SYNOPSIS
Polyphenol oxidase (PPO) enzyme catalyzes the hydroxylation of monophenols to o-diphenols through monophenol oxidase activity and a subsequent oxidation of these o-diphenols to the corresponding o-quinones by a catecholase/diphenolase activity in the presence of its co-substrate oxygen. The structure of the active site of the enzyme contains two copper ions, which is bound by six histidine residues and a single cysteine residue. The enzyme has been reported to be universally distributed in animals, plants, fungi and bacteria. In animals it is responsible for melanosis, which protects skin against UV damage. In plants this enzyme is required for defense against predators like herbivores and insects. However, its role in fungi and bacteria is not conclusive. It is involved in enzymatic browning in plant products, seafood, and melanin formation in
skin. Therefore, it has received significant attention from researchers working in area of food science, plant physiology, and cosmetics. In plant cell, PPO and phenolics were reported to be present in chloroplast and vacuoles, respectively (Mayer and Harel, 1979). During physical cutting, cellular structure gets disrupted leading to release of PPO enzyme and its substrate (phenolics) resulting in their physical contact. In presence of oxygen, the enzymatic reaction produces the quinone which autopolymerises to produce melanin like brown coloured pigments eventually resulting in enzymatic browning. This post-processing enzymatic browning is a major problem for industries dealing with cut fruits and vegetables including brinjal and its inhibition has remained a challenging issue. Many cultivars of brinjal (*Solanum* genus) are available in India with varying morphological features such as colour, shape, and spiny nature (Raigón et al., 2008). These cultivars differ in the extent of post-cut browning which could be due to variations in PPO activity or level of soluble phenolics. Further purification of PPO to homogeneity was also found to be difficult especially in case of brinjal due to high phenolic content and their irreversible binding to PPO during purification process. Due to this, there are a few reports on partial purification and characterization of PPO from brinjal (Roudsaria et al., 1981; Pérez-Gilabert and Carmona, 2000; Doğan et al., 2002; Concepción et al., 2004).

**Objective of the thesis**

The study of PPO activity, phenolics and browning in different brinjal cultivars will help understanding the relative contribution of PPO enzyme and its substrate in overall browning process in cut brinjal. Further the purification and characterization of the PPO enzyme in brinjal could help find suitable methods for controlling its activity. The control of enzymatic browning in fresh cut brinjal is important for postharvest food processing
industry. For a detailed understanding of the browning process and its control it is intended to purify the PPO enzyme to homogeneity and further characterize its kinetic properties. The sequencing of PPO gene from different brinjal cultivars and their comparative analysis will also be performed which could be helpful in revealing its genetic makeup and for establishing a correlation with its enzyme activity.

**Organization of the thesis**

The thesis is organized into six chapters. Chapter 1 provides introduction to the topic and review of the scientific literature available in the area. Chapter 2 describes studies on comparative evaluation of PPO activity, browning, and phenolics in major Indian cultivars. Chapter 3 of the thesis describes a method developed for controlling the enzymatic browning in fresh cut brinjal. Chapter 4 deals with purification of PPO from a brinjal cultivar that showed the maximum activity and its biochemical characterization. Chapter 5 describes the genetic polymorphism of PPO gene in different brinjal cultivars. Chapter 6 provides the summary and conclusion of the work.

**Chapter 1:** The introduction and literature review covered in this chapter explain the problems associated with postharvest handling and storage of different cultivars of brinjal. The relevant information about taxonomy, origin, distribution, agricultural production, biochemical composition along with information about the existence of a large number of cultivars in India is detailed here. The PPO related introduction includes its mechanism of action, methods of assay, and standard approach of its purification from brinjal. The mechanism of action is detailed which involves a catalytic cycle where the active site changes among three forms (met, oxy, and deoxy) for converting phenols to quinone. The different methods of assay, their pros and cons, and the routinely followed
spectrophotometric assay using 4-methyl catechol are discussed. The reported literature about purification and characterization of PPO from fruits and vegetables and difficulties associated also included. The enzymatic browning is known to be the major problem and inhibition of PPO has remained a major challenge. It also describes the general methods used for inhibition of PPO and related browning in cut fruits and vegetables. The methods include both physical such as heat treatment, refrigeration, irradiation, high pressure treatment, and ultrafiltration and chemical methods used are based on application of antioxidants, acidulants, chelators, complexing agents (Marshall et al., 2000). The enzymatic and molecular approaches of PPO inhibition include use of proteases and antisense RNA technology. Many industrially used inhibitors and newly found natural compounds are also described. The chapter also describes structural features of PPO including its amino acid sequence, its copper binding domain, crystal structures from other sources (Eicken et al., 1999). The relevance of copper atom in its active site is detailed along with the information and physiological role of different types of copper proteins. The genetic features are detailed with the information about existing gene families in other plants, chromosomal location, expression of PPO and its regulation, and cloning and expression of PPO in E.coli. (Thygesen et al., 1995; Sommer et al., 1994).

The posttranslational modification including the literature about transport of PPO precursor protein to chloroplasts, N- and C-terminal processing, and other modifications are detailed including information reported in tomato and few other plants.

PPO or its substrate phenolics or both together was addressed by studying these cultivars of brinjal. The maximum PPO activity and browning was observed in ‘Kalpatharu’ cultivar. The level of phenolics increased in all these brinjal cultivars. When such stored brinjals were cut, browning index was always found to be increased. Thus the findings indicated that in around a week ambient or low temperature stored brinjal, where post harvest physiological changes and gradual senescence leading to homoeostatic imbalance takes place, phenolics concentration and browning index always increased, whereas, PPO activity either increased or reduced depending upon cultivars. Further, browning index was always found to be maximally correlated with phenolics but not with PPO in case of stored samples, indicating the major role of phenolics in post-cut browning of raw stored brinjal. However, in case of fresh raw sample both PPO as well as phenolics were found to be equally important with respect to browning index. The chlorogenic acid estimated in using HPLC showed it as the major phenolic with range of ~50-70% of total phenolics (Luthria et al., 2010). No correlation of chlorogenic acid content was observed with PPO activity and browning in these cultivars.

**Chapter 3:** This chapter deals with inhibition of browning in fresh cut brinjal using a novel but simple approach. This phenomenon of browning is markedly observed in brinjal which immediately turns brown after cutting. The mechanics of cutting and further processing were found to have very profound effect on the browning process. Interestingly, browning was significantly inhibited by cutting using a sharp blade (thickness, 0.04 mm) and immediate dipping in water for 10 min, followed by ambient air drying and packaging. The scanning electron and fluorescence microscopic examinations showed that sharp blade cutting caused lesser physical injury and cellular death, resulting in reduced leaching of phenolics and polyphenol oxidase activity and hence lesser
browning. For commercial acceptability of the technique, storage studies were performed at ambient, 10 and 4 °C, which indicated that fine cut samples could be stored up to 5, 12, and 16 days at these temperatures, respectively, with organoleptically acceptable scores.

**Chapter 4:** This chapter of the thesis deals with purification and characterization of PPO enzyme purified from brinjal. The browning of protein extract was inhibited by addition of PVPP (2%), PVP (1%) and ascorbic acid (30 mM) in the extraction solution. The ammonium sulphate fractionation showed PPO activity in two discrete fractions (20-30% and 50-70%) indicating presence of two isoforms which was confirmed by native PAGE. The 50-70% fraction showing higher specific activity was used for purification. During DEAE ion exchange chromatography column binding took place at pH 8.0 and gradient elution was performed (0-0.5M NaCl) which resulted in 13% yield and ~9 fold purification. During phenyl Sepharose chromatography column binding took place at 18% ammonium sulphate saturation and gradient elution was performed with decreasing concentration of ammonium sulphate (18-0%) which resulted in 2.7% yield and 44 fold purification. The gel filtration chromatography resulted in 0.02% yield and 259 fold purification. The molecular weight determined with gel filtration chromatography and SDS PAGE showed PPO to be a 112 kDa homodimer. The enzyme showed very low Km (0.34 mM) and high catalytic efficiency (3.3 x 10⁶) with 4-methyl catechol. The substrate specificity was of the order of 4-methyl catechol > tert-butylcatechol > dihydrocaffeic acid > pyrocatechol > DOPA > caffeic acid > chlorogenic acid > pyrogallol > 3,4-dihydroxybenzaldehyde > Gallic acid > p-Cresol. The Km for tert-butylcatechol, dihydrocaffeic acid, and pyrocatechol was found to be 0.44, 0.48, and 0.54 mM respectively. Cysteine hydrochloride, potassium metabisulfite, ascorbic acid, erythorbic acid, resorcylic acid and kojic acid showed competitive inhibition, whereas, citric acid...
and sodium azide showed mixed inhibition of PPO activity. The inhibitor constant for these inhibitors was determined and GRAS (Generally Recognized As Safe) compound cysteine hydrochloride was found to be an excellent inhibitor with low inhibitor constant of 1.8 µM.

**Chapter 5:** This chapter deals with comparative evaluation of genetic polymorphism and structural features of PPO gene in brinjal cultivars. The PPO gene from six cultivars were sequenced and submitted to Genbank (Genbank accession numbers JQ621948, JQ621949, JQ621950, GQ149349.1, JQ621951, JQ621952). The PPO nucleotide sequence was found to contain 1773-1788 bp and hence predicted conceptual protein to have 590-595 aa, as there is no intron in this gene. The presence of a 40 amino acid long N-terminal chloroplast targeting sequence and 40 amino acid thylakoid lumen targeting sequence was deduced from bioinformatics analysis. The PPO active site containing two copper binding regions (A and B), each coordinated by three histidine (H) residues also observed. Nucleotide sequence and conceptual protein sequence were found to be significantly conserved in these cultivars. However, two cultivars (‘Kalpatharu’ and ‘Raveena’) which have shown highest post-cut browning were found to have difference in a stretch of 38 amino acid in the region close to 301 to 338 of total ~593 residues with respect to other four cultivars. In four of the stored raw brinjal cultivars level of PPO was found to get reduced. The nucleotide blast search revealed the brinjal PPO showed high similarity with potato (86%) and tomato (84%), which are its closest member in Solanaceae family. Further it showed about 80% similarity with tobacco PPO. The NCBI protein blast results showed about 97% similarity with potato (AAA85122.1), 96% with tomato (AAB22610.2), 98% with tobacco (ABE96885.1), and 97% with sweet potato (AAW78869.1). The conserved domain search showed the presence of domains from
three super families including Tyrosinase; pfam00264 superfamily (CDD 189478), PPO1-DWL superfamily pfam12142 (CDD 192942) and PPO1-KFDV superfamily pfam12143 (CDD192943). The PPO gene from ‘Kalpatharu’ brinjals cultivars was cloned in PET 28a vector and expressed in E.coli (BL-21) under IPTG promoter. The expressed protein was purified and mol. wt. was found to be of 66 kDa which is 10 kDa higher than the size of native PPO observed during standard protein purification. This could be due to presence of 81 amino acid long N-terminal signal peptide present in precursor PPO.

Chapter 6: This chapter of the thesis summarizes the conclusion of the entire thesis work. The brinjal cultivars showed significant difference in PPO activity, phenolics content and browning. In fresh brinjal samples both PPO as well as phenolics were found to be equally important for browning. Browning was always found to be maximally correlated with phenolics but not with PPO in case of stored samples, indicating the major role of phenolics in post-cut browning. The chlorogenic acid was found to be the major phenolic with range of ~50-70% of total phenolics. The change in chlorogenic acid its concentration was independent of PPO activity. Fine blade cutting and water dip inhibited browning in fresh-cut brinjal. Fluorescence and SEM studies showed lesser tissue damage in fine blade-cut brinjal. Shelf life of fine blade-cut, water dipped, and packaged brinjal increased up to 16 days at 4 °C. PPO was significantly (259 fold) purified. Two isoforms of PPO were observed in all the studied cultivars of brinjal. The PPO enzyme showed highest specificity towards 4-methyl catechol with very low Km (0.34 mM) and high catalytic efficiency (3.3 x 10^6). Cysteine hydrochloride was found to be an excellent inhibitor of PPO with low inhibitor constant of 1.8 μM. (competitive inhibition). Nucleotide sequences were found to be significantly conserved. Two cultivars which have shown highest post-cut browning and PPO activity were found to have difference in
amino acid sequence of 38 amino acid stretch in the region close to 301 to 338 of total ~593 residues. PPO gene was also cloned and expressed in E.coli BL-21. The recombinant protein was purified and mol. wt. was found to be of 66 kDa due to presence of signalling peptide sequence of 80 amino acids at N-terminal end. To the best of our knowledge this work describes the novel findings characterizing PPO from brinjal and its possible inhibition which could be an important mean to control post processing browning in many fruits and vegetables including brinjal.

References


**Publications:**

**a. Published**


**Genbank submissions**


Synopsis


b. Communicated:


Signature of Student: 

Date: 09.05.2012

Doctoral Committee:

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