CHAPTER 5

PROTEIN AND PROTEIN OXIDATION

Mature Fruits of *Syzygium cuminii*
Introduction

Plant seeds are rich source of variety of proteins including dietary proteins. Seed proteins are known to perform variety of functions; structural, enzymic, transcriptional factors, chaperons etc. Massive synthesis of storage proteins during seed development is necessarily required for the supply of reduced nitrogen at early phases of germination (Morton et al., 1995; Galili, 1997; Shewry and Halford, 2002). Many proteins are stage specific as they are regulatory and associated with particular stages of seed development (Bettey et al., 1998; Gumilevskaya and Azarkovich, 2010; Tunnaciffe et al., 2010), dormancy (Ahmed and Dennis, 1994; Chibani et al., 2006; Oracz et al., 2007), germination (Gallardo et al., 2002; Job et al., 2005; Gumilevskaya and Azarkovich, 2010; Nonogaki et al., 2010; Tunnaciffe et al., 2010; Chen et al., 2012) and longevity/ageing (Wechsberg et al., 1994; Chaitanya et al., 2000a; Murthy and Sun, 2000; Rajjou et al., 2008; Xin et al., 2011; Yao et al., 2012). For example, the low molecular weight late embryogenic abundant proteins/heat shock proteins are synthesized during maturation phase of the seed development that confers desiccation tolerance to post-harvested seeds during drying in storage (Rosenberg and Rinne, 1988; Dure et al., 1989; Tunnaciffe and Wise, 2007). These proteins are accumulated in response to several other abiotic stresses and may be involved in the ion sequestration, membrane stabilization, water binding, and chaperon activity (Veeranagamallaiah et al., 2011). Alteration in the levels of proteins participating as transcriptional factors in the signal transduction have been identified using proteome analysis during seed ageing (Rajjou et al., 2008; Xin et al., 2011; Yao et al., 2012). Ageing or low vigour seeds are characterized by reduced capacity of protein biosynthesis both
at transcription and translation levels (Peumans and Carlier, 1981; Gidrol et al., 1990). Decreasing amounts of protein, at any particular stage, may be related to, 1-reduced protein synthesis, 2-de novo activation of proteases or declining amounts of protease inhibitors and, 3-oxidation or modification of proteins (Anderson, 1970; Hallman et al., 1973; Szczotka, 1975; Bewley and Black, 1982; Espindola et al., 1994; Barba-Espin et al., 2011). Accelerated ageing mediated loss of viability is associated with enhanced activity and synthesis of proteolytic enzymes which is correlated with simultaneous decline in total protein content (Saxena et al., 1985; Misra and Kar, 1990; Chaitanya et al., 2000a; Xin et al., 2011). Proteomic study revealed down regulation of several regulatory and enzymic proteins involved in glycolysis, tricarboxylic acid cycle, mitochondrial electron transport chain and phosphorylation in deteriorating seeds undergoing dehydration (Xin et al., 2011). Substantial decline in protein content is reported during ageing seeds of Citrus reticulata, Phaseolus aureus and Pisum sativum (Samshery and Banerjee, 1979), Oryza sativa (Prabakar and Mukherjee, 1980) and Shorea robusta (Nautiyal and Purohit, 1985). In recalcitrant sal (Shorea robusta) seed, the loss of protein was correlated with the rate and magnitude of dehydration rather than the percent germination (Chaitanya et al., 2000a). For example, dehydration of sal seeds from 42.1 to 36.7% moisture content resulted in substantial loss of total protein in axis and cotyledon with no change in germinability. Often, the low vigour (Byrd and Delouche, 1971) and desiccation intolerant (Stewart and Bewley, 1981) seeds are characterized by low protein content.

AOS induced oxidative damage to biomolecules such as proteins, lipids or DNA is deleterious to cell functions and consequently responsible for cellular damage (Dalle-Donne et al., 2003; Davies, 2005;
Job et al., 2005; Rinalducci et al., 2008). Accumulation of the oxidative products is vital in promoting the ageing related changes (Stadtman, 1992; Agarwal and Sohal, 1994). Oxidative stress induced protein modification caused severe alterations or malfunctioning of cellular activity. The oxidized or damaged proteins are susceptible to proteolytic degradation (Stadtman and Oliver, 1991), unfolding and protein aggregation (Grune et al., 1997; Butterfield et al., 2006). Several types of free radicals such as superoxide, hydroxyl, alkoxyl, hydroperoxyl and non-radicals such as hydrogen peroxide and peroxynitrite are known to mediate protein oxidation (Buxton et al., 1988; Neta et al., 1988). During oxidation carbonyl (CO) groups are produced on protein side chains especially of proline, arginine, lysine, and threonine (Job et al., 2005). These carbonylated proteins are chemically stable and thus considered markers for the detection of protein oxidation (Ballesteros et al., 2001; Das et al., 2001; Mostertz and Hecker, 2003; Johansson et al., 2004) using sensitive techniques standardized for its detection (Levine et al., 1990, 1994). The derivatives of carbonylated proteins may also be formed by the oxidative cleavage of proteins by either the α-amidation pathway or by oxidation of glutamyl side chains, leading to formation of a modified peptide in which the N-terminal amino acid is blocked by α-keto acyl derivative (Cabisco et al., 2000; Luxford et al., 2000; Nystrom, 2005; Rinalducci et al., 2008). The aldehydes (4-hydroxy-2-nonenal, malondialdehyde, 2-propenal - (acrolein)) generated during lipid peroxidation are also likely source of incorporating CO groups in proteins by secondary reactions to the nucleophilic side chains of cysteine, histidine, and lysine (Murthy and Sun, 2000; Liu and Wang, 2005). Proteins can be degraded through metal-catalyzed oxidation (MCO) system that trigger oxidation in the presence of O2/H2O2 and
Fe(III)/Fe(II) (Stadtman, 1994) thus rendering them highly susceptible to proteolytic degradation.

Drying induced AOS mediated oxidative stress during maturation, post-harvest storage and germination may be considered important in the oxidation of proteins that, in turn, is responsible for reduced seed vigour (Job et al., 2005). Direct and active role of AOS mediated protein oxidation during accelerated ageing was reported in *Arabidopsis thaliana* (Rajjou et al., 2008). Proteomic analysis of *Arabidopsis thaliana* seeds undergoing natural (slow rate) or accelerated (rapid) ageing exhibited significant loss of storage proteins mainly due to increased protein carbonylation with reduced translation during accelerated ageing. AOS induced alleviation of seed dormancy in *Helianthus annuus* has been correlated with increased formation of protein carbonylation (Oracz et al., 2007).

Total and soluble protein was determined in the axis and cotyledon of the dehydrating seeds of jamun. Pattern of protein profile was also studied in seeds exposed to different drying rates. Further, the level of carbonyl protein, a product of protein oxidation, was also monitored to determine its relationship in the axis and cotyledon of dehydrating jamun seeds.

**Materials and Methods**

**Total Protein**

Total protein extracts were prepared from dry mature viable and deteriorating seeds at different stages of ageing following the method of Wang et al. (2004). The seed samples were grinded into fine powder using mortar and pestle under liquid nitrogen. 0.3 g of powder was
transferred into 2 ml micro-centrifuge tube and the tube was filled with 1 ml chilled 10% TCA prepared in acetone. The contents were mixed well by using vortex mixture, and then centrifuged at 10,000 rpm for 25 minutes at 4°C. After centrifugation the residue part was collected, and the tube was filled with 1 ml of 80% methanol having 0.1 M ammonium acetate. The contents were mixed well by using a vortex mixer and centrifuged at 10,000 rpm for 25 minutes at 4°C. Once again, the supernatant was discarded and the tube was filled with 1 ml chilled 80% acetone and after a thorough mixing of the components, the samples were placed for centrifugation at 10,000 rpm for 25 minutes at 4°C. Later, the supernatant was discarded and the residue was kept for air drying at room temperature (25°C), to remove the residual acetone from the sample.

In an air dried sample, 600 µl: 600 µl of Phenol: SDS buffer of pH 8.0 was added. The samples were mixed thoroughly and incubated for 1 hour at room temperature, and centrifuged at 10,000 rpm for 25 minutes at 4°C. The upper phenol phase was taken into a fresh micro centrifuge tube and then the tube was filled with 1 ml methanol containing 0.1 M ammonium acetate and then the tube was stored at -20°C for whole night. After this, the sample was centrifuged at 10,000 rpm for 25 minutes at 4°C, so as to, get clear white pellet on the bottom of the tube. The pellet was washed first with 1 ml chilled methanol and then with 1 ml chilled 80% acetone. During each washing step, the pellet was mixed well with respective washing media by using vortex mixture and centrifuged at 10,000 rpm for 25 minutes at 4°C. After complete drying of the white pellet, it was dissolved in Laemmli buffer (pH 6.8) and was used for estimation of protein and also loaded in to SDS-PAGE gels (12%) after proper mixing of bromophenol blue (as a tracking dye) and glycerol (10%). The purified samples were heated for 15 minutes in a boiling
water bath, to denature the protein. Protein concentrations in the various extracts were measured according to Bradford (1976). Bovine serum albumin was used as a standard and expressed as mg protein g\(^{-1}\) FW of the cotyledon or axis.

**Soluble Protein**

Soluble Protein was estimated by following the method of Bradford (1976). This method relies on the binding of dye Comassie Brilliant Blue G-250 to protein. The more protein is present, the more the dye Comassie blue binds. At low pH, the dye has adsorption maxima at 470 and 650 nm but when bound to protein has the adsorption maxima at 595 nm.

**Dye concentrate**

100 mg Comassie Brilliant Blue G-250 was dissolved in 50 ml of ethyl alcohol and 100 ml of 85% orthophosphoric acid. The final volume was made up to 1000 ml with distilled water. The solution was filtered, and stored in a dark amber bottle in a cool and dry place.

**Working solution**

Soluble protein was estimated by adding 20 µl of protein sample and 80 µl of distilled water to make final volume 100 µl. 2 ml of Bradford dye was added to the solution, it was mixed and incubated for 15 minutes in dark at room temperature. The absorbance was recorded at 595 nm by spectrophotometer, and values were expressed as µg protein g\(^{-1}\) FW.
**SDS-PAGE**

**Gel preparation**

Stock Solutions:

(a) Acrylamide - Bis Acrylamide (30%)

- Acrylamide: 29.2 g
- Bisacrylamide: 0.8 g

Final volume was made up to 100 ml with distilled water. The solution was filtered through Whatman No.1 filter paper in dark at low temperature and stored in cool and dark place in amber bottle.

(b) Resolving Gel Buffer (1.5 M Tris-HCl, pH - 8.8)

- Tris – base: 36.4 g
- Distilled water: 80 ml

Adjusted the pH to 8.8 and made the final volume to 100 ml with distilled water.

(c) Stacking Gel Buffer (0.5 M Tris HCl, pH - 6.8)

- Tris – base: 6 g
- Distilled water: 80 ml

Adjusted the pH to 6.8 and made the final volume to 100 ml with distilled water.

(d) Sodium Dodecyl Sulphate (10%)

- SDS: 10 g

It was dissolved in 100 ml of distilled water.

**Procedure of SDS-PAGE**

Electrophoresis was carried out using vertical gel electrophoresis unit. A monomer solution for the 12.5% resolving gel and 3.9% stacking gel was prepared by combining all reagents including 10% SDS, APS and TEMED added, before pouring. Polymerization was achieved in 30 - 45 minutes. Electrophoresis apparatus was assembled and filled with
electrode buffer (0.025 M Tris, 0.192 M Glycine, 0.1% SDS). Purified protein sample (50 µg) was loaded into each well. Electrophoresis was carried out at 4°C temperature at a constant current of 20 mA. The run was continued till the tracking dye reached above the bottom edge of the gel.

**Composition of Gel**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Stock Solution</th>
<th>Resolving Gel (7%)</th>
<th>Stacking Gel (3.9%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Acrylamide-Bis Acryl amide (30%)</td>
<td>2.5 ml</td>
<td>0.36 ml</td>
</tr>
<tr>
<td>2.</td>
<td>Stacking Gel Buffer</td>
<td>1.5 M (pH-6.8)</td>
<td>0.69 ml</td>
</tr>
<tr>
<td>3.</td>
<td>Resolving Gel Buffer</td>
<td>3.0 M (pH-8.8)</td>
<td>0.75 ml</td>
</tr>
<tr>
<td>4.</td>
<td>1.5% APS</td>
<td>270 µl</td>
<td>248 µl</td>
</tr>
<tr>
<td>5.</td>
<td>TEMED</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>6.</td>
<td>Water</td>
<td>2.45 ml</td>
<td>1.68 ml</td>
</tr>
<tr>
<td>7.</td>
<td>10% SDS</td>
<td>60 µl</td>
<td>27.5 µl</td>
</tr>
<tr>
<td></td>
<td><strong>Total Volume</strong></td>
<td><strong>6.0 ml</strong></td>
<td><strong>3.5 ml</strong></td>
</tr>
</tbody>
</table>

**Staining of the Gel**

Staining of protein was done by using Commassie Stain (250 mg of Commassie Brilliant Blue R-250 in Methanol; Distilled water; Glacial Acetic Acid (45:45:1)). The gel was soaked in staining solution for 6-8 hours for staining.

**De-staining of the Gel**
Gel were destained by immersing the gel in De-Staining solution (Methanol; Distilled water; Glacial Acetic Acid (45:45:1)) for about 6-8 hours till bands were distinctly visible.

**Carbonylated Protein**

Protein was extracted by the method of Wang *et al.* (2004). The protein extracts were used for the measurement of protein oxidation by the method of Levine *et al.* (1994). The 0.5 ml protein extracts were incubated with 0.03% (v/v) Triton X-100 and 1% (w/v) Streptomycin sulphate for 20 minutes at room temperature, to remove the nucleic acids. After centrifugation at 10,000 rpm, clear supernatant 200 µl was mixed with 300 µl of 10 mM DNPH prepared in 2 M HCl.

The blank was incubated only with 2 M HCl. After the passage of 1 hour incubation at room temperature, proteins were precipitated with 10% (w/v) trichloroacetic acid and the pellets were washed three times with 500 µl of ethanol: ethyl acetate (1:1) mixture. Then, the pellets were finally dissolved in 6 M Guanidine hydrochloride prepared in potassium phosphate buffer (20 mM, pH 2.3) and the absorption was measured at 370 nm using a spectrophotometer. The protein carbonyl content was calculated using a molar absorption coefficient 22000 min⁻¹ cm⁻¹ and expressed as mM µg⁻¹ protein.
Results

Total Protein – Slow Drying

Figure 5.1 Decline in the total protein content in the embryonic axis and cotyledon of Syzygium cumini seeds with loss in water content during slow drying. Correlation between total protein content and loss of water content of dehydrating seeds was $r = 0.91$ (axis) and $r = 0.97$ (cotyledon), while the correlation between total protein content and days of slow drying was $r = -0.93$ (axis) and $r = -0.98$ (cotyledon). Data are mean of 4 replicates ± SD, where no bars are shown, the spread of ± SD is less than the size of the symbol.

Amount of total protein, calculated on fresh weight basis, was similar in the axis and cotyledon of dehydrating jamun seeds monitored throughout the period of the analysis. The total protein was initially high in the axis (2.84 mg protein g$^{-1}$ FW) and cotyledon (2.70 mg protein g$^{-1}$ FW) of fresh undesiccated seeds. Levels of total protein declined gradually and equally to 0.24 mg protein g$^{-1}$ FW in the axis and 0.19 mg protein g$^{-1}$ FW in the cotyledon when desiccated to 0.23 g H$_2$O g$^{-1}$ DM. A positive correlation was obtained between loss of water content of dehydrating seeds and total protein estimated in axis ($r = 0.91$) and cotyledon ($r = 0.97$), and a negative correlation was established between total protein in the axis ($r = -0.93$) and cotyledon ($r = -0.98$) with the days of slow drying.
Total Protein – Rapid Drying

![Graph showing decline in total protein content with water content during rapid drying.](image)

**Figure 5.2** Decline in the total protein content in the embryonic axis and cotyledon of *Syzygium cuminii* seeds with loss in water content during rapid drying (by silica gel). Correlation between total protein content and water content of dehydrating seeds was $r = 0.96$ (axis) and $r = 0.95$ (cotyledon), while the correlation between total protein content and days of rapid drying was $r = -0.98$ (axis) and $r = -0.97$ (cotyledon). Data are mean of 4 replicates ± SD, where no bars are shown, the spread of ± SD is less than the size of the symbol.

The estimated total protein content and pattern in axis and cotyledon of rapid-dried seed was almost similar at all stages of analysis. The axis and cotyledon of fresh and 100% viable seeds recorded highest total protein content whereas it was reduced with rapid desiccation of seeds. As a result, lowest amount of total protein were observed in the axis and cotyledon of non-viable seeds containing 0.12 g H$_2$O g$^{-1}$ DM water content. Total protein detected in the fresh seeds was 2.84 in the axis and 2.70 mg protein g$^{-1}$ FW in the cotyledon which was reduced respectively to 0.42 and 0.54 mg protein g$^{-1}$ FW in seeds rapidly dried to 0.12 g H$_2$O g$^{-1}$ DM. A positive correlation was established between total protein in the axis ($r = 0.96$) and cotyledon ($r = 0.95$) with the decline in water content and a negative correlation was established between total protein in the axis ($r = -0.98$) and cotyledon ($r = -0.97$) with the days of rapid drying.
Soluble Protein – Slow Drying

Figure 5.3 Decline in the soluble protein content in the embryonic axis and cotyledon of Syzygium cumini seeds with loss in water content during slow drying. Correlation between soluble protein content and loss of water content of dehydrating seeds was $r = 0.97$ in the axis and cotyledon both, while the correlation between soluble protein content and days of slow drying was $r = -0.98$ (axis) and $r = -0.99$ (cotyledon). Data are mean of 4 replicates $\pm$ SD, where no bars are shown, the spread of $\pm$ SD is less than the size of the symbol.

Slow drying induced loss of seed water content in jamun seeds resulted in concomitant rapid loss of soluble protein content in axis and cotyledon, both. The amount of soluble protein was slightly higher in cotyledon (560.46 µg protein g$^{-1}$ FW) than in the axis (490.36 µg protein g$^{-1}$ FW) of freshly harvested seeds. Although the soluble protein content declined both in the axis and cotyledon, it was far more rapid in cotyledon compared to axis. For example, dehydration of seeds from 0.93 to 0.23 g H$_2$O g$^{-1}$ DM exhibited almost 28-fold loss of soluble protein in cotyledon i.e. from 560.46 to 20.47 µg protein g$^{-1}$ FW than nearly 10-fold in the axis i.e. 490.36 to 50.73 µg protein g$^{-1}$ FW. Rapid loss of seed water content during slow drying showed strong positive correlation with the loss of soluble protein content in axis ($r = 0.97$) and cotyledon ($r = 0.97$), and a negative correlation was established between soluble protein in the axis ($r = -0.98$) and cotyledon ($r = -0.99$) with the days of slow drying.
Soluble Protein – Rapid Drying

**Figure 5.4** Decline in the soluble protein content in the embryonic axis and cotyledon of *Syzygium cuminii* seeds with loss in water content during rapid drying. Correlation between soluble protein content and water content of dehydrating seeds was $r = 0.94$ (axis) and $r = 0.96$ (cotyledon), while the correlation between soluble protein content and days of rapid drying was $r = -0.98$ in both axis and cotyledon. Data are mean of 4 replicates ± SD, where no bars are shown, the spread of ± SD is less than the size of the symbol.

The pattern of soluble protein estimated in the axis and cotyledon of rapid-dried seed was similar to total protein although the loss was gradual. The amount of soluble protein was substantially higher in the cotyledon than the axis. The axis and cotyledon of fresh seeds observed respectively 490.36 and 560.46 µg protein g$^{-1}$ FW, soluble protein. The soluble protein was reduced to 110.24 µg protein g$^{-1}$ FW in the axis and 90.08 µg protein g$^{-1}$ FW in the cotyledon in seeds rapidly dried to 0.12 g H$_2$O g$^{-1}$ DM. Correlation calculated between loss of water content and amounts of soluble protein in axis ($r = 0.94$) and cotyledon ($r = 0.96$) was strongly positive, while a negative correlation developed between days of rapid drying and amounts of soluble protein in axis ($r = -0.98$) and cotyledon ($r = -0.98$)
**SDS PAGE**

**Storage Protein- Axis**

**Slow Drying**

*Fig.5.5a* SDS PAGE generated electrophoresis banding pattern of storage proteins in the embryonic axis of *Syzygium cumini* seeds with loss in water content during slow drying.

*Fig.5.5b* Decline in number and intensity of polypeptide bands of storage proteins in the embryonic axis of *Syzygium cumini* seeds with loss in water content during slow drying.
**Fig. 5.5c** Decline in the intensity of polypeptide bands of storage proteins in the embryonic axis of *Syzygium cuminii* seeds with loss in water content during slow drying.

**Fig. 5.5d** Changes in the intensity of polypeptide bands of storage proteins in the embryonic axis of *Syzygium cuminii* seeds at different distances during slow drying.
Fig. 5.6a SDS PAGE generated electrophoresis banding pattern of storage proteins in the cotyledon of *Syzygium cuminii* seeds with loss in water content during slow drying.

Fig. 5.6b Decline in number and intensity of polypeptide bands of storage proteins in the cotyledon of *Syzygium cuminii* seeds with loss in water content during slow drying.
**Fig. 5.6c** Decline in the intensity of polypeptide bands of storage proteins in the cotyledon of *Syzygium cuminii* seeds with loss in water content during slow drying.

**Fig. 5.6d** Changes in the intensity of polypeptide bands of storage proteins in the cotyledon of *Syzygium cuminii* seeds at different distances during slow drying.
Fig. 5.7a SDS PAGE generated electrophoresis banding pattern of storage proteins in the embryonic axis of *Syzygium cuminii* seeds with loss in water content during rapid drying (by silica gel).

Fig. 5.7b Decline in number and intensity of polypeptide bands of storage proteins in the embryonic axis of *Syzygium cuminii* seeds with loss in water content during rapid drying (by silica gel).
**Fig. 5.7c** Decline in the intensity of polypeptide bands of storage proteins in the embryonic axis of *Syzygium cumini* seeds with loss in water content during rapid drying (by silica gel).

**Fig. 5.7d** Changes in the intensity of polypeptide bands of storage proteins in the embryonic axis of *Syzygium cumini* seeds at different distances during rapid drying (by silica gel).
Fig. 5.8a SDS PAGE generated electrophoresis banding pattern of storage proteins in the cotyledon of *Syzygium cumini* seeds with loss in water content during rapid drying (by silica gel).

Fig. 5.8b Decline in number and intensity of polypeptide bands of storage proteins in the cotyledon of *Syzygium cumini* seeds with loss in water content during rapid drying (by silica gel).
Fig. 5.8c Decline in the intensity of polypeptide bands of storage proteins in the cotyledon of *Syzygium cuminii* seeds during rapid drying (by silica gel).

Fig. 5.8d Changes in the intensity of polypeptide bands of storage proteins in the cotyledon of *Syzygium cuminii* seeds at different distances during rapid drying (by silica gel).
SDS-PAGE

The number and intensity of protein bands decreased gradually in the axis and cotyledon of seeds desiccated from 0.93 to 0.23 g H₂O g⁻¹ DM. Total 7 and 9 protein bands were detected respectively in the axis and cotyledon of undesiccated jamun seeds. In axis, band 2 (≈14 kDa) and 3 (≈20 kDa) were present constantly throughout the analysis in seeds exposed to slow drying. Similarly band 4 and 8 were present, respectively, in the axis and cotyledon of all the viable seeds dried from 0.93 to 0.26 g H₂O g⁻¹ DM but remained absent in non-viable seeds (0.23 g H₂O g⁻¹ DM). The polypeptide 5 and 6 appeared in the axis of seeds with water content 0.93 and 0.86 g H₂O g⁻¹ DM during slow drying. Band 7 disappeared in the axis slow dehydrated after 0.34 g H₂O g⁻¹ DM. Band 2 and 3 in axis and Band 3, 4, 6, 7 and 9 in cotyledons were present continuously at all the stages of analysis, during rapid drying. The band 1 in the axis and band 5 and 6 in the cotyledons appeared only in undesiccated and desiccated seeds with water content 0.86 g H₂O g⁻¹ DM.

Slow desiccation of the seeds showed no change in the banding pattern initially up to 3 DoS of storage but further drying for 14 DoS resulted in the disappearance of low molecular weight band 1 (≈6.5 kDa). Band 1 and 5 (≈29 kDa) remained disappeared throughout the period of analysis in the cotyledon of the seeds dehydrated from 20 to 30 DoS. Similarly band 9 (≈116 kDa) disappeared from 27 DoS whereas band 8 (≈97 kDa) and 6 (≈45 kDa) were absent in cotyledon of non-viable seeds. Band 2 (≈14 kDa), 3 (≈20 kDa) and 4 (≈24 kDa) were continuously present throughout the period of analysis. Intensity of all the bands was highest in the cotyledons of the seed with high water content i.e. 0.93 and 0.86 g H₂O g⁻¹ DM but declined gradually with the progress of exposure period of drying. In cotyledons, the positive effect of rapid
drying on the banding pattern of protein was clearly visible by recording higher number and intensity of bands even in seeds dried to low water contents. Bands 3, 4, 6, 7 and 9 were present at all the stages of dehydrating seeds. Even band 1, 2, 5 was present in the cotyledon of seeds dried to 0.4 g H_2O g^{-1} DM.
Carbonylated Protein – Slow Drying

Figure 5.9 Decline in the carbonylated protein content in the embryonic axis and cotyledon of Syzygium cumini seeds with loss in water content during slow drying. Correlation between carbonylated protein content and water content of dehydrating seed was $r = -0.95$ (axis) and $r = -0.93$ (cotyledon) whereas correlation between carbonylated protein content and days of slow drying was $r = 0.98$ in the axis and cotyledon both. Data are mean of 4 replicates ± SD, where no bars are shown, the spread of ± SD is less than the size of the symbol.

Carbonylated protein, a product of protein oxidation, was promoted in the axis and cotyledon of dehydrating seeds. Higher content of carbonyl protein was discernible in the axis than the cotyledon. The accumulation of carbonyl protein due to protein oxidation was altered when the undesiccated seeds were dehydrated from 0.93 g H$_2$O g$^{-1}$ DM. Dehydration from 0.86 to 0.23 g H$_2$O g$^{-1}$ DM resulted in promotion of carbonylated protein from 510.48 to 970.08 mM µg$^{-1}$ protein. Similarly, the carbonylated protein was increased from 50.60 to 550.36 mM µg$^{-1}$ protein in the cotyledon of seeds dried from 0.93 to 0.23 g H$_2$O g$^{-1}$ DM. A negative correlation was established between carbonyl protein and water content in axis ($r = -0.95$) and cotyledon ($r = -0.93$), and a positive correlation was established between carbonylated protein in the axis ($r = 0.98$) and cotyledon ($r = 0.98$) with the days of slow drying.
Carbonylated Protein – Rapid Drying

Figure 5.10 Decline in the carbonylated protein content in the embryonic axis and cotyledon of Syzygium cumini seeds with loss in water content during rapid drying. Correlation between carbonylated protein content and water content of dehydrating seed was $r = -0.97$ (axis) and $r = -0.95$ (cotyledon), whereas correlation between carbonylated protein content and days of rapid drying was $r = 0.97$ in both axis and cotyledon. Data are mean of 4 replicates ± SD, where no bars are shown, the spread of ± SD is less than the sizes.

Unlike the total and soluble protein content the carbonylated protein, a modified protein due to oxidation, was minimum in the axis and cotyledon of fresh undried jamun seeds. The levels of carbonylated protein were promoted both in the axis and cotyledon of seeds subjected to rapid dehydration. Comparatively the amounts of carbonylated protein was significantly higher (more than 5-fold) in the axis than the cotyledon throughout the analysis. For example the carbonylated protein in the axis of fresh seeds observed 400.31 mM µg⁻¹ protein whereas it was only 50.60 mM µg⁻¹ protein in the cotyledon. Dehydration of seeds from 0.93 to 0.12 g H₂O g⁻¹ DM resulted in the promotion of carbonylated protein from 400.31 to 780.09 mM µg⁻¹ protein in the axis and 50.60 to 350.88 mM µg⁻¹ protein in the cotyledon respectively. Although the amounts of carbonylated protein was substantially high in the axis the rate of increase of carbonylated protein was relatively higher in the dehydrating cotyledon (7-fold) than the axis (2-fold) of seeds dehydrated from 0.93 to
0.12 g H₂O g⁻¹ DM. A negative correlation was established between carbonylated protein in axis (r = -0.97) and cotyledon (r = -0.95) with the decline in water content and a positive correlation was developed between carbonylated protein in axis (r = 0.97) and cotyledon (r = 0.97) with the days of rapid drying.

**Discussion**

The level of total protein decreased gradually in the cotyledon and axis of jamun seed with the advance in drying or storage period although the rate of decline varied with the seed tissue and drying rate. Higher rates of protein loss during ageing may be due to, 1-high protease activity as reported in dehydrating and ageing *Abelmoschus esculentus* (Gill et al., 1981), *Cajanus cajan* (Kalpana and Madhava Rao, 1997), *Shorea robusta* (Chaitanya et al., 2000a), *Raphanus sativus* (Jain et al., 2006) seeds or, 2-modification or oxidation of protein (Job et al., 2005) or 3- combination of both. The total protein estimated in the cotyledon and axis of jamun seeds observed relatively faster loss in the cotyledon (14-fold) than the axis (11-fold) during slow drying. The rapid drying of seeds effectively reduced the rate of loss of total protein compared to slow drying in both the tissues; cotyledon and axis. The amount of total protein was reduced from 2.7 to 0.54 mg protein g⁻¹ FW and 2.84 to 0.42 mg protein g⁻¹ FW, respectively, in cotyledon and axis when the seeds were exposed to rapid drying from 0.93 to 0.12 g H₂O g⁻¹ DM.

The pattern of soluble protein detected in the cotyledon and axis was similar to total protein although significant variation in the rate of loss of soluble protein was discernible especially during slow drying in cotyledon and axis. Cotyledon of the fresh undesiccated seeds (0.93 g H₂O g⁻¹ DM) observed very high amounts of soluble protein (560.46 µg
protein g\(^{-1}\) FW) that was reduced to nearly 27-folds (20.47 \(\mu g\) protein g\(^{-1}\) FW) in the seeds desiccated to 0.23 g H\(_2\)O g\(^{-1}\) DM. Whereas soluble protein declined by 9-fold during this drying period (0.93 to 0.23 g H\(_2\)O g\(^{-1}\) DM) in the axis of slow dried seeds. Throughout the analysis, the rapid drying reduced similar amounts of soluble protein both in the cotyledon and the axis. During rapid drying the soluble protein was reduced from 560.46 to 90.08 \(\mu g\) protein g\(^{-1}\) FW and 490.36 to 110.24 \(\mu g\) protein g\(^{-1}\) FW respectively in cotyledon and axis of seeds desiccated from 0.93 to 0.12 g H\(_2\)O g\(^{-1}\) DM. Our data is in agreement with the findings of Connor and Sowa (2003) showing loss of viability and vigour in recalcitrant acorns of *Quercus alba* as a result of gradual loss of protein in response to dehydration during storage. The differential loss of protein in the cotyledon and axis of dehydrating jamun seeds may be related to higher rates of lipid peroxidation associated deterioration (Refer chapter 2) in the cotyledon than the axis. The products of lipid peroxidation are capable of interacting with the proteins and modifying them by incorporating the carbonyl groups thus are responsible for damaging proteins (Murthy and Sun, 2000; Refsgaard *et al.*, 2000; Liu and Wang, 2005). Thus, it is concluded that dehydration; slow or rapid, of jamun seeds during storage resulted in net decline in soluble and total protein content and is responsible for reduced seed viability and vigour. Significant decline in protein content was linked with seed deterioration in *Citrus reticulata*, *Phaseolus aureus* and *Pisum sativum* (Samshery and Banerjee, 1979), *Oryza sativa* (Prabhakar and Mukherjee, 1980) and *Shorea robusta* (Nautiyal and Purohit, 1985; Chaitanya *et al.*, 2000a). Seeds with lower protein content have been shown to exhibit reduced vigour (Byrd and Delouche, 1971; Jain *et al.*, 2006) and reduced desiccation tolerance (Stewart and Bewley, 1981). Substantial decline in the levels of proteins in dry seeds of *Shorea robusta* (Nautiyal and Purohit, 1985; Chaitanya *et al*.
al., 2000a), *Arabidopsis thaliana* (Job et al., 2005; Rajjou and Debeaujon, 2008; Rajjou et al., 2008), *Zea mays* (Xin et al., 2011), mediated loss of seed vigour (Bailly et al., 2008).

The SDS-PAGE analysis conducted in the axis and cotyledon of drying jamun seeds; slow and rapid, was in conformity to quantitative changes in the protein; soluble and total. Several polypeptides disappeared as the jamun seeds were dehydrated below CWC. For example, polypeptides 5 and 6 were absent in the axis of seeds slow dehydrated below CWC. Similarly, the fastest moving and smallest molecular weight protein band 1 was absent in the axis of seeds rapid dried below CWC. Several polypeptides, such as 4, 5, 6 and 7 during rapid drying and 1, 4 and 7 during slow drying, disappeared in the axis of non-viable jamun seeds. Seeds undergoing ageing exhibit enhanced activities of protease that, in turn, are responsible for disappearance of several enzymic proteins involved in the maintenance of germinability (Chaitanya et al., 2000a; Xin et al., 2011). For example, activity of protein L-isoaspartyl methyltransferase (PIMT), an enzyme catalyzing the repair of damaged L-isoaspartyl residues accumulated in proteins during ageing in response to oxidative stress, is linked with longevity (Ingrosso et al., 2002; Clarke, 2003; Xu et al., 2004; Oge et al., 2008). In contrast, polypeptide 2 and 3 registered their presence in the axis of all stages including non-viable seeds during slow and rapid drying. Like axis, the number of polypeptides decreased gradually in cotyledons of the seeds subjected to slow and rapid drying. The polypeptides 2, 3, 4, 7 and 3, 4, 6, 7 and 9 were continuously present, although with reducing intensity in the cotyledon of slow and rapid dried seeds. The polypeptides disappeared fast in the slow dried seeds than the rapid dried. For example, protein band 5 disappeared in cotyledon of rapid dried seeds with germinability of 41% whereas, in the cotyledons of slow dried seeds,
bands 5 and 9 disappeared as the germinability was reduced, respectively, to 46 and 20%. Invariably, the intensity of all the polypeptides observed in the cotyledons and axis of jamun seeds exposed to slow and rapid drying declined gradually with the advance in dehydration. Relatively higher number of polypeptides registered their presence in the axis and cotyledons of rapid dried seeds than the slow dried. Massive loss of several polypeptides and the intensity of almost all the polypeptides in aged seeds (both during slow and rapid drying) may be due to reduced protein biosynthesis (Peumans and Carlier, 1981; Gidrol et al., 1990) or increased rates of protein oxidation (McDonough et al., 2004; Rajjou et al., 2008) was in conformity with the loss of antioxidant enzymes activity reported in the previous chapter. Controlled deterioration of pea seeds leads to massive changes in the protein including several proteins of antioxidant enzymes (due to reduced transcription of respective genes) (Yao et al., 2012).

Massive increase in carbonylated protein, an irreversible measurable product of protein oxidation as a result of seed deterioration, was observed in seeds exposed to unfavourable storage conditions (Job et al., 2005; Rajjou et al., 2008). Irrespective of axis or cotyledon, in desiccating jamun seeds, the levels of carbonylated protein were promoted. Our results corroborate the findings of Terskikh et al., (2008) who have detected excessive amounts of oxidized proteins during seed deterioration of western red cedar (Thuja plicata). A substantially higher amount of carbonylated protein was discernible in all the desiccating stages of the axis than the cotyledon. The slow drying promoted levels of carbonylated protein from 50.6 to 550.36 mM µg⁻¹ protein-carbonyl in cotyledon and 400.31 to 970.8 mM µg⁻¹ protein-carbonyl in axis of jamun seeds slow-dried from 0.93 to 0.23 g H₂O g⁻¹ DM. Comparatively less amounts of carbonylated protein were promoted in the cotyledon and
axis of seeds exposed to rapid drying. The cotyledon and axis registered net increase, respectively, of 300.28 and 379.78 mM µg⁻¹ protein carbonyl. Significantly low amounts of protein oxidation, as indicated by recording carbonylated protein, registered in the axis than in the cotyledon indicated differential responses of these tissues to drying. Net loss of regulatory and enzymatic proteins during protein oxidation in accelerated aged seeds is responsible for reduced germination percent and seedling growth (Job et al., 2005; Rajjou et al., 2008). It appears that very high rates of accumulation of carbonylated protein in response to slow dehydration in cotyledon is responsible for dehydration induced loss of viability as the accumulation was significantly dropped in the cotyledon of the seed exposed to rapid drying showing comparatively higher germinability.

Data analysis of soluble, total and carbonylated protein in the axis and cotyledon of jamun seeds revealed dehydration induced decline in soluble and total protein with corresponding increase in carbonylated protein. The rate of drying irrespective of storage period influenced the rapidity of the loss of protein; soluble and total and promotion of carbonyl protein contents. The reduced levels of proteins were further substantiated by examining the number and intensity of the polypeptides in the axis and cotyledon of jamun seeds. Additionally, as a result of dehydration the rapid activation of protein oxidation caused loss of targeted proteins that are involved in the seed germination or longevity. Strong correlation of loss of protein and simultaneous increment in the levels of carbonylated protein in dehydrating jamun seeds can be used as one of the reliable tools to assess losses in viability and vigour of the seeds during storage.