a 10 μl reaction volume.
6. Two μl of 10X G5 reaction buffer (provided by the manufacturer) and 1 μl of EndoH (500 units/μl) enzyme was added to denatured protein and incubated overnight in 37 °C.

7. To the reaction, laemmli buffer was added and boiled for 5 min. Then the samples were electrophoresed with untreated (control) and blotted on to nitrocellulose membrane to detect it with anti-SIAMS antibody or the gel stained with commassie R-250.

2.3.16 2-Dimensional gel electrophoresis, SDS-PAGE

1. The soluble fraction of proteins were diluted in dilution buffer and boiled for 5 min and allowed to cool at room temperature (25°C), precipitated with 9 volumes of 100% chilled acetone for 2 hrs.

2. The precipitates were recovered by centrifugation at 15,000 rpm at 4°C for 15 min. Isolelectric focusing was carried out with 20 μg of purified protein sample in 250 μL 2-DE rehydration buffer for 13 cm gel strips.

3. Proteins were first separated according to their charge after passive rehydration (in-gel rehydration) of 13 cm long immobiline dry strips, pH 4-7 for 12-16 hrs.

4. Electrofocusing was performed using IPGphor system (Amersham Biosciences) at 20°C for 20,000 Vhrs. The focused strips were subjected to reduction with 1% (w/v) DTT in 10 ml of equilibration buffer followed by alkylation with 2.5% (w/v) iodoacetamide in the same buffer.

5. The strips were then loaded on top of 12.5% polyacrylamide gels for second dimensional separation. The electrophoresed proteins were stained with silver stain plus kit (Bio-Rad, CA, USA).

6. Gel images were digitized with a FluorS imaging system (Bio-Rad, CA) equipped with a 12-bit camera. Experimental molecular mass and pi were calculated from digitized 2-DE images using standard molecular mass marker proteins.

7. The spots were cut from the gel and analyzed by electrospray ion trap time-of-flight mass spectrometry (LC-MS/MS) (Q-Star Pulsar i, Applied Biosystems). The spectra were analyzed by Mascot sequence matching software (www.matrixscience.com) against the Viridiplantae (green plants) database.

2.3.17 Antibody preparation

1. The purified SIAMS was emulsified with Freund's complete adjuvant and the thick emulsion was injected subcutaneously in the New Zealand white rabbit.

2. Subsequent boosters were given in Freund's incomplete adjuvant subcutaneously after a period of three weeks.
3. Test bleeds were taken with ear vein checked for the production of specific antibodies.
4. Antibody titer was checked by immunoblot analysis and once antibody titer was good the rabbit was bled to collect 20 ml blood sample.
5. The serum was separated from the blood and stored in -20°C in presence of azide.

2.3.18 Affinity purification of antibodies

Purification of antibodies was done using protein A sepharose according to Harlow and Jane, 1988.
1. The crude antibody was quantitated using Bradford reagent.
2. 100 µl of the beads, which had the capacity of 10mg/ml, were packed in a syringe and washed with 10 column volumes of 100mM Tris-Cl, pH 8.0 followed by 10 column volumes of 10mM Tris-Cl, pH 8.0.
3. To the washed beads 1mg of crude antibody was loaded manually and washed similar to step 2.
4. The antibodies were eluted with 100mM glycine (pH 3.0, adjusted with HCl). This buffer was added stepwise, 500 µl each time. The elutant was collected in 1.5 ml eppendorf containing 50 µl of 1M Tris-Cl (pH 8.0).
5. The tubes were mixed gently to bring the pH back to neutral and during the process care were taken to avoid bubbling or frothing. Then the presence of immunoglobulins in the fractions was checked by absorbance at 280nm or by Bradford reagent. Approximately 1 OD corresponds to the concentration of 0.8 mg/ml.
6. This affinity purified antibody was used for library screening as well as immunoblot analysis.

2.3.19 Immunoblot analysis

For immunoblot analysis 50 µg of the protein from different stages of ripening was quantified using Bio-Rad protein assay kit (Bradford, 1976) and precipitated with 20% TCA for 1 hr at 4°C. The samples were centrifuged at 13,000 rpm for 10 min, the supernatant was discarded and the pellet was air dried. Then 5 µl of 1M Tris base was added to the samples. Further the samples were boiled for 5 min after adding 10 µl of laemmli buffer. The samples were loaded on 12.5 % SDS–PAGE and resolved at 70 V constant. After the run the protein/s were transferred to nitrocellulose Hybond membrane (Amersham-GE) using Towbins buffer (Towbin et al., 1979).
1. After SDS-PAGE the gel was soaked in Towbins buffer for 5-10 min. Then the gel was sandwiched between the cassette in such a way that the gel was towards the negative electrode and the nitrocellulose membrane (Hybond C extra, Amersham)
towards the positive electrode, supported by 3MM Whatman paper of size equal of the gel.

2. The transfer was carried out in an electro transfer apparatus (Hoefer) at 150mA constant current for 3 hr at room temperature or at 4°C overnight at 15 mA.

3. After the transfer, the blots were stained with 0.5% Ponceau S (Salinovich and Montelaro, 1986) in 1% acetic acid for 5 min. The position of the molecular weight markers and the positive control was marked and then the blots were destained completely by repeated washing in water.

4. The blots were rinsed in 1X TBS and blocking was done for overnight in 5 % non-fat dry milk in TBST at 4°C or using Blotto in TBS (PIERCE).

5. The blocking solution was replaced by primary antibody diluted 1:5000 in TBST. It was incubated for an hour at RT.

6. The blots were washed twice with TBST for 5 min and then three times, each of 15 min duration in gyrotary shaker at 50 rpm at room temperature.

7. Secondary antibody, anti-rabbit IgG alkaline phosphatase conjugate (Sigma), was added to the blot at a dilution of 1:20,000 in TBST and incubated for 1 hr at RT. In case of chemiluminiscence kit (West Pico, PIERCE) horse radish peroxidase conjugated secondary antibody was used in the ratio of 1:20,000.

8. The blots were developed using NBT/BCIP (Bio-Rad) in AP buffer as a substrate for alkaline phosphatase. Once the colour is developed, the reaction was stopped by rinsing the blots in MQ H₂O. For detecting Horse radish peroxidase the blot was incubated in the substrate provided by the manufactures for 5 min in dark. Then the blot was placed in between saran wrap and exposed to the film. The time of exposure varied depending on the intensity of the signal.

2.3.20 Immunoprecipitation of antibodies

Immunoprecipitation was done using protein A sepharose beads (Amersham) according to the manufacturers protocol.

1. The first step in immunoprecipitation is to couple antigen to antibody. Here 20 µg of the crude protein was taken in 500 µl of extraction buffer and to that 10 µl of antibody was added. Another reaction wherein crude protein was incubated with pre-immune serum as a negative control.

2. These tubes were incubated at 4°C for 1 hr with gentle shaking. To this mixture 50 µl of Protein A Sepharose 4 fast flow (Amersham) was added and again incubated at 4°C for 1 hr with gentle shaking. This step ensures the binding of antibody to the sepharose beads.
3. Then the tubes were centrifuged at 6000 rpm for 20 sec and the supernatant was discarded. The pellet was washed with extraction buffer containing 0.25M NaCl.

4. Step 3 was repeated 3-4 times. Then the pellet was resuspended in Laemmli buffer, boiled for 5 min and the supernatant was loaded on 12% SDS-PAGE. Then the gel was stained with comassie blue R-250 or the gel was electroblotted to nitrocellulose membrane and analysed by immunoblot analysis.

2.3.21 Immunotitration

1. Purified Alpha-mannosidase (1 μg) was incubated with different volumes of immune as well as Pre-immune serum (Negative control) for 4 hrs at room temperature.

2. The antigen-antibody complexes were spun down at 13,000 rpm for 10 min at 4°C and the enzyme activity was checked in the supernatant.

3. The % activity remaining in the supernatant was plotted against the amount of antiserum/ pre immune serum added to the reaction as compared to the activity of native protein stored at 0°C

2.3.22 Immunolocalization

Immunolocalization was done according to Sauer et al., 2006 with some modifications.

1. Fruits at green and breaker stage were harvested and 5 mm X 5 mm pieces of pericarp were fixed in 4% paraformaldehyde overnight. In case of capsicum stage 8 fruits were used for the purpose.

2. Next day the plant material was washed with 1X PBS twice. The plant material was mounted on to the cryostat (Lieca CM1510S) and frozen, then 10-15μm slices were cut and taken on the slides. Then the slides were allowed to dry at room temperature for 1 hr.

3. The slides were washed with 1X PBS, and incubated for 1 hr at room temperature with 3% BSA to block the non specific sites. Further washed with 1X PBS for three times.

4. The slides with the fruit pericarp sections were incubated with polyclonal antibody of SIAMS/CaAMS overnight at 4 °C. This was done by placing strip of parafilm over the sections so that a thin layer of antibody is spread over it and kept inside a humid chamber to prevent it from drying.

5. Next day the sections were washed three times and secondary antibody labeled with FITC was added to it and kept in the humid chamber for three hours. Then the sections were washed with 1X PBS for 8 times at an interval of 10 min each. Further the samples were mounted under the cover slip and viewed in fluorescence microscope with appropriate filter.
2.4 Results

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<td>.8</td>
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2.4.1 Mannosidase purification and characterization from tomato

2.4.1.1 Purification

First step in purification was to identify the stage of ripening at which maximum SLAMS enzyme accumulated. The flowers were tagged at anthesis and fruits were harvested at green (the surface of the tomato is completely green, the shade of the colour vary from light to dark), breaker (there is definite break in colour from green to yellow or red), pink (when more than 30% but not more then 60% of the surface shows pink or red colour) and red (when more than 90% of the surface shows red colour). Using PNP-alpha-D-mannopyranoside as substrate we found maximum activity at breaker followed by green, pink and red stages (Fig. 2.1). Taking the breaker stage tomatoes of cv. Pusa Ruby extraction was done in 100mM Tris-Cl pH 7.8. Crude extract was precipitated with ammonium sulphate (0-80%) and the pellet obtained was dialysed with one change to remove all the salt present in the protein so as to facilitate its binding to the anion exchanger. The dialysed protein was loaded on DEAE sepharose and eluted with the linear salt gradient (Fig. 2.2A). The mannosidase enzyme got eluted at 100 mM salt concentration as revealed by enzyme activity assay, further no activity was found throughout the elution up to 1M gradient elution of salt. This result excluded the
Figure 2.1 Curve showing specific activity of α-mannosidase in fruits at different stages in cultivar Pusa Ruby. Four different stages of ripening were identified and samples collected after tagging the flowers at anthesis. Breaker stage showed maximum enzyme activity.
possibility of isoforms in the pericarp for tomato mannosidase unlike other reports who found two isoforms in tomato (Suvarnalatha and Prabha, 1999), and three isoforms in cotyledon tissue of P. vulgaris (Wilden and Chrispeels, 1983). Parts of the fractions with activity were checked on 12.5 % SDS-PAGE (Fig. 2.2B) and remaining was precipitated and loaded on to 120 cm long gel filtration column. The enzyme eluted just after the void volume of the column and the fractions rich in the activity were checked on the PAGE (Fig. 2.2 C-D). The different steps involved in purification of tomato mannosidase are depicted in the Figure 2.2E. Final yield of the enzyme was 14.32% and was purified up to 45 fold (Table 2.1). The purified enzyme showed a single band of 360 kD on 6% native PAGE (Fig. 2.3A), had two subunits of 70 kD and 45 kD on SDS-PAGE (Fig. 2.3B).

2.4.1.2 Purity of the mannosidase protein
Purity of the sample was checked by two fold serially diluting 5 µg of the protein (Fig. 2.3C) and we found two subunits devoid of any contaminants. The commassie stain was able to detect about 78 ng of the protein. Further to check the purity of the sample, 20µg of the purified protein was separated on two dimensional gel Electrophoresis (2DE) which revealed that 70 kD subunit was a single spot and the 45 kD subunit further separated into three proteins with different pls (Fig. 2.3D). The migration distance of the proteins in the second dimension SDS-PAGE exactly correlated with that of two subunits seen in the single dimension SDS-PAGE. We identified the spots by LC/ MS-MS analysis and found that each was a different protein, the 70kD represented the alpha-mannosidase (spot no.1, pi 6.8) and the 45 kD which splits into three proteins identified putative monosaccharide transporter (spot no.2, pi 6.4), geraniol responsible factor spot no.3, pi 6.3) (which is the part of the gene) and the third was the ATPase (spot no.4, pi 6.2). The probability based Mowse score and the peptide peaks are depicted in the Fig. 2.4A-B. Therefore, we predicted, two of the three proteins found with the lower 45 kD subunit may be interacting proteins and not the glycoforms.

2.4.1.3 Activity calculation by using standard curves
To calculate the activity we constructed two standard curves. First curve was plotted using different concentrations of BSA which we used for quantitation of total protein. Serial dilutions of the BSA (1-15 µg) quantified spectrophotometrically by using Bradford reagent. OD values taken at A_595 were plotted on Y-axis and concentration on X-axis (Fig. 2.5A); the R^2 value of the curve was 0.9325. We calculated the concentration of unknown protein (x) using the slope of the curve passing through the origin, y = 0.0718 x. Where y = OD value, and 0.0718 is the slope.
Figure 2.2 Purification of α-mannosidase from tomato. (A) Chromatogram showing elution profile on DEAE Sepharose ion exchange column attached to AQT- FPLC. The column was equilibrated with 20mM Tris-Cl pH 7.8 and fractions were eluted with linear gradient of 0-50 % NaCl. Activity (Units/mg) histogram is shown in brown broken line which represents the elution of the mannosidase. Green line indicates the linear gradient of NaCl (B) Fractions with enzyme activity were resolved on 12.5% SDS-PAGE. M is molecular weight markers, C is Crude protein and numbers indicates the fractions.
Figure 2.2 (C) Elution profile on Sephadex G-100 gel filtration column equilibrated and eluted with 20mM Tris-Cl pH 7.8 with a flow rate of 10 ml/hr. (D) Commassie blue stained 12.5% SDS-PAGE gel of the fractions (85-99) showing enzyme activity. (E) Silver stained 12.5% SDS-PAGE gel with protein obtained at different stages of purification. Lane 2: Crude extract, Lane 3: Fraction from ion exchange chromatography, Lane 4: Fraction from gel filtration chromatography, Lane 1: Molecular weight markers.
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Values are averages of three independent experiments. One unit is equivalent to 1 µm PNP released/min. Specific activity is expressed as 1 µmol PNP released/min/mg protein.

**Table 2.1** Summary of purification of α- mannosidase from tomato.
Figure 2.3 (A) Purified α-mannosidase separated on 6% native gel showing single band. (B) Purified protein denatured and separated on 12.5% SDS-PAGE. (C) Two fold serial dilution of 5 μg tomato purified protein and resolved on 12.5% SDS-PAGE to assess the purity. (D) 20 μg of the α-mannosidase protein was focused on 4-7 IPG strips in the first dimension and resolved on 12.5% SDS-PAGE in the second dimension. The 70 kD protein, represented α-mannosidase (Spot 1) and the 45 kD band resolved into three spots representing monosaccharide transporter (Spot 2), geraniol responsible factor 15 (Spot 3) and ATPase (Spot 4). Left side of gel shows the purified mannosidase denatured and directly loaded on to the second dimension as markers.
Figure 2.4 (A) The probability mowse score and (B) the peptide peaks of two of the peptides obtained by LC MS/MS analysis of tomato protein.
The second curve was plotted using PNP (Para-nitro phenol) for calculating the activity of the enzyme. Serial dilutions of PNP (1-50 μM) in water was quantified at A₄₀₅ and the OD values were plotted on Y-axis and concentration on X-axis. The R² value of the curve was 0.9988 (Fig. 2.5B).

2.4.1.4 Molecular mass determination
The molecular weight of the protein was determined by gel filtration using superdex 200 10/300 GL analytical column. This column is a Tricorn™ high performance pre packed column made of cross linked agarose and dextran with average particle size of 13 μm with column dimensions of 10 cm diameter and 300mm length. The approximate bed volume is 24 ml with pH stability of 3-12 and temperature stability of +4 to +30 °C. The exclusion limit of the column for globular protein was approx. 1.3 x 10⁶ and optimum separation range was 10 kD to 600 kD with maximum column pressure limit of 1.5 MPa. Using this column the calculated size of tomato mannosidase was 290 kD. The retention time or the elution volume of the standard proteins as well as the mannosidase is depicted in Figure 2.6A. To calculate the molecular weight of the protein we plotted a standard curve using log MW (Molecular weight) on Y-axis and Kav (Partition coefficient) on X-axis (Fig. 2.6B). The size of the protein determined by gel filtration never matched with size on the native gel, which depicted 360 kD. In order to confirm that the native protein corresponds to mannosidase we performed activity band correlation. This was done by excising the gel slice from the unstained non denaturing 6% native gel corresponding to the band position in the stained gel and mashed it in the extraction buffer to check the mannosidase activity. The assay gave mannosidase activity in the supernatant confirming the protein. Further, we performed activity assay by cutting the gel pieces at equal intervals throughout the lane and found no activity (Fig. 2.3A).

2.4.1.5 Effect of temperature and pH on the activity
The enzyme was stable at 60°C without considerable reduction in the activity up to 1 hr. At 65°C only 20% reduction in the activity was seen after 1 hr of incubation. The activity reduced by 60% after 10 min of incubation at 75 °C and further after 10 min complete activity was lost (Fig. 2.7A). Optimum temperature for activity was determined to be 45°C (Fig. 2.7B).

The pH optimum for the enzyme activity was 6.0 (Fig. 2.8A). Further preincubation of enzyme at different pH and the activity measured at optimum pH had no effect towards the basic side. But the activity reduced drastically at acidic pH less than 4. This may be due to effect of pH on the ionizable groups of the active site or substrate.
Figure 2.5 Standard curves for protein quantitation. (A) Purified BSA was serially diluted to form a series of concentrations (1-15 μg) and detected spectrophotometrically at A_{280} using Bradford reagent. (B) Para-nitrophenol was serially diluted from 100 μM stock to form a series of concentration (1-50 μM) and detected at A_{405}. The concentration was plotted against the absorbance.
Figure 2.6 (A) Elution profiles of the native molecular weight standards run on the superdex 200 column with a flow rate of 0.1 ml/ min.
Figure 2.6 (B) Standard curve plotted using the elution volume of the standard molecular weight proteins run on superdex 200 gel filtration column to determine the molecular weight of the unknown. The calculated molecular weight of tomato mannosidase was 290 kD.
Figure 2.7 (A) Effect of temperature on mannosidase. A known quantity of enzyme was incubated at different temperatures for 1 hr and activity assay was performed. The activity was expressed in percentage relative to the activity of the protein stored at 0°C. At 37°C enzyme did not lose any activity and incubation at 75°C for 10 min rendered the enzyme inactive. (B) Optimum temperature of the enzyme was 45°C as depicted in the graph.
2.4.1.6 Km of tomato mannosidase enzyme
From Lineweaver Burk plot (Fig. 2.8B), the apparent Km value of 4.6 mM was calculated for para nitrophenyl α-D Mannopyranoside as the substrate. This gave a Vmax of 0.86 m mol/L/min.

2.4.1.7 Immunoblot analysis
We raised antibody against the 70 kD subunit of the protein as well as for native soluble protein which showed homology to mannosidase. We did immunoblot analysis for all the ripening stages; 100μg of protein was loaded on SDS-PAGE and transferred to nitrocellulose membrane and HRP conjugated secondary antibody was used for the analysis. Immunoblot analysis was similar to activity curve showing maximum protein accumulation at breaker stage followed by green, pink and red (Fig. 2.9A). The antibody raised against the SIAMS subunit was able to detect the protein at a titre of 1: 5000.

2.4.1.8 Glycoproteic nature
Tomato α-mannosidase stained positive with glycoprotein (Periodic Acid Schiff) stain. Further we confirmed the presence of glycan moiety by Endo H digestion (Fig. 2.9B-C). This enzyme cleaves the carbohydrate moiety and the protein migrates faster than the undigested sample. In case of mannosidase the Endo H could break the glycan/carbohydrate moiety only when the mannosidase was denatured by boiling. Endo H digestion products were resolved on 12.5 % SDS PAGE and transferred to the nitrocellulose membrane and detected by anti-AMS antibody which showed a shift of 2-3 kD (Fig.2.9B). We performed another test for glycoproteins by binding mannosidase to ConA sepharose and eluting with linear gradient of methyl mannosidase which further confirmed it to be a glycoprotein (data not shown).

2.4.1.9 Immunological characterization
Antibodies raised against AMS could immunoprecipitate the enzyme from the crude protein using protein A sepharose. In immunotitration experiment the antibody was able to precipitate the enzyme activity as compared to pre-immune serum (Fig. 2.10). Further, protein localization was detected using anti-SIAMS and the signal was found to be in the cell wall (Fig. 2.11).

2.4.2 Mannosidase purification and characterization from capsicum
2.4.2.1 Purification
Capsicum being non climacteric fruit, fruit development and ripening was divided into 8 stages. Taking matured fruits protein extraction was done in Tris-Cl pH 7.0. Activity assay was performed at all the different stages of development and ripening of capsicum and
Figure 2.8 (A) Optimum pH and stability for mannosidase. Bell shaped curve represents the optimum pH for the activity which is 6.0. The overlapping curve represents the stability of the enzyme at different pH. This was done by pre incubating the enzyme at different pH and performing activity assay at the optimum pH (B) Lineweaver burk plot represents the $K_m$ of the enzyme which is 4.6 mM. Reciprocal of the velocity ($1/V$) and substrate concentration ($1/S$) were plotted on Y and X axis respectively.
Figure 2.9 (A) Shows the Immunoblot analysis of α-Mannosidase at different stages of ripening (G, Green, B, Breaker, P, Pink, R, Red Ripe) with a purified enzyme as the +ve control. Total protein from all the ripening stages were separated on 12.5 % SDS-PAGE and detected by anti-AMS. (B) Endo H digestion of the purified protein (PP+EndoH) showing a shift as compared to undigested (PP), detected by anti-SIAMS. (C) Glycoprotein staining of mannosidase (lane 4 and 5) with negative control (lane 3), positive control (lane 2 ) and protein markers (lane 1).
Figure 2.10 Immunotitration of purified SIAMS antigen with the antiserum. Pre-immune serum was used as a negative control which was not able to precipitate the activity.
Figure 2.11 Localization of AMS in tomato. Tomato pericarp from breaker stage was used for the analysis after sectioning and processing. A, B and C shows the cell wall localization of AMS using FITC conjugated secondary antibody, E, F and G represent the respective bright field images. D is the negative control wherein pre-immune serum was used as the primary antibody and H represents the corresponding bright field image.
maximum activity was found in stage 8 fruits (Fig. 2.12). For capsicum different purification strategy was used because of its unusual behavior of not binding to any of the matrix (Cation and Anion exchanger) between the pH 5.0 and 9.0. For capsicum a compromise buffer was used where in the protein was loaded on to the column of Q-Sepharose equilibrated and collected in the flow through using 20mM Tris-Cl pH 7.0 (Fig. 2.13A). And this strategy ensured that most of the protein contaminants were bound to the matrix and mannosidase eluted in the flow through. Activity containing fractions were resolved on 12.5 % SDS PAGE (Fig. 2.13B) and precipitated with ammonium sulphate (0-90%). The pellet thus obtained was dissolved in minimum amount of buffer and directly loaded on to Sephadex G-100 gel filtration column (1.5 cm x 120 cm) (Fig 2.14A). The eluted fractions containing the activity were concentrated and resolved on the gel (Fig. 2.14B). The various steps in the purification are depicted in the Figure 2.14C. The purified protein was a single band of ~500 kD on 6% native gel (Fig. 2.15A) and on SDS-PAGE two subunits of 70 and 50 kD were resolved (Fig. 2.15B). The summary of purification is depicted in Table 2.2. Overall yield of the protein was 6.73% and purified upto 37 fold. Unlike tomato, there are no isomers for capsicum mannosidase other than what is reported here.

2.4.2.2 Purity of the protein
Two fold serial dilution of 5 µg of the protein revealed that protein has two subunits without any contaminants. Commassie stain was able to detect 76 ng of protein on SDS-PAGE (Fig. 2.15C). Another similar gel was transferred to nitrocellulose membrane and detected by anti-AMS. Electrofocusing of purified protein on 2DE revealed it to be a complex and gave a hint of interacting proteins. The lower 50 kD band separated in to three proteins with different pls very much similar to tomato mannosidase (Fig. 2.16A). Identification of proteins by LC/ MS-MS analysis revealed that the 70kD represented the alpha-mannosidase (spot no.1, pl 6.8) and the 50 kD which splits in to three proteins had putative monosaccharide transporter (spot no.2, pl 6.4), geraniol responsible factor spot no.3, pi 6.3) (which is the part of the gene) and the third was the ATPase (spot no.4, pi 6.2). The probability Mowse score and peptide peaks are depicted in Fig. 2.17A-B.

2.4.2.3 Oligomerization
The mannosidase from capsicum when resolved on native non-denaturing PAGE migrated as a single band with the apparent molecular mass of 500 kD. On 12.5% SDS-PAGE, the protein showed two subunits of 70 and 50 kD confirming it to be hetero dimer. When the protein sample was boiled in the presence or absence of a reducing agent (2-Mercaptoethanol) and resolved on SDS-PAGE, it migrated as dimer with two polypeptides of 70 and 50 kD (Fig 2.16B).
Figure 2.12 Graph showing specific activity of α-Mannosidase at different stages of capsicum development and ripening. Stage 8 (S8) fruits showed maximum activity for mannosidase.
Figure 2.13. Elution profile on (A) Q-Sepharose ion exchange column, equilibrated and eluted with 0.05, 0.10 and 0.15 M NaCl, pH 7.0 (flow rate 15 ml/hr). Arrows indicate the change of gradient. (B) SDS-PAGE of the fractions showing enzyme activity. M is Markers and numbers indicate the fractions.
Figure 2.14 (A) Elution profile on sephadex G-100 gel filtration column. (B) Fractions (85-101) with enzyme activity were loaded on 12.5% SDS-PAGE (C) SDS-PAGE with protein from different stages of purification, crude (lane 1), fractionated (lane 2), dialyzed (lane 3) and purified (lane 4, 5 & 6) protein of capsicum mannosidase. M is molecular weight markers.
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Values are averages of three independent experiments.
One unit is equivalent to 1 µm PNP released/ min.
Specific activity is expressed as 1 µmol PNP released/min/mg protein.

Table 2.2 Summary of purification of α-mannosidase from capsicum.
Figure 2.15 (A) Native PAGE showing the migration of mannosidase protein as a single band of ~500 kD (Lane 3 and 4). Lane 2: Crude, Lane 1: Native molecular weight markers. (B) Silver stained 12.5% SDS-PAGE gel showing two bands of 70 and 50 kD. (C) Two fold serial dilutions (lane 1-7) starting from 5 µg of purified protein was resolved on 12.5% SDS-PAGE. (D) Immunodetection of serially diluted mannosidase using anti-AMS.
Figure 2.16 (A) Iso-electric focusing of capsicum mannosidase on IPG strip of pH 4-7 revealed that the 50 kD subunit has other interacting proteins. The 70 kD protein, represented α-mannosidase (Spot 1) and the 50 kD band resolved into three spots representing monosaccharide transporter (Spot 2), geraniol responsible factor 15 (Spot 3) and ATPase (Spot 4). (B) Oligomeric organization of mannosidase determined on 12.5% SDS-PAGE by subjecting the protein to denaturing and non-denaturing conditions as indicated in the legend.
Figure 2.17 (A) The probability Mowse score and (B) the peptide peaks of two of the peptides obtained by LC MS/MS analysis of capsicum protein.
2.4.2.4 Molecular mass determination
Molecular weight of complex as determined by superdex 200 analytical column was similar to that of tomato about ~290 kD. The elution of mannosidase protein on superdex 200 column is depicted in figure 2.18. This was very much different from the size as depicted on the 6% native gel. We confirmed the native band to be mannosidase by activity band correlation assay as done in case of the tomato protein.

2.4.2.5 Effect of temperature and pH
Unlike tomato, capsicum mannosidase was more stable. The enzyme was stable upto 65 °C for 1 hr without losing any activity. At 70 °C, 75 % of the activity was lost after 1 hr of incubation. Further at 75 °C almost all the activity was lost after 30 minutes of incubation (Fig. 2.19A). The optimum temperature for activity was around 55 °C (Fig. 2.19B).

The pH optimum for the enzyme was 6.0, similar to tomato mannosidase (Fig. 2.20 A). Preincubation of the enzyme at different pH had no effect on the activity (Fig. 2.20 A).

2.4.2.6 Km of Capsicum mannosidase enzyme
Using PNP-α-D Mannopyranoside as the substrate we found that the Km for the enzyme was 1.6 mM and Vmax to be 0.64 m mol/L/min (Fig. 2.20 B).

2.4.2.7 Glycoproteic nature
Capsicum α-mannosidase was also positive for glycoprotein stain. Endo H digestion revealed the presence of carbohydrate moiety which migrated faster than the undigested sample (Fig 2.21A-C).

2.4.2.8 Immunological characterization
Immunoblot analysis of the total protein isolated from different stages of ripening revealed that stage 8 fruits had the maximum activity (Fig. 2.22A). Antibodies raised against AMS could immunoprecipitate (Fig. 2.22B-C) the enzyme from the crude protein using protein A sepharose. In immunotitration experiment the antibody was able to precipitate the enzyme activity as compared to pre-immune serum (Fig. 2.22D). Immunolocalization of the protein using the polyclonal anti-CaAMS showed its localization in the cell wall (Fig. 2.23).
Figure 2.18 Elution profile of purified capsicum mannosidase (in replicate) on superdex 200 gel filtration column equilibrated and eluted with a flow rate of 0.1 ml/min. This was used to calculate the molecular weight of the protein.
Figure 2.19  (A) Effect of temperature on capsicum mannosidase. A known quantity of enzyme was incubated at different temperatures for 1 hr and activity assay was performed. The activity is expressed in percentage relative to the activity of the protein stored at 0°C. At 37°C enzyme did not lose any activity and incubation at 75°C for 10 min rendered the enzyme inactive. (B) Optimum temperature of the enzyme was 55°C as depicted in the graph.
Figure 2.20 (A) Graph shows optimum pH and stability of capsicum mannosidase at different pH. (B) Lineweaver Burk plot showing the Km for purified protein to be 1.6 mM using PNP-α- mannopyranoside as the substrate.
Figure 2.21 (A) Two µg of denatured purified mannosidase enzyme was treated (lane 2) with 50 units of Endo H for 18 hrs and resolved on 12.5% SDS-PAGE and compared with untreated mannosidase (lane 1). Horse radish peroxidase (HRP) was used as the positive control (lane 3 is untreated, lane 4 is treated). (B) Glycoprotein staining of mannosidase (lane 3) with negative control (lane 2) and positive control (lane 1). (C) Similar gel was run and stained with commassie blue.
Figure 2.22 (A) Immuno blot analysis of all the developmental and ripening stages of capsicum. S8 stage of capsicum showed maximum expression of mannosidase. (B) Immunoprecipitation of mannosidase using polyclonal antibodies and resolving it on 12.5% SDS-PAGE and (C) detected by Anti-AMS antibodies. Lane 1 – Purified protein (P), Lane 2- Immunoprecipitated protein with anti-mannosidase antibody (Ab), Lane 3- Immunoprecipitation with pre-immune serum (PIS), Lane 4- Only serum (S), The arrow indicates the immunoprecipitated protein from the crude in lane 2. (D) Immunotitration of the enzyme by anti-mannosidase antibody and Pre-immune serum.
Figure 2.23 Localization of AMS in Capsicum. Capsicum pericarp was used for the analysis after sectioning and processing. A, B, and C show the cell wall localization of AMS using FITC conjugated secondary antibody. E, F, and G represent the respective bright field images. D is the negative control wherein pre-immune serum was used as the primary antibody and H represents the corresponding bright field image.
2.5 Discussion

Alpha-mannosidase was isolated from the pericarp of tomato and capsicum by series of steps including ammonium sulphate precipitation, ion exchange and gel filtration chromatography. In tomato, breaker stage fruits showed highest activity among the other ripening stages unlike in capsicum which showed increase in the last stage of ripening but otherwise constant throughout the development and ripening stages. This expression pattern indicates towards its differential regulation in both capsicum and tomato, which differ in the respiratory behavior. During the course of purification we found that the SIAMS was able to bind to DEAE sepharose ion exchange matrix unlike CaAMS which was unable to bind to any of the ion exchange matrix from pH ranging from 4-10. This was the reason different approach was adopted for purification, involving a compromise buffer with pH 7.0. Although there were reports of mannosidase purification from tomato (Suvamalatha and Prabha, 1999) and capsicum (Priya Sethu and Prabha, 1997) earlier, but the reports do not confirm them to be mannosidase. This prompted us to confirm the purified enzymes by N-terminal as well as LC MS/MS analysis before proceeding for further experiments. In this study, no isoforms of the enzyme was found unlike other reports, where two isozymes in tomato was reported (Suvamalatha and Prabha, 1999). The enzyme was found to be glycosylated with very high molecular weight both in capsicum and tomato. On 6% native gel, we estimated molecular weight of 360 kD and ~500kD for tomato and capsicum, respectively. But on superdex 200 gel filtration column both the enzymes had the same retention time. And also the molecular weight determined by gel filtration was different from that of the native gel. This apparent molecular size obtained by gel filtration could be due to the tendency of certain glycoproteins to interact noncovalently in solution (Kleinman et al., 1986; Farasch-Carson and Carson, 1989). On SDS-PAGE two polypeptides were observed in both the cases, but the lower subunit of tomato differed from the capsicum (Fig 2.24A). This difference may be due to heterogeneity in glycosylation among both the proteins. This heterogeneity is due to modification of the protein by glycosylation ranging from single saccharides on protein to the complex and densely packed branched structure. Therefore, the apparent molecular size of 70 and 50 kD in capsicum and 70 and 45 kD in tomato on SDS-PAGE using Laemmli buffer system should be considered an approximation since the enzyme has carbohydrate moiety attached to it. Moreover glycoproteins bind SDS in a ratio different from that of other proteins and are known to form asymmetric SDS-protein complexes thus resulting in anomalous migration when compared to other molecular size standards (Cozens and Reithmeier, 1984).

Iso-electro focusing of the AMS from both capsicum and tomato revealed presence of interacting proteins, as the lower 45 kD of tomato and 50 kD of capsicum separated into three more spots. Among the predicted interacting proteins, one showed homology to monosaccharide transporter and another to ATPase. The presence of monosaccharide
Figure 2.24 (A) Similarities and dissimilarities between the two subunits of AMS. (B-C) Cross reactivity of native capsicum antibody with the tomato.
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transporter with the complex made sense, as it is known that higher plants possess two distinct sugar transporters that import monosaccharide and may function in the recovery of monosaccharide liberated by cell wall turnover. Recycling of sugars that are released as a consequence of cell wall turnover and utilization of salvaged breakdown products would require their import (Vicente et al., 2007). The extent of the recycling of simple sugars generated by plant cell wall degradation is still unclear but association of monosaccharide transporter with the mannosidase complex for the same purpose can not be excluded. Further, sugar transporters play such a key role in source-sink interactions; it is likely that its activity is tightly controlled by the activity of H+ ATPase. This may be the possible reason of ATPase being associated with monosaccharide transporter. The above explanation indirectly indicates the possible role of the enzyme in cell wall degradation and recovery of monosaccharides by the transporter, liberated by cell wall turnover, associated with it.

The pH is known to influence the velocity of an enzyme catalyzed reaction. The active sites on enzymes are frequently composed of ionizable groups that must be in the proper ionic form in order to maintain the conformation of the active site that binds the substrates or catalyses the reaction. The stability of the enzyme depends on the temperature, ionic strength, chemical nature of the buffer, concentration of various preservatives, concentration of contaminating metal ions, concentration of the substrate and also enzyme concentration. The decline in activity away from the optimum pH can be ascribed to the effect of pH on ionizable groups of the active site or substrate. The stability of both the proteins, in particular CaAMS, to a wide pH range can be attributed to its resistance to pH denaturation. Another important post translational modification of the protein which influences many properties is glycosylation. In plants, N-linked glycans strongly influence the glycoprotein conformation, stability and biological activity. The presence of N-glycans affects both the co and post-translational folding of the protein. Numerous works have also demonstrated that the N-glycans can protect the protein from proteolytic degradation, as well as the fact that they are responsible for the thermal stability, solubility and biological activity of glycoproteins (Rayon et al., 1998). This is likely true in both the proteins, which were found to be stable over a wide range of pH and were moderately thermo stable. This can be directly related to the glycoproteic nature of the enzymes. Further, 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. Thus, the stability of the mannosidase to pH and temperature change can be attributed to presence of glycan units. CaAMS showed more stability to temperature and pH than its counterpart. This can be attributed to the differential glycosylation or heterogeneity in the glycosylation. The structural diversity inherent in the branched sugar moieties characteristics of the glycan element of glycoproteins enables subtle changes in protein shape, charge and volume that
have the potential to modify its function both temporally and spatially (Packer and Keatinge, 2002). In many cases, a substrate may induce a conformational change in the enzyme to a form that is more or less resistant to pH or temperature denaturation.

The antiserum against the mannosidase was able to precipitate the protein from the crude and also precipitate the activity of the purified mannosidase after incubation in the solution. The capsicum antibody (both raised from native protein as well as from 70 kD subunit) cross reacted with tomato protein and vice-versa (Fig. 2.24 B-C). This is because both the proteins are very much similar to each other as revealed by the conserved peptides obtained by LC/MS-MS analysis. Although both the protein organization and properties look similar still they differ post translationally in their heterogeneity and degree of glycosylation. Even difference at molecular level can not be excluded. CaAMS had more affinity towards the substrate than the tomato (SIAMS) as the Km value of former was less than the latter. We found localization of the protein using polyclonal antibodies raised against both the mannosidases and secondary antibody coupled with FITC. Both the enzymes were found to be localized in the cell wall.

The present study on α-mannosidase is a new addition to ‘tomato literature’ as well as in relation to the fruit ripening since this is one of the most active enzymes in most of the fruits showing enhanced activity during ripening of many fruits including capsicum and tomato (Priya Sethu and Prabha, 1997). Therefore, its role in ripening and or softening needs to be understood. Suppression of this enzyme both by antisense or RNAi technology would elucidate the function of this gene and assign it a role during ripening. To answer the questions raised during the discussion necessitates the gene cloning.