Isozymes of antioxidative enzymes during ripening and storage of ber (*Ziziphus mauritiana* Lamk.)

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Abstract Isozyme profile of antioxidative enzymes viz. superoxide dismutase (SOD), peroxidase (POX), catalase (CAT) and ascorbate peroxidase (APX) was studied during ripening and storage of two cultivars of ber fruit (*Ziziphus mauritiana* Lamk.) differing in their shelf-lives viz. Umran (shelf-life, 8–9 d) and Kaithali (shelf-life, 4–5 d). The profile revealed that Umran variety exhibited three bands each of SOD and POX while in Kaithali, these enzymes had two isoenzymes throughout ripening. CAT and APX, however, showed two isozymes each during ripening of both the varieties and the pattern remained the same at all the stages of ripening except at the initial stage i.e. immature green stage where single CAT isozyme was visible. During storage, one extra band each of SOD and POX present only in Umran got disappeared at later stages of storage, whereas in Kaithali, the pattern remained unchanged. Also, there was no change in the pattern of CAT and APX isozymes during storage of both the varieties. One isozyme of CAT could be considered as ripening related while one isozyme each

of SOD and POX could be related to higher shelf life of fruits.

Keywords *Ziziphus mauritiana* Lamk. · Antioxidant isozymes · Lyophilization · Ripening · Storage

Introduction

Fruits, being a rich source of essential nutrients, are generally important in our diet for their high content of fibers, vitamins and minerals. However, they contain a very high percentage of their fresh weight as water and consequently exhibit relatively high metabolic activity which continues post harvest and makes most fruits highly perishable commodities. It is this perishability and inherent short shelf-life that poses greatest problem for their successful transportation and marketing. Efforts have been made to increase the shelf-life of fruits using antisense RNA technology to inactivate ethylene biosynthetic (Gómez et al. 2009; Johnston et al. 2009) and cell wall degrading (Jimenez-Bermudez et al. 2002) enzymes. In spite of considerable efforts and huge resource commitments world over, enhancement of fruit shelf-life has not met with more than limited success. If a big breakthrough in this field has to be achieved, the problem has to be handled from different angles and, therefore, there is a continuous search for alternative targets. One of the important areas in this direction appears to be that of scavenging of reactive oxygen species (ROS) such as O_2^- (superoxide anion), H_2O_2 (hydrogen peroxide), $\text{OH}^\cdot$ (hydroxyl radical) and O_2 (singlet oxygen) which are essentially generated in

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normal metabolism as byproducts of inevitable leakage of electrons on molecular oxygen during the electron transport activities in chloroplast, mitochondria and plasma membrane (Foyer 1997; Kumar and Malhotra 2008; Kumar et al. 2010a, b). Accumulation of ROS is linked to deteriorative changes such as DNA mutation, protein denaturation, enzyme inactivation and lipid peroxidation (Regoli and Winston 1999). Accumulation and overproduction of ROS is generally high during various stress conditions. Senescence in green plants is accompanied by increased contents of malondialdehyde (Ye et al. 2000), higher production of ROS (Kumar et al. 2010a) and gradual loss in the ability of scavenging enzymes to neutralize the free radicals (Kanazawa et al. 2000). Fruit ripening is a stressful process and the overall process of fruit ripening is considered as functionally modified protracted from of senescence, the mechanisms operating during senescence might also operate during ripening and thus help in decreasing/affecting shelf life of fruits (Mondal et al. 2004). Plants, however, possess an impressive array of enzymatic and non-enzymatic defense mechanisms against oxidative stress. The anti-oxidant enzymes include superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), while the non-enzymatic antioxidants include water soluble (ascorbate, glutathione, phenolic compounds and flavonoids) and lipid soluble (α -tocopherol, β -carotene, lycopene) metabolites. In spite of such an efficient defense system, oxidative stress may still occur due to over accumulation of ROS under various stress conditions including, fruit ripening and storage (Mondal et al. 2003, 2004, 2006, 2009) because of gradual loss in the ability of ROS scavenging enzymes to neutralize the free radicals leading to over accumulation of ROS. Studies carried out in our laboratory have revealed that tomato (Mondal et al. 2004), guava (Mondal et al. 2009) and ber (Kumar and Malhotra 2008; Kumar et al. 2009; Kumar et al. 2010a, b) fruit cultivars with higher shelf-life exhibit higher activities of ROS scavenging enzymes and so experience less oxidative stress. These studies further revealed that efficient antioxidative system can protect the fruit from the deleterious effect of ROS only during initial stages of storage and not until later where over accumulation of ROS occurs either because of their over production or because of the inability of the scavenging system. To ascertain the previous observations, the present investigations were conducted with a view to find out any ripening related isoenzyme which could be further manipulated by modern molecular tools. Isoenzymic profile of these enzymes has been studied during storage of two cultivars of ber (*Ziziphus mauritiana* Lamk.) fruit differing in their shelf-lives viz. Umran (shelf-life 8–9 d) and Kaithali (shelf-life 4–5 d) and

efforts have also been made to correlate the number of isoenzymes with the shelf-life of fruits.

Materials and methods

Plant material and chemicals Ber (*Ziziphus mauritiana* Lamk.) fruits of two varieties differing in their shelf-lives viz. Umran and Kaithali were harvested from ten year old trees grown at Horticulture farm, CCSHAU, Hisar. Based on visual observations, fruits were categorized as immature green (IG), mature green (MG), colour turning (CT), ripe (R) and overripe (OR). For studies during storage, fruits harvested at mature green (MG) stage were stored at room temperature and analyzed at two d interval until complete decay. All chemicals used during present investigations were of analytical grade and obtained from HiMedia Pvt. Ltd., Mumbai (KH_2PO_4 , K_2HPO_4); E-Merck (benzidine-HCl); Sisco Research Laboratories Pvt. Ltd., Bombay (TCA, nitroblue tetrazolium, riboflavin).

Extraction Preliminary experiments were conducted to optimize the extraction conditions with respect to pH, molarity and type of buffer, concentration of stabilizing agent(s) and other constituents of extraction medium. Unless stated otherwise, all extraction procedures were carried out at 0–4°C. The standardized extraction medium for SOD and POX consisted of 0.1 M Tris-HCl buffer (pH 7.5) containing 3% (w/v) polyvinylpyrrolidone, 1 mM EDTA and 1 mM CaCl_2 while for CAT and APX, it consisted of 0.1 M potassium phosphate buffer (pH 7.5) in place of Tris-HCl buffer, the rest extractants were the same. The homogenate was prepared by macerating 1 g tissue with 4 ml of ice cold extraction medium in a pre-chilled pestle and mortar using acid washed sand as abrasive. The homogenate was filtered through four layers of cheese cloth and the filtrate centrifuged at 15,000 g for 20 min in a refrigerated centrifuge at 4°C. The supernatant was carefully decanted and used as crude enzyme extract. Ten ml each of protein samples (extracted in Tris and PO_4^{3-} buffer) was filled in scintillation vials and lyophilized for 96 h at -28°C with vacuum pump temperature of $-78 \pm 2^\circ\text{C}$. The samples lost more than 90% moisture by sublimation and became concentrated enough for isozymic studies. Total soluble protein before and after lyophilization was estimated by the method of Bradford (1976).

Staining of isozymes Isoenzymes of SOD, POX and APX were resolved on 10% while those of CAT on 7% polyacrylamide gel. Electrophoresis was carried out for 4 h using 25 mA current. Gels were stained with specific staining solutions for each enzyme by employing the following methods:

Superoxide dismutase Gel was soaked in 2.45 mM solution of nitroblue tetrazolium for 20 min followed by illumination for 2–4 h in a solution containing 28 mM TEMED, 0.5 μ M riboflavin in 0.036 M potassium phosphate buffer at pH 7.8 (Kumar et al. 2006). During illumination, the gel became uniformly blue except at positions having SOD activity. Illumination was discontinued when maximum contrast between achromatic zones and the general back ground blue colour had achieved. The gel was maintained in distilled water till photographed.

Peroxidase For peroxidase staining, method of Guikema and Sherman (1980) was employed. The gels were stained in solution of 25% acetic acid containing 0.3% benzidine and 0.5% H_2O_2 . Within 2 min, blue coloured bands appeared which turned brown after 10–15 min.

Catalase Gel was stained as per the method of Rucinska et al. (1999). It was rinsed thrice in distilled water for 15 min every time in a refrigerator. Following this, gel was placed in 3 mM H_2O_2 for 5 min, once again rinsed in distilled water and stained in a solution of 1% potassium ferricyanide (w/v) and 1% ferric chloride (w/v) mixed in equal volume. Gel was incubated for 10 min in above solution under airtight and dark conditions to develop background colour. White coloured bands appeared against bluish/greenish background.

Ascorbate peroxidase Isozymes of APX were detected by the procedure described by Mittler and Zilinskas (1993). Electrode buffer containing 2 mM ascorbate was prepared and the gel was run for 30 min before the samples were loaded. The gel was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min. Then, the gel was incubated in a solution composed of 50 mM sodium phosphate buffer (pH 7.0), 4 mM ascorbate and 2 mM H_2O_2 for 20 min. Further, the gel was washed in the buffer for 10 min and finally submerged in a solution of 50 mM sodium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT for 15 min with gentle agitation. White colored achromatic zones against bluish background represented APX activity. The gel was washed with distilled water to remove excess stain and photographed.

Results and discussion

Reduction of relatively un-reactive molecular oxygen requires four electrons during electron transport chain but this reduction is accomplished via supply with a partial one

to three electrons at a time. Thus, all in between partially reduced states of oxygen give rise to active/reactive oxygen species (Blokhina et al. 2003). In general, accumulation of ROS, decreased activity of antioxidants and antioxidative enzymes, increased lipid peroxidation, membrane damage and tissue leakage are the major causes of less shelf-life of fruits (Mondal et al. 2004; Regoli and Winston 1999) during ripening and storage. The results during the present investigation for antioxidative isozymes were as follow:

Isozyme profile during ripening Isoenzymic pattern of SOD (Fig. 1a) indicated that two isoenzymes (SOD1 and SOD2) were common to both the varieties and were present throughout ripening of the two cultivars. However, in Umran—a higher shelf-life variety, one extra band (SOD3) appeared at MG stage which remained active till the later stages of ripening. The appearance of one extra isoform of SOD in Umran during ripening might account for higher SOD activity in this cultivar and for its better shelf-life. Similarly, the isoenzymic profile of POX (Fig. 1b) depicted that Umran had three while Kaithali had two isozymes. However, at R and OR stage, the band unique to Umran disappeared. Presence of one extra band each for SOD and POX in Umran indicated that these isoenzymes might provide tolerance against high temperature encountered during ripening of this variety. Similar to SOD, this extra variant of POX also might be responsible for protecting the fruit against deleterious effects of oxidative stress and thus for higher shelf-life of Umran. Not much information is available on the isoenzymic pattern of antioxidative enzymes during ripening. However, the number of isozymes of SOD has been reported to vary from three to four in maize leaves (Matters and Scandalios 1987), seedlings and needles of norway spruce (Kroniger et al. 1993), olive leaves (Corpas et al. 2006), tomato fruit (Kumar et al. 2006) and watermelon cotyledons (Rodriguez-Serrano et al. 2007). Similarly, Ahn et al. (2002) while studying the activity profile of POX during tomato fruit ripening reported two major isozymes whereas Ullrich et al. (2004) observed a total of 6 isozymes in *Agrocybe aegerita* (a mushroom). Number of isoforms have been reported to be four in roots of 5 month old *Withania somnifera* (AGB002) (Johri et al. 2005) and in the skin of maturing tomato fruit (Andrews et al. 2000). Bernardi et al. (2004), in two poplar clones differing in sensitivity towards ozone exposure, found six POX isozymes in young leaves and seven in mature leaves, indicating expression of one new POX during maturity.

Figure 1c depicted that there was a single band of CAT isozyme at IG stage in both the varieties. However, after that, another variant of CAT got expressed at MG

Fig. 1 Isozyme profile of (a) superoxide dismutase (b) peroxidase (c) catalase and (d) ascorbate peroxidase during various stages of ripening. IG: Immature Green; MG: Mature Green; CT: Colour Turning; R: Ripe; OR: Over ripe

stage which was present throughout ripening. Since this band appeared during ripening and that also in both the

varieties could be suggested to be a ripening induced isozyme. Kumari et al. (2008) reported four while Bailly

Fig. 2 Isozyme profile of (a) superoxide dismutase (b) peroxidase (c) catalase and (d) ascorbate peroxidase during storage

et al. (2004) found two isoforms of CAT during development and desiccation of pea and sunflower seeds, respectively. They also have reported the appearance of one extra isozyme of 55 kDa in response to dehydration. Ali et al. (2005) localized two isoforms in tissue cultured roots of *Panax ginseng* and *Panax quinquefolium* subjected to methyl jasmonate stress whereas Rucinska et al. (1999) observed five in lupin roots exposed to lead. Mittler et al. (2004) have characterized four genes for CAT in *Arabidopsis*. Isoenzyme pattern of APX during ripening (Fig. 1d) showed that there were two isoforms in both the varieties at all the stages of ripening. No new isozyme could be observed during ripening. These results are in agreement to those reported by Ahn et al. (2002) where two major isozymes of APX have been reported to be present in tomato during fruit development. Similarly, Chen and Asada (1989) and Rucinska et al. (1999) found two isozymes of APX in tea leaves and lupin roots, respectively. Bernardi et al. (2004) found young leaves of poplar to have two isozymes while mature leaves had only one. However, Narendra et al. (2006) observed three isozymes of APX in *Arabidopsis* during growth and development.

Isozyme profile during storage Since the fruits harvested at MG stage were stored and the isoenzymic profile was studied at two d interval until deterioration, the pattern obtained at 0 d of storage was the same as observed at MG stage of fruit ripening. Isozyme profile of SOD (Fig. 2a) showed that Umran had three isozymes of SOD from 0 to 6th d of storage; thereafter only two bands could be visualized. It may be either because of the down regulation of that isoform or because of very low activity not to be detected by the staining method. In variety Kaithali, two isoforms of SOD were present throughout storage, though the intensity of bands decreased during storage. Similarly, the variety, Umran exhibited three POX isozymes from 0 to 6th d of storage (Fig. 2b) and thereafter, upto 10th d, there were only two major bands of POX, while Kaithali had two POX isozymes throughout the storage period. The number of SOD and POX isoenzymes observed during the present investigations was less than that observed by Dama et al. (2010) who found 4 to 6 SOD isoforms and 2 to 4 POX isoforms in six different accessions of mushroom stored at 5 and 10°C. The difference observed in SOD and POX isozymes was in terms of intensity and size of bands and apparently no new addition or deletion of isozymes occurred throughout the storage. Laugesen et al. (2007) found three distinct POXs in barley while Veljovic-Jovanovic et al. (2006) found four POXs in senescing leaves of *Ramonda serbica*.

Isozyme profile for CAT (Fig. 2c) during storage indicated that there are two loci in both the varieties and

the pattern remained the same throughout storage period. Kuk et al. (2003) reported two isozymes of CAT which were shown to offer protection from chilling damage in rice plants. Similar to CAT, two loci have been identified for APX (Fig. 2d) in both the varieties and the status remained unchanged throughout the storage period. These results are in agreement with the results reported earlier in sunflower seeds (Bailly et al. 2004; Ali et al. 2005), tea leaves (Chen and Asada 1989) tomato fruit (Ahn et al. 2002), lupin roots (Rucinska et al. 1999), young leaves of poplar (Bernardi et al. 2004) and harvested broccoli (Nishikawa et al. 2003).

Conclusion

From the results presented here, it could be documented that one isoform of CAT which appeared only during ripening at MG stage and remained active throughout ripening of both the cultivars could be considered as ripening related isoform. Modern molecular tools can be employed to manipulate the activity of this isoform so as to extend the ripening process vis-à-vis higher duration for the fruit to remain on the tree to achieve better taste, flavour and aroma. The results further reveal that one isoform each of SOD and POX is present only in Umran and not in Kaithali. This may be regarded as a form responsible for combating the oxidative stress better and so higher shelf-life of the variety. Disappearance of this extra band unique to Umran during storage and decreased intensity of SOD and POX isoforms during storage of Kaithali further confirm our previous observations that antioxidative system can provide protection against oxidative stress only during the initial stages of storage and not until later when oxidative damage may occur due to over accumulation of ROS.

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