STUDIES ON POLYPHENOL OXIDASE IN BRINJAL (EGGPLANT; *Solanum melongena*)

By

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As members of the Viva Voce Board, we certify that we have read the dissertation prepared by Bibhuti Bhusan Mishra entitled "Studies on polyphenol oxidase in brinjal (eggplant; *Solanum melongena*)"and recommend that it may be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

(Bibhuti Bhusan Mishra)

Dedicated to.....

.... the motivations that shaped

this accomplishment.....

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Synopsis

Homi Bhabha National Institute

Ph. D. PROGRAMME

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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; Concellón 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.

Chapter 2: This chapter details comparative evaluation of PPO enzyme activity, phenolic content, and enzymatic browning in eight different brinjal cultivars namely ‘Pusa purple long’, ‘Ravaiya’, ‘Azad kranti’, ‘Arka navneet’, ‘Kalpatharu’, ‘Raveena’, ‘Anupam’, ‘Silki’. The basic question what regulates the extent of browning: the inherent level of

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 ºC, 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 (3.3×10^6) 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 metabilsulfite, 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 ^{0}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.

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Publications:

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1. Mishra, B. B., Mukhopadhyaya, R. Sharma, A. (2008). *Solanum melongena* polyphenol oxidase mRNA, partial cds. NCBI Accession no. GQ149350.
2. Mishra, B. B. Sharma, A. (2008). *Solanum melongena* polyphenol oxidase gene, complete cds. NCBI Accession no. GQ149349.1.
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7. Mishra, B. B., Gautam, S., Sharma, A. (2012). *Solanum* melongena (CV. Anupam) polyphenol oxidase gene, complete cds NCBI Genbank Accession no. JQ621952

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Date: 09.05.2012

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3	Dr. (Mrs.) M. Krishna	Member		9/5/12
4	Dr. T. P. A. Devasagayam	Member		9-5-12

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Chapter 1

Introduction

1.1. General

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. Enzyme nomenclature differentiates the monophenol oxidase as laccase (EC 1.10.3.2) and the o-diphenol:oxygen oxidoreductase as catechol oxidase (EC 1.10.3.1). However, depending on the source of enzyme it may act on two general types of substrates, monophenols and diphenols, hence in general polyphenol oxidase (Tyrosinase; EC 1.14.18.1) name has still been in use in literature (Mayer, 2006). For example, tyrosinase from mushroom shows both monophenol oxidase and o-diphenol oxidase activity, whereas, the catechol oxidase from most plant sources shows only o-diphenol oxidase activity.

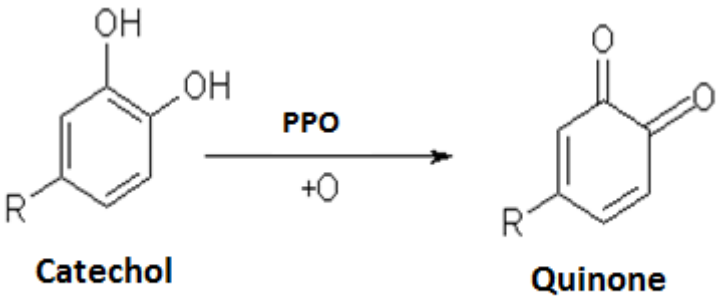


Figure 1.1. Polyphenol oxidase (Catechol oxidase, EC 1.10.3.1) enzyme reaction showing diphenol oxidase activity found mostly in plants (Mayer and Harel, 1979)**.** R in the figure represents any functional group.

The naming of this enzyme has not been consistent and many a times it has been parallely named on the basis of individual substrate upon which it has been found to work such as tyrosinase, phenolase, catechol oxidase, catecholase, o-diphenol oxidase, monophenol

oxidase, and cresolase. The structure of the active site of the enzyme contains two copper ions, which are bound by six histidine residues and a single cysteine residue. The enzyme seems to be of almost universal distribution in animals, plants, fungi and bacteria. In plants this enzyme is required for defense against predators like herbivores and insects.

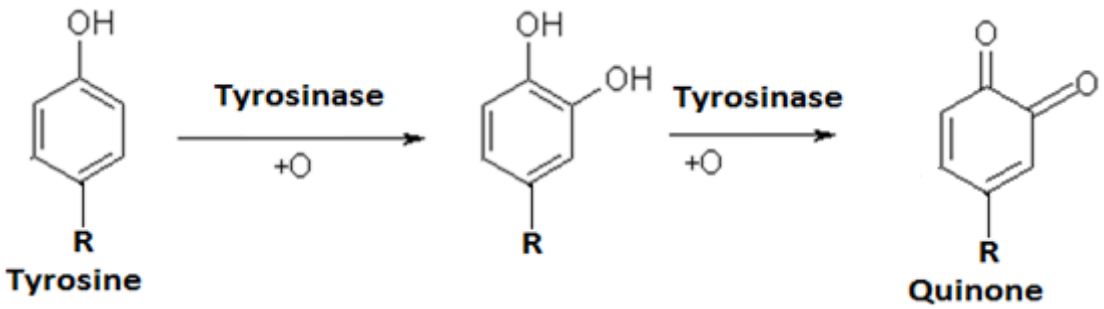


Figure 1.2. Polyphenol oxidase (tyrosinase, EC 1.14.18.1) enzyme action showing both monophenol oxidase and diphenol oxidase activity found mostly in animals and fungi (Mayer and Harel, 1979). R in the figure represents any functional group.

However, its role in fungi and bacteria is not clear. This enzyme is responsible for undesirable enzymatic browning in plant products, seafoods, and melanin formation in human skin. Therefore, it has received significant attention from researchers working in area of food science, plant physiology, and cosmetics development. Though in plants it is considered to be related for defense, not much is known about its exact biological functions. In plant cell, PPO and phenolics were reported to be present in chloroplast and vacuoles, respectively (Yoruk and Marshall, 2003). During physical cutting, cellular structures get disrupted leading to release of PPO enzyme and its phenolic substrate and allowing their physical contact. In the presence of oxygen, the enzymatic reaction produces the quinone which autopolymerises to produce melanin like brown coloured pigment resulting in enzymatic browning (Lozano, 2006). This post-processing enzymatic browning is a major problem for industries dealing with cut fruits and vegetables, juices,

shrimps and related products as it causes loss of natural eye appeal in these products (Oms-Oliua et al., 2010). The control of enzymatic browning by inhibiting PPO enzyme activity has remained an exciting and challenging problem.

1.2. Brinjal (Eggplant): Taxonomy, cultivation, and nutritional value

The brinjal (*Solanum melongena*) is a popular vegetable of Indian origin. Its many cultivars are available in India and other parts of world. It belongs to the family Solananceae with chromosome number 24 (2n= 24). In different countries it is known by different names like eggplant, aubergine, melongene, and guinea squash (Dhaliwal, 2007).

Taxonomic classification of brinjal

Kingdom	Plantae– Plants
Subkingdom	Tracheobionta– Vascular plants
Superdivision	Spermatophyta– Seed plants
Division	Magnoliophyta– Flowering plants
Class	Magnoliopsida– Dicotyledons
Subclass	Asteridae
Order	Solanales
Family	Solanaceae– Potato family
Genus	*Solanum* L.– nightshade
Species	*melongena* L.– brinjal

In India the local name in different states is different but in English language it is known by the name “Brinjal”. The name brinjal is popular in Indian subcontinents and is derived from Arabic and Sanskrit, whereas, the name eggplant has been derived from the shape of the fruit of some varieties, which are white and resemble in shape to chicken eggs. The history of brinjal cultivation in India dates back to about 3000 years. From India it was carried to neighboring country China in 500 B.C. The vegetable got spread by Arab and Persian traders to Africa and rest of world. In USA brinjal was introduced quite late in year 1800. Though this vegetable is worldwide cultivated, India ranks second after China in total production. The global area under brinjal cultivation has been estimated at 1.85

million ha with a total production of about 32 million MTs (FAO data, 2005, http://faostat.fao.org/). India accounts for about 8.7 million MTs with an area of 0.53 million hectares under cultivation. West Bengal is the highest producer of brinjal (26%) followed by Orissa (20%) and Bihar (10%) (http://agriexchange.apeda.gov.in). Brinjal is exported in the fresh or frozen form. In 2007-08, 34 million kg worth of Rs. 19 million was exported mainly to UK, Netherland, Saudi Arabia and Middle East (http://dbtbiosafety.nic.in/guidelines /brinjal.pdf).

The fruit is pendent fleshy berry borne singly or in clusters. The varieties of brinjal display a wide range of shapes and colours, starting from pure white to purple, black, green, and variegated in different shades (Hazra and Banerjee, 2005). Purple colour is due to anthocyanin pigment and white brinjal lacks anthocyanin pigment. The green colour is due to chlorophyll pigment. The fruit shape also varies from long to oval, and oblong to round. The fruits of cluster type are small and primarily cooked as stuffed. Brinjal fruit (unripe) is primarily consumed as cooked vegetable in various ways. In Indian sub-continent large round varieties are preferred as '*bhartha'* and long ones are used for mixed vegetable preparation in combination with other vegetables. Brinjal is low in calories and fats, and contains mostly water, some protein, and carbohydrates. It is a good source of minerals and vitamins and is rich in sugars, and amide proteins among other nutrients. The composition of edible portion of brinjal is given in Table 1 (Gopalan et al., 2007). It has been reported that the long fruited cultivars contain higher amount of glycoalkaloids (such as solasodine), and amide proteins. High anthocyanin and low glycoalkaloid contents are considered essential attributes of the vegetable varieties. For processing purposes, the fruit of high dry matter content and low level of phenolics are preferred. Bitterness in brinjal is due to the presence of glycoalkaloids which are of wide

Table 1.1. Composition of brinjal vegetable per 100 g of edible portion*.

Components	Quantity	Components	Quantity
Calories	24	Sodium (mg)	3
Moisture content (%)	92.7	Copper (mg)	0.12
Carbohydrates (%)	4	Potassium (mg)	2
Protein (g)	1.4	Sulfur (mg)	44
Fat (g)	0.3	Chlorine (mg)	52
Fiber (g)	1.3	Vitamin A (I.U.)	124
Oxalic acid (mg)	18	Folic Acid (µg)	34
Calcium (mg)	18	Thiamine (mg)	0.04
Magnesium (mg)	15	Riboflavin (mg)	0.11
Phosphorus (mg)	47	B-carotene (µg)	0.74
Iron (mg)	0.38	Vitamin C (mg)	12
Zinc (mg)	0.22	Amino Acids	0.22

(*Source: Gopalan et al., 2007. National Institute of Nutrition report)

occurrence in plants of Solanaceae family. Generally, a high content of glycoalkaloids (above 20 mg/100 g fresh weight) produces a bitter taste and off flavor. The glycoalkaloid contents in the Indian commercial brinjal cultivars vary from 0.37-4.83 mg/100 g fresh weight (Das, 1993). Brinjal is also known to have medicinal properties and is considered good for diabetic patients in Ayurveda. It has also been recommended as an excellent remedy for individuals suffering from liver complaints (Das, 1993).

1.2.1. Postharvest handling and storage

Most common brinjal varieties are harvested when they reach a dark, glossy, uniform purple/black/green colour. Overly mature fruit becomes pithy and bitter, reducing market value. After harvest the vegetable is shifted to cooler place and sprinkled with water to

maintain the turgidity and luster. It is also wiped for cleaning or washed and then cooled to extend shelf life. The packaging is mostly performed in bamboo baskets or gunny bags for supplying to nearby markets and cardboard boxes for distant markets. Because the fruit is delicate and bruises easily, it is required to be handled carefully. The spoilage of the vegetable is mostly caused by water loss resulting in weight loss and appearance of wrinkles on skin. The bacterial and microbial growth also result in rotting and spoilage during storage. In India during summer and winter season the average shelf life is reported to be 1-2 and 3-4 days, respectively (Dhaliwal, 2007). Under ambient temperature (26 ± 2 ºC) and 80% relative humidity the shelf life is about 8-10 days. The vegetable with bigger size has higher shelf life compared to the smaller varieties. The green brinjal is reported to have higher shelf life of four weeks at 8-10 ºC at a modified atmosphere with 5% carbon dioxide. The purple varieties can be stored for 7-10 days at similar temperature at 85-95% relative humidity (Dhaliwal, 2007). Even under ideal conditions, brinjal stored longer than 14 days showed ripening related physiological changes and loses its typical culinary traits.

1.2.2 Cultivars of brinjal

Many brinjal cultivars are available in India. The planned and natural hybridization and continuous selection by man could have played an important role in the evolution of the present day cultivated varieties of brinjal. Variability of brinjal fruit can be characterized in shape, size, colour, fruit bearing habit and presence of spines on calyx (Hazra and Banerjee, 2005). Among these the shape is considered to be very stable genotype dependent character. Based upon these characteristics Indian brinjal varieties are listed in Table 1.2. These cultivars were developed in different agricultural research institutes and universities of India and are currently cultivated.

Table 1.2. Commonly available brinjal cultivars in India*.

Sr. no.	Fruit type	Cultivated varieties
1.	Long fruit variety	Pusa Purple long, Hisar pragati, Swetha (SM-6-6), Pusa Bhairav, KS 331, JB-15, Green long, Punjab Barsati, PH-4, Pant Samrat, Azad kranti, Azad B1, Arka Sheel, Arka Shirish, Punjab Sadabahar, Krishanagar green long, NDB-25
2.	Round or oval fruit variety	IDU-1, BR-11, Surya (SM- 6-7), CHBR – 1, DBR-8, Azad B2, Pusa Uttam, Pusa Upkar, Pusa Purple Round, Pant Rituraj, T-3, Jamuni Gole, Punjab Neelam, Krishnanagar Purple Round, Hisar Shyamal, Silki, Kalpatharu, Arka Navneet
3.	Oblong fruit variety	CO-1(Coimbatore), Annamalai, CO-2, Bhagyamati, Utkal Tarini, Hisar Jamuni
4.	Longish oblong (cylindrical) fruit variety	Utkal Madhuri, Utkal Keshari, Pusa Kranti, Pusa Anupam,
5.	Small fruit variety	PKM-1, Shyamala, Aruna, JB 64-1-2, Pusa Ankur, Pusa Bindhu, Manjari Gota, Ravaiya
6.	Cluster bearing variety	PLR – 1, Arka Neelkanth, KKM- 1, ARU- 19, Arka Nidhi, Utkal Jyoti, Gulabi, Vaishali, Pragati, Pusa Purple Cluster, ARU- 2C, Arka Kushumkar, Punjab Barsati, Raveena

(* Source: Hazra and Banerjee, 2005)

1.3. Mechanism of PPO enzyme action

In general PPO oxidizes phenolic substrates in the presence of oxygen. The active site of PPO undergo transitions among met, oxy, and deoxy forms in a cyclic manner (Fig. 1.3) and in each cycle two molecules of catechol are oxidized and one molecular oxygen is reduced to water resulting in formation of two quinone products (Eicken et al., 1999). The dioxygen (O_2) binds to the copper metal center of the enzyme replacing the solvent molecule (H_2O) bonded to CuA in the reduced enzyme form (Deoxy or reduced form).

UV/Vis spectroscopy suggests that molecular oxygen binds first as a peroxide which is followed by binding of the catechol substrate (Eicken et al., 1999). The catechol molecule undergoes deprotonation of one of the two hydroxyl groups and binds to CuB (Oxy form). The transfer of two electrons from the substrate to the peroxide is followed by protonation of the peroxide group and cleavage of the O-O bond (Fig. 1.4).

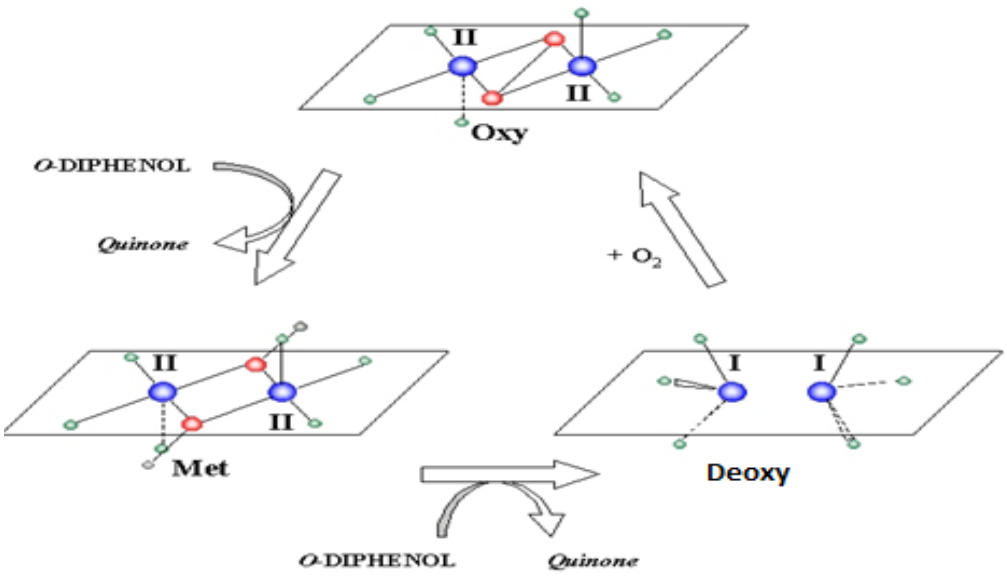


Figure. 1.3. The cyclic transitions of the active site of PPO among met, oxy, and deoxy forms during catalysis (Adapted from Eicken et al., 1999). Coloured circles in blue, red, and green represents copper, oxygen, and histidine, respectively.

Glutamate 236 and the second non-coordinating hydroxyl group of the substrate donate a proton and promote the loss of water and the formation of the o-quinone product. Protonation of the bridging group by solvent brings the active site into the resting hydroxide-bridged dicupric state (met form). Another molecule of catechol can then serve as 'co-substrate' reducing the hydroxide-bridged dicupric (CuII) state back to the dicuprous form (CuI). This step of the proposed reaction pathway is supported by data on the ortho-diphenol oxidase activity of tyrosinase (Fig. 1.4). The dicuprous state Cu(I)-Cu(I) form of active site again repeat the catalytic cycle (Eicken et al., 1999).

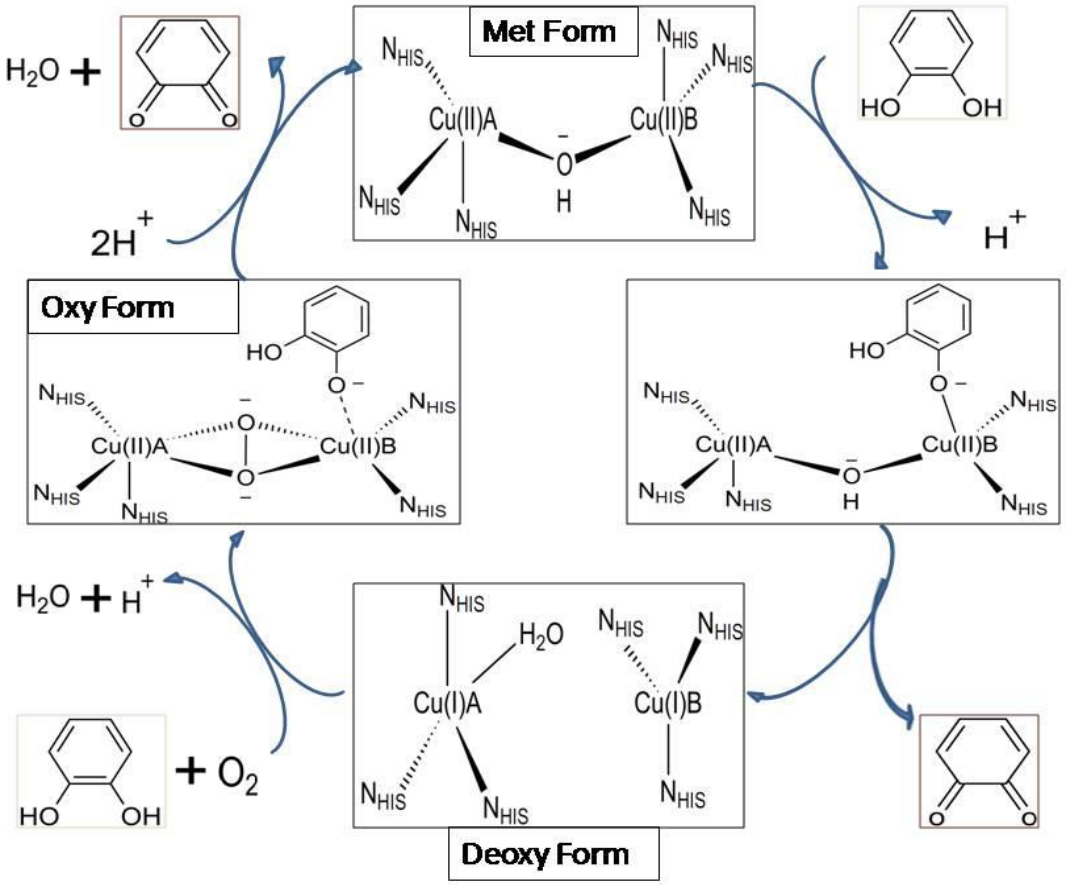


Figure 1.4. Mechanism of PPO enzyme action showing the binding of catechol and oxygen to the copper in the active site and formation of quinone and water during the catalytic cycle (adapted from Eicken et al., 1999).

1.4. Assay of PPO

PPO uses phenolic substrate and molecular oxygen for activity. The PPO oxidized phenolics undergo many secondary reactions. The different methods for estimating PPO activity have been reviewed by Yoruk and Marshall (2003). The PPO activity is determined by measuring the initial rates because the enzyme undergoes inactivation during its catalysis due to suicidal inactivation. This is due to the irreversible binding of the oxidized products to the active site of PPO resulting in enzyme inactivation. This

suicidal inactivation occurs due to reaction of o-semibenzoquinone free radicals (intermediate products in the oxidation reaction), with the active site's histidyl groups bound to copper. Free radical catalyzed fragmentation of the active site's histidine residue(s) results in release of the enzyme bound copper and consequently PPO becomes inactive. Therefore, the initial rate of PPO enzyme catalyzed reaction with o-diphenols is linear for only a short period. The absorbance near 280 nm, where o-diphenols show strong absorbance, is one way of measuring the product formation from monophenol substrates. Another method where, MBTH (3-methyl-2-benzothiazolinone hydrazone) binds PPO reaction product o-quinone is used to assay the activity of PPO (Yoruk and Marshall, 2003). However, some MBTH-quinone adducts showed solubility problems under certain assay conditions. The radioassay technique which measures the rate of formation of tritiated water (^3H_2O) from (3,5^{3}H)-tyrosine is an alternative and highly sensitive method for assaying the rate of the hydroxylation reaction. However, this assay of measuring the rate of tritium removal from the o-position of a monophenol during the reaction is a better approach for monitoring monophenol hydroxylation only. The most convenient way to follow the enzyme reaction is to follow the initial rate of quinone formation using a spectrophotometer. This simplest method of assaying PPO activity is by recording the final colour yield when the enzyme is incubated with a substrate such as catechol, DOPA, or 4-methylcatechol. Among different substrates, 4-methyl catechol is the most frequently used substrate in colorimetric assays because it yields a dark brown end product (Mayer and Harel, 1979). In this reaction, PPO catalyzes the conversion to quinone which then autopolymerizes to yield dark brown melanin type pigments. However, this simple procedure has a limitation, as it measures the end product of a sequence of reactions rather than the true initial reaction rate. In order to overcome this limitation, several workers developed a chronometric assay that involves measuring the rate of loss of ascorbate in an o-PPO/phenolic substrate/ascorbate coupled system;

however, this is a cumbersome procedure. The most reliable method of PPO assay is measurement of the rate of O_2 uptake using an O_2 electrode. During PPO enzyme reaction oxygen is used and its concentration decreases in the reaction mixture. The oxygen electrode measures the oxygen concentration in the reaction mixture. The polarographic O_2 electrode has a rapid response and coupled to a potentiometric recorder or data logger for the immediate display of results. This method has been used routinely by many workers in biochemical studies of PPO. Most of laboratories are currently using the simplest and accurate method of monitoring quinone formation where the increase in absorbance at about 420 nm is monitored for a few minutes and the average increase in absorbance per min is expressed as PPO activity (Yoruk and Marshall, 2003).

1.5. Purification of PPO

The purification of PPO to homogeneity has remained difficult in brinjal possibly due to high phenolic content and the irreversible binding of phenolics to PPO during purification process. Therefore, there are only a few reports on partial purification and characterization of PPO from brinjal. Roudsaria et al. (1981) reported only 15 fold partial purification using ion exchange and gel filtration chromatography. This PPO from a purple cultivar showed catechol oxidase activity. Pérez-Gilabert and Carmona (2000) characterized the 40% ammonium sulphate precipitated fraction without the use of chromatographic purification methods. Doğan et al. (2012) have characterized the PPO activity of ammonium sulphate precipitated fraction in three different cultivars without any further purification. Concellón et al. (2004) have also reported the PPO activity of crude extract during low temperature storage of brinjal without use of any chromatographic purification method. However the PPO purification was found to be less difficult in a few other plants. In potato, the PPO was 5 fold purified with 18% yield,

whereas, in banana peel the PPO was 460 fold purified with 2.2% yield (Sánchez-Ferrer et al., 1993; Yang et al., 2001). In loquat fruit the PPO was 422 fold purified with about 35 % yield (Ding et al., 1998). The PPO has been 183 fold purified from mamey sapota (*Pouteria sapota*) with an 11% yield using ammonium sulphate precipitation, ion exchange and phenyl Sepharose chromatography (Palma-Orozco et al, 2011). Marri et al. (2003) reported 22 fold purification of PPO with 0.5% yield from potato (*Solanum tuberosum*). Gao et al. (2011) reported 37 fold purification of PPO with 44% yield from spiderflower (*Cleome gynandra*) leaves. Mdluli (2005) reported 58 fold purification of PPO with 1.3% yield from marula fruit using ion exchange and gel filtration chromatography. The kinetic characteristics of PPO purified from a few of reported sources are included in Table 1.3 (Quiroz et al., 2008).

These variations in fold purification and yield could be due to differences in concentration and types of phenolics affecting their overall binding to the PPO protein which has been found to interfere with protein purification in certain fruits and vegetables (Papadopoulou and Frazier, 2004). The phenolics bound to the enzyme change its ionic and hydrophobic characteristics resulting in variation in elution behavior during purification steps (Papadopoulou and Frazier, 2004). The PPO was reported to elute with wide peaks. The collection of fractions only with maximum PPO activity for better fold purification resulted in loss of PPO protein from shoulder region of the peak, ultimately affecting the total yield of purified protein (Yoruk and Marshall, 2003).

The browning of crude enzyme is partially prevented by extraction under N_2 at low temperature (4 ºC). The phenol binding agents like polyethylene glycol, polyamide, or polyvinyl pyrrolidone are sometimes used to inhibit the phenolics binding to the enzyme during extraction (Mayer and Harel, 1979). However, a few of these inhibit the enzyme

Table 1.3. Kinetic parameters of PPO extracted from different vegetable sources*.

Source	Substrates with higher affinity	Km (mM)	Optimum pH	Optimum temperature (°C)
Apple	4-Methylcatechol	3.1	7.0	7.0
	Catechol	34.0		
Artichoke	Catechol	10.2	6.0	25
	4-Methylcatechol	12.4		
Aubergine	Catechol	8.7	7.0	20
Banana	Catechol	8.5	7.0	30
Grape	Chlorogenic acid	3.2	5.0	25
	Catechin	4.3		
Loquat	Chlorogenic acid	1.0	6.5	30
	4-tert-catechol	1.2		
Mango	Catechol	6.3	7.0	30
	Pyrogallol	47.8		
Medlar	Epicatechin	4.0		
	L-DOPA	4.7		
Mulberry	Pyrogallol	1.2	7.5	20
	4-Methylcatechol	9.2		
	Catechol	19.8		
Thymus	Pyrogallol	5.5	6.5	35
	4-Methylcatechol	9.8		
	Catechol	18.0		
Peppermint	Catechol	6.3	7.0	30
Strawberry	Catechol	5.9	5.0	25
Persimon	Catechol	12.4	7.5	20-40
	4-Methylcatechol	14.6		
Mamey Sapota	Catechol	44	7.0	
	Pyrogallol	1.3		

*(Adapted from Quiroz et al., 2008)

irreversibly. The PPO existing in membrane bound form is insoluble in extraction solution. In such cases, solubility is increased by addition of detergents or by use of cold acetone for extraction. However, in a few cases these agents modify the structure and activity of the enzyme. Another obstacle in the purification procedure is the presence of different isozymes and interconversion between these forms during extraction and purification process. Procedures which have been used for purification of PPO in general is ammonium sulphate precipitation, ion exchange chromatography, hydrophobic interaction chromatography, and gel filtration chromatography. Celite and hydroxyapatite chromatography have been used in a few cases of PPO purification (Mayer and Harel, 1979).

1.6. Inhibition of PPO and enzymatic browning

Brinjal along with many other fruits and vegetables including potato, sweet potato, breadfruit, jackfruit, yam, mushroom, apple, avocado, banana, grape, peach are prone to postharvest and processing induced browning and therefore causes economic loss (Lozano, 2006). The control of browning from harvest of the vegetable to the consumer is therefore very critical for minimizing losses and maintaining economic value. Browning also adversely affects flavour and nutritional value. The phenomenon of enzymatic browning and the approaches used for its prevention have been elaborately discussed by Marshall et al. (2000). Various methods have been developed for inhibiting or preventing PPO activity over the years for the control of this undesirable enzyme activity. These techniques eliminate one or more of the essential components (oxygen, enzyme, copper, or substrate) required for PPO reaction.

a) Browning of the cut fruit occurs rapidly upon exposure to oxygen. The elimination of oxygen from the cut surface of the vegetables prevents the

browning reaction, which is also possible by immersion in water, syrup, brine, or by vacuum treatment (Marshall et al., 2000).

b) Copper is present in the active site of PPO enzyme. Elimination of copper by using chelating agents makes PPO inactive and browning can be prevented (Lozano, 2006).

c) Inactivation PPO by physical treatments (high pressure, steam blanching) is used for the control of browning in canned or frozen fruits and vegetables. Heat treatments are not practically applicable in the storage of fresh produce (Marshall et al., 2000).

d) PPO catalyses the oxidation of phenolic substrates such as caffeic acid, protocatechuic acid, chlorogenic acid, and tyrosine. Chemical modification of these substrates can prevent their oxidation and browning (Iyengar and McEvily, 1992).

e) Certain chemical compounds react with the products of PPO activity and inhibit the formation of the melanin like pigments resulting in inhibition of browning (Raju and Bawa, 2006).

1.6.1. Physical methods

Following physical treatments have been used to control enzymatic browning in different fruits and vegetables.

Heat treatment is the most widely utilized method for stabilizing foods because of its capacity to destroy microorganisms and to inactivate enzymes. However, steam blanching is not practical for prevention of browning in fresh foods (Yemeniciogïlu et al., 1997). Refrigeration is also used for inhibition of enzymatic browning as it is known that for every 10 ºC temperature increase (in biological important ranges), there is a two fold

increase in the rate of an enzyme catalysed reaction (Q_{10}). Low temperature can inhibit browning by inhibiting PPO activity (Lozano, 2006). However, fruits like banana undergo chilling injury mediated browning under refrigerated storage. Use of irradiation (Low dose gamma radiation) for inhibition of browning in potato tubers and tropical fruits has been extensively reviewed by Thomas (1984; 1986). Mondy and Gosselin (1989) reported that a radiation dose of 1 kGy caused less darkening in potatoes. High pressure treatment has also been tested for inhibition of browning. Weemaes et al. (1998) reported inactivation of PPO in apple, grape, avocado and pear at room temperature (25 ^{o}C) at 600, 700, 800 and 900 Mpa, respectively. Ultrafiltration (UF) is used for stabilizing the colour of white wines and other fruit juices and is believed to remove PPO, but not lower molecular weight polyphenols (Giovanelli and Rasasini, 1993). The molecular weight of PPO is more than 30 kDa, which is much more than molecular weight cut-offs of UF membranes. UF was used for improving the colour stability of banana juice without the application of heat, which is known to alter its flavor (Flores et al., 1988).

1.6.2. Chemical methods

The use of browning inhibitors in food processing is restricted due to concerns like toxicity, effect on taste, flavour, and texture (Iyengar and McEvily, 1992). Browning inhibitors may be classified in accordance with their primary mode of action. PPO inhibitors could be grouped in six catagories based their mode of inhibition of enzymatic browning (Table 1.4).

1.6.2.1. Reducing agents/Antioxidants

Reducing agents play a role in the preventing enzymatic browning either by reducing o-quinones to colourless diphenols, or by reacting irreversibly with o- quinones to form stable colourless products. Reducing compounds are very effective in the control of

browning and include sulfiting agents, ascorbic acid and analogs, cysteine, and glutathione (Molnar-Perl and Friedman, 1990).

Sulfiting agents are currently applied for the inhibition of melanosis (blackspot) in shrimp, potatoes, mushrooms, apples, and other fruits and vegetables. Sulfite concentrations necessary for controlling enzymatic browning vary widely in accordance with the food material and the time required for inhibition of the browning reaction (Taylor et al., 1986). Sulfites no longer have "Generally Required as Safe Status" (GRAS) status for use on fruits and vegetables served raw, sold raw or presented to the consumer as raw in the United States.

According to the United States Food and Drug Administration (US FDA) Federal Register foods containing ≥10 ppm of sulfiting agent, regardless of source, must declare

Table 1.4. Inhibitors of enzymatic browning (Adapted from McEvily et al., 1992).

Reducing agents/antioxidants	Sulfiting agents, ascorbic acid and analogs, cysteine, glutathione, phenolics
Chelating agents	Phosphates, EDTA, organic acids
Acidulants	Citric acid, phosphoric acid
Enzyme inhibitors	Aromatic carboxylic acids, aliphatic alcohol, anions, peptides, substituted resorcinols
Enzyme treatments	Oxygenases, *o*-Methyl transferase, proteases
Complexing agents	Cyclodextrins
Transgenic approach	Introduction of antisense gene

the content on the ingredient label (Marshall et al., 2000). More regulatory restrictions are likely to be applied globally to the use of sulfites in foods since sulfite allergies pose a health risk in many populations. Regulations enacted by the US FDA in 1995 prohibit the

use of sulfites in salad bars. As a result, there has been a considerable focus on identifying appropriate sulfite substitutes for use in foods. The FDA has proposed maximum residual sulfur dioxide levels for certain foods. In accordance with these proposed limits, residual sulfur dioxide levels for fruit juices, dehydrated potatoes, and dried fruit, are 300, 500, and 2000 ppm, respectively (Marshall et al., 2000). Shrimp products having residual sulfite levels in excess of 100 ppm are considered adulterated, since these levels are considered unsafe.

Ascorbic acid is a moderately strong reducing compound, which is acidic in nature, forms neutral salts with bases, and is highly water-soluble. L-ascorbic acid (vitamin C) and its various neutral salts and other derivatives have been the leading GRAS antioxidants for use on fruits and vegetables, juices, for the prevention of browning. PPO inhibition by ascorbic acid has been attributed to the reduction of product o-quinones to their precursor diphenol substrates (Walker, 1977). More stable forms of ascorbic acid derivatives, such as erythrobic acid, 2- and 3-phosphate derivatives of ascorbic acid, phosphinate esters of ascorbic acid, and ascorbyl-6-fatty acid esters of ascorbic acid, have been developed to overcome these problems (Sapers and Garzarella, 1989). Ascorbic acid esters release ascorbic acid upon hydrolysis by acid phosphatases (Seib and Liao, 1987). Their relative effectiveness as browning inhibitors varies in accordance with the food product. Compounds containing reactive amino or thiol groups can greatly affect the reactivity of o-quinones.

Erythorbic acid and its salt, sodium erythorbate are strong reducing agents with GRAS status. They both act as oxygen scavengers, thus eliminating oxygen as a substrate for browning reactions (Sapers and Ziolkowski, 1987). Erythorbic acid is the D-isomer of ascorbic acid but does not have vitamin C activity. Its use in conjunction with citric acid has often been suggested as a substitute for sulfites in the control of enzymatic browning.

Current research suggests that L-ascorbic acid and erythorbic acid both possess equivalent antioxidant properties. A combination of both acids is applied for inhibiting oxidative rancidity and discolouration in vegetables, salads, apples, and frozen seafood. Erythorbic acid or sodium erythorbate can suppress browning reactions in frozen fruits (Raju and Bawa, 2006).

Cysteine is an effective inhibitor of enzymatic browning. It is reported to be more effective than sodium bisulfite as an antibrowning agent (Kahn, 1985). Concentrations of cysteine and other thiols required for the achievement of acceptable levels of browning inhibition have however been shown to have negative effects on taste. The inhibition of melanosis by cysteine is thought to be due to the formation of colourless thiol-conjugated o-quinones. Cysteine has also been shown to reduce o-quinones to their phenol precursors (Walker, 1977; Cilliers and Singleton, 1990). Cysteine-quinone adducts serve as competitive inhibitors of PPO.

Phenolic antioxidants: Antioxidants are defined by the US FDA as substances, which may be applied in preserving food by retarding deterioration, rancidity or discolouration due to oxidation. Antioxidants inhibit oxidative processes by reacting with free radicals, through metal chelation, and by scavenging singlet oxygen (Raju and Bawa, 2006). Both synthetic and naturally occurring phenolic antioxidants are used in food applications. Several synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiarybutyl hydroxyquinone (TBHQ) and propyl gallate (PG) are permitted for use in food. Plant phenolic compounds such as tocopherols, flavonoid compounds, cinnamic acid derivatives, and coumarins are naturally occurring compounds, which have antioxidant and PPO inhibition properties (Raju and Bawa, 2006).

1.6.2.2. Chelating agents

PPO enzyme has copper metal ions at its active site. Chelating of copper ions by chelating agents can inactivate the enzyme. Chelating agents have been applied in various food processing applications, for enzyme inactivation (McEvily et al., 1992). Chelators used in the food industry include sorbic acid, polycarboxylic acids (citric, malic, tartaric, oxalic, and succinic acids), polyphosphates (ATP and pyrophosphates), macromolecules (porphyrins, proteins), and EDTA. Other non-GRAS chelating agents which are capable of inhibiting PPO include cyanide, diethyldithiocarbonate, sodium azide and 2-mercaptobenzothiazole, carbon monoxide, mercaptobenzthiazol, dimercaptopropanol, and potassium methyl xanthate. Ascorbic acid also has a chelating effect on the prosthetic group of PPO (McEvily et al., 1992).

EDTA: Calcium disodium EDTA (Ethylenediaminetetraacetic acid) and disodium EDTA have been approved for use as food additives by the US FDA (Raju and Bawa, 2006). It inhibits PPO by chelating the copper metal ion. EDTA is generally used in combination with other chemical treatments for the prevention of enzymatic browning in foods. A typical combination of anti-browning agents might consist of a chemical reducing agent (ascorbic acid), an acidulant (citric acid) and a chelating agent (EDTA).

Phosphates: Polyphosphates, sodium acid pyrophosphate, and metaphosphate are chelating agents of limited cold water solubility. They have been used as antibrowning agents for fresh peeled fruits and vegetables at concentrations as low as 0.5 to 2 percent (final concentration in the dip solution) (McEvily et al., 1992). SporixTM, an acidic polyphosphate mixture (sodium acid pyrophosphate, citric acid, ascorbic acid, and calcium chloride) has been observed to delay the onset of oxidation and enzymatic browning in fruits and vegetables.

Polysaccharides: Different sulfated polysaccharides, including carrageenans, amylose sulfate, and xylan sulfate, were reported to inhibit browning in apple juice and diced apples (Tong and Hicks, 1991). Pectin, a naturally occurring anionic polysaccharide at a concentration of 0.5 % is also reported to inhibit browning in apple juice (Tong et al., 1995). Carboxyl groups present in pectin are believed to be capable of chelating the copper moiety of PPO, thus preventing browning.

Carbon monoxide (CO) is a known inhibitor of many copper containing oxidases and behaves as a non-competitive inhibitor of PPO. It has been studied in preventing the discolouration of mushrooms (Fujimoto et al., 1972). This inhibition was, however, reversible and removal of CO led to restoration of the initial activity. A two-step gas treatment of potato strips with SO_2 followed by CO resulted in 99.9 percent inactivation of PPO (Kramer et al., 1980). A number of safety problems are, however, associated with the use of carbon monoxide gas.

1.6.2.3. Acidulants

Ionizable functional groups of the enzyme structure are affected by the pH of the food medium. These groups must be in appropriate ionic form in order to maintain the conformation of the active site, bind substrates, or catalyse the enzymatic reaction (Raju and Bawa, 2006). Changes in the ionization status of enzymes are generally reversible. Irreversible denaturation can occur under conditions of extreme pH. The stability of the substrate is also affected by changes in pH, since substrates can undergo chemical breakdown under extreme conditions of pH. Degraded substrates often behave as enzyme inhibitors, since they share the molecular features of the substrate (Tipton and Dixon, 1983). Acidulants are generally applied in order to maintain the pH well below that required for optimum catalytic activity of an enzyme. Acidulants such as citric, malic, and

phosphoric acids are capable of lowering the pH of a system, thus rendering PPO inactive (Richardson and Hyslop, 1985). Acidulants are often used in combination with other antibrowning agents.

Citric acid is the one of the most widely used acidulants in the food industry. It is typically applied at levels ranging between 0.5 and 2 percent (w/v) for the prevention of browning in fruits and vegetables. In addition, it is often used in combination with other antibrowning agents such as ascorbic or erythorbic acids and their neutral salts, for the chelation of prooxidants and for the inactivation of PPO. Recommended levels for citric acid typically vary between 0.1 and 0.3 percent (w/v) with the appropriate antioxidant at levels ranging between 100 and 200 ppm (Raju and Bawa, 2006). Citric acid exerts its inhibitory effect on PPO by lowering the pH as well as by chelating the copper at the active site of the enzyme (Pizzocaro et al., 1993).

1.6.2.4. Enzyme inhibitors

Four-hexylresorcinol (4-HR) has been used in pharmaceuticals and is considered to be safe and effective in use as an anti-browning agent (Jiménez and Garcáa-Carmona, 1997). It has several advantages over sulfites when applied in the control of browning in foods. Four-hexylresorcinol was reported to control browning in fresh and hot-air dried apple slices as well as in apple juice (McEvily et al., 1992). Several studies have shown the effectiveness of 4-HR in controlling enzymatic browning in shrimp, mushroom and apple slices (Marshall et al., 2000). Four-hexylresorcinol is a chemically stable, water-soluble compound. Toxicological, mutagenic, carcinogenic, and allergenic studies have shown that there are no risks associated with the levels of 4-HR used in the treatment of shrimp (Mayer, 2006). Four-hexylresorcinol has obtained GRAS status from the United States Food and Drug Administration, for use on shrimp (Jiménez and Garcáa-Carmona, 1997).

Halide salts: Inorganic halides are known to inhibit PPO, for example NaF is the most potent inhibitor of apple PPO, followed by NaCl, NaBr, and NaI (Raju and Bawa, 2006). The inhibition of enzymatic browning by halides decreases with increase in pH. Sodium chloride and calcium chloride at concentrations of ranging between 2 and 4 percent (w/v) are most commonly used in the food industry for the inhibition of browning (Marshall et al., 2000). Poplyphenol oxidase activity was observed to decrease with increasing concentrations of NaCl for peach, brinjal and avocado. Sodium and zinc chloride was shown to be a highly effective browning inhibitor when used in combination with calcium chloride, ascorbic acid, and citric acid (Raju and Bawa, 2006).

Amino acids, peptides and proteins inhibit PPO either by inhibition of the enzyme or by reacting with the product quinones (McEvily et al., 1992). Proteins, peptides and amino acids are capable of forming stable complexes with Cu^{2+}. In addition, they are also capable of chelating copper at the active site of PPO. Histidine and cysteine have high affinities for Cu^{2+}. Imidazole ring of histidine and thiol group of cysteine, both have metal binding capacity. Mushroom PPO was weakly inhibited by mM concentrations of L-lysine, glycine, L-histidine and L-phenylalanine. L-cysteine was reported to be the most effective amino acid in inhibiting o-dihydroxyphenolase activity (Marshall et al., 2000)

Aromatic carboxylic acids such as benzoic acid and cinnamic acid are structurally similar to phenolic substrates and known to inhibit PPO. These acids inhibit PPO by forming complex with copper at the active site. The degree of PPO inhibition by carboxylic acids is pH dependent, and increases with a decrease in pH. Cinnamic acid at levels of 0.01 percent was observed to be effective in long term inhibition of PPO in apple juice (Walker, 1976). Benzoic acid and its derivatives showed inhibitory effect on PPO activity in mushrooms (Kermasha et al., 1993).

Aliphatic alcohols: Although the inhibition of PPO by ethanol has been reported, there are no extensive studies describing the effect of aliphatic alcohols on PPO. Valero et al. (1990) studied the effects of natural aliphatic alcohols on grape PPO. Inhibition was observed by increasing chain length of the aliphatic alcohol.

1.6.2.5. Complexing agents: Cyclodextrins and Chitosan

The cyclodextrins (CDs) are a class of cyclic oligosaccharides containing 6-8 glucose units per cycle. The central cavity of CD is hydrophobic while the outer region of the oligosaccharide is hydrophilic due to the presence of primary and secondary hydroxyls at both the narrow and wide bases (Raju and Bawa, 2006). CD's are highly insoluble. CD inhibits browning of fruit juice by binding with PPO substrates. Polyphenols can be removed by CD and by insoluble polyvinyl polypyrrolidone or polyethylene glycol (Mayer and Harel, 1979). The adsorption of flavour or colour compounds by cyclodextrins poses a major drawback to their use in food systems. The applicability of cyclodextrins in fruit and vegetable juices has been patented (Marshall et al., 2000) However, cyclodextrins have not yet been approved for food use by the United States FDA.

Chitosan, a natural polymer of β-(1→4)-N-acetyl-D-glucosamine, is present in chitin of shellfish. It has been shown to inhibit enzymatic browning in apple and pear juices (Sapers, 1989). The browning in apple juice was reported to be inhibited by addition of 200 ppm of chitosan. The exact mechanism of chitosan action is not known. The inhibitory effect is considered to be due to adsorption of positively charged polymer to PPO, its substrates, or products. Zhang and Quantick (1997) also showed potential inhibitory activity of chitosan coating on PPO and POD activity in lychee (*Litchi chinensis* Sonn.) fruit.

Edible Coatings minimize undesirable changes due to minimal processing and has been reported for several commodities (Baldwin et al., 1996). Zhang and Quantick (1997) found that an edible coating based on sucrose esters of fatty acids significantly delayed pericarp browning of litchi fruit. Carboxymethyl cellulose/soy protein coating formulations containing 0.5 percent ascorbic acid applied to freshly cut apples were more effective in antibrowning activity than aqueous solutions of 0.5 percent ascorbic acid alone (Baldwin et al., 1996).

1.6.2.6. Enzyme treatments

Killer enzymes can be used to control browning either by substrate and/or product modification, and direct inactivation by use of proteases. Apple juice treated with the bacterial enzyme protocatechuate-3, 4-dioxygenase prevented browning by modifying its phenolic constituents which resulted in PPO enzyme deprived of substrates. The plant proteases including ficin, papain and bromelain are reported to be very effective inhibitors of browning (Labuza et al., 1992). Ficin was observed to be effective in preventing black spot formation in shrimp under refrigerated conditions. Commercial application of enzymes in the control of browning is precluded by their high cost. Chemical anti-browning agents and their combinations are more affordable and effective in commercial use.

1.6.2.7. Transgenic approach: antisense gene introduction

One of the recent approaches for controlling browning is inhibition of gene expression in plants by introduction of antisense genes (Marshall et al., 2000). It involves insertion of a gene or a significant part of it, into the cell in a reverse orientation. Messenger RNA encoded by this antisense gene undergoes hybridization with mRNA encoded by the endogenous PPO gene, preventing production of the protein product. Gene silencing or

the elimination of expected phenotypic characteristics, through antisense techniques has received much attention in recent years. Bachem et al. (1994) reported that the expression of PPO in potatoes was decreased through the use of vectors carrying antisense PPO cDNAs. Approximately 70 percent of the transformed plants had lower PPO activity than controls, and on visual scoring, a significantly low level of discolouration. Insertion of PPO in the sense orientation resulted in very high PPO activity in the lines expressing the construct. This technology of genetically preventing enzymatic browning in a wide variety of food crops can be used without resorting to chemical and physical treatments (Marshall et al., 2000).

1.7. Structure of PPO

Many PPOs are believed to function *in vivo* as monomers, but a few fungal PPOs may associate to function as small oligomers (e.g. tetramers). The subunit molecular masses reported in the literature for plant and fungal PPOs vary considerably, even for a single species. The monomers have independent active site and are enzymatically active, but for structural stability they often exist as oligomers. For a few species lower molecular mass subunits have been shown to result from proteolysis of a larger precursor form. Among plants, the crystal structure of PPO, sweet potato (*Ipomoea batatas*) (Klabunde et al., 1998) and grapes (greenache; *Vitis vinifera*) (Virador et al., 2010) has been resolved and published in Protein Data Bank (PDB). The ribbon drawings with active site details are detailed in Figure 1.5. From sweet potato three structures are published, out of which two are in native Cu(I)-Cu(I) state (PDB accession no. 1BT1, 1BT3), one is in reduced Cu(II)-Cu(II) state (1BT2) and the last one in complex with phenylthiourea (1BUG). In grapes (greenache variety) the native PPO structure is published (2P3X).

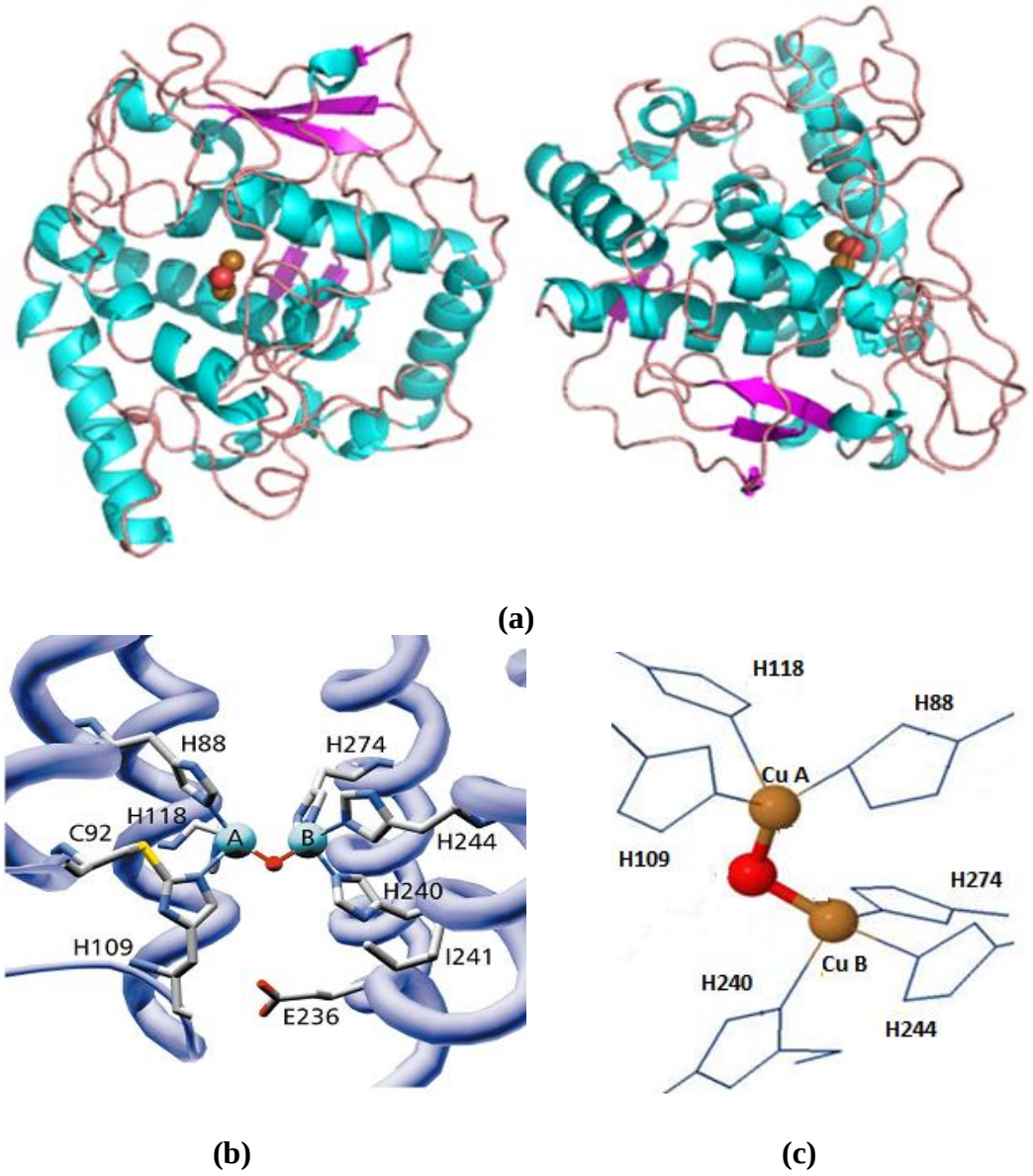


Figure 1.5. Ribbon drawings of sweet potato PPO and its active site region (Adapted from Eicken et al., 1999). (a) Drawing of PPO dimer; each monomer showing two copper atoms linked to the oxygen in oxidized state. The pink colour represents strands and helices are in green. (b) The active site region with both copper sites, CuA (left) and CuB (right), showing three histidine ligands and the bridging solvent molecule. The disulfide bridges between C92 and H109 are displayed in yellow (d) The active site region showing oxidized Cu(II)–Cu(II) with bound oxygen and each copper bound to six histidine residues.

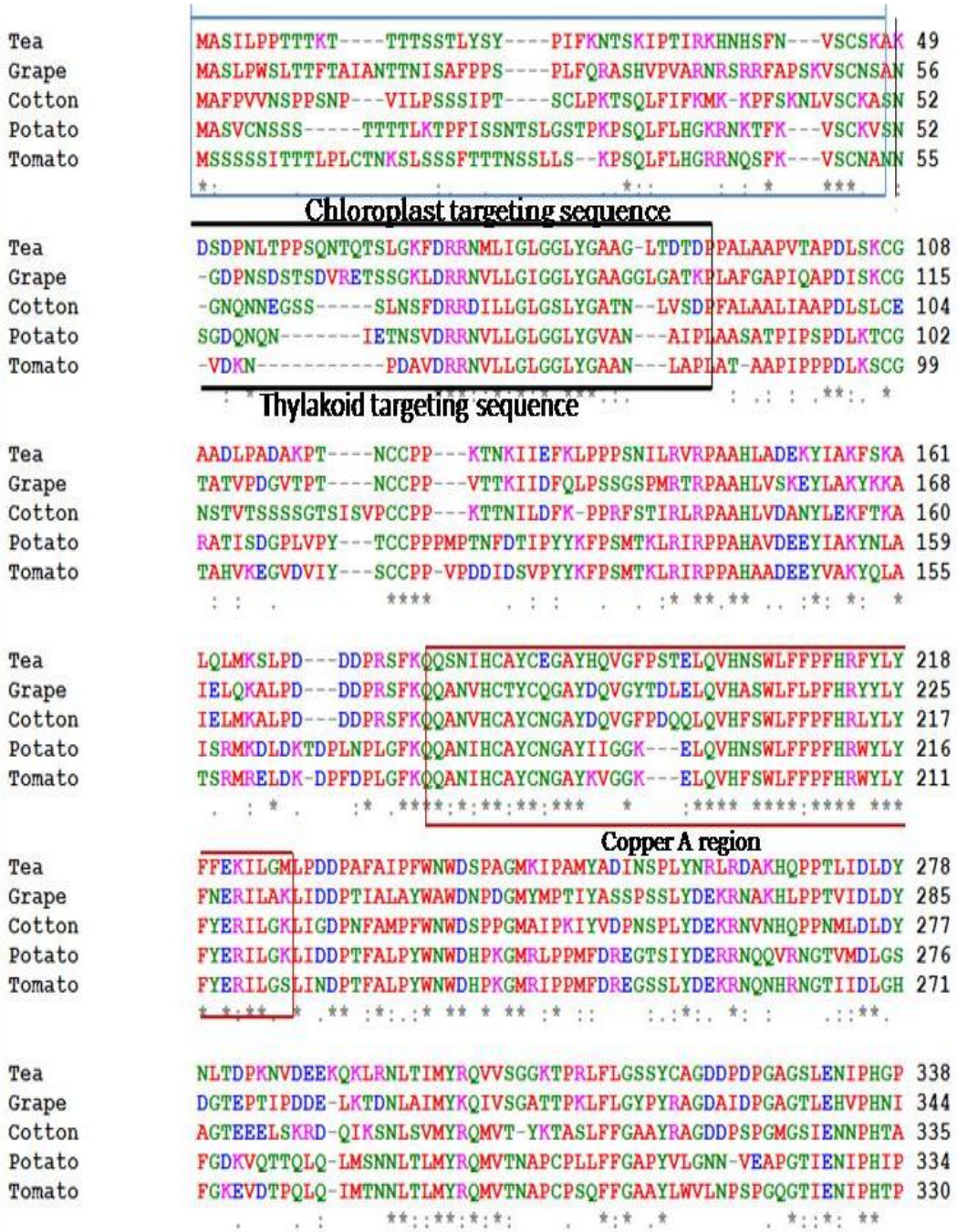
In fungi two structures of mushroom (*Agaricus bisporus)* tyrosinase are published, both are in deoxy state, contain an unknown lectin-like subunit and bind to inhibitor tropolone (Ismaya et al., 2011). The search of PDB with tyrosinase keyword yielded additional bacterial sources like *Streptomyces castaneoglobisporus*, *Citrobacter freundii*, and *Bacillus megaterium.* From *Streptomyces* as many as 22 different structures were submitted to PDB which included the tyrosinase caddie protein complex PPO differently soaked in copper solution (8 nos. starting from 3AWS-Z and3AXO), in deoxy-from (4 nos. from 2ZWD to 2ZWG, 2ZMZ, 2AHL), met-form (2ZMX, 2ZMY), and oxy-form (1WX4, 1WX5) (Matoba et al., 2006). These structures are also published in copper free form (1WXC, 1WX5). This study explained that the dinuclear copper centre in active site remain flexible during catalysis and can accommodate both the monophenolase and diphenol oxidase activity, whereas, the catechol oxidase from other sources including plants have rigid active site and do not show the monophenolase activity. The six structures of PPO from *Bacillus megaterium* published were prepared in the presence and absence of zinc ion and kojic acid inhibitor explained the plasticity of copper binding, which means copper ion position varies in active site (Sendovski et al., 2011). The histidine residue position is also shown to play a role in accessibility of substrate to the active site of enzyme. In sahara scorpion (*Androctonus*), the structure of oxygen carrier hemocyanin, a similar type 3 copper protein with dinuclear copper centre was shown to have phenol oxidase activity upon SDS activation (Cong et al., 2009). The SDS treatment causes twisting of one flexible domain resulting in access of substrate to catalytic active site.

1.7.1. Sequence and structural features

The list of plants in which PPO has been sequenced and at least partly characterized is growing steadily. The nucleotide sequence of PPO is available in close relatives of brinjal

including potato, tomato, tobacco, sweet potato and many other plants including cereals like wheat, and legumes like pea. In most of the dicot plants reported so far including tomato and potato the introns are reported to be absent. Monocots like banana and wheat, which have at least four and six distinct o-diphenol oxidase genes, respectively, show the presence of intervening sequences (Shetty et al., 2011). In pineapple (*Ananas comosus*) sequence analyses showed that both PPO genes contained single intron (Zhou et al., 2003). The presence of two introns was also reported in barley (*Hordeum vulgare*) PPO (Takeda et al., 2010).

Alignment of nucleotide sequences showed considerable homology among close family relatives. The homology is limited among the phylogenetically distantly placed plants (Wong, 1995). For example, potato nucleotide sequence (Genbank accession U22921.1) showed 89% homology with tomato (Z12836.1), whereas, the homology was 77% with relatively distant relative persimmon (*Diospyros kaki*) PPO (AF039165.1). PPOs of closely related plants, such as potato (AAA85122.1) and tomato (AAB22610.1) showed approximately 74% homology in amino acid sequence. However, potato showed 43, 44, and 42% homology with cotton, tea, and grape, respectively (Fig. 1.6). Though the sequence length varies in different plants, in general the nucleotide sequence of PPO gene was about 1800 bp long with the translated PPO precursor protein about 600 amino acid long with a molecular weight of about 66 kDa. The protein sequence of PPO can be classified into three regions. The N-terminal is the chloroplast targeting sequence region and spans about 80 amino acids. The next is the copper binding region (dinuclear copper centre) which again divided into Cu A (170-220 aa) and Cu B (350-410 aa) region. The remaining part of the sequence up to about 600 residues constitutes the C-terminal region.


Chloroplast targeting sequence
Thylakoid targeting sequence
Copper A region

Contd…..

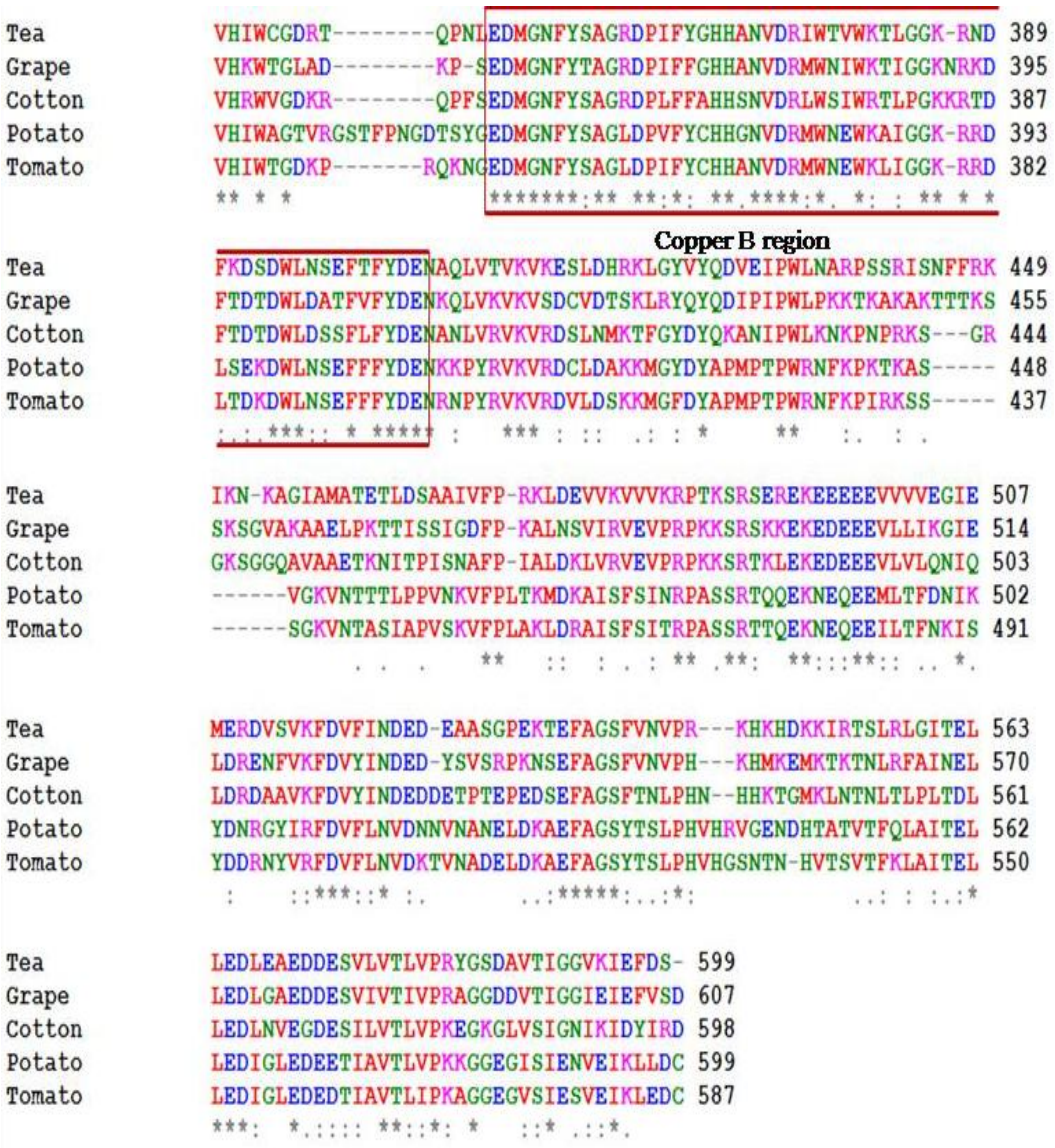


Figure 1.6. Multiple sequence alignment of conceptual amino acid sequence of potato (Genbank accession AAA8512 2.1), tomato (AAB22610.1), cotton (AFC36521.1), tea (ACJ04908.1), and grape (A AB41022.1) PPO showing the N-terminal targeting sequences and copper binding regions.

The comparative analysis of PPO protein sequence in plants and fungi has been done by Marusek et al. (2006). They have shown that a number of important structural features are conserved in the N-terminal domains of PPOs from various plants and fungi, including a tyrosine motif which can be considered a landmark indicating the beginning of the linker region connecting the N- and C-terminal domains. The sequence alignments and secondary structure predictions indicated that the C-terminal domains of PPOs are likely to be similar in tertiary structure to that of hemocyanin. Detailed bioinformatics analyses of the linker regions predicted that this section of the polypeptide chain is intrinsically disordered (lacking fixed tertiary structure) and contains a site of proteolytic processing as well as a potential phosphorylation site. All species possess the six histidine residues that associate with the two copper ions of the active site. The first three histidine residues encompass a region termed the CuA site, and the following three encompass the CuB site. An unusual thioether bridge was identified between a cysteine residue and the second histidine of the CuA site. This covalent bridge appears to have a structural role in which it puts additional structural restraints on the CuA center. In sweet potato (*Ipomoea batatas*) PPO, the cysteine involved in the thioether bridge is located four residues after the first histidine of the CuA site. The multiple sequence alignments revealed that this cysteine is conserved in the other plant PPOs. The same is true of fungal (*Neurospora crassa*) PPO cysteine, and this cysteine is conserved in the other fungi too. Due to the conservation of these cysteines, it is likely that all plant and fungal PPOs have a thioether bridge as a structural component of their CuA sites (Marusek et al., 2006). The Phenylalanine (at 261 position) of sweet potato PPO has been termed the gate residue because of its location above the active site, partially blocking the substrate entrance. The bulky phenylalanine side chain must rotate to allow substrate binding to occur. Other plants either have phenylalanine or leucine at this position, whereas, fungal PPO have leucine or proline at this position. Immediately after the CuB region, a tyrosine motif (Y/FxY) is located (at

331-333 amino acid position in sweet potato PPO). The hydroxyl group of tyrosine side chain form hydrogen bonds to an aspartate side chain located four residues after the third histidine of the CuB site. This aspartate (at 288 position in sweet potato PPO) is reported to be conserved in all plant and fungal PPOs. Again the first tyrosine/phenylalanine from tyrosine motif interacts (pi-cation interaction) with an arginine (at 49 position in sweet potato PPO) from N-terminal domain. The conservation of this tyrosine/phenylalanine, aspartate, and arginine indicated their importance to the structural integrity of N-terminal domain (Marusek et al., 2006).

The glycosylation sites could not be predicted with confidence for plant and fungal polyphenol oxidases by Marusek et al. (2006), however, such sites were recently predicted in a cultivar of brinjal (Shetty et al., 2011). The primary sequence of the linker region is much longer for plant and fungal PPOs, ranging from 52 (in rice), 80 residues in potato and faba bean, and 59-98 residues for fungi (Van-Gelder et al., 1997).

1.7.2. Copper as PPO cofactor

Copper is an essential nutrient in most plants and all animals and it is used as a cofactor in enzymes. In trace amounts it is necessary for life, but in higher amounts it is toxic and potentially deadly. Copper is found in PPO and other essential proteins such as cytochrome c oxidase, catechol oxidase, and ascorbate oxidase, a Cu/Zn superoxide dismutase, plastocyanin, ethylene receptor, and many other oxidoreductases, and monooxygenases. It is responsible for the oxidation-reduction processes that involve electron transfer, dioxygen chemistry, and reduction of nitrogen oxides. The review by Fontecave and Pierre (1998) explained why copper was evolutionarily selected as the metal of choice for oxidases like PPO. Iron and copper are the two most familiar redox metals serving biological functions. Both appear to ably catalyze similar reactions and

either iron or copper sites are found in oxygen carriers, mono-oxygenases, di-oxygenases or oxidases. Prior to the photosynthetic generation of an oxidizing environment, water insoluble copper (I) prevailed and was, thus, not biologically available. Soluble ferrous iron was present in large amounts in the oceans and was used for biological functions. The production of O_2 by photosynthetic organisms resulted in the oxidation of cuprous (Cu I) to soluble cupric (Cu II) and of soluble ferrous (Fe II) to insoluble ferric (Fe III). This is suggested to be the reason why living organisms selected copper instead of iron. Copper and iron display distinct features in terms of their coordination chemistry and their redox properties. One of the major differences resides in their distinct electrochemical properties, which explain most of their specific reactivity. As a matter of fact, the reduction potentials of the Fe(III)–Fe(II) system can be finely tuned by small modifications in the metal coordination sphere so that iron sites can encompass the entire biologically significant redox potential range: from about −0.5 V to about +0.6 V. In contrast, a relatively high redox potential for the Cu(II)–Cu(I) system is found in copper enzymes, most of them working between +0.25 and +0.75 V. This high potential can be utilized for a direct oxidation of certain substrates, easy to oxidize, such as superoxide (in superoxide dismutase) or ascorbate (in ascorbate oxidase) or catechols (in tyrosinase or in laccases).

The transition metal containing active sites are small regions contained within a much larger protein structure. The metal cofactors are usually not covalently bound to the rest of the protein molecule, but are kept in place by ligation of amino acid side chains that have unpaired electrons (histidine, cysteine) with empty axial orbitals of the transition metal cofactor. The position that the ligand binding between the transition metal and the amino acid side chain gives to the cofactor will determine what types of reactions it can participate in and the degree to which they are reversible. Copper exists mainly in two

oxidation states, Cu(I) and Cu(II), and often changes between these two states while catalyzing reactions. It can also, like other transition metals, serve as a structural support for the larger protein molecule that it is a part of, thereby affecting activity of a protein in another fashion.

The copper needs to be transported inside thylakoid lumen of chloroplast organelle in pant cell. In plants, relatively little is known about copper transport into and within cells, although several families of heavy metal transporters have been identified (Shikanai et al., 2003). Chloroplasts have a complex internal structure, and Cu must be delivered in a regulated manner to defined locations within the organelle. The Arabidopsis COPT1 gene and its four homologs encode Cu transporters that allow the entrance of Cu into cells. Components of intracellular Cu transport and trafficking identified in *Arabidopsis* include metallothioneins, a Cu chaperone (CCH, P-type ATPases that function in the endomembrane system) (Shikanai et al., 2003). In *Synechocystis*, a cyanobacterium that shares evolutionary ancestry with chloroplasts, two Cu transporting P-type ATPases have been characterized (CtaA and PacS). CtaA functions in Copper import into the cell, whereas, PacS is involved in thylakoid import. A copper chaperone, designated bacterial Atx1, has been discovered which interacts with the N-terminal domains of both CtaA and PacS (Abdel-Ghany et al., 2005). The study by Shingles et al. (2004) showed the possible presence of at least two copper transporters in the thylakoid membrane of pea (*Pisum sativum*) chloroplasts.

1.7.3. Copper proteins

Copper proteins are proteins that contain one or more copper ions as prosthetic groups. The metal centres in the copper proteins can be classified into several types depending

upon the number of copper atoms present and the geometry of the copper centre. Some of the copper proteins interacting with oxygen are listed in Table 1.5.

Table 1.5. Copper proteins that bind and/or activate dioxygen*.

protein (abbreviation)	Copper ions present	reaction performed
Tyrosinase (Tyr)	2	hydroxylation of aromatic ring
Catechol oxidase (CO)	2	oxidation of catechol to o-quinone
Hemocyanin (Hc)	2	reversible binding of O2
Laccase (Lc)	3	oxidative coupling of catechols
Ascorbate oxidase (AO)	3	oxidation of ascorbate
Ceruloplasmin (Cp)	3	oxidation of Fe(II) to Fe(III)
Cytochrome C oxidase	1	establishment of membrane proton gradient
Amine oxidase (AO)	1	aldehydes and H_2O_2 from primary amines
Galactose oxidase (GAO)		

(*Source: Lewis and Tolman, 2004)

- Type 1 copper centres (T1Cu) are characterized by a single copper atom coordinated by two histidine residues and a cysteine residue in a trigonal planar structure, and a variable axial ligand. In class I T1Cu proteins (e.g. amicyanin, plastocyanin and pseudoazurin) the axial ligand is the sulfur of methionine, whereas, aminoacids other than methionine (e.g. glutamine) give 600 nm (due to S→Cu charge transfer), which usually gives rise to a blue colour. Cupredoxins are therefore often called "blue copper proteins". This may be misleading, since some T1Cu centres also absorb around 460 nm and are, therefore, green. When studied by EPR spectroscopy, T1Cu centres show small hyperfine splittings in the parallel

region of the spectrum (compared to common copper coordination compounds) (Solomon et al., 1996).

- Type 2 copper centres (T2Cu) exhibit a square planar coordination by N or N/O ligands. They exhibit an axial EPR spectrum with copper hyperfine splitting in the parallel region similar to that observed in regular copper coordination compounds. Since no sulfur ligation is present, the optical spectra of these centres lack distinctive features. T2Cu centres occur in enzymes, where they assist in oxidations or oxygenations (Solomon et al., 1996).
- Type 3 copper centres (T3Cu) consist of a pair of copper centres, each coordinated by three histidine residues. These proteins exhibit no EPR signal due to strong antiferromagnetic coupling (i.e. spin pairing) between the two S = 1/2 metal ions due to their covalent overlap with a bridging ligand. These centres are present in some oxidases and oxygen-transporting proteins (e.g. hemocyanin and tyrosinase) (Solomon et al., 1996).
- Binuclear Copper A centres (CuA) are found in cytochrome c oxidase and nitrous-oxide reductase (EC 1.7.99.6). The two copper atoms are coordinated by two histidines, one methionine, a protein backbone carbonyl oxygen, and two bridging cysteine residues (Solomon et al., 1996).
- Copper B centres (CuB) are found in cytochrome C oxidase. The copper atom is coordinated by three histidines in a trigonal pyramidal geometry.
- Tetranuclear Copper Z centre (CuZ) is found in nitrous-oxide reductase. The four copper atoms are coordinated by seven histidine residues and bridged by a sulfur atom (Solomon et al., 1996).

1.8. Molecular characteristics of PPO

The PPO gene is transcribed to form the PPO mRNA which is translated to the precursor PPO protein inside cell. The PPO gene and its transcript has the following features.

1.8.1. PPO multigene family

Gene family is a set of several similar genes, formed by duplication of a single original gene, and generally with similar biochemical functions. Genes are categorized into families based on shared nucleotide or protein sequences. PPO gene families have been described in several species, with individual PPO genes typically showing distinct expression patterns. They have been most extensively investigated in potato, tomato, brinjal (5 genes), poplar (9 genes), faba bean, red clover (6 genes), and in apple (Newman et al., 1993; Thygesen et al., 1995; Shetty et al., 2012; Tran et al., 2011; Cary et al., 1992; Sullivan et al., 2004). Again, five PPO genes from potato and seven from tomato, four from banana were isolated especially from fruit, flowers, and leaves of these crop plants (Hunt et al., 1993; Shahar et al., 1992; Thipyapong et al., 1997). Monocots like banana and wheat, have shown at least four and six distinct o-diphenol oxidase genes, respectively, and show the presence of intervening sequences (Gooding et al., 2001; Massa et al., 2007). Though genes homologous to o-diphenol oxidase are absent in Arabidopsis genome, laccases with at least 17 genes constituting the multigene family are present (McCaig et al., 2005).

Although constitutively expressed in plants, PPO activity is often up-regulated in wounded tissues, and thus PPOs are often considered to be plant defense proteins (Constabel et al., 2000). Gene expression profiling in poplar (*Liriodendron tulipifera*) tissues and organs revealed that the PPO genes are all differentially expressed during normal development, but that only a small subset of PPO genes are significantly

upregulated by wounding, methyl jasmonate or pathogen infection. Hexaploid wheat kernels have six genes coding for PPO, of which at least three are expressed during development of the kernel. These six genes fall in two clusters with three similar sequences in each.

1.8.2. Chromosomal location

In wheat (*Triticum aestivum)* the genetic mapping of PPO exclusively performed and has implications for selection for wheat variety with low PPO activity (Jimenez and Dubcosky, 1999). The Quantitative Trait Loci (QTL) for PPO in *T. aestivum* indicated that a number PPO genes are present on different chromosomes. One gene from of *T. turgidum* coding for PPO was mapped to chromosome 2D (Jimenez and Dubcosky, 1999). In Barley the PPO or phenol reaction (Phr) gene has been mapped to the long arm of barley chromosome 2H (Takeda et al., 2010). In grape variety (*Pinot nori*) the search of the complete genome showed the presence of four homologues on chromosome 10 (Virador et al., 2010). The SOL (Solanum abbreviated) Genomics Network (SGN) is a website which provides information about genomic, genetic, phenotypic and taxonomic information for plant genomes, especially the Solanaceae family members (e.g. tomato, potato, brinjal, pepper and petunia). The search for PPO gene in SOL genomics network website resulted in finding the physical locus PPO gene on chromosome number 8 both in potato and tomato. Further, in comparison to tomato chromosome 8, the chromosomal rearrangements (inversions and translocations) are absent in brinjal which indicated the similar localization of PPO in chromosome number 8.

1.8.3. Expression of PPO

The presence of multiple genes of PPO and its differential expression in different parts of plant and at different stages of development is one of the most important phenomena

reported (Steffens et al., 1994). Differential tissue specific expression of six genes coding for PPO in potatoes has been reported by Thygesen et al. (1995). Again, the expression of seven genes in different tissues of tomatoes has been reported by Thipyapong et al. (1997). Other early contributions to this aspect are the observations that apple PPO is encoded by a multiple gene family, whose expression is up-regulated by wounding of the tissue. Two different genes are expressed at different stages of apple flower development, one gene coding for PPO being expressed only at the post-anthesis stage (Kim et al., 2001). There are few evidences that implicates PPO as defence genes possessing both constitutive and inducible modes of expression and play multiple functions. The constitutively abundant PPO in glandular trichomes of *Lycopersicon* spp. and *Solanum* spp. is reported to be responsible for entrapment of small-bodied insects via oxidative polymerization of trichome exudate. Alternatively, direct toxicity of quinones and covalent modification of nucleophilic amino acids by quinones as an antinutritive defense strategy have been proposed. Systemic induction of PPO expression might act as an additional line of defense to protect the growing parts of plants against further attack by pathogens and insects (Thipyapong et al., 1995). Recently, it has been shown that antisense down regulation of constitutive and inducible PPO activity resulted in pathogen hypersusceptibility, suggesting that PPO may possess a key role in plant defence systems. Vickers et al. (2005) manipulated the levels of PPO in transgenic sugarcane using constructs of sense and antisense to the native PPO gene and found change in the degree of browning.

Fungal PPO, as plant PPO, can be present in latent form which is activated by SDS or proteolysis or acid shock. SDS activation of PPO in beet root is reported to be reversible (Perez-Gilabert et al., 2004). Gandia-Herrero et al. (2005) suggested that a common peptide is involved in activation by SDS and trypsin. The evidence is not totally

compelling and it should be remembered that very old work on sugar beet chloroplast PPO already showed that even peptides with molecular weight of around 10 kDa still retain considerable PPO activity. This shows that by no means the entire protein is required for activity (Mayer, 1979). Further insight into the activation of a latent PPO comes from the work of Kanade et al. (2006) on PPO from *Dolichos lablab*, which shows that both acid and SDS change the environment of a single glutamic residue, close to the di-copper active site. As a result the active site is unblocked or opened and enzyme activity enhanced. An intriguing suggestion by the authors, as yet unproven, is that wounding or methyl jasmonate could cause localized acidification which results in conversion of a latent to an active enzyme.

1.8.4. Regulation of transcription

The promoter studies of PPO have been reported in tomato (Thipyapong et al., 1997) and pineapple (Zhou et al., 2003). In pineapple southern blot analyses suggested that pineapple contained two PPO genes, *PINPPO1 and PINPPO2*. Analysis of expression of PINPPO1 promoter GUS fusion constructs showed this promoter had a low basal activity and was cold and wound inducible. Striking homologies to gibberellin response complexes (GARC) were observed in sequences of both the PINPPO1 and PINPPO2 promoters, which indicated these promoters respond gibberellins. In tomato, analysis of PPO promoters as GUS fusion constructs showed that mechanical wounding and infection by fungal and bacterial pathogens induced transcription of one isozyme of PPO (PPOf). The PPO transcript levels systemically increased in young leaves when mature leaflets were injured. Different injuries, salicylic acid, ethylene, and jasmonates elicited developmental stage specific patterns of PPO expression. It was demonstrated in their study that cis-elements are sufficient for PPO induction and reside in the 5’ flanking region.

Again Newman et al. (2011) reported similar results in another isozyme of tomato PPO (PPOB). The PPOB promoter was sequenced (1975 bp 5' upstream) and subjected to homology analysis. Sequence similarity of this 5' upstream sequence was observed with nucleotide sequences of genes encoding enzymes/proteins active in phenylpropanoid biosynthesis, signal transduction and responsiveness to hormones and stresses. The putative cis-elements were also reported. Chimeric gene fusions were constructed linking PPOB 5' regions to the reporter gene, β-glucuronidase (GUS). The resultant transgenic plants were analyzed for GUS activity in various vegetative and reproductive tissues. It was shown that PPO B expression was tissue specific, developmentally regulated, and localized to mitotic or apoptotic tissues.

In barley (*Hordeum vulgare*), two PPO enzymes were identified and their expression was differentially expressed in seed coat (Taketa et al., 2010). An insertion of a hAT-family transposon in the promoter region of PPO2 was indicated to be responsible for different expression patterns of the duplicate PPO genes in barley.

1.8.5. Cloning and expression

In potato (*Solanum tuberosum*) and pokeweed (*Phytolacca americana*) two PPO cDNAs clones from leaves were reported which were used for nucleotide sequence characterization (Hunt et al., 1993; Joy et al., 1995). In these studies The PPO cDNA was made from mRNA using reverse transcriptase enzyme and oligo dT primer. The second primer was made from the conserved copper binding region. The part of the PPO gene was PCR amplified using these two primers. This fragment of PPO gene was used as a probe for screening the cDNA library. The successful clones were sequenced and used for characterization of the PPO gene. In grape berry the PPO cDNA clone was constructed from the sequence of two overlapping cDNA clones using 5' and 3'-RACE (Rapid

Amplification of cDNA Ends) PCR (Dry and Robinson, 1994). A degenerate oligonucleotide primer based on the N-terminal amino acid sequence of purified grape PPO protein was used for this study. In case of apple (*Malus pumila*) PPO gene was expressed in *E. coli*, and the gene product (56 kDa) without a transit peptide was immunochemically detected and was the same size (*ca*. 65 kDa) as the main PPO of apple fruit by SDS-PAGE (Haruta et al., 1998). The DNA coding region for one of the PPOs from apple fruit was cloned and expressed in *E. coli*. The PPO contained a transit protein and was processed to a mature PPO, Mw 56 kDa. Although the protein expressed in *E. coli*, Mw 56 kDa, was detected using antibodies, the gene product was enzymically inactive (Haruta et al., 1998). Molecular cloning and characterization of PPO gene has been reported in apricot (*Prunus armeniaca*) fruit (Chevalier et al., 1999), hybrid poplar (*Populus trichocarpa* x *Populus deltoides*) plant (Constabel et al., 2000) and red clover (*Trifolium pretense*) leaves (Sullivan et al., 2004). In apricot, the PPO gene was isolated from cDNA library and cloned into T-tailed pBluescript vectors and transformed in *E.coli.* However, the overexpression studies in *E.coli* were not reported. In poplar also the PPO cDNA clone was isolated from cDNA library and the probe was used in northern hybridization to study the expression of PPO in response to wounding and herbivory. In red clover grass leaves PPO cDNA was prepared, cloned and expressed in *E. coli.* However, the expressed protein in *E. coli* formed insoluble inclusion bodies. The protein could not be purified from inclusion bodies using nickel metal affinity chromatography. This PPO gene when expressed in alfa alfa (*Medicago sativa*) surprisingly showed significant enzyme activity. In tomato, overexpression of polyphenol oxidase in transgenic tomato plants resulted in enhanced bacterial disease resistance (Li and Steffens, 2002). In order to investigate the role of PPO in plant disease resistance, transgenic tomato (*Lycopersicon esculentum*) plants that overexpressed a potato cDNA under control of the cauliflower mosaic virus 35S promoter. The transgenic plants

expressed up to 30-fold increase in PPO transcripts and 5 to 10-fold increases in PPO activity and immunodetectable PPO. Arican and Gozukirmizi, N. (2003) reported reduced PPO activity in transgenic potato plants associated with reduced wound inducible browning phenotypes. Wounded transgenic plants with reduced PPO activity exhibited a great amount of reduced browning when compared with control plants. Wang and Constabel (2004) studied the defensive role of leaf PPO in transgenic poplar plants overexpressing PPO from another variety. The transgenic plants showed high PPO enzyme activity and resistance to forest tent caterpillar larvae. Thipyapong et al. (2007) reviewed the experiments in which the roles of PPO in disease and insect resistance were investigated using transgenic tomato (*Lycopersicon esculentum*) plants with modified PPO expression levels (suppressed PPO and overexpressing PPO). These transgenic plants showed normal growth, development and reproduction under laboratory conditions. Antisense PPO expression dramatically increased susceptibility while PPO overexpression increased resistance of tomato plants.

1.9. Posttranslational modification

The synthesis of PPO and its transport to its site in chloroplasts, where plant PPOs are thought to be located, is a complex process, but it has the general features of import of nuclear coded proteins into sub-cellular organelles. The PPO is translated as a precursor protein and undergoes N-terminal and C-terminal processing along with phoshorylation, N-myristoylation, and O-glycosylation sites. In greenache grapes amino acid sequence of leaf PPO was deduced together with predicted sites of posttranslational modification (Virador et al., 2010). Possible phosphorylation sites are abundant and consistent with some reports on phosphorylation of PPOs. However, the mature active form that was crystallized was devoid of phosphorylation and glycosylation. The occurrence of six

potential myristoylation sites in this grape was also predicted which indicated the probable association with membrane (Virador et al., 2010).

1.9.1. Targeting of PPO and N-terminal processing

The synthesis of PPO and transport to its site in chloroplasts is a complex process, but it has the general features of import of nuclear coded proteins into sub-cellular organelles. Plant PPOs contain a bipartite N-terminal plastidic transit peptide directing the protein to the plastid lumen. This N-terminal transit peptide is cleaved off in two steps to generate the mature protein. The pathway by which plant PPO reaches the chloroplast was investigated by Sommer et al. (1994). Using an in vitro system it was found that tomato PPO, coded by cDNA, was processed in pea chloroplasts in two steps during its import. The precursor PPO with Mw, 67 kDa was imported into the stroma of the chloroplasts by an ATP-dependent step. It was then processed into a 62 kDa form by a stroma peptidase. Subsequent transport into the lumen was light dependent and resulted in the mature 59 kDa form. Apparently, such processing is a feature of all chloroplast located PPOs. The precursor protein contains a transit peptide, which must be removed in order that the PPO reaches its site in the chloroplast. The processing is carried out by a stromal peptidase, which was purified and characterized (Koussevitzky et al., 1998). The peptide bond cleaved by the processing proteases was identified as alanine-serine or alanine-alanine in most plant PPO examined so far. The transit peptide is characterized by the presence of three domains: The N-terminal 25 amino acids contain many hydroxyl containing amino acids, the middle part is more loosely conserved and called the ‘n’-region, while the C-terminal 25 amino acids form a hydrophobic domain, which is the thylakoid transfer domain (lumen targeting). The import and processing did not require Cu^{2+}, but import was inhibited by micromolar concentrations of Cu^{2+}. Further studies revealed that this inhibition was probably due to this inhibition of the stromal peptidase involved in the

processing of the precursor protein (Sommer et al., 1995). N-terminal protein sequencing by Edman degradation of the mature protein has established the transit peptide cleavage site for PPO from sweet potato (*I. Batatas*), tomato (*L. esculentum*; PPOE), potato (*S. tuberosum*; POT32) tubers, spinach (*S. oleracea*) thylakoid, wheat (*Triticum aestivum*), bean (*V. Faba*), and grape (*V. vinifera*). The transit peptide cleavage site for many other plants has been predicted based on homology.

The location of fungal PPO is not entirely clear. Generally it appears to be a cytoplasmic enzyme. The products of fungal and bacterial genes lack the N-terminal domain, since these organisms also lack the plastids to which the plant PPO is targeted. However, a PPO from *Pycnoporus* over produced in *Aspergillus niger*, could be targeted to the extracellular growth medium (Halaouili et al., 2006). An additional PPO is present in the mycelium of *Pycnoporus saguineus*, which has a very high tyrosinase activity and is able to convert coumaric acid to caffeic acid *in vitro* (Halaouili et al., 2005). An interesting feature of tyrosinase from *Agaricus* is that it contains putative glycosylation and phosphorylation sites, although no glycosylation or phosphorylation has so far been reported for fungal PPO (Wichers et al., 2003). Although fungal PPOs does not have transit peptides, two reports indicated that PPO from some fungi do undergo N-terminal proteolysis of a few residues (Flurkey et al., 2008). N-terminal protein sequencing of the active enzymes from *Pycnoporus sanguineus* and *Trichoderma reesei* revealed that 4 and 18 residues had been cleaved from their N-termini, respectively. In the case of *T. reesei* PPO, these 18 residues constitute a signal peptide. Unlike all fungal PPO studied to date, *T. Reesei* PPO is excreted. On the other hand, N-terminal protein sequencing of *Neurospora* crassa PPO indicated that this protein did not undergo any N-terminal proteolysis except the initial methionine.

1.9.2. C-Terminal processing

Most of the PPOs that have been studied are known to undergo significant processing to cleave the C-terminal domain, and gene sequences suggested the existence of similar processing sites in a host of related proteins. The C-terminal cleavage is thought to be carried out by enzymes in the thylakoid lumen. The C-terminal processing takes place in the linker sequence (WLPKNTKAKAK), which begins shortly after a tyrosine motif (YQY). A proline residue in this linker sequence was found to be the actual site of proteolysis (Virador et al., 2010). For *V. faba* and *V. vinifera* PPOs the 40 kDa active form has been shown to result from proteolytic cleavage of a C-terminal fragment from the 60 kDa latent form (Flurkey et al., 2008). C-terminal processing occurs for the PPO from the sweet potato (*I. batatas*) as well. The crystal structure has been determined for an active 39 kDa form of *I. batatas* PPO which is missing a C-terminal fragment, apparently as a result of *in vivo* proteolysis. Similarly, the 75 kDa precursor of tyrosinase from the ascomycete *N. crassa* is C-terminally processed to a 46 kDa active form (Flurkey et al., 2008).

CHAPTER 2

Comparative Evaluation of PPO Activity in Different Brinjal Cultivars

2.1. Introduction

The polyphenol oxidase (PPO) enzyme in plants oxidizes phenolics in the presence of oxygen on cut surface of fruits and vegetables, producing quinones which autopolymerise to form brown colour pigments (Madinez and Whitaker, 1995). During physical cutting, disruption of cellular structure leads to the release of PPO enzyme and its phenolic substrate. Many cultivars of brinjal (*Solanum melogena*) are available with varying morphological features such as colours (purple, green, purple with white and green stripes and patches), shapes (ovoid, obovate, oblong, cylindrical, club shaped), and calyx (spiny, non-spiny) (Raigón et al., 2008). These cultivars differ in the extent of post-cut browning which could be due to the variations in PPO activity or level of soluble phenolics. There are a few reports on characterization of PPO from brinjal. Roudsaria et al. (1981) reported purification of PPO using chromatography. Pérez-Gilabert and Carmona (2000) and Doğan et al. (2002) have characterized the ammonium sulfate precipitated PPO from brinjal. Concellón et al. (2004) have also reported the PPO activity of the crude extract during low temperature storage of brinjal. There are also reports about the phenolic compounds present in brinjal with chlorogenic acid as the major component (Luthria et al., 2010). In the current study, comparative evaluation of PPO enzyme activity, substrate status, and the overall browning index in different brinjal cultivars have been performed. The findings will help in understanding the contribution of these factors in post-cut browning process in fresh as well as stored brinjal.

2.2. Materials and methods

2.2.1. Chemicals

Agarose, ammonium sulfate, ascorbic acid, Bradford reagent, cetyl trimethyl ammonium bromide, chloroform, chlorogenic acid, disodium hydrogen phosphate, Folin-Ciocalteu reagent, gallic acid, isoamyl alcohol, 4-methyl catechol, sodium dihydrogen phosphate, polyvinyl pyrrolidone (PVP), polyvinyl polypyrrolidone (PVPP), sodium carbonate, and triton X-100 were procured from Sigma-Aldrich Inc., St. Louis, MO.

2.2.2. Brinjal cultivars

Eight major popular cultivars including 'Pusa purple long', 'Ravaiya', 'Azad kranti', 'Arka navneet', 'Kalpatharu', 'Raveena', 'Anupam', 'Silki' (listed in Table 2.1) of brinjal (*Solanum melongena*) were procured from a local supplier (4 kg of each variety). The vegetables were cleaned in potable water and stored at ambient (26 ±2 ºC) as well as 10 ºC temperature and 62% humidity. The samples were analysed periodically for browning index, PPO activity, and total soluble phenolics.

2.2.3. Determination of extent of browning

For browning measurement of cut brinjal, reflectance in visible spectrum region (360 to 780 nm) was recorded at 10 nm wave length interval using a Minolta CM-3600D spectrophotometer (Konica Minolta Sensing, Inc., Osaka, Japan). D65 lamp was used as reference light source and the detector was fixed at an angle of 10º with respect to the light source (Ramírez-Jiménez et al., 2000). The equipment was calibrated prior to analysis with a standard white tile and a black box for 100 and 0% reflectance,

respectively. The colour parameter used was CIE L (Lightness) which denotes the amount of light or luminance reflected from the sample. The browning/darkening was calculated as (100-L), which is opposite of lightness (Ramírez-Jiménez et al., 2000).

2.2.4. Extraction of PPO

Cut brinjal pieces (30 g) were frozen in liquid N_2, ground to fine powder and homogenized in 100 ml of extraction solution for better extractability using a polytron homogenizer (Model PT3100, Kinematica AG, Switzerland). The extraction solution contained sodium phosphate buffer (pH 6.8, 0.05 M) with polyvinyl pyrrolidone (PVP) (1%), polyvinyl polypyrrolidone (PVPP) (2%), triton X-100 (1%) and ascorbic acid (30 mM). The extract was stirred for 30 min, filtered using muslin cloth, and centrifuged (5810R, Eppendorf, Hamburg, Germany) at 10000 g for 15 min at 4ºC. The supernatant was collected and fractionated using ammonium sulfate precipitation (up to 80%). Initially, to remove most hydrophobic proteins, 20% ammonium sulfate precipitation was performed. Later, the supernatant of this fraction was saturated upto 80% ammonium sulfate and precipitated. This precipitate represented the total PPO. Based on earlier findings supernatant of 20% fraction was also parallelly fractionated using 10% sequential increase of ammonium sulfate which resulted in precipitation of two isoforms of PPO called PPO 1 (precipitated at 30% ammonium sulfate fractionation) and PPO 2 (precipitated at 70% ammonium sulfate fractionation) (Englard and Seifter, 1990). The individual fractions were solubilized in 10 ml of phosphate buffer (pH 6.8, 20 mM) and dialysed using 10 kDa cut off membrane in 3 litre phosphate buffer (pH 6.8, 2 mM) at 4ºC with three buffer changes at 4 h interval for removal of salt.

2.2.5. Assay of protein and PPO activity

The polyphenol oxidase (PPO) enzyme activity was determined spectrophotometrically using 4-methyl catechol as a substrate (Concellón et al., 2004). The enzyme assay was carried out taking 0.88 ml of phosphate buffer (pH 6.8, 50 mM), 0.1 ml substrate (0.1M) and 0.02 ml of enzyme extract (prepared as discussed later). The increase in absorbance at 420 nm was monitored at 30 sec interval for 3 min using a spectrophotometer (Model UV 4-100, Unicam, Cambridge, UK) and the average change in absorbance per min was calculated. One unit of enzymatic activity was defined as the amount of enzyme which caused a change in absorbance of 0.1/min. The PPO activity was expressed as U/g of brinjal weight. The specific activity was determined by expressing PPO activity/mg protein. Protein content of the brinjal extract was determined by the Bradford method (1976), using bovine serum albumin (BSA, Sigma Chemical, St. Louis, USA) as standard.

2.2.6. Estimation of total soluble phenolics

Brinjal pieces (7.5 g) were soaked in 50 ml of 80% methanol and homogenized using a polytron homogenizer (Model PT3100, Kinematica AG, Switzerland). The suspension was centrifuged at 10,000 g for 20 min and the supernatant was collected. A 25 µl of aliquot of the supernatant was mixed with equal volume of milli Q water and further mixed with 50 µl of 0.2 N Folin-Ciocalteu reagent. The suspension was incubated at ambient temperature (26±2ºC) for 15 min and later mixed with 0.15 ml of sodium carbonate solution (0.2 g/ml). The reaction mixture was incubated in a water bath at 40ºC for 20 min. Later placed on ice for 5 min and then the absorbance was measured at 755 nm using a spectrophotometer (Model UV 4-100, Unicam, Cambridge, UK). The total

phenolic content was calculated using gallic acid as a standard and expressed as mg GA equivalent/g of brinjal (Luthria et al., 2010).

2.2.7. Estimation of chlorogenic acid and HPLC profile of other phenolics

Chlorogenic acid was estimated using HPLC by the method of Luthria et al. (2010). Brinjal (7.5 g) was soaked in 50 ml of 80% methanol and homogenized using a polytron homogenizer. The suspension was centrifuged at 10,000 g for 20 min, the supernatant was collected and membrane (0.2 µm) filtered. A 60 µl aliquot was loaded on a reverse phase C18 column. The elution was carried out using a gradient of formic acid (0.1%) from 90 to 55% over methanol for 26 min at a flow rate of 1 ml/min. The detection was performed at 350 nm using a UV detector. The chlorogenic acid concentration in brinjal was calculated using pure commercially available chlorogenic acid (retention time 16 min in specified mobile phase) as standard. The phenolics profile of cut brinjal before and after browning was performed using HPLC of 80% methanol extract and using a gradient of acetic acid (0.2%) from 100% to 0% over methanol for 30 min at flow rate of 1 ml/min and the detection was carried out at 285 nm.

2.2.8. Statistical analysis

Experiments were repeated in three sets independently, each set having 3 replicates. The means and SD were calculated taking all the readings into consideration. Two way ANOVA was performed to ascertain the significance of difference of the means. Statistical analysis was performed using BioStat 2009 Version Professional 5.8.0.0 (AnalystSoft Inc., Canada) .

Table 2.1. The different brinjal cultivars studied and their characteristics.

	Fruit characteristics*	Picture	Cultivar	Fruit characteristics	Picture
Pusa purple long (V1)[xx]	Club shaped Purple (60 ± 20 g)		Kalpatharu MEBH 10 (V5) [xx]	Ovoid reddish purple with white stripes, spiny calyx (50 ± 15 g)	
Ravaiya MEBH39 (V2) [xx]	Ovoid Purple (40 ± 10 g)		Raveena (V6) [xx]	Cylindrical Green (60±15 g)	
Azad kranti (V3) [xx]	Club shaped Purple (300±50 g)		MHB Anupam (V7) [xx]	Club shaped green (300±50 g)	
Arka navneet (V4) [xx]	Obovate Purple (400±100 g)		Silki (V8) [xx]	Ovoid, spiny calyx purple with green stripes (40 ± 10 g)	

*Fruit calyx is without spines unless mentioned. Values in parenthesis indicate average weight per fruit ± standard deviation.

[xx] Coding of different cultivars used for ease of description

3. Results and Discussion

The varieties of brinjal display a wide range of shapes and colours, starting from pure white to purple, black, green, and variegated in different shades (Hazra and Banerjee, 2005). The fruit characteristics (shape, colour, and average weight) and photographs of different brinjal cultivars used in this study are detailed in Table 2.1. The local names of these cultivars differ in different parts of India. However, the shape is considered as a very stable genotype dependent character and cultivars can be identified from the morphology. Among the cultivars, the 'Kalpatharu' is maximally grown and available in most parts of India. In India during summer and winter season the average shelf life is reported to be 1-2 and 3-4 days, respectively (Dhaliwal, 2007). Under ambient temperature and storage (26 ± 2 ºC) and 80% relative humidity the shelf life is about 8-10 days. The vegetable with bigger size have higher shelf life compared to the smaller varieties.

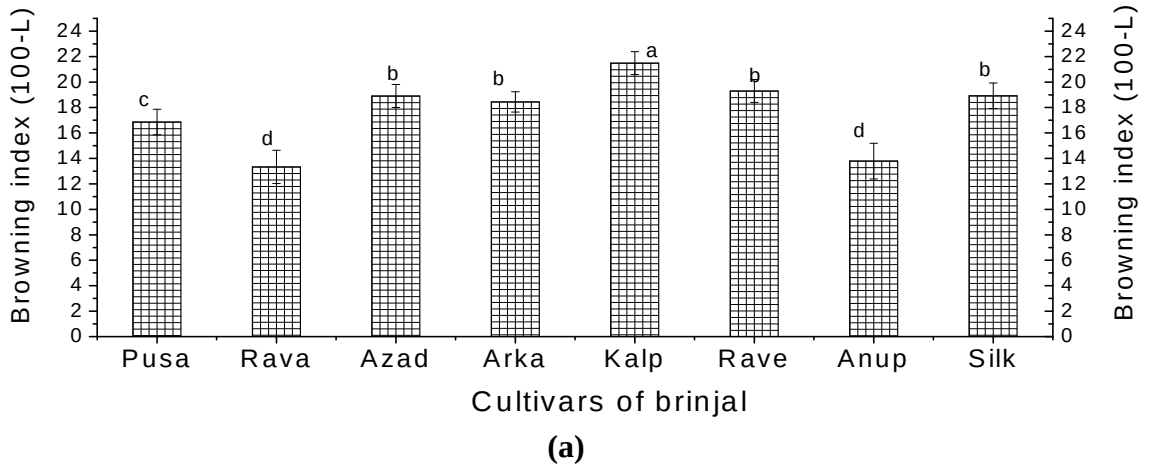
2.3.1. Extent of browning varied among cultivars

Browning discolouration started immediately after cutting and increased with time. The measurement of browning after 20 min of cutting was found to be suitable for comparison among the cultivars and shown in Figure 2.1a. The 'Kalpatharu' (V5) showed maximum browning followed by other cultivars (V3, V4, V6, and V8) which showed similar level browning and the overall extent was about 11 % lesser than V5. The browning in 'Ravaiya' (V2) and 'Anupam' (V7) was found to be further less and difference among them was insignificant ($P \leq 0.05$). These two cultivars showed minimum browning and were about 39 and 35% less compared to V5, showing maximum browning. As PPO and its phenolic substrates have been reported as major factors responsible for post-cut

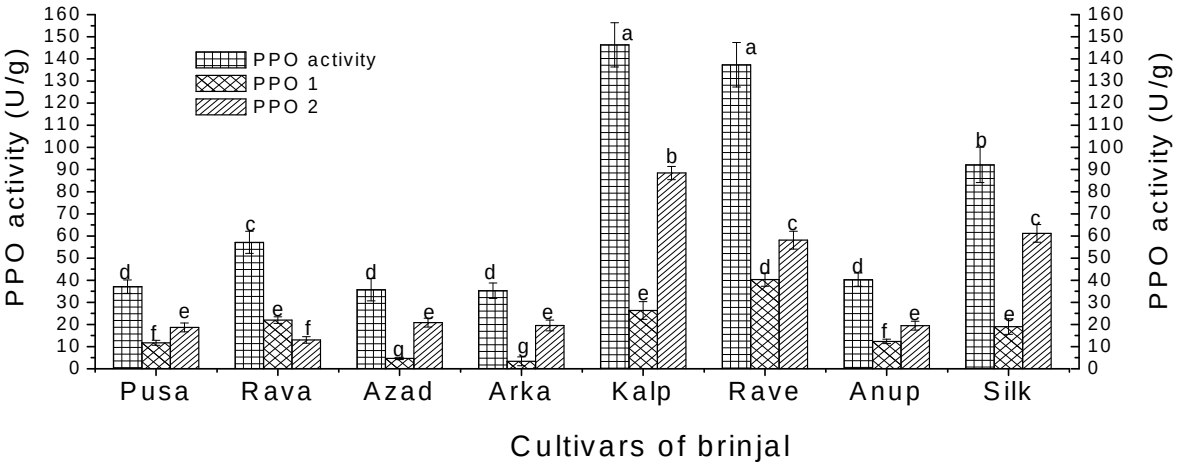
browning in various other fruits and vegetables, it was interesting to know their status in these brinjal cultivars too.

2.3.2. Cultivars showing maximum browning exhibited higher PPO activity

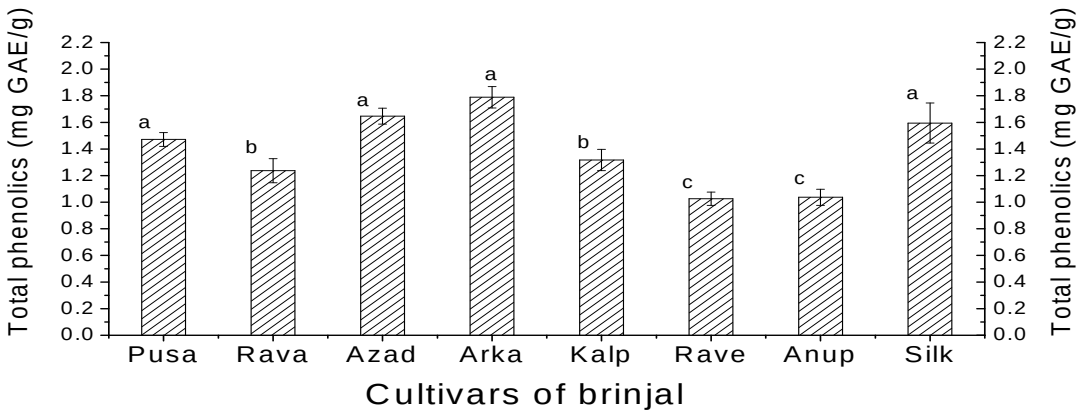
The maximum PPO activity was observed in brinjal cultivars 'Kalpatharu' (V5) and 'Raveena' (V6) followed by 'Silki' (V8) (Fig. 2.1b). The cultivar V2 showed lesser PPO activity, whereas, others (V1, V3, V4, V7) showed the least PPO activity. A significantly high PPO activity was observed in two discrete ammonium sulfate precipitated fractions (20-30% and 50-70%) compared to other fractions ($P \leq 0.05$) and could be due to the presence of two isoforms of PPO (Fig. 4.1a). These two fractions after running on native PAGE were stained with the substrate (4-methyl catechol) in the presence and absence of inhibitor potassium metabisulfite (PMS) for ruling out the enzyme activity by any other proteins including peroxidase (Mayer, 2006). The results detailed in chapter 4 showed that these two are indeed PPO isoforms localized at different positions on gel (Fig. 4.1b). In all these cultivars PPO2 was found to be predominant, except 'Ravaiya' (V2). Comparatively, the overall activity of PPO2 was significantly less in 4 cultivars (V1, V4, V3, and V7). The total PPO activity has been observed more than the individual activity of PPO1 and PPO2 in all the cultivars. This also indicated that possibly total PPO activity could be representing both these activities together. The varietal difference in PPO activity has been reported in other fruits and vegetables including litchi which probably could be due to variations in its level of expression or bioactivity. Existence of isozymes of PPO has been reported in other fruits and vegetables including potato (Thygesen et al., 1995). In brinjal too different alleles of PPO have been reported in root, leaf and fruit (Shetty et al., 2011).



(a)



(b)



(c)

Figure 2.1. (a) Browning index in different brinjal cultivars. **(b)** The total PPO activity and activity of PPO1 and PPO2. **(c)** The total soluble phenolic content of different brinjal cultivars. The different letter superscript indicated significance of difference ($P \leq 0.05$). The initial 4 letter of cultivar name was used as their abbreviation in figures. U–unit of PPO activity (change in 0.01 absorbance/ min).

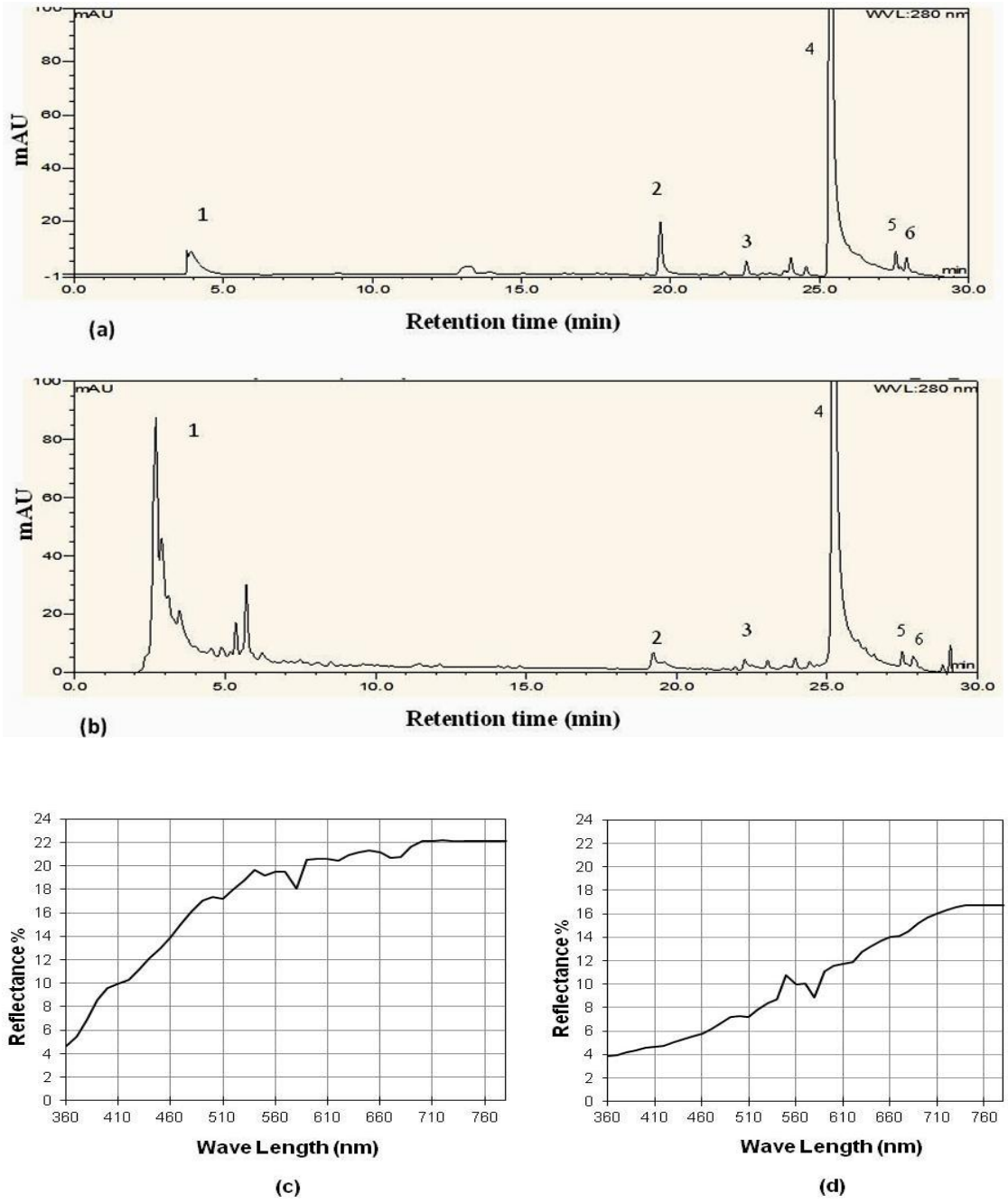


Figure 2.2. The phenolics profile of freshly cut brinjal (a) and brinjal after browning (b) using HPLC with detection at 280 nm. [1; benzoquinone and adducts ($t_{R,}$ 2-6 min), 2; dihydroxycinnamoyl amide ($t_{R,}$ 19 min), 3; dicaffeoylspermidine ($t_{R,}$ 22.5 min), 4; chlorogenic acid (5-caffeoylquinic acid, $t_{R,}$ 25.5 min)), 5; 5-caffeoylquinic acid ($t_{R,}$ 27.5 min), 6; 5-cis-caffeoylquinic acid($t_{R,}$ 28 min)]. The reflectance sprectrum (360-780 nm) of fresh (c) and brown (d) cut brinjal.

2.3.3. Phenolic concentration varied significantly among cultivars

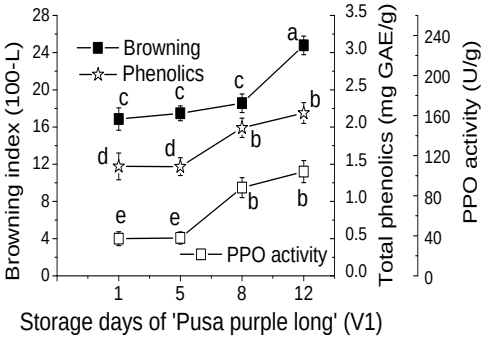
The phenolic content in brinjal cultivars has been shown in Figure 2.1c. It was found to be the highest in brinjal 'Arka navneet' (V4). In the case of cultivars 'Pusa purple long' (V1), 'Azad kranti' (V3), and 'Silki' (V8) too, phenolic content was statistically similar to that of V4. The cultivar V2 and V5 showed about 28% less, whereas, V6 and V7 showed about 45% less phenolics than V4. Phenolic content has been reported to vary in different cultivars of fruits and vegetables (Camm and Towers, 1973).

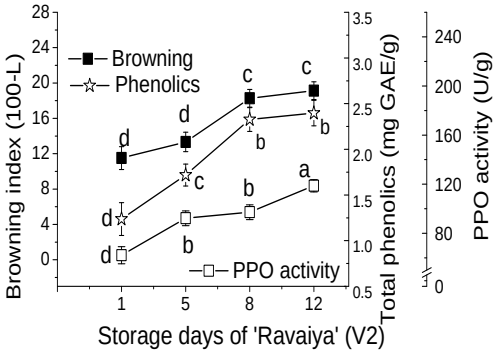
2.3.4. Kinetics of changes in browning index, PPO activity and phenolic content in stored brinjal

The change in browning, phenolics and PPO activity was evaluated during ambient temperature (26 ±2º C) storage of different brinjal cultivars up to 12 days (Fig. 2.2). The dynamics of these parameters were found to significantly change during storage and profound cultivar based variations were observed.

2.3.4.1. Browning index, PPO activity and soluble phenolics increased during ambient temperature storage in four cultivars

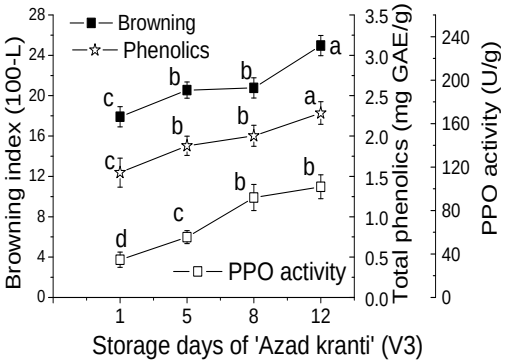
Four cultivars 'Pusa purple long' (V1), 'Ravaiya' (V2), 'Azad kranti' (V3), and 'Arka navneet' (V4) showed increase in browning index and phenolics along with increase in PPO activity and phenolic content during storage (Fig. 2.2a, 2b, 2c, and 2d). Cultivar 'Pusa purple long' (V1) with respect to fresh brinjal samples did not show any significant increase in browning until day 8 and then increased significantly by about 50% on day 12 (Fig 2.2a). The PPO activity and soluble phenolics during the same period was increased by 160 and 44%,

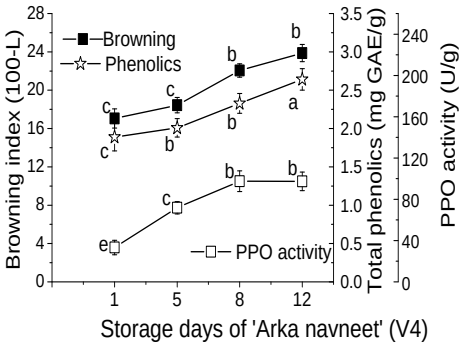

Browning
Phenolics
PPO activity
Browning index (100-L)
Total phenolics (mg GAE/g)
PPO activity (U/g)
Storage days of 'Pusa purple long' (V1)


Browning
Phenolics
PPO activity
Browning index (100-L)
Total phenolics (mg GAE/g)
PPO activity (U/g)
Storage days of 'Ravaiya' (V2)

(a)

(b)


Browning
Phenolics
PPO activity
Browning index (100-L)
Total phenolics (mg GAE/g)
PPO activity (U/g)
Storage days of 'Azad kranti' (V3)


Browning
Phenolics
PPO activity
Browning index (100-L)
Total phenolics (mg GAE/g)
PPO activity (U/g)
Storage days of 'Arka navneet' (V4)

(c)

(d)

Contd……

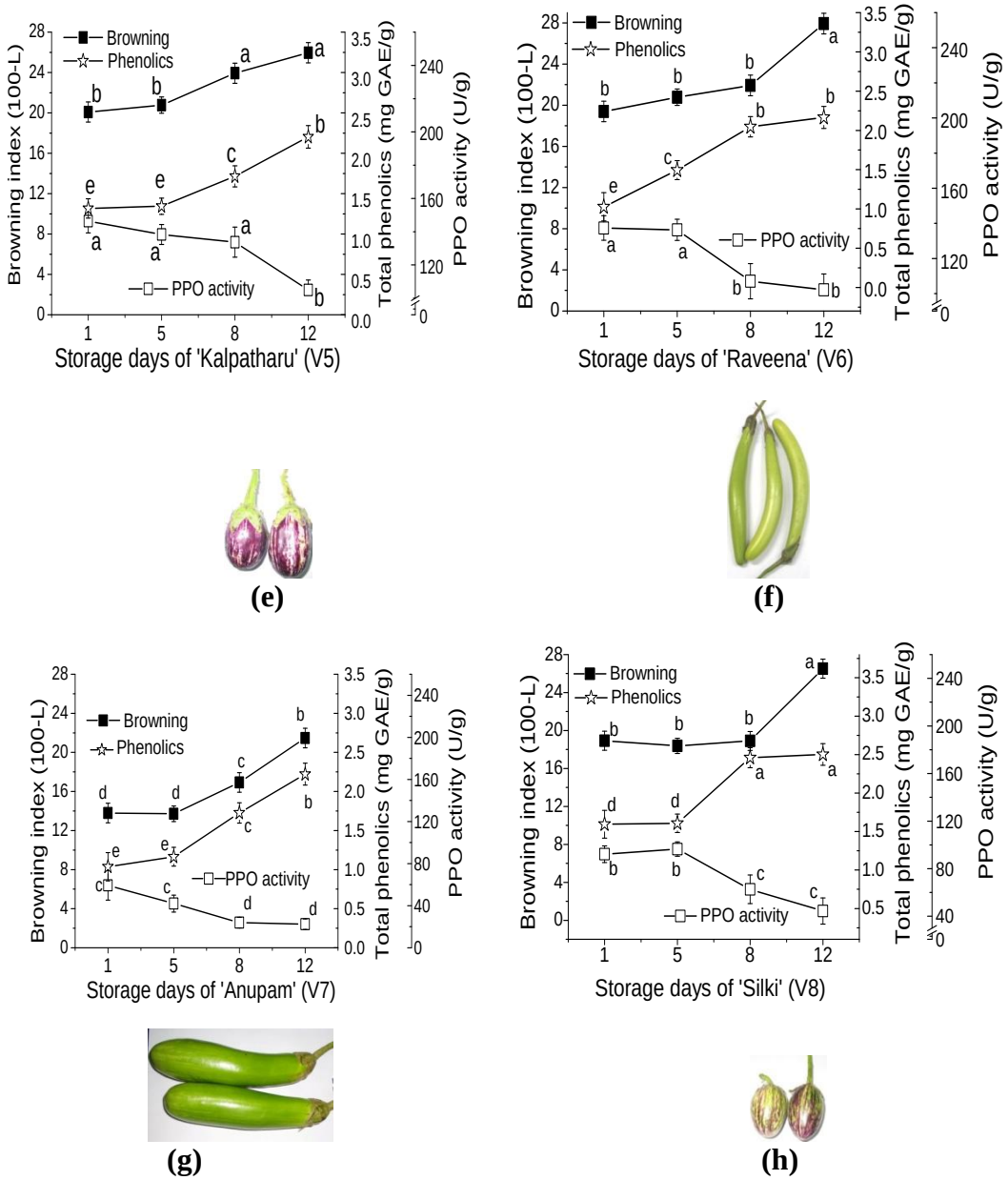
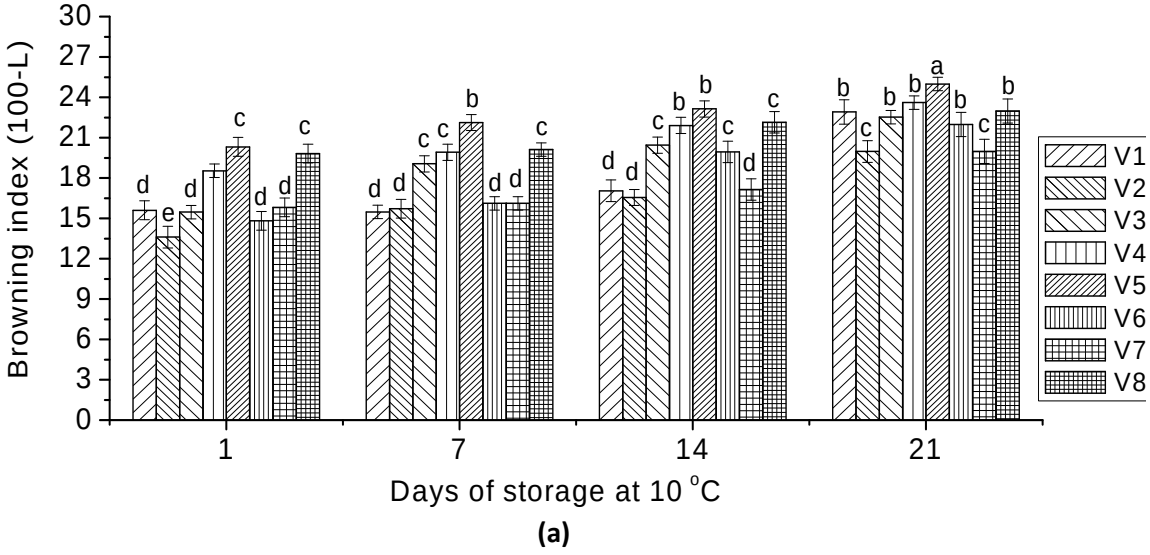


Figure 2.3. Comparative profile of browning index, PPO activity, and total soluble phenolic content of brinjal cultivars (a–h). For each parameter, data points with different letter superscripts are significantly different (P≤0.05). GAE – Gallic acid equivalent. U – unit of PPO activity (change in 0.01 absorbance/ min). U – unit of PPO activity (change in 0.1 absorbance/ min).

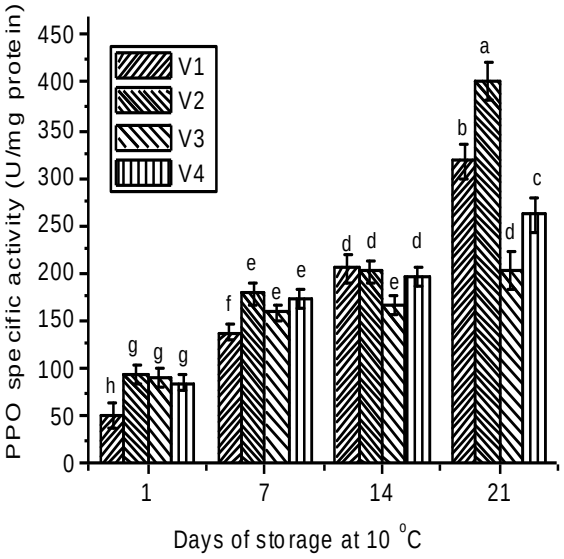
respectively (Fig. 2.2a). The increase in PPO activity was found to be comparatively very high than the phenolic content and browning index. In case of 'Ravaiya' (V2), an increase in these parameters was observed during storage. The browning and PPO activity were steadily increased to about 72 and 100% during 12 days of storage (Fig. 2.2b). The increase in browning index and phenolic content was highest in this variety. The phenolic level was increased by 125% on day 12 (Fig. 2.2b). In brinjal 'Azad kranti' (V3), the browning and PPO activity were increased by 38 and 185%, respectively (Fig. 2.2c). Among these cultivars the increase in PPO activity was found to be highest in V3. The phenolic content was increased by about 50% during 12 days of storage (Fig. 2.2c). In cultivar V4, the browning, PPO activity and phenolics were increased by 41, 180, and 36%, respectively (Fig. 2.2d). The activity of oxidative enzymes like PPO is known to increase during storage in fruits (Jiang et al., 2004). Such increase in PPO activity, phenolics and browning during storage was reported in two cultivars of litchi fruit (Mishra et al., 2011). The change in total phenolics during storage could be due the physiological changes associated with senescence of brinjal. The increase in PAL activity, which is a regulatory enzyme in phenolics biosynthesis, could also be the reason for such an increase in total phenolics (Camm and Towers, 1973).

2.3.4.2. Browning index and soluble phenolics increased but PPO activity decreased in rest of the four cultivars during ambient temperature storage

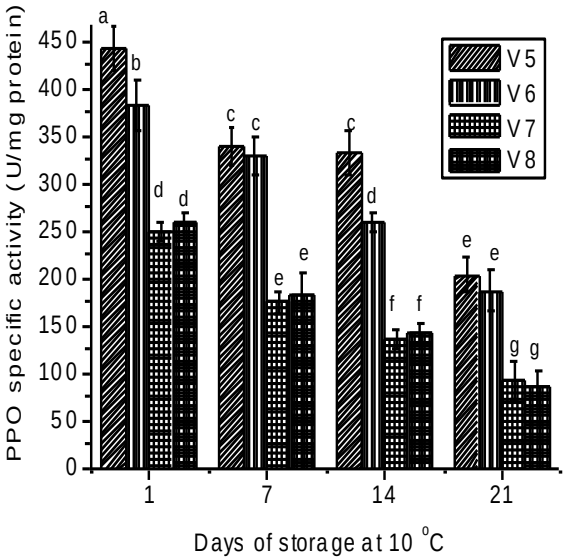
In brinjal 'Kalpatharu' (V5), 'Raveena' (V6), 'Anupam' (V7), and 'Silki' (V8), both browning index and phenolic content were found to increase during storage, however, PPO activity decreased (Fig. 2.2e, 2f, 2g, and 2h). Such a decrease in PPO activity has been reported earlier at low temperature storage of brinjal (Concellón et al., 2004). With respect to fresh brinjal samples, the browning and phenolics were increased by 30 and



(a)



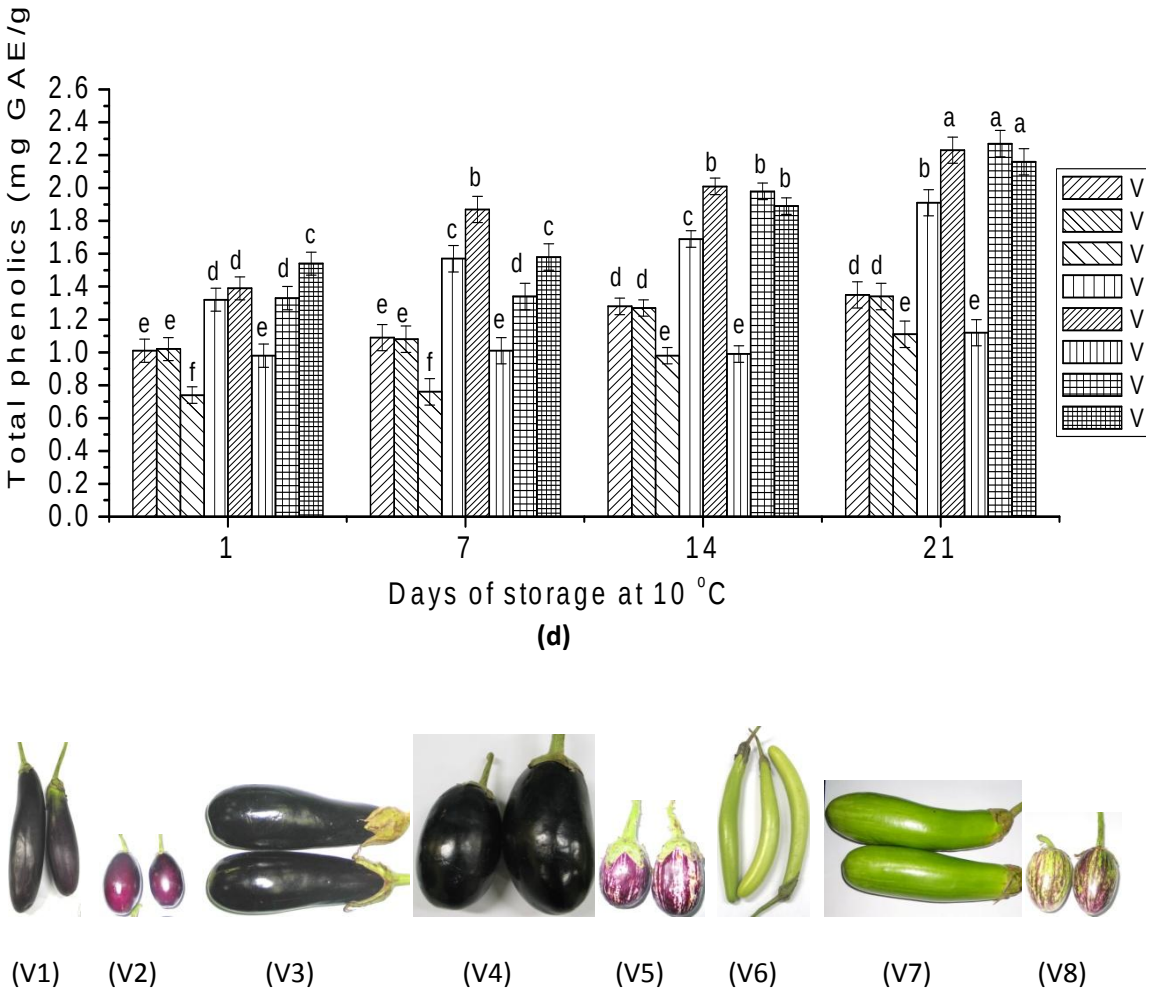
(b)



(c)

(V1) (V2) (V3) (V4) (V5) (V6) (V7) (V8)

Contd……



(V1) (V2) (V3) (V4) (V5) (V6) (V7) (V8)

Figure 2.4. Comparative profile of browning index (**a**), polyphenol oxidase (PPO) specific activity (**b, c**), and total soluble phenolics (**d**) in different eggplant cultivars during storage at 10 (±2)°C. For each parameter, data points with different letter superscripts are significantly different (P≤0.05). GAE – Gallic acid equivalent. U – unit of PPO activity (change in 0.1 absorbance/ min).

61%, respectively in V5, 36 and 56%, respectively in V6, 50 and 109%, respectively in V7, and 42 and 100%, respectively in V8 (Fig. 2.2e- h). The PPO activity decreased by 28% in V5, 46% in V6, 36% in V7, 27% in V8 (Fig. 2.2e- h). The increase in browning index even after the decrease in PPO activity signifies the role of free phenolics in post-cut browning in stored brinjal. Similar observation with respect to PPO activity has also been reported earlier in case of rambutan fruit (Yingsanga et al., 2008). In another report browning was reported to increase with increase in phenolic content where PPO activity remained unchanged during storage in longkong (*Aglaia dookkoo*) fruit (Lichanporn et al., 2009).

2.3.4.3. Cultivar based variation in PPO activity, phenolics and browning was storage temperature independent

During ambient temperature storage, all these cultivars underwent senescence which was determined in terms of loss of weight and firmness. The percentage weight loss and firmness in these cultivars after 12 days were found to be in the range of 12-23 and 18-31%, respectively. Similar percentage weight loss and firmness was observed after 21 days of storage in samples stored at 10ºC. This indicated that at low temperature storage eggplant senescence was delayed and an extension of shelf life up to 21 days was observed. The variation in PPO sp. activity, phenolic content, and browning was also analysed in eggplant fruit stored at 10ºC to assess if these changes were similar to those observed in case of ambient temperature stored samples. As observed in ambient temperature stored sample in group 1 cultivars (V1-V4), the browning, soluble phenolic content, and PPO sp. activity increased during storage (Fig. 2.4). However, in group 2 cultivars (V5-V8), the browning and soluble phenolics increased but PPO sp. activity

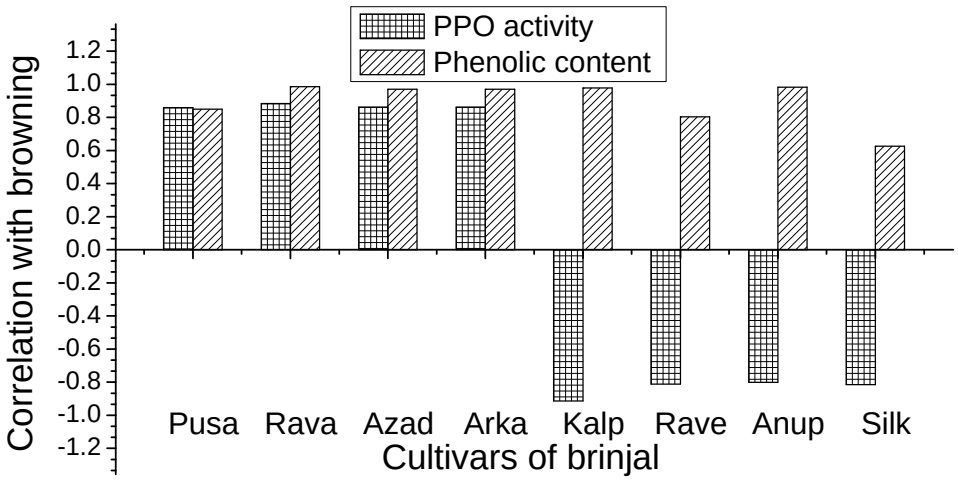


Figure 2.5. Correlation analysis of browning index with PPO activity and total soluble phenolics in different brinjal cultivars. The initial four letter of cultivar name was used as their abbreviation in figures.

decreased during storage. These findings indicated that the change in these parameters was senescence independent and could be the characteristic features of the specific cultivars. The decrease in storage temperature did not affect the enzyme activity and thus indicated the variety specific variation in the kinetics of PPO activity during storage.

2.3.4.4. Correlation analysis of browning with PPO activity and phenolics

In fresh samples of the majority of varieties, PPO activity was found to be comparatively well correlated with browning index. However, in stored samples phenolics were found to be the major contributors to the browning. In four cultivars (V1-V4) the PPO activity showed a positive correlation (*r*) in the range of 0.86 to 0.88, whereas, phenolics showed marginally higher correlation (*r*) in the range of 0.85 to 0.97 with browning (Fig. 2.4). The correlation of browning with phenolics was observed to be higher than the correlation between PPO activity and browning. Contrary to this in other four cultivars (V5-8) the browning increased with the increase in phenolics during storage but PPO activity decreased. In these four cultivars a strong negative correlation (*r*) was observed in the range of -0.80 to -0.91 among PPO activity and browning during storage (Fig. 2.4).

2.3.5. Chlorogenic acid content showed no correlation with browning and PPO activity

The concentration of chlorogenic acid in brinjal was reported to vary between 40-70% (Shetty et al., 2011). The HPLC chromatogram of 80% methanol extract which contained most of the soluble phenolics showed a single major peak which matched with chlorogenic acid standard compound and is shown in Figure 2.5. The chlorogenic acid content in brinjal cultivars has been compared with the phenolic content to observe the relative difference in their ratio among cultivars (Fig. 2.6). In most of the cultivars

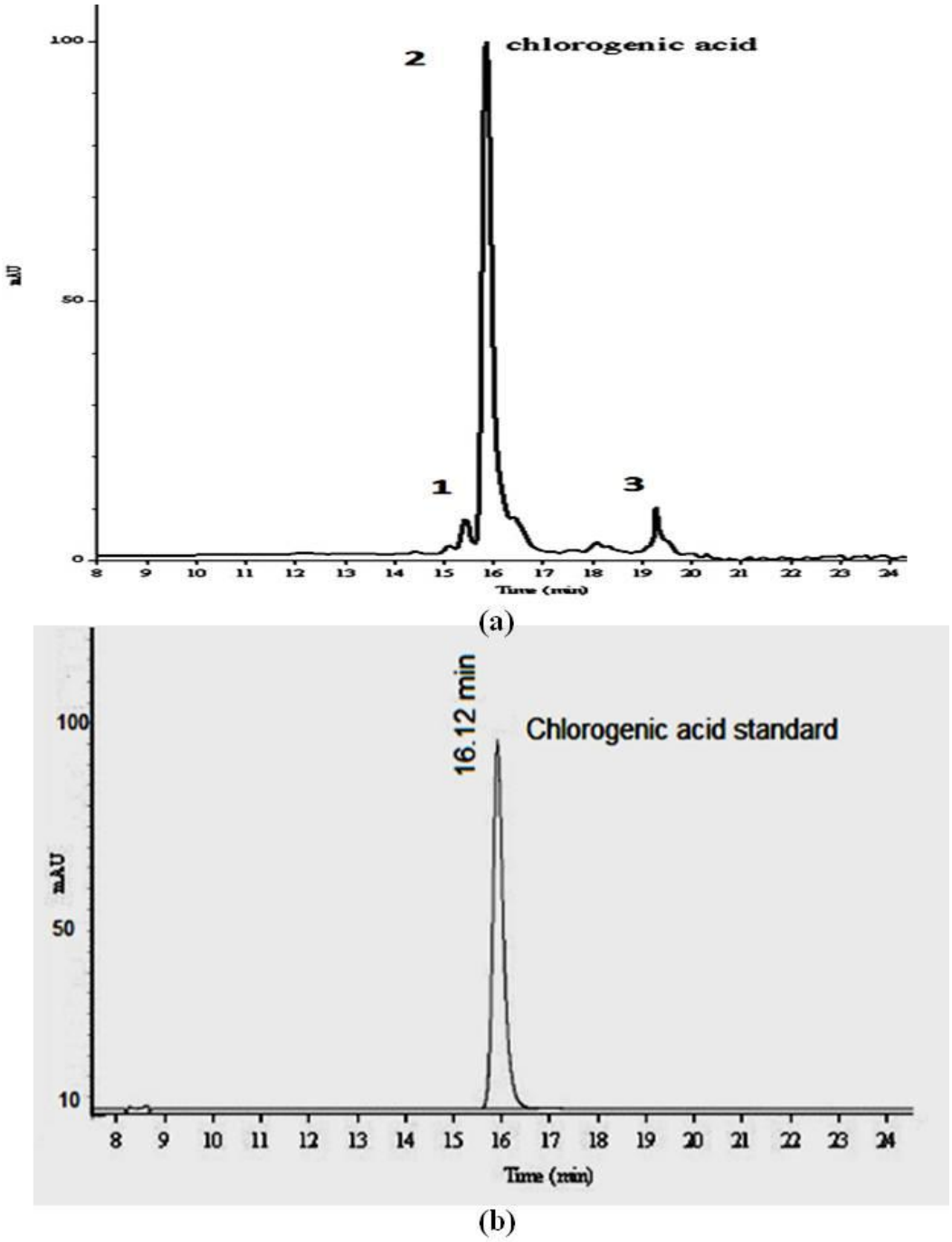


Figure 2.6. **(a)** The HPLC profile of methanol extract of brinjal showing chlorogenic acid as the major phenolic (peak 2). The other major peaks found were N, N'-dicaffeoylspermidine (peak 1), and 3-acetyl-5-caffeoylquinic acid (peak 3) (Singh et al., 2009). **(b)** The HPLC chromatogram of standard chlorogenic acid.

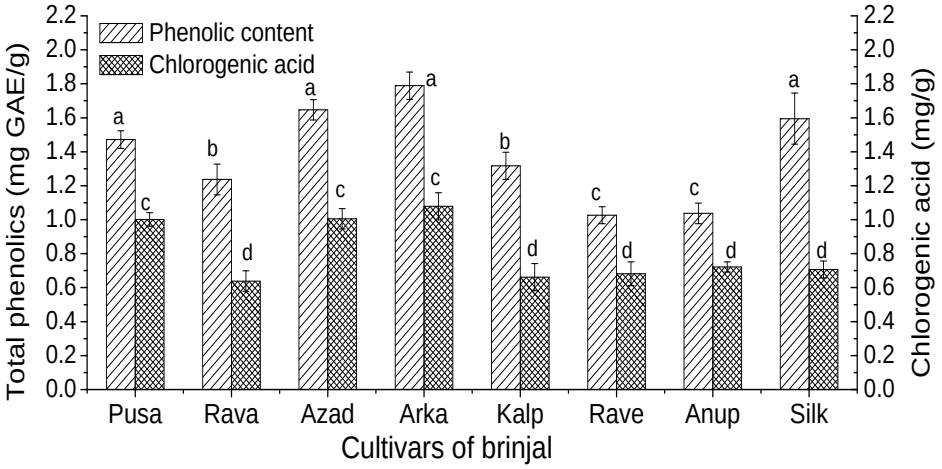
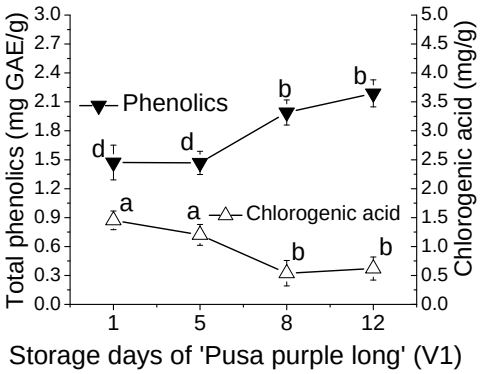
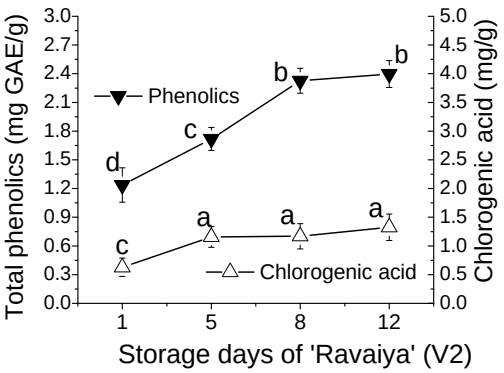


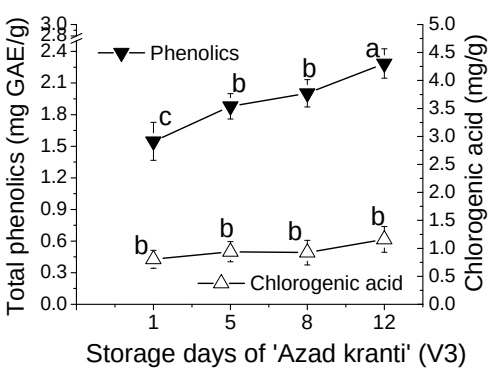
Figure 2.7. Comparison of chlorogenic acid and total phenolics among cultivars of brinjal. For each parameter, data points with different letter superscripts are significantly different ($P \leq 0.05$). The initial four letter of cultivar name was used as their abbreviation in figures. GAE – Gallic acid equivalent.


Total phenolics (mg GAE/g)
Chlorogenic acid (mg/g)
Phenolics
Chlorogenic acid
Storage days of 'Pusa purple long' (V1)

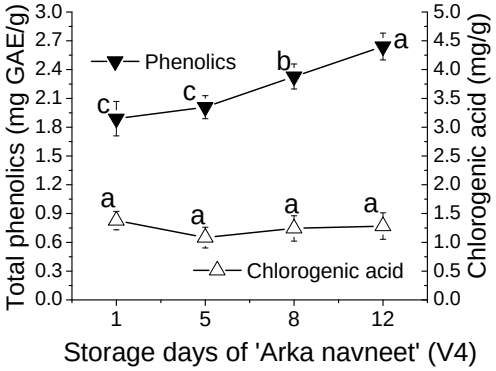
(a)


Total phenolics (mg GAE/g)
Chlorogenic acid (mg/g)
Phenolics
Chlorogenic acid
Storage days of 'Ravaiya' (V2)

(b)


Total phenolics (mg GAE/g)
Chlorogenic acid (mg/g)
Phenolics
Chlorogenic acid
Storage days of 'Azad kranti' (V3)

(c)


Total phenolics (mg GAE/g)
Chlorogenic acid (mg/g)
Phenolics
Chlorogenic acid
Storage days of 'Arka navneet' (V4)

(d)

Contd…..

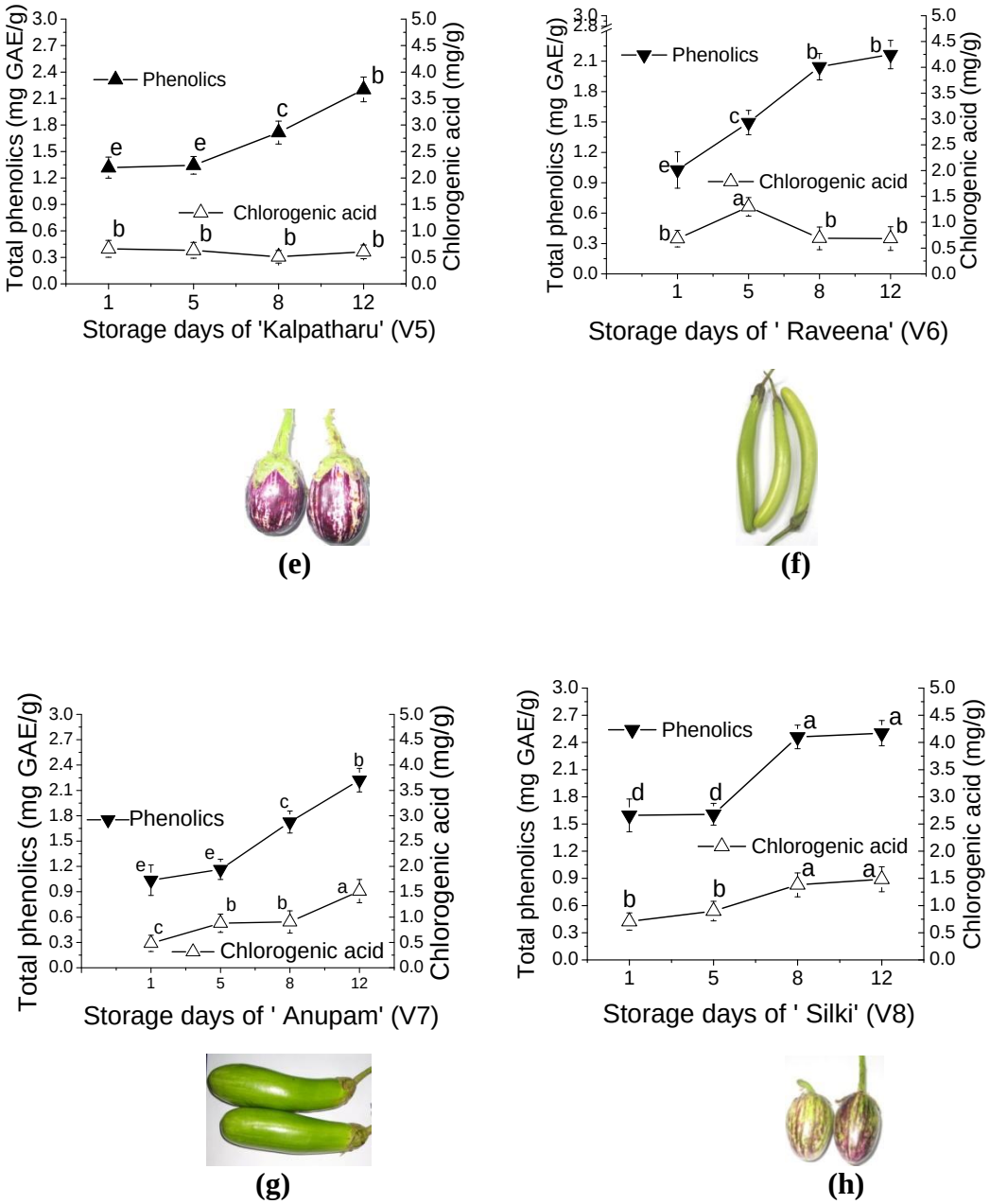


Figure 2.8. The comparison of change in phenolics and chlorogenic acid during storage in brinjal cultivars (a- h). For each parameter, data points with different letter superscripts are significantly different (P≤0.05). GAE – Gallic acid equivalent.

including V3 (‘Azad kranti’), V4 (‘Arka navneet’), (V5 ‘Kalpatharu’), and V8 (‘Silki’), the chlorogenic acid concentration was about 45-60% of the total soluble phenolics. In a few cultivars including V1 (‘Pusa purple long’), V6 (‘Raveena’), and V7 (‘Anupam’) the chlorogenic acid concentration was as high as about 70% of the total phenolics (Fig 2.6). Each fruit and vegetable was known to differ in concentration of phenolics. In apple the chlorogenic acid content was shown to differ among cultivars (Awad et al., 2000). The varietal difference is also common in many fruits and vegetables. The change in the concentration of chlorogenic acid compared to phenolic content in cultivars during storage is shown in Figure 2.7. Change in chlorogenic acid was also found to be different from change in phenolics during storage in majority of cultivars. In ‘Pusa purple long (V1), phenolics increased but chlorogenic acid content decreased during storage (Fig. 2.7a). In V2 (‘Ravaiya’), V3 (‘Azad kranti’), V7 (‘Anupam’), and V8 (‘Silki’) the increase in chlorogenic acid concentration was less compared to increase in phenolic content (Fig. 2.7b, c, g, h). In V4 (‘Arka navneet’) and V5 (‘Kalpatharu’), the chlorogenic acid concentration was almost stable, whereas, the phenolic content increased significantly during storage (Fig. 2.7d, e). Similarly, chlorogenic acid content did not show considerable correlation with PPO activity and browning in these cultivars.

2.4. Discussion

The extent of PPO activity and phenolic content differ in fruits and vegetables. These two factors seem to be the major determinants of browning in processed fruits and vegetables. The brinjal (*Solanum melogena*) is one of the vegetable displaying intense post-cut browning. Interestingly the post-cut browning in brinjal was found to be independent of its outer skin colour. The green and purple skin colour could be due to chlorophyll and anthocyanins, respectively. The ‘Kalpatharu’ (V5) and ‘Raveena’ (V6) are purple and

green in colour, respectively, and showed higher browning. The highly purple 'Ravaiya' (V2) brinjal on cutting showed comparatively less browning. Again the comparison among cultivars indicated the major role of PPO and phenolics in browning, particularly in case of fresh raw brinjal. For example in V5 and V6, high PPO activity reflected in the maximum browning with comparatively lesser concentration of phenolics. In cultivar 'Raveena' (V6) and 'Anupam' (V7) the phenolic concentration was almost same, but V6 showed higher browning which seems to be due to higher PPO activity. However, in V3 and V4, although less PPO activity was observed, browning was found to be higher, which could be due to the high concentration of phenolics (Fig. 2.1). Thus both these factors were found to play a role in browning of fresh and raw brinjal and the lesser concentration of any of these two components got compensated if other one was on higher side. If both these two factors are on comparatively lower side, as in case of cultivar V2 and V7, browning index was also low.

The profile of PPO activity change during storage resulted in assortment of these brinjal cultivars in two groups. The one group comprising of V1-V4 showed increase in PPO activity, whereas, the other group counting V5-V8 showed decrease in PPO activity during storage. The study of browning, phenolic content and PPO activity at lower temperature (10 ºC) of storage in two representative cultivars ('Arka navneet' (V4) and 'Pusa purple long' (V5), one each from these two groups, also endorsed the findings that profile of change in PPO activity during postharvest storage and processing was variety specific and not related to the senescence of brinjal.

In stored brinjal, the correlation of browning with phenolics in V2, V3, and V4 was observed to be marginally higher than the correlation between PPO activity and browning. In other four cultivars (V5-V8) during storage, browning showed negative

correlation (*r*) with PPO activity and high correlation with the phenolic content (Fig. 2.4). This indicated that the lower concentration of PPO is not a limiting factor for browning particularly in case of stored brinjal. The presence of a few molecules of enzyme could be sufficient to cause browning if level of phenolics is good enough.

The chlorogenic acid was found to be the major phenolic in brinjal and is also reported to be the major phenolic in potato (a close taxonomic relative). The varietal differences in the content of chlorogenic acid are common in many fruits and vegetables (Mishra et al., 2011). In apple the chlorogenic acid content was shown to differ among cultivars (Awad et al., 2000). The change in the content of chlorogenic acid during storage did not show any correlation with change in phenolics, PPO activity, or browning. This observation could be due to the fact that chlorogenic acid may not be the major natural substrate for PPO in brinjal. Chlorogenic acid is reported to be the major phenolic in potato (90% of total phenolics) and it did not show correlation with browning (Friedman, 1997). However, in their studies as in our finding the browning showed a correlation with PPO activity. Our observation that chlorogenic acid showed about only 31% substrate specificity with PPO (shown in chapter 3) compared to other phenolics also supported this hypothesis. The chlorogenic acid might be having other metabolic roles and less involved in the enzymatic browning of brinjal. The chlorogenic acid was shown to participate in the regulation of shoot, root and root hair development in *Hypericum perforatum* (Franklin and Dias, 2011). The inhibition of chlorogenic acid biosynthesis also showed inhibition of shoot, root and root hair development. Chlorogenic acid also reported to be antimicrobial and confers resistance to several major foliage-feeding insect pests (Friedman, 1997).

CHAPTER 3

Processing Strategy to Control Browning in Fresh-cut Ready To Cook (RTC) Brinjal

3.1. Introduction

The post-processing enzymatic browning of brinjal is a major problem for industries dealing with cut vegetables. Browning results in loss of eye appeal for the consumers and may adversely affect the nutritional and sensory properties of fresh-cut brinjal (Oms-Oliua et al., 2010). These reasons have also negatively affected the commercial availability of shelf-stable cut brinjal. The level of phenolics and its oxidizing enzyme polyphenol oxidase (PPO) are known to play a key role in the browning process of various raw and cut fruits and vegetables (Mayer, 2006; Hodges and Toivonen, 2008; Mishra et al., 2011). Several studies have suggested measures to control browning in fruits and vegetables. These included chemical dip treatment and modified atmosphere packaging excluding oxygen from the environment of the vegetable (Kang and Saltveit, 2003; Saxena et al., 2009; Oms-Oliua et al., 2010). In the present study a novel but a simple approach has been used, where cutting of brinjal was performed using a sharp blade which caused lesser physical injury to the tissues. This was followed by immediate dipping in water that helped wash away the leached phenolics and enzymes from the cut surface. The air drying followed by packaging in commercially used styrofoam trays wrapped with stretchable film and storage at ambient temperature (26±2 ºC) as well as low temperatures (10±2 ºC and 4±2 ºC) were further explored to achieve the maximum shelf life and improved microbiological quality of the product.

3.2. Materials and methods

3.2.1. Chemicals

Chlorogenic acid, disodium hydrogen phosphate, Folin-Ciocalteu reagent, gallic acid, 4-methyl catechol, polyvinyl pyrrolidone (PVP), polyvinyl polypyrrolidone (PVPP),

potassium metabisulfite (PMS), sodium carbonate, sodium dihydrogen phosphate, and triton X-100 were procured from Sigma-Aldrich Inc., St. Louis, Mo., U.S.A. The plate-count agar and potato dextrose agar were procured from Himedia Laboratories, Mumbai, Maharashtra, India.

3.2.2. Sample preparation

Brinjal (*Solanum melogena* L.) cv. Kalpatharu (12 kg) was procured from a local market and cleaned in potable water. The fruit was cut using a sharp blade into cubes of approximately size of 2 cm^3 (~3 g) each. To know the effect of cutting procedure on the browning of brinjal a sharp blade (thickness about 0.04 mm) and conventional vegetable cutting knife (blade thickness about 0.25 mm) were separately used for cutting. The blade thickness was measured using a digital caliper (Digimatic caliper, Mitutoyo, Kanagawa, Japan). The pieces were immediately submerged in potable water in a plastic bowl for 10 min, air dried on a clean surface, packaged in styrofoam trays and wrapped with stretchable film. The samples were then stored at ambient (26±2 ºC) and low temperatures (10±2 ºC and 4±2 ºC) and periodically examined for browning parameters and related biochemistry.

3.2.3. Scanning electron microscopy (SEM)

The sample preparation for SEM was carried out using a modified method by Dwivedi and Ahmad (1985). The thin sections from mesocarp portion of brinjal vegetable were immediately fixed in cold 2% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.0) for 48 h, washed twice in cold phosphate buffer, and then stained in 1% osmium tetroxide (w/v) in 0.1 M phosphate buffer (pH 7.0) overnight. Sections were then washed in deionized water for 20 min and sequentially dehydrated with increasing concentrations

(40, 60, and 80%) of ethanol for 2 h each and finally in 100% ethanol overnight. The sections were then air dried at ambient temperature and stored in a desiccator until use. The SEM was carried out using a facility (SEM Model Quanta 200 SEM, FEI Company, Oregon, USA) available with Icon Analytical Equipments Pvt Ltd., Mumbai, India. The sample was fixed on a carbon tape and observed at different magnifications using high vacuum mode at 90 Pa and 20 kV voltage.

3.2.4. Fluorescence microscopy

The thin section of mesocarp portion of brinjal was stained with propidium iodide solution and fluorescein diacetate solution for 10 min as per the procedure described by Regan and Moffatt (1990). A stock solution of fluorescein diacetate (2 mg mL^{-1}) was made in acetone and diluted to 0.01 mg mL^{-1} with 70 mg mL^{-1} sucrose. The propidium iodide stock solution (1 mg mL^{-1}) was prepared in milli Q water and diluted to 0.1 mg mL^{-1} solution with 70 mg mL^{-1} sucrose. The brinjal sections were stained for 10 min, later rinsed in 70 mg mL^{-1} sucrose, transferred to a glass slide, and gently covered with a coverslip. The stained sections were then observed under a fluorescence microscope (model Axiolab, Carl Zeiss, Germany) equipped with HBO 50 mercury lamp, a camera (Axiocam MRc, Carl Zeiss, Germany) and filter sets no. 9 and 15 (Carl Zeiss, Germany) for fluorescein diacetate and propidium iodide, respectively. The images were captured and processed using Axiovision AC.4.1. software (Zeiss, Haller-bergmoos, Germany).

3.2.5. Determination of the extent of browning

For browning measurement of cut brinjal, reflectance in visible spectrum region (360 to 780 nm) was recorded at 10 nm wave length interval using a Minolta CM-3600D spectrophotometer (Konica Minolta Sensing, Inc., Osaka, Japan). D65 lamp was used as

reference light source and the detector was fixed at an angle of 10º with respect to the light source (Ramírez-Jiménez et al., 2000). The equipment was calibrated prior to analysis with a standard white tile and a black box for 100 and 0% reflectance, respectively. The colour parameter used was CIE L (Lightness) which denoted the amount of light or luminance reflected from the sample. The extent of browning/darkening was calculated as (100-L), which is opposite of lightness (Ramírez-Jiménez et al., 2000).

3.2.6. Determination of PPO activity

The PPO enzyme activity was determined spectrophotometrically using 4-methyl catechol as substrate (Concellón et al., 2004). The brinjal samples (30 g) was frozen in liquid $N_{2,}$ grounded to fine powder and homogenized in 100 mL of extraction solution using a polytron homogenizer (Model PT3100, Kinematica AG, Switzerland). The extraction solution contained sodium phosphate buffer (pH 6.8, 0.05 M) with polyvinyl pyrrolidone (10 mg mL^{-1}), polyvinyl polypyrrolidone (10 mg mL^{-1}), triton X-100 (1 mg mL^{-1}) and ascorbic acid (30 mM). The enzyme extract was stirred for 30 min, filtered using 3 layered muslin cloth, and centrifuged (CMF 15 KR, Tigra, Poland) at 10,000 g for 15 min at 4 ºC. The supernatant was collected and ammonium sulphate precipitated. The 20-80% saturation fraction showing PPO activity was solubilized in 8 mL of phosphate buffer (pH 6.8, 20 mM) and dialysed for removal of salt. The enzyme assay was carried out by taking 0.85 mL of phosphate buffer (pH 6.8, 50 mM), 0.1 mL substrate (0.1M) and 0.02 mL of the enzyme extract. The increase in absorbance at 420 nm was monitored at 1 min interval for 3 min using a spectrophotometer (Model UV 4-100, Unicam, Cambridge, UK) and the average change in absorbance per min was calculated. One unit of enzymatic activity was defined as the amount of enzyme which caused a change of 0.1 in

absorbance/min. The PPO activity was expressed as U/g of brinjal weight. The specific activity was determined by expressing PPO activity/mg protein. The protein content of the brinjal extract was determined by the Bradford method (1976), using bovine serum albumin (BSA; Sigma Chemical, St. Louis, USA) as standard.

3.2.7. Determination of leached PPO activity from cut surface of brinjal

The leached PPO activity from the cut surface of brinjal was determined spectrophotometrically using 4-methyl catechol as substrate. The sample (20 g) was dipped in 26 mL of phosphate buffer (50 mM, pH, 6.8) containing 4 mL substrate (0.1 M) for 4 min. The increase in absorbance at 420 nm of buffer component due to leached enzymes from the cut surface of the brinjal was monitored at 1 min interval for 3 min using a spectrophotometer (Model UV 4-100, Unicam, Cambridge, UK) and the average change in absorbance per min was calculated. The PPO activity was expressed as discussed above.

3.2.8. Estimation of total soluble phenolics

Brinjal sample (7.5 g) was soaked in 50 mL of 80% methanol and homogenized using a polytron homogenizer (Model PT3100, Kinematica AG, Switzerland). The suspension was centrifuged at 10,000 g for 20 min and the supernatant was collected. A 25 µl of aliquot of the supernatant was mixed with an equal volume of milli Q water and further mixed with 50 µl of 0.2 N Folin-Ciocalteu reagent. The suspension was incubated at ambient temperature (26±2ºC) for 15 min and later mixed with 0.15 mL of sodium carbonate solution (0.2 g mL^{-1}). The reaction mixture was incubated in a water bath at 40ºC for 20 min, and later placed on ice for 5 min. Finally the absorbance was measured at 755 nm using a spectrophotometer (Model UV 4-100, Unicam, Cambridge, UK). The

total phenolic content was calculated using gallic acid as a standard and expressed as mg GA equivalent/g of brinjal (Luthria et al., 2010).

3.2.9. Estimation of chlorogenic acid

Chlorogenic acid was estimated using HPLC by the method of Luthria et al. (2010). Brinjal sample (7.5 g) was soaked in 50 mL of 80% methanol and homogenized using a polytron homogenizer. The suspension was centrifuged at 10,000 g for 20 min. The supernatant was collected and membrane (0.2 µm) filtered. A 60 µl aliquot was loaded on a reverse phase C18 column. The elution was carried out using a gradient of formic acid (0.1%) from 90 to 55% over methanol for 26 min at a flow rate of 1 mL/min. The detection was performed at 350 nm using a UV detector. The chlorogenic acid concentration in brinjal was calculated using pure commercially available chlorogenic acid (retention time 16 min in specified mobile phase) as standard.

3.2.10. Microbiological studies

The microbiological analyses were performed at regular intervals as per the methods detailed in ICMSF (International Commission for the Microbiological Specifications of Foods) (2002). The total bacterial count (TBC) and yeast and mould count (YMC) of fresh-cut brinjal was determined using standard pour plate method. The 10 g of brinjal was suspended in 90 mL sterile saline (8.5 mg mL^{-1} NaCl) and homogenized using a stomacher lab blender (model 400, Seward, U.K). An aliquot (1 mL) of the suspension was withdrawn, serially diluted using sterile saline and pour plated in duplicate on plate count agar and potato dextrose agar plates (Himedia Laboratories, Mumbai, India) to determine TBC and YMC as colony forming units (cfu) /g, respectively.

3.2.11. Organoleptic evaluation

Organoleptic or sensory evaluation of fresh-cut brinjal vegetable was performed by a panel of 15 trained and experienced panelists from Food Technology Division, Bhabha Atomic Research Centre, Mumbai, India in a taste panel laboratory in individually partitioned compartments of a controlled environment room (Meilgaard et al., 1999). The sensory panel scored different quality attributes such as appearance, colour and browning for fresh-cut brinjal. Besides these features, texture, taste, and overall acceptability were also evaluated for cooked samples. The cooking of brinjal was performed uniformly for 10 min using a frying pan under low heat condition. Initially, 20 ml of refined cooking oil in a frying pan was heated on a cooking gas stove. The brinjal sample (250 g) was added and stirred for 1 min. Water (200 ml) and cooking salt (3 g) was then added and lid was covered for 10 min. Scoring was done based on a 9-point hedonic scale (9-like extremely; 8-like strongly; 7-like very well; 6-like fairly well; 5-like moderately; 4-like slightly; 3-dislike slightly; 2-dislike moderately; 1-dislike extremely). Sensory analysis was performed till the samples were visibly acceptable during storage period. Visible fungal growth was considered as the indicator of spoilage and hence, no further sensory evaluation was carried out with such samples.

3.2.12. Statistical analysis

Experiments including PPO activity, phenolic estimation, browning measurements were repeated in three sets independently, each set having 3 replicates. Chlorogenic acid was estimated using HPLC in three replicates. Organoleptic evaluation was performed in three independent experiments. Microbiological analysis was performed in two sets of experiments each having three replicates. The means and standard deviations were

calculated taking all the readings into consideration. As two factors (temperature and storage days) were used in this study, two way ANOVA (analysis of variance) at the level of significance P≤0.05 using Tukey test was performed for comparison of means. For other experiments, one way ANOVA was performed to ascertain the significance of the means. Statistical analysis was performed using BioStat 2009 Version Professional 5.8.0.0 (AnalystSoft Inc., Canada).

3.3. Results and Discussion

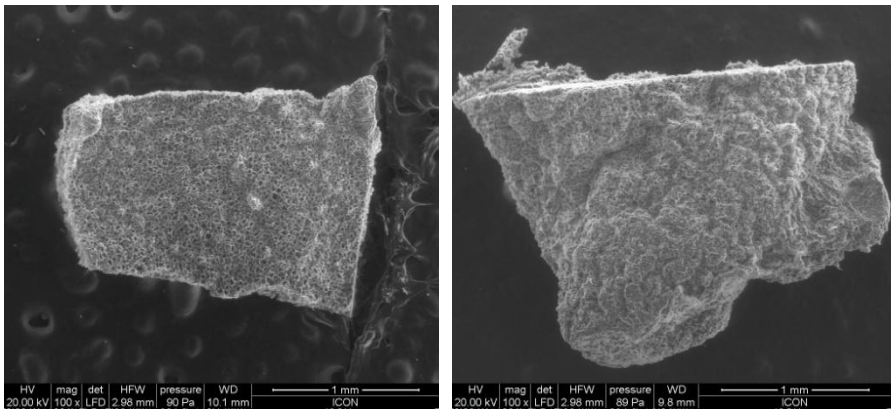
3.3.1. Effect of cutting on physical injury of brinjal

The thickness of thin blade used for cutting was about 0.04 mm and that of conventional knife 0.25 mm, more than 6 fold thicker than the thin blade. More physical damage is expected with thicker knife due to increase in contact area, whereas, fine cutting is expected to do lesser physical injury possibly, limited to the plane of cutting (Watada and Qi, 1999). The sharp blade-cut and water dipped brinjal with markedly low browning, as well as the conventional knife-cut brinjal samples showing significant browning are shown in Fig 3.1(a) and (b), respectively.

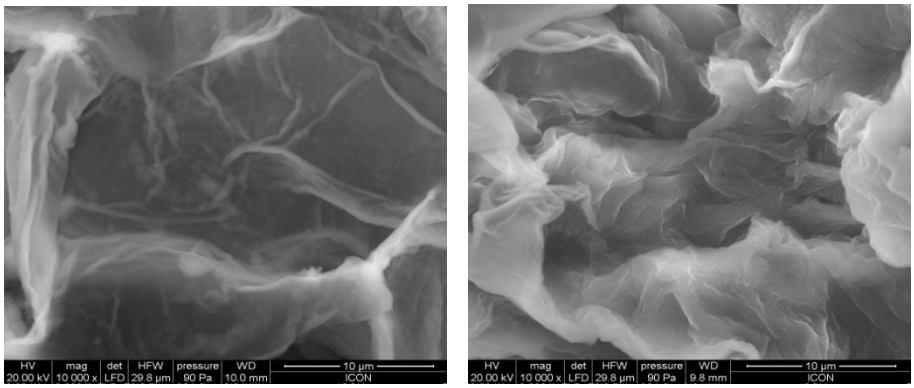
3.3.1.1. SEM analysis showed lesser abrasive injury due to thin blade cutting

The scanning electron microscopy images of surface of brinjal cut with the sharp blade and knife have been shown in Fig 1(c-f). The Fig 3.1(d) showed comparatively higher uneven and corrugated features in knife-cut sample than blade-cut sample (Fig 1c) when analysed using 100X magnification. Such a higher abrasive impact indicated increased physical injury. The 10000X magnification of the same blade and knife-cut samples has been provided in Fig. 3.1(e) and (f), which clearly established that the abrasiveness and wrinkle formation in knife cutting was much more than that in blade-cut sample. Thus the

(a) **(b)**

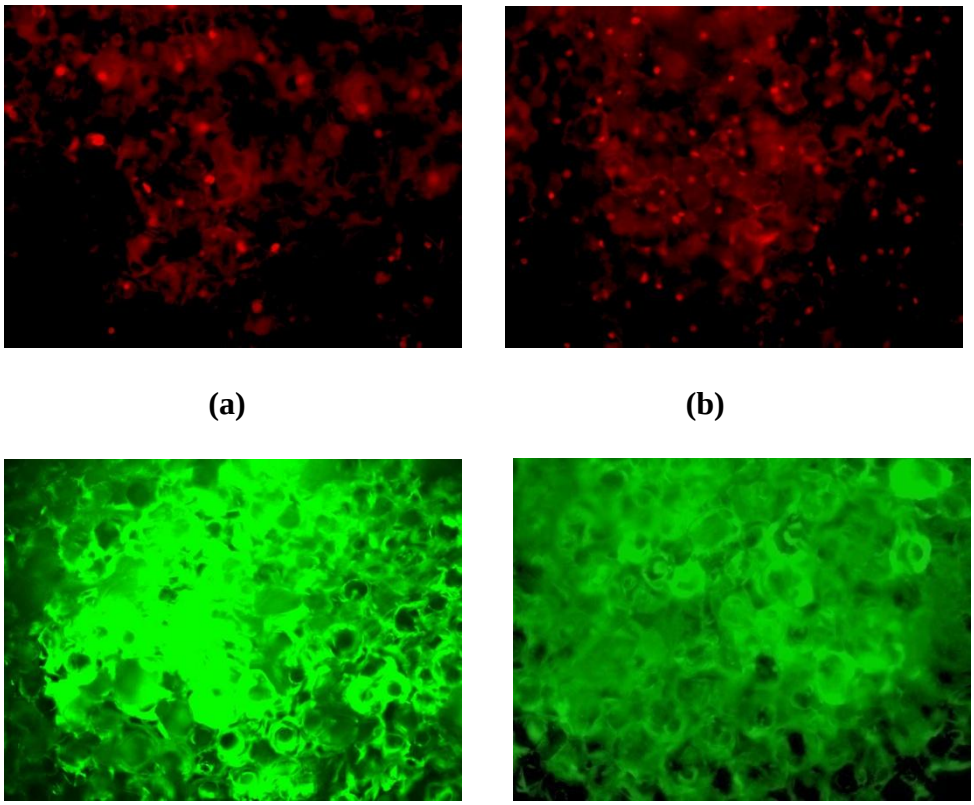


(c) **(d)**



(e) **(f)**

Figure 3.1. The photograph of cut brinjal showing; **(a)** reduced browning in blade-cut water dipped sample, **(b)** increased browning in knife-cut but not water dipped sample. The scanning electron microscopy image of blade-cut **(c** and **e**) and Knife-cut **(d** and **f)** pieces of brinjal, (**c**) and (**d**) 100 X; (**e**) and (**f**) 10,000X.



(a) **(b)**

(c) **(d)**

Figure 3.2. The propidium iodide (red) and fluorescein diacetate (green) staining of blade-cut **(a, c)** and knife-cut **(b, d)** brinjal. Propidium iodide, a nuclear dye indicating increased dead cell population **(b)**. Fluoroscein diacetate staining indicated the physiologically live cells showing intense bright green fluorescence **(c)**.

knife cutting of brinjal seems to be quite harsh and rough which eventually resulted in higher physical injury compared to fine blade-cut sample.

3.3.1.2. Fluorescence microscopy indicated reduced cell death due to thin blade cutting

Profile of dead cell population in brinjal cut by these two methods of cutting was analyzed by propidium iodide (PI) staining [Fig 3.2(a) and (b)]. The dead cells with damaged membrane allowed entry of the fluorescent dye into cells which stained the nucleus red making them prominently visible in knife-cut sample (Fig 3.2b) under a fluorescence microscope. The fine blade-cut sample showed lesser stained nuclei due to comparatively lesser cellular injury and death (Fig 3.2a). The fluorescein diacetate (FDA) staining of brinjal cut with sharp blade and knife has been shown in Fig 3.2(c) and (d), respectively. The non-fluorescent FDA stain is lipid soluble and hence easily enters the cells. In metabolically active viable cells reactive oxygen species are frequently produced, which activate the cytoplasmic esterase enzymes which in turn are reported to cleave FDA to yield a fluorescent product. The product is retained within the cell if membrane function is intact. Hence, live cells fluoresce bright green and nonviable cells are dim or less fluorescent (Coder, 1997). The fine blade-cut sample showed brighter fluorescence upon FDA staining compared to the knife-cut sample indicating higher number of viable cells in fine blade-cut sample. The higher physical injury in knife-cut sample might have resulted in higher loss of cellular viability due to excessive membrane damage.

3.3.1.3. Effect of cutting and water dip on browning

The extent of browning in differently cut and water dipped samples has been shown in Table 3.1. The thin blade-cut samples showed 39% less browning compared to the knife (thick blade) cut samples. The noticeable difference in browning was visible after 2 h of

cutting. The blade-cut and water dipped sample showed 56% lesser browning compared to knife-cut samples. When blade and knife-cut samples were both dipped in water, the former showed 45% less browning. Thus it was evident from the data that blade-cut and water dipped samples showed significantly lesser browning compared to the other combinations ($P \leq 0.05$).

3.3.1.4. Higher PPO activity in knife-cut samples

The PPO enzyme activity and the specific activity in brinjal were found to be significantly influenced by the methods of cutting (Table 3.1). The PPO activity observed to be increased by more than 57 and 25% in knife and blade-cut samples, respectively. The browning is reported to be caused by the activity of the enzyme PPO in brinjal (Mayer and Harel, 1979). In native brinjal the PPO and phenolics were reported to be present in chloroplast and vacuoles, respectively (Thipyapong et al., 1995). During physical cutting, the cells are damaged along the line of cutting and adjacent cells could also get mechanically injured resulting in disrupted cellular structure. This eventually leads to release of PPO enzyme and its phenolic substrates and allow their physical contact. In the presence of oxygen, this enzymatic reaction takes place leading to the formation of melanin like brown coloured pigment (Thipyapong et al., 1995). The pigment turns the colour of cut surface brown, and thereby, the original appearance is lost. The specific activity of PPO was also found to increase by 94 and 41% with knife and blade cutting, respectively, which meant an increase in ratio of the active PPO protein to the total protein. This could be due to the cutting induced increase in synthesis or activity of PPO enzyme in brinjal. Injury induced increase in PPO activity has been reported in case of potato and other plants (Thipyapong et al., 1995).

Table 3.1. The browning related parameters of fresh-cut brinjal prepared using different cutting method.

	Browning index (100-L)	PPO activity (U/g)	PPO sp. activity (U/mg protein)	Leached PPO activity (U/g)	Total soluble Phenolics (mg GAE/g)	Leached soluble Phenolics (mg GAE/g)	Chlorogenic acid (mg/g)
Whole brinjal	-	145^{c}±11	17^{c} ±1.6	-	1.23^{d} ±0.05	-	0.74^{c} ±0.02
Knife-cut	36^{a} ±2	229^{a}±19	33^{a} ± 1.8	7.51^{a}±0.38	1.57^{a} ±0.06	0.35^{a} ±0.02	0.95^{a} ±0.03
Knife-cut water dip	35^{a} ±2	218^{a}±17	30^{a} ± 1.5	5.93^{b}±0.29	1.45^{b} ±0.04	0.24^{c} ±0.02	0.84^{b} ±0.02
Blade-cut	22^{b} ±1	182^{b}±14	24^{b} ± 1.6	6.65^{c}±0.31	1.52^{a} ±0.03	0.28^{b} ±0.01	0.94^{a} ±0.03
Blade-cut water dip	16^{c} ±2	172^{b}±16	22^{b} ± 1.8	4.99^{d}±0.25	1.39^{b} ±0.04	0.22^{c} ±0.01	0.81^{b} ±0.02
Knife-cut PMS dip	14^{c} ±1	108^{d}±14	12^{d} ± 1.6	1.98^{e} ±0.15	1.43^{c}±0.04	0.24^{c} ±0.02	0.82^{b} ±0.03
Blade-cut PMS dip	14^{c} ±1	102^{d}±12	11^{d} ± 1.6	1.79^{e} ±0.18	1.38^{c}±0.04	0.22^{c} ±0.01	0.80^{b} ±0.03

Mean values in a column with different letter superscript are significantly different (P≤0.05). – Symbol denote unavailable sample. U – Units, L – Lightness, GAE -Gallic acid equivalent. PMS, potassium metabisulfite.

The analysis of leached PPO activity from cut surface of brinjal also showed similar results where 12% higher PPO activity was observed in knife-cut samples compared to the blade-cut sample (Table 3.1). Further the water dipping reduced the leached PPO activity by 22 and 25% in knife and blade-cut samples, respectively, compared to non-dip samples and resulted in significantly reduced browning ($P \leq 0.05$). These results reconfirmed that dipping in water contributed to decrease in browning.

3.3.1.5. Higher phenolic level in knife-cut samples

The data on total soluble phenolics in differently cut brinjal samples has been shown in Table 3.1. In knife-cut brinjal, an increase of more than 27% was observed in soluble phenolics compared to whole brinjal vegetable, which was found to be statistically significant ($P \leq 0.05$). This is due to mechanical injury leading to decompartmentalization of phenolics and wound induced increase in the synthesis of phenolics by the key regulatory enzyme phenylalanine ammonia lyase (PAL) (Kang and Saltveit, 2003). The post-cutting dipping in water marginally lowered the phenolics level by nearly 8% both in knife and blade-cut samples. This could be due to the diffusion or leaching of soluble phenolics into water through cut surface. This was confirmed by measuring the leached out phenolics in the leftover water after the dipping step. The leached phenolic level was about 32 and 22% higher in knife and blade-cut samples, respectively, compared to the respective water dipped samples. The results showed that significant amount of phenolics leached out to water during the dipping step (Table 3.1). The leaching of phenolics was found to be significantly higher (20%) in knife-cut samples compared to blade-cut samples which could be due to the higher tissue damage ($P \leq 0.05$).

3.3.1.6. Higher level of chlorogenic acid in knife-cut samples

The increase in chlorogenic acid was observed in cut brinjal compared to the whole vegetable (Table 3.1). The major phenolic compound present in brinjal is chlorogenic acid (5-caffeoylquinic acid) and contributed to about 60% of the total soluble phenolics (Luthria et al., 2010). The 80% methanolic extract of brinjal when analysed by HPLC showed a single major peak of this compound with retention time of 16 min in specified mobile phase at 350 nm. The chlorogenic acid in fresh-cut brinjal was found to be nearly 27% higher compared to whole brinjal. This could be due to physical injury or wound induced increased synthesis of chlorogenic acid by PAL enzyme as discussed above (Kang and Saltveit, 2003). A significant decrease of 12 and 14% in chlorogenic acid content was observed in knife and blade-cut water dipped samples, respectively, which could be due to its leaching in water. The difference in its content between blade-cut water dipped and knife-cut water dipped samples was not found to be significant ($P \leq 0.05$).

3.3.1.7. Inhibition of browning in cut brinjal by PPO inhibitor

The knife and blade-cut samples were dipped in potassium metabisulfite (PMS) (2 mg mL^{-1}, 10 min), an inhibitor of PPO**,** to study the comparative contribution of phenolics and PPO activity on browning process (Table 3.1). The inhibitor dipped knife and blade-cut samples were compared with the respective water dipped control samples. Due to inhibitor dip treatment, the browning was inhibited by 60 and 13% in knife and blade-cut samples, respectively, compared to water dip control samples. Similarly, the PPO activity was inhibited by 51 and 41% in knife and blade-cut samples, respectively. The leached PPO activity also significantly reduced in PMS dipped samples by 67 and 65% in knife and blade-cut samples, respectively,

compared to water dip controls. However, the change in phenolics and chlorogenic acid concentration, both in knife and blade-cut samples, due to PMS dip was found to be only about 2% and was insignificant ($P \leq 0.05$). Thus, it was observed that browning showed a high correlation with PPO activity and little correlation with the concentration of phenolics including chlorogenic acid level, which justified the observation that indeed PPO was the major contributor towards browning than the phenolics in fresh sample.

3.3.2. Status of browning parameters during storage

Since fine blade cutting was found to significantly inhibit the browning in cut brinjal, further studies were performed with these samples where analysis of browning related parameters as well as microbiological and organoleptic status were performed during storage at different temperatures to assess the commercial acceptability of the process and product.

3.3.2.1. Browning index

The change in browning status of sharp blade-cut brinjal during storage at different temperature is shown in Table 3.2. The increase in browning was found to be insignificant until day 4 of storage. However, a significant increase of about 25% was observed on day 6 at ambient temperature storage ($P \leq 0.05$). Such an increase in browning in other fresh-cut stored vegetables has also been reported earlier even at low temperatures (Aguila et al., 2010). The physical injury is known to induce physiological responses including browning which is further manifested if storage temperature is high. However, the kinetics of browning were found to be slower at lower storage temperatures of 10 and 4 ºC (Table 3.2). In these samples, the increase in browning was found to be insignificant until day 8 and 10 at 10 and 4 ºC storage

temperatures, respectively. Although, browning was found to be increase significantly by 25% at the end of low temperature storage, still this was 44% lesser compared to knife-cut samples on day 1 ($P \leq 0.05$). The low temperature storage inhibited the biochemical reactions involved in the browning process and has been considered as the most general approach for shelf-life improvement of many fresh-cut vegetables. The temperature of about 5 ºC has also been recommended earlier for storage of fresh-cut produce (Aguila et al., 2010).

3.3.2.2. PPO activity

The change in PPO activity of fine blade-cut brinjal during storage at different temperatures is shown in Table 3.2. A gradual increase in PPO activity was observed during ambient storage until day 4 which was found to be insignificant ($P \leq 0.05$). But on day 6 of storage a significant increase of 33% was observed in PPO activity. This change could be due to increase in activity of oxidative enzymes during senescence process in fruits and vegetables which accelerated at ambient temperature storage (Concellón et al., 2004). Similarly, 41% increase in the specific activity of PPO was also observed on day 6. In whole (uncut) brinjal also the increase in PPO activity has been reported even at low temperature storage which could be due to physiological senescence (Concellón et al., 2004). The PPO activity and the specific activity did not increase significantly until day 8 and 10 of storage at 10 and 4ºC, respectively (Table 3.2). However, significant ($P \leq 0.05$) increase was observed during the end of storage period on day 12 and 16 at low temperature (10 and 4ºC) stored samples. Though such an increase in PPO activity was also observed in ambient temperature stored samples, the lower temperature storage has been found to slow down the process (Concellón et al., 2004). .

Table 3.2. Status of browning related parameters and microbial load of sharp blade-cut and water dipped brinjal during storage at different temperatures.

Storage (Day)	Browning (100-L)	PPO activity (U/g)	Specific activity (U/mg protein)	Total soluble phenolics (mg GAE/g)	Chlorogenic acid (mg/g)	Microbiology TBC log (cfu/g)	Microbiology YMC(cfu/g)
26 ±2 ºC							
1	16^{b}±0.9	172^{b} ±10.2	24^{b} ±1.7	1.39^{a} ±0.06	0.82^{a} ±0.04	3.7^{c} ±0.2	3.4^{b} ±0.4
2	17^{b}±0.8	182^{b} ±13.4	25^{b} ±1.8	1.33^{a} ±0.05	0.77^{a} ±0.03	3.4^{c} ±0.2	3.5^{b} ±0.4
4	18^{b}±0.7	192^{b} ±12.4	27^{b} ±1.4	1.26^{a} ±0.07	0.68^{b} ±0.03	4.4^{b} ±0.3	3.7^{b} ±0.5
6	20^{a}±0.9	229^{a} ±11.2	34^{a} ±1.6	1.06^{b} ±0.06	0.57^{c} ±0.02	6.0^{a} ±0.4	5.8^{a} ±0.4
10 ±2 ºC							
1	16^{b} ± 0.8	174^{b} ±15	26^{b} ±1.7	1.40^{a} ±0.03	0.82^{a} ±0.03	3.5^{c} ±0.4	2.0^{d} ±0.2
4	17^{b} ± 0.5	184^{b} ±14	28^{b}±1.4	1.36^{a} ±0.03	0.78^{a} ±0.04	4.5^{b} ±0.5	2.5^{c} ±0.2
8	18^{b} ± 0.6	189^{b} ±11	29^{b} ±1.3	1.31^{a} ±0.02	0.64^{b} ±0.03	4.9^{b} ±0.3	3.8^{b} ±0.2
12	20^{a} ±0.8	218^{a} ±12	33^{a} ±1.6	1.01^{b} ±0.06	0.59^{c} ±0.04	5.7^{a} ±0.3	5.5^{a} ±0.2
4 ±2 ºC							
1	16^{b} ± 0.8	164^{b} ±14	25^{b} ±1.9	1.38^{a} ±0.05	0.81^{a} ±0.04	2.4^{d} ±0.2	1.9^{d} ± 0.2
5	17^{b} ± 0.7	172^{b} ±15	27^{b} ±1.7	1.27^{a} ± 0.06	0.76^{a} ±0.03	3.4^{c} ±0.3	2.4^{c} ± 0.1
10	18^{b} ± 0.6	183^{b} ±12	28^{b} ±1.3	1.21^{a} ±0.05	0.66^{b} ±0.04	4.5^{b} ±0.4	2.6^{c} ± 0.1
16	20^{a} ±0.7	215^{a} ±15	34^{a} ±1.8	1.06^{b} ±0.06	0.55^{c} ±0.03	5.5^{a} ±0.2	3.7^{b} ± 0.2

Mean values in a column with different letter superscript are significantly different (P≤0.05). U – Units, L – Hunter's Lightness colour parameter, GAE -Gallic acid equivalent, TBC - Total bacterial count, YMC - Yeast and mold count, cfu- Colony forming unit.

3.3.2.3. Total soluble phenolics

The change in total soluble phenolic content in blade-cut stored brinjal is shown in Table 3.2. There was no significant decrease in the phenolics observed in ambient temperature stored sample till day 4. However, on day 6, a significant (17%) decrease in phenolic content was observed ($P \leq 0.05$). This is attributed to the oxidation of phenolics to insoluble forms due to increased PPO activity. Similar decrease in phenolics has also been reported earlier in fresh-cut jack fruit during storage (Saxena et al., 2009). The phenolic level did not change significantly until day 8 and 10 of storage at 10 and 4 ºC, respectively (Table 3.2). The insignificant change in PPO activity at low temperature storage could be the reason associated with unchanged level of total soluble phenolics during this storage period. However, a significant ($P \leq 0.05$) decrease of 28 and 23% in soluble phenolic content was observed during end of the storage period at 10 and 4 ºC, respectively, which was well correlated with the increase in PPO activity (Table 3.2).

3.3.2.4. Chlorogenic acid

The chlorogenic acid content gradually decreased in blade-cut brinjal during 6 days of shelf life at ambient temperature storage (Table 3.2). On day 4 and 6 of storage, significant decrease of 17 and 30% in chlorogenic acid concentration was observed ($P \leq 0.05$). Such a decrease could be due to increase in PPO activity, which leads to oxidation of chlorogenic acid to insoluble phenolics. The decrease in chlorogenic acid content was found to be 28% during a shelf life of 12 days at 10ºC. Again, at 4 ºC the decrease was found to be 32% during 16 days of shelf life (Table 3.2). As discussed above, this could be due to the biochemical changes associated with senescence of brinjal involving increase in activity of the oxidative enzymes (Saxena et al., 2009).

3.3.3. Microbiological analysis

The analyses were periodically performed during storage to determine the microbial load in blade-cut and stored ready-to-cook brinjal. The visible mold growth was considered as the indicator of spoilage making the product unusable. The total bacterial count (TBC) and yeast and mold count (YMC) were found to be in the range of 3-4 log cfu/g on day 1 of storage at ambient temperature. The bacteria, yeast and mold are known to be the important causes of spoilage in fresh-cut vegetables. During the preparatory steps of minimal processing, the natural protection of vegetable is compromised and hence, they become highly susceptible to microbial spoilage. In addition, microbial contamination also occurs during cutting process. The high water activity and near neutral pH of brinjal make it suitable hosts for many types of microorganisms (Oms-Oliua et al., 2010). During 6 days of ambient storage the bacterial and yeast mold count were found to increase by more than 2 log cycles (Table 3.2). However, the increase in counts was found to be within the permissible limit and may be acceptable. The legislations in different countries have set the limit of microbial load as 4.7 log cfu/g at production stage and 7.7 log cfu/g at consumption stage for fresh cut vegetables (Erturk and Picha, 2006). However, beyond 6 days of storage, a few samples started showing visible mold growth and hence could not be accepted. On day 1 of storage the counts in 10 and 4 ºC stored sample were found to be less than that of ambient temperature stored samples. This could be due to inhibition of microbial growth at low temperature (Oms-Oliua et al., 2010). During 12 days of storage at 10 ºC the bacterial and yeast mold counts were found to increase by 2 and 3 log cycles, respectively. During 16 days of storage at 4 ºC the bacterial and yeast mold count were found to show nearly similar increase (Table 3.2). It has been observed that the increase in bacterial count was higher compared to yeast and mold

count. However, towards the end of storage period the mold growth became prominent rendering the product unacceptable. In general cutting process was not found to have any profound effect on microbiological profile of brinjal.

3.3.4. Organoleptic analysis

The results of organoleptic evaluation of fresh knife-cut and blade-cut stored sample (at different temperatures) after cooking are shown in Table 3.3. The fresh knife-cut samples analyzed within 1 h of cutting (Day 0) did not show any significant difference ($P \leq 0.05$) in surface browning compared to day 1 blade-cut water dipped samples. However, the fresh knife-cut cooked samples did not show any significant difference with day 1 blade-cut water dipped samples during remaining storage days. The day 1 knife-cut samples were rated as 'like slightly,' whereas, blade-cut brinjal as 'like strongly' when analysed for visual observations like appearance, colour and browning on day 1 of storage. The knife-cut samples stored at 26 ± 2, 10 ± 2, 4 ± 2 ºC were not appreciated (Overall acceptability score < 4) even on day1, and hence not studied further due to poor acceptability. During storage only a marginal decrease in these ratings was observed in blade-cut water dipped samples. This could be due to the physiological changes associated with senescence and water loss during storage Most of the vegetables are known to lose water during storage resulting in dryness on their skin (Toivonen and Brummell, 2008). However, these changes get reduced in low temperature stored samples. The brinjals with bruising and scars are known to taste bitter and astringent. Such physical injury is known to cause increase in phenolic biosynthesis and oxidation, which lead to astringency and bitterness in cooked brinjal (Es-Safi et al., 2003). Since cutting involves physical injury, the cooked vegetable

Table 3.3. Organoleptic evaluation of knife-cut and blade-cut water dipped brinjal during storage at different temperatures.

Storage	Knife-cut control		Blade-cut								
			26 ±2 ºC			10 ±2 ºC			4 ±2 ºC		
(Days)→	0*	1#	0	3	5	0	8	12	0	8	16
Raw											
Appearance	6.8^{a}±0.6	4.2^{c}±0.3	8.0^{a}±0.8	7.7^{a}±0.7	7.1^{a}±0.4	8.0^{a}±0.8	7.7^{a}±0.8	7.2^{a}±0.5	8.2^{a}±0.7	7.6^{a}±0.7	7.1^{a}±0.7
Colour	6.7^{a}±0.5	4.1^{c}±0.3	7.8^{a}±0.8	7.7^{a}±0.8	7.0^{a}±0.6	8.0^{a}±0.7	7.9^{a}±0.8	7.0^{a}±0.6	8.2^{a}±0.8	7.7^{a}±0.6	7.2^{a}±0.7
Browning	6.7^{a}±0.5	2.9^{c}±0.3	7.7^{a}±0.8	7.8^{a}±0.7	6.9^{a}±0.5	7.8^{a}±0.7	7.5^{a}±0.8	7.1^{a}±0.5	7.8^{a}±0.8	7.6^{a}±0.7	7.2^{a}±0.6
Cooked											
Appearance	6.9^{a}±0.6	6.7^{a}±0.6	7.1^{a}±0.6	7.3^{a}±0.6	6.7^{a}±0.5	7.1^{a}±0.6	6.7^{a}±0.5	6.9^{a}±0.5	7.1^{a}±0.7	7.1^{a}±0.7	6.7^{a}±0.4
Colour	6.9^{a}±0.7	6.7^{a}±0.5	7.3^{a}±0.7	7.3^{a}±0.7	6.8^{a}±0.6	7.4^{a}±0.8	6.8^{a}±0.4	6.6^{a}±0.6	7.4^{a}±0.8	7.2^{a}±0.6	7.1^{a}±0.6
Browning	7.1^{a}±0.7	6.6^{a}±0.6	7.5^{a}±0.8	7.8^{a}±0.8	7.3^{a}±0.7	7.5^{a}±0.7	6.7^{a}±0.5	6.6^{a}±0.6	7.3^{a}±0.6	6.6^{a}±0.5	6.7^{a}±0.5
Aroma	6.7^{a}±0.5	6.6^{a}±0.6	6.7^{a}±0.5	6.8^{a}±0.5	7.0^{a}±0.6	6.9^{a}±0.7	7.2^{a}±0.6	7.1^{a}±0.6	7.2^{a}±0.5	7.2^{a}±0.4	6.9^{a}±0.6
Taste	7.1^{a}±0.3	4.1^{c}±0.4	7.3^{a}±0.6	7.2^{a}±0.6	6.6^{a}±0.6	7.3^{a}±0.4	7.2^{a} ±0.7	6.8^{a}±0.5	7.4^{a}±0.6	7.1^{a}±0.6	6.6^{a}±0.6
Texture	5.2^{b}±0.4	5.2^{b}±0.4	5.3^{b}±0.4	5.4^{b}±0.4	5.2^{b}±0.5	5.1^{b}±0.4	5.2^{b}±0.4	5.1^{b}±0.4	5.1^{b}±0.4	5.3^{b}±0.4	5.2^{b}±0.4
Overall Acceptability	6.9^{a}±0.6	3.6^{c}±0.4	7.3^{a}±0.7	7.3^{a}±0.7	6.6^{a}±0.6	7.3^{a}±0.7	7.1^{a}±0.6	6.9^{a}±0.8	7.2^{a}±0.7	7.2^{a}±0.7	6.8^{a}±0.6

The data presented as mean ± SD of scores given by 15 panelists. Mean values in a row with different letter superscript are significantly different (P≤0.05). * Samples were analysed within 1 h after cutting. # The knife-cut samples stored at 26 ±2, 10 ±2, 4 ±2 ºC were not appreciated (Overall acceptability score < 4) even on day1.

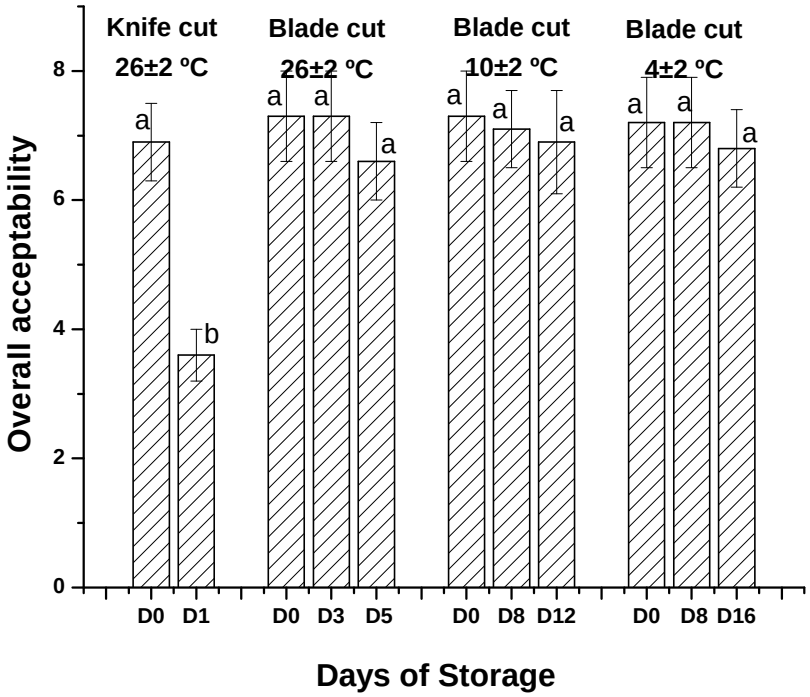


Figure 3.3. Organoleptic analysis indicating overall acceptability of knife-cut and blade-cut water dipped brinjal during storage at different temperatures. The data presented as mean ± SD of scores given by 15 panelists. Mean values with different letter superscript are significantly different (P≤0.05). The knife-cut samples stored at 26 ±2, 10 ±2, 4 ±2 ºC were not appreciated (Overall acceptability score < 4) even on day1.

was also organoleptically evaluated to judge its effect on taste. The cooked blade-cut brinjal samples were found to score little better than the knife-cut samples in appearance colour and browning. This is because during cooking brinjal is known to lose its whitish colour and turn brown. The difference in the texture of knife-cut and blade-cut cooked brinjal was found to be insignificant. However, the taste scores in knife-cut samples were significantly lesser than the blade-cut samples ($P \leq 0.05$). The knife-cut sample was bitter in taste compared to the blade-cut samples. This could be due to higher physical injury induced oxidation of phenolics resulting in bitterness of cooked brinjal (Es-Safi et al., 2003). In overall acceptability category, the knife-cut samples scores showed less acceptability than the blade-cut samples on day 1 of storage. Blade-cut water dipped brinjal samples were found to have shelf-life of 6 days at 26 ºC, 12 days at 10 ºC, and 16 days at 4 ºC. All these samples scored about 7 (‘like very well’) during organoleptic analysis.

CHAPTER 4

Purification and Characterization of PPO from Brinjal Cultivar Kalpatharu

4.1. Introduction

The postharvest processing of brinjal (*Solanum melongena*) is one of the major problems for industries dealing with cut vegetables (Madinez and Whitaker, 1995). Polyphenol oxidase has been reported to be one of the important factors contributing to the browning during postharvest processing. In brinjal the PPO and phenolics were reported to be present in chloroplast and vacuoles, respectively (Mayer and Harel, 1979; Güllçin, et al., 2005). Disruption of cellular structures during physical cutting leading to release of PPO enzyme and its phenolic substrate has been demonstrated using electron and fluorescence microscopy (Mishra et al., 2012). PPO catalyzes the hydroxylation of monophenols to o-diphenols (EC 1.14.18.1) through a monophenolase activity and a subsequent oxidation of these o-diphenols to the corresponding o-quinones (EC 1.10.3.1) by a catecholase/diphenolase activity (Mayer, 2006). In the presence of oxygen, this enzymatic reaction takes place leading to the formation of melanin like brown coloured pigment (Madinez and Whitaker, 1995). The pigment turns the colour of cut surface brown, and thereby, the original appearance is lost. The inhibition of this enzyme activity and associated browning has remained a challenge for the processed fruits and vegetables industry (Mayer, 2006). The purification and characterization of the enzyme in brinjal could help find suitable methods for controlling its activity. The purification of PPO to homogeneity has remained difficult in brinjal due to its high phenolic content and their irreversible binding to PPO during purification process. However, 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; Concellón, et al., 2004). Roudsaria et al. (1981) reported 15 fold partial purification using ion exchange and gel filtration chromatography. Pérez-Gilabert and Carmona (2000) characterized the 40% ammonium sulphate precipitated fraction without the use of chromatographic purification

methods. Doğan et al. (2002) have characterized the PPO activity of ammonium sulphate precipitated fraction. Concellón et al. (2004) have also reported the PPO activity of the crude extract during low temperature storage of brinjal without any purification process. In the current study, PPO enzyme from brinjal was purified to homogeneity and the kinetic parameters were determined using the purified PPO. The kinetics of enzyme inhibition of the purified PPO was also studied using natural and synthetic inhibitors.

4.2. Materials and methods

4.2.1. Chemicals

Ammonium sulphate, ascorbic acid, Bradford reagent, bovine serum albumin, caffeic acid, chlorogenic acid, citric acid, cysteine hydrochloride, dihydrocaffeic acid, 3,4-dihydroxybenzaldehyde, disodium hydrogen phosphate, D and L-DOPA (dihydroxyphenylalanine), EDTA (Ethylenediaminetetraacetic acid), erythorbic acid, gallic acid, glycine, kojic acid, 4-methyl catechol, p-Cresol, polyvinyl pyrrolidone (PVP), polyvinyl polypyrrolidone (PVPP), potassium metabisulfite, pyrocatachol, pyrogallol, α-resorcylic acid, tert-butylcatechol, tris buffer, sodium azide, sodium dihydrogen phosphate, sodium chloride, and triton X-100 were procured from Sigma-Aldrich Inc., St. Louis, MO. The DEAE Sepharose CL6B, phenyl Sepharose CL4B, and Sepharose 6B were procured from GE Healthcare Bio-Sciences Uppsala, Sweden.

4.2.2. Assay of protein and PPO activity

The polyphenol oxidase (PPO) enzyme activity was determined spectrophotometrically using 4-methyl catechol as substrate (Concellón et al., 2004). The enzyme assay was carried out taking 0.88 ml of phosphate buffer (pH 6.8, 50 mM), 0.1 ml substrate (0.1M)

and 0.02 ml of enzyme extract (prepared as discussed later). The increase in absorbance at 420 nm was monitored at 30 sec interval for 3 min using a spectrophotometer (Model UV 4-100, Unicam, Cambridge, UK) and the average change in absorbance per min was calculated. One unit of enzyme activity was defined as the amount of enzyme which caused a change of 0.1 unit absorbance/min. The PPO activity was expressed as U/g of brinjal weight. The specific activity was determined by expressing PPO activity/mg protein. Protein content of the brinjal extract was determined by the Bradford method (1976), using bovine serum albumin (BSA, Sigma Chemical, St. Louis, USA) as standard.

4.2.3. Purification of native PPO

The popular 'Kalpatharu' brinjal variety (1 kg) procured from a local vegetable market was frozen in liquid N_2, ground to fine powder, and homogenized in 3 litre of extraction solution for better extractability using a polytron homogenizer (Model PT3100, Kinematica AG, Switzerland). The extraction solution contained sodium phosphate buffer (pH 6.8, 0.05 M) with polyvinyl pyrrolidone (PVP) (1%), polyvinyl polypyrrolidone (PVPP) (2%), triton X-100 (1%) and ascorbic acid (30 mM). The extract was stirred for 30 min, filtered using muslin cloth, and centrifuged (5810R, Eppendorf, Hamburg, Germany) at 10000 g for 15 min at 4ºC. The supernatant was collected and its total activity was considered as 100% for the ease of enzyme yield calculation. The yield of each purification step was calculated as its percentage. The specific activity of this crude extract was considered as basal and the fold increase in each step of purification was considered as fold purification. The fractionation of crude extract was performed using ammonium sulphate precipitation method with 10% concentration increase in each step (Englard and Seifter, 1990). The individual fractions were solubilized in 50 ml of phosphate buffer (pH 6.8, 20 mM) and dialysed using 10 kDa cut off membrane in 5 litre

phosphate buffer (pH 6.8, 2 mM) at 4ºC with three buffer changes at 4 h interval for removal of salt.

4.2.3.1. DEAE anion exchange chromatography

The DEAE (Diethylaminoethyl) Cl-6B Sepharose column material (40 ml) was prepared by washing initially with distilled water (250 ml) and then with phosphate buffer (pH 8.0, 2 mM). The dialyzed enzyme extract (70 ml) was diluted to 700 ml, pH adjusted to 8.0 and mixed with DEAE column material, kept at slow stirring for 30 min at 4ºC. This slurry was then washed twice with 500 ml phosphate buffer (pH 8.0, 2 mM) and loaded onto the column. The elution was carried out using increasing gradient of NaCl from 0 to 0.5 M in 150 ml of phosphate buffer (pH 8.0, 2mM). The fractions (5 ml each) were tested for enzyme activity and the protein was estimated. The fractions having maximum activity were pooled.

4.2.3.2. Phenyl Sepharose hydrophobic interaction chromatography

The phenyl Sepharose column material (10 ml) was prepared by washing initially with distilled water (50 ml) and then with phosphate buffer (pH 6.8, 20 mM). The enzyme extract (15 ml) was saturated with 18% ammonium sulphate and centrifuged. The supernatant was loaded onto phenyl Sepharose column, washed with 25 ml of 17% ammonium sulphate in 20 mM phosphate buffer. The elution was carried out with decreasing gradient of ammonium sulphate from 18 to 0% in phosphate buffer (50 ml, pH 6.8, 20 mM). The enzyme assay and protein estimation (Bradford reagent) of each fraction (2 ml) was carried out. The fractions with peak of PPO activity were pooled.

4.2.3.3. Superdex gel filtration chromatography

Further purification of PPO and molecular weight determination was carried out using gel filtration chromatography in a Superdex™200 (GE Healthcare) column using HPLC equipped with a quaternary pump (Model PU-2089, Jasco, Tokyo, Japan) and a UV detector (Model 2500, Knauer, Berlin, Germany). The protein was loaded into HPLC column and the elution was carried out in phosphate buffer with 150 mM NaCl. The flow rate was maintained at 0.5 ml/min and the detection was carried out using an UV detector set at 280 nm. The molecular weight was determined using a calibration curve prepared from the retention time of proteins of known molecular weight. The PPO activity and protein estimation were also performed for different fractions collected at 30 sec interval. The molecular weight was determined for the single peak showing PPO activity based on its retention time.

4.2.4. SDS and native PAGE

The protein samples for native and SDS-PAGE (10%) were prepared, electrophoresed and visualized as described by Davis (1964) and Laemmli (1970). The protein samples were prepared by mixing 2X (double strength) gel loading buffer (0.1 M Tris-C1, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and β-mercaptoethanol (0.2 M) mixed in proportion of 1:1, boiled for 5 min, centrifuged (10000 g) for 2 min, and then loaded on to gel along with molecular weight marker mix. The total protein for each well was approximately calculated to be between 6-20 μg. After electrophoresis the gel was fixed in acidified methanol water (40% methanol and 10% acetic acid) for 1 h and then stained for 3 h with 0.1% coomassie brilliant blue R-250 on a rocker (Neolab, Mumbai, India). Later, the gel was de-stained with the same acidified methanol water until the protein

bands became distinguishable and prominent. The native PAGE (10%) was performed as above except the use of SDS and β-mercaptoethanol. The substrate staining of the gel was performed with 4-methyl catechol (0.2M) substrate in phosphate buffer (pH-6.8, 50 mM) for 1 h till the bands became prominent.

4.2.5. Determination of substrate specificity

The enzyme assay was performed by taking 0.9 ml of phosphate buffer (pH 6.8, 50 mM), 0.1 ml substrate (0.1M) and 5 µl of purified enzyme. The change in absorbance for substrates 4-methyl catechol, tert-butylcatechol, pyrocatechol, chlorogenic acid, L-DOPA (dihydroxyphenylalanine), and cresol was monitored at 420 nm using a spectrophotometer (Pérez-Gilabert and Carmona, 2000). For dihydrocaffeic acid, caffeic acid, and dihydroxybenzaldehyde, the absorbance was monitored at 400 nm (Ding et al., 1998). The absorbance for pyrogallol, D-DOPA, and gallic acid was monitored at 334, 480, and 350 nm, respectively. The absorbance was recorded at 30 sec interval for 4 min and the average change in absorbance per min was calculated and termed as activity. The relative activity of these substrates was expressed as percentage with respect to that of 4-methyl catechol substrate (considered 100%).

4.2.6. Determination of kinetic parameters of PPO

The enzyme activity was monitored with fixed purified enzyme concentration (10 µl containing ~ 4 units) and increasing substrate [S] concentration (0.04 - 0.2 mM) in the reaction mixture. The reaction velocity [V] was determined by monitoring change in absorbance per min. The reciprocal plot (1/[V] vs. 1/[S]) was plotted and the Km and Vmax were determined from the slope and intercept of the straight line (Yang, et al., 2001; Lineweaver and Burk, 1934). The total enzyme molecule concentration was

determined by dividing estimated total protein by molecular weight of PPO. The turnover number (kcat) was calculated by dividing total substrate molecules changed per min by the total molecules of PPO enzyme in the reaction mix. The catalytic efficiency was calculated by dividing kcat by Km.

4.2.7. Inhibitor studies

The inhibitor studies were carried out with ascorbic acid (10-30 µM), α-resorcylic acid (20-50 mM), erythorbic acid (10-30 µM), cysteine hydrochloride (6- 10 µM), potassium metabisulfite (5- 20 µM), kojic acid (0.05-0.2 mM), citric acid (20 -40 mM), and sodium azide (0.1-10 mM) using the method described by Janovitz-klapp et al., (1990). The enzymatic reactions were monitored with a fixed amount of purified enzyme (10 µl containing ~ 4 units) and increasing concentrations of 4-methyl catechol substrate (0.02-0.2 mM) along with different concentrations of the inhibitors under test. The reciprocal plot 1/[S] on X-axis vs 1/[V] on Y-axis was plotted for each concentration of inhibitor. The inhibition mechanism was determined from the nature of graphs. The compound with competitive inhibition mechanism had similar Vmax values. For each straight line graph the apparent Km was calculated from x-axis intercept (-1/Km). For determination of inhibitor constant (Ki), inhibitor concentration [I] on X-axis was again plotted against apparent Km value on Y-axis. The Ki value was calculated from the value of X-axis intercept of this straight line (-Ki). Similarly, for compounds with mixed inhibition mechanism, the Ki value was calculated from the plot of inhibitor concentration on X-axis against apparent Vmax on Y-axis.

4.2.8. Statistical analysis

Experiments were repeated in three sets independently, each set having 3 replicates. The means and standard deviations were calculated taking all the readings in consideration. One way ANOVA (at the level of significance $P \leq 0.05$) was performed to ascertain the significance of the means. Statistical analysis was performed using BioStat 2009 Version Professional 5.8.0.0 (AnalystSoft Inc., Canada).

4.3. Results

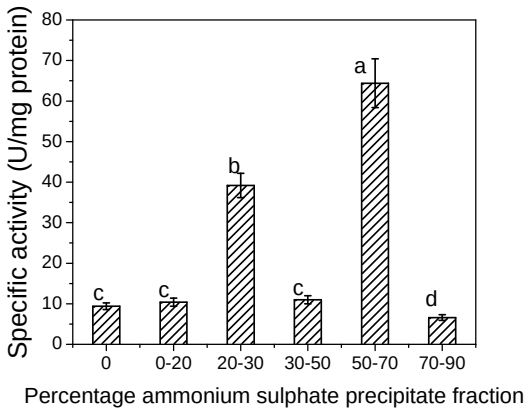
4.3.1. Optimization of parameters for extraction of PPO

Although PPO has been purified from many plant sources, there is scarcity of reports describing purification of PPO from brinjal. Plant sources rich in phenolics pose problems during purification due to irreversible binding of PPO with its phenolic substrates that affects the ionic and hydrophobic characteristics of the enzyme, and, that in turn affects the elution pattern during chromatographic purification (Mayer, 2006). In the current study the problem was solved by adding a combination of polyvinyl pyrrolidone (PVP) (1%), polyvinyl polypyrrolidone (PVPP) (2%), and ascorbic acid (30 mM) in the extraction solution. This significantly inhibited the binding of phenolics to PPO enzyme. However, addition of excess ascorbic acid showed negative effect on the enzyme activity. The extraction was performed at 4 ºC. The crude sodium phosphate buffer extract from brinjal showed extensive browning during the extraction process. The extract showed 90% of activity in the supernatant. Addition of detergent (Triton X-100, 1%), salt (NaCl, 0.5 M), or SDS (0.5%) during extraction did not increase PPO activity which indicated the absence of strongly membrane bound, weakly ionic bound, or latent forms of PPO in brinjal, respectively.

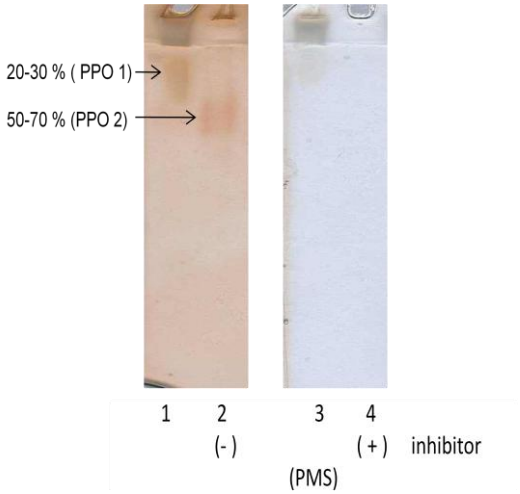
In most of the reports 20-80% ammonium sulphate precipitation fraction was chosen for purification purpose. But in the current study, the ammonium sulphate precipitation was performed with gradual increase of 10-20% in each step. A significantly high PPO activity was observed in two discrete fractions (20-30% and 50-70%) compared to other fractions ($P \leq 0.05$) and could be due to the presence of two isoforms of PPO (Fig. 4.1a). These two fractions after running on native PAGE were stained with the substrate (4-methyl catechol) in the presence and absence of inhibitor potassium metabisulfite (PMS) for ruling out the enzyme activity by any other proteins including peroxidase (Mayer, 2006). The results showed that these two are indeed PPO isoforms localized at different positions on gel (Fig. 4.1b). In addition to this, the PPO activity of these two fractions was also tested by enzyme assay in the presence and absence of inhibitor (Fig. 4.1b). The results also showed complete inhibition by PMS in both the fractions. This indicated that PPO in these two fractions may differ in its hydrophobic characteristics resulting in precipitation with two different ammonium sulfate concentrations. Pérez-Gilabert and Carmona (2000) have characterized the crude 20-30% ammonium sulphate precipitated fraction and showed the presence of cresolase and catacholase activity. In the present study a basal PPO activity was also observed in other remaining fractions, which indicated the probable nonspecific precipitation of this protein due to binding of phenolic compounds as reported earlier by Papadopoulou and Frazier (2004). However, the specific activity in 50-70% fraction was the highest and was about 62% more than that of 20-30% fraction and hence was used for further purification and characterization (Fig. 4.1a).

4.3.2. Purification of PPO

The PPO enzyme was observed to bind DEAE column material at pH 8.0 with low buffer concentration (2 mM). During gradient elution (0-0.5M NaCl) a high PPO activity at fraction nos. 25 to 35 (NaCl concentration about 0.15 M) was observed with peak activity at fraction no. 32 (Fig. 4.1c). The protein content increased gradually with peak at about fraction number 40 and a gradually decreased towards the end of the gradient elution. The peak region was therefore pooled and used for phenyl Sepharose chromatography. The PPO binding to phenyl Sepharose was observed at 18% ammonium sulphate saturation in phosphate buffer (20 mM, pH 6.8). The active PPO was eluted early in the gradient at 15% of ammonium sulphate concentration in eluent buffer with the peak activity in fraction no. 10 (Fig. 4.1d). The protein content in eluted fraction showed two peaks. The first sharp peak coincided with the PPO activity peak and the second broad one appeared in 20-25 fractions with ammonium sulphate concentration of about 7-8%. This peak could be due to other interfering proteins. The fractions with peak PPO activity were pooled and were used for gel filtration chromatography for further purification and approximate molecular weight determination. The gel filtration showed a single peak eluted at 27 min, whereas, the protein content peak was observed at 32 min (Fig. 4.2a). The eluent at 27 min on SDS PAGE showed multiple peaks (data not shown), therefore, the peak PPO activity was collected, concentrated and injected again which helped in achieving further improvement in purity.



(a)



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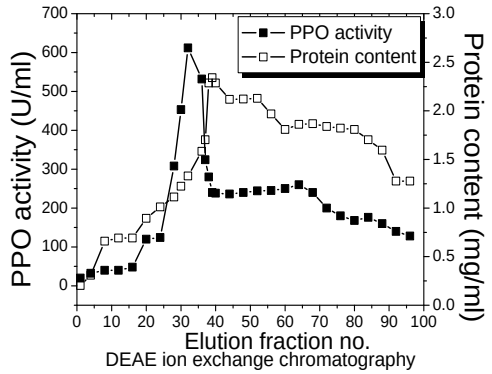
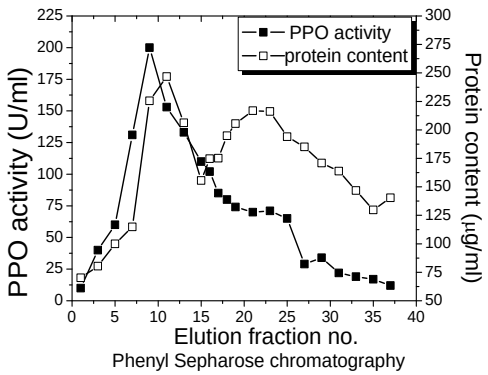
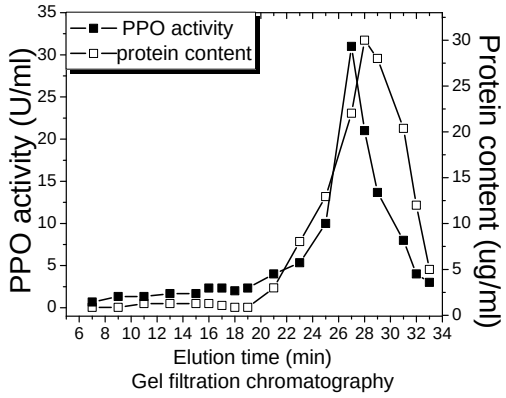


(c)

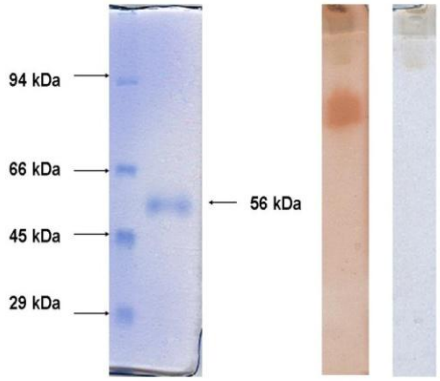


(d)

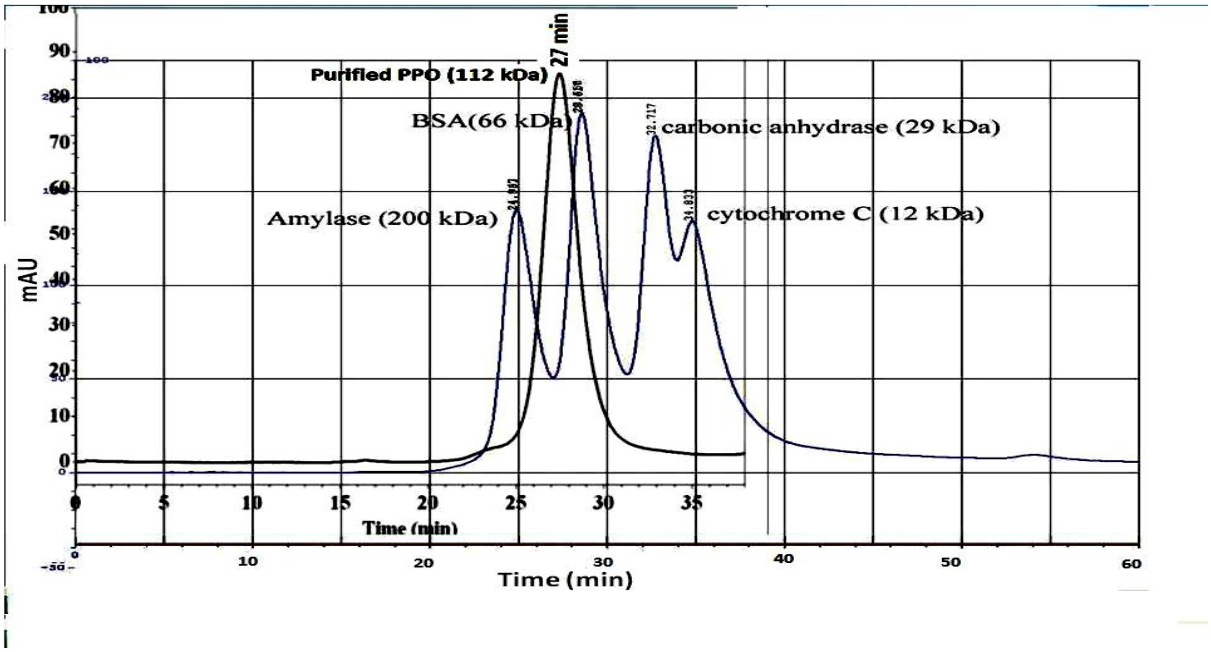
Figure 4.1. The different steps of purification and profile of PPO from brinjal. **(a)** Ammonium sulphate precipitation (different letters in the superscript indicate significance of difference in the mean), **(b)** native PAGE indicating PPO activity in brinjal crude extract showing presence of two isoforms (PPO1 in lane 1 and PPO2 in Lane 2) while incubated with buffer and substrate (4-methyl catechol) without (lane 1 and 2) and with (lane 3 and 4) potassium metabisulfite, a PPO inhibitor **(c)** DEAE ion exchange chromatography, **(d)** Phenyl Sepharose hydrophobic interaction chromatography.



(a)



(b) **(c)** **(d)**



(e)

Figure 4.2. Molecular weight determination and native PAGE profile of PPO. Superdex gel filtration chromatography **(a)**, SDS-PAGE exhibiting purified PPO with molecular weight markers **(b)**, native PAGE indicating PPO activity in PPO2 extract while incubated with buffer and substrate (4-methyl catechol) without **(c)** and with **(d)** potassium metabisulfite, a PPO inhibitor, HPLC profile indicating gel filtration purified PPO superimposed with proteins of known molecular weight **(e)**.

4.3.3. PPO is a dimer of 112 kDa molecular weight

The molecular weight of purified PPO was observed to be about 112 kDa, based on a calibration curve prepared using proteins of known molecular weight (Fig. 4.2b and 4.2c). The SDS-PAGE profile of the finally purified protein showed a single band of about 56 kDa (Fig. 4.2d). This indicated the active protein to be a homodimer of 112 kDa.

4.3.4. Native PAGE of 50-70% fraction showed presence of a single band

The two isoforms of PPO observed in 20-80% ammonium sulphate precipitation only resolved on native PAGE when run on two different wells (Fig. 4.1b). The gel run with 20-80% fraction in a single well resulted in unresolved smear after substrate staining. The 50-70% ammonium sulphate precipitated extract, was analysed for presence of different isoforms of PPO enzyme on a native PAGE without addition of denaturing (SDS) and reducing (β-mercaptoethanol) agent. The substrate staining with 4-methyl catechol (0.1 M) immediately after electrophoresis showed the presence of a single band (Fig. 4.2e). The substrate staining of a gel in the presence of potassium metabisulfite, a PPO inhibitor showed no visible band, confirming the identity of a PPO (Fig. 4.2f).

4.3.5. Fold purification and yield of PPO

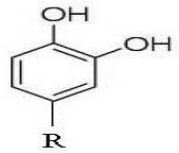
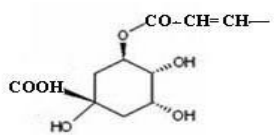
During each subsequent steps of purification the PPO activity/ml increased and as expected the protein content decreased. This resulted in increase in specific activity to 4925 units/mg protein the total yield was significantly reduced to about 0.02%. In 1 kg of brinjal, the finally purified PPO fraction contained 0.37 mg of active PPO protein (Table4.1) which is 259 fold after gel filtration chromatography (Table 4.1). However, in each step of purification a loss in total activity was noticed because only peak fraction displaying PPO activity was collected purposely. This significantly increased the

Table 4.1. The purification of PPO from brinjal.

Purification step	Volume (ml)	Activity (Units/ ml)	Total activity (Units)	Protein (mg/ml)	Specific activity (U/ mg protein)	Yield (%)	Purification (fold)
Crude	3000^{a} ±50	28^{d} ±5	$(8.4^{a}\pm0.6)$ x10^{4}	1.47^{a}±0.1	19^{e}±5	$100^{a}\pm0^{*}$	1^{e}±0
50-70% Ammonium sulphate saturation	400^{b} ±20	133^{c} ±12	$(3.8^{b}\pm0.3)$ x10^{4}	2.05^{b}±0.2	65^{d}±5	45^{b}±5	3.42^{d}±0.3
Ion exchange (DEAE)	43^{c} ±5	256^{b} ±30	$(1.1^{c}\pm0.2)$ x10^{4}	1.5^{a}±0.1	170^{c}±16	13^{c}±1.1	8.94^{c}±0.7
Hydrophobic (Phenyl Sepharose)	6^{d} ±1	378^{a} ±35	2268^{d}±226	0.45^{c}±0.04	840^{b}±55	2.7^{d}±0.2	44^{b}±3
Gel filtration (Superdex)	4.7^{d} ±0.5	394^{a} ±32	1851^{e}±154	0.08^{d}±0.01	4925^{a}±460	0.02^{e}±0.002	259^{a}±23

* Activity of extract prepared by homogenizing 1 kg of brinjal in 3 litre of extraction solution. The values are expressed as mean ± SD of three parallel experiments. The values in column with different letter superscript are significantly different ($P\leq0.05$).

Table 4.2. Substrate specificity of native PPO purified from brinjal.

 Substrate	Functional group (R) at meta position of –OH group	Relative Activity* (%)
4-Methylcatechol	-CH_3	100^{a} ±0
tert- butylcatechol	-$C(CH_3)$	83^{b} ±4
Dihydrocaffeic acid	-CH_2-CH_2-COOH	82^{b} ±3
Pyrocatachol	Nil	70^{c} ±3
D-DOPA, L-DOPA	-CH_2-$CH(NH_2)$-COOH	40^{d} ±2, 22^{f} ±2
Caffeic acid	-CH=CH-COOH	32^{e} ±2
Chlorogenic acid		31^{e} ±2
Pyrogallol	-OH at *ortho* position	17^{g} ±2
3,4-dihydroxybenzaldehyde	-CHO	5^{h} ±1
Gallic acid	-COOH (and -OH at *ortho*)	1.5^{h} ±0.3
p-Cresol	Nil, (-CH_3 at *para* position)	0

* Relative activity was expressed with respect to 4-methyl catechol considering it as 100%. The values were expressed as mean ± SD of three parallel experiments. The values in column with different letter superscript are significantly different ($P \leq 0.05$).

purification fold, however, the total yield was significantly reduced to about 0.02%. In 1 kg of brinjal, the finally purified PPO fraction contained 0.37 mg of active PPO protein (Table 4.1).

4.3.6. PPO substrate specificity showed higher catechol oxidase activity

Purified PPO was characterized for its substrate specificity using eleven well known substrates namely, 4-methyl catechol, tert-butylcatechol, pyrocatechol, chlorogenic acid, D and L-DOPA, cresol, dihydrocaffeic acid, caffeic acid, dihydroxybenzaldehyde, pyrogallol and gallic acid. Out of these 4-methyl catechol showed the maximum activity and considered as 100% to compare its activity with other substrates (Table 4.2). The specificity of each substrate was found to be significantly different ($P \leq 0.05$). Higher enzyme activity was observed with substrates having catechol ring structure and a functional group (-R) at meta position with respect to –OH group. A smaller functional group ($–CH_3$) present in 4-methyl catechol probably rendered it with the maximum activity. With an increase in the size of this functional group the activity decreased as was observed in the case of tert-butylcatechol, chlorogenic acid, and DL-DOPA (Table 4.2). On the contrary, the absence of this functional group also decreased activity by 30% as observed in case of pyrocatechol. These observations indicated the important role of the $–CH_3$ group and its location in catechol structure in determining its specificity. Most of the substrates with catechol ring structure and a functional group at other position of the ring (-OH at *ortho* position as in pyrogallol, gallic acid, and $-CH_3$ at *para* position as in p-cresol) showed very less or absence of activity. The enzyme did not show any activity against monophenol like tyrosine (data not shown).

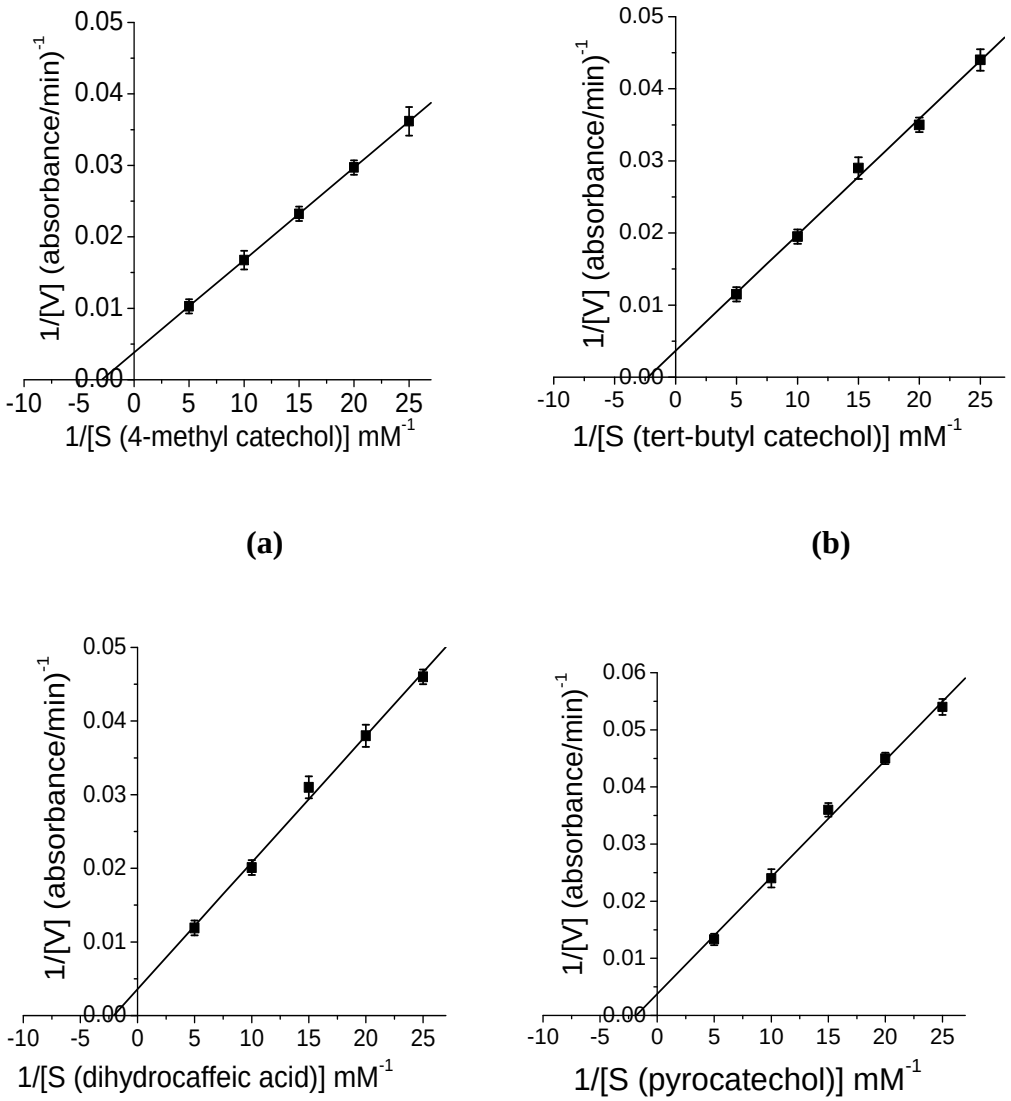


Figure 4.3. The Lineweaver-Burk plot (reciprocal plot of 1/[V] against 1/[S]) of native PPO with different substrates to determine Km values.

Table 4.3. The kinetic characteristics* of purified brinjal PPO.

Substrate	Km (mM)	Vmax (ΔOD/min)	Substrate concentration (μM) at Vmax	kcat	kcat/Km ($mM^{-1)}$
4-methyl catechol	0.34^{d} ±0.05	280^{a}± 26	212^{a} ± 10	$(1.14^{d}$±0.1)x10^{6}	$(3.3^{c}$±0.2) x10^{6}
tert-Butyl catechol	0.44^{c} ±0.02	277^{a}±24	209^{a} ± 12	$(3.8^{a}$±0.2)x10^{5}	$(8.6^{a}$±0.5) x10^{5}
Dihydrocaffeic acid	0.48^{b} ±0.01	280^{a}±25	205^{a} ± 11	$(2.3^{b}$±0.2)x10^{5}	$(4.7^{b}$±0.4) x10^{5}
Pyrocatechol	0.54^{a} ±0.04	267^{a}±21	203^{a} ± 10	$(1.6^{c}$±0.1) x10^{5}	$(2.9^{d}$±0.1) x10^{5}

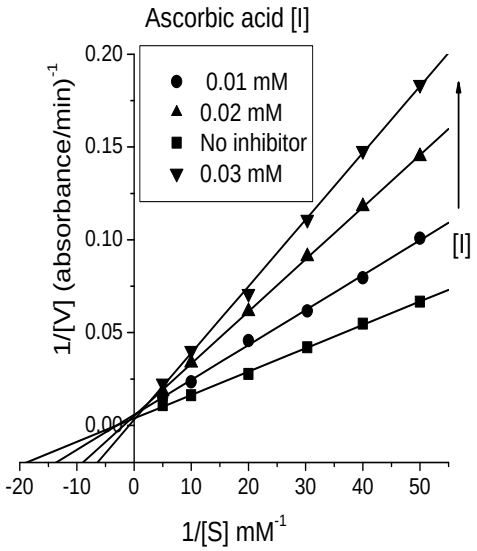
*Km – Michaelis-Menten constant, Vmax- maximum reaction velocity, kcat- turn over number of enzyme, kcat/Km- catalytic efficiency. The values were expressed as mean ± SD of three parallel experiments. The values in column with different letter superscript are significantly different ($P \leq 0.05$).

4.3.7. Kinetic characteristics of PPO

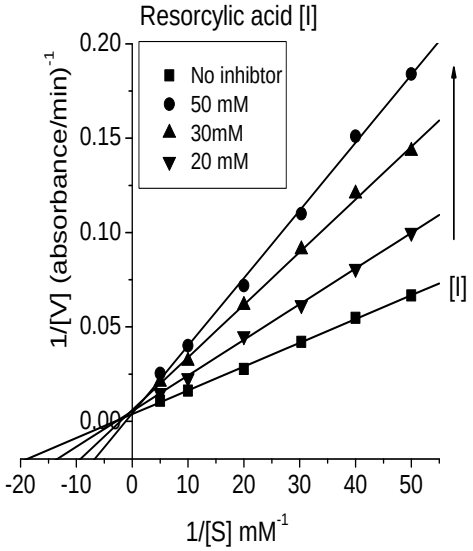
The Km and Vmax values were determined for substrates having higher activity using Lineweaver-Burk plot (1/S and 1/V) (Fig 4.3a-d). The lowest Km was observed for 4-methyl catechol (0.34 mM) and Vmax was 212 µM, with absorbance change of about 280 units/min (Table 4.3). The Km value increased with less specific substrates. However, the Vmax remained almost constant. The tert-butyl catechol, dihydrocaffeic acid, and pyrocatechol showed 29, 41, and 58% higher Km value compared to 4-methyl catechol and the difference was found to be significant ($P \leq 0.05$). The turnover number (kcat) and catalytic efficiency (kcat/Km) were also found to be maximum for 4-methyl catechol and minimum for pyrocatechol (Table 4.3).

4.3.8. Inhibitor studies

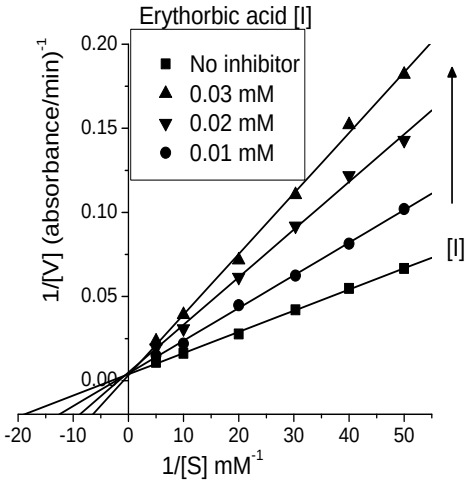
The optimum concentration range for each inhibitor to perform the kinetic study was determined by performing preliminary experiments (data not shown) and the values were found to vary significantly among different inhibitors. The eight different inhibitors namely ascorbic acid, α-resorcylic acid, erythorbic acid, cysteine hydrochloride, potassium metabisulfite, kojic acid, citric acid, and sodium azide were studied. Out of these inhibitors, citric acid and sodium azide showed mixed inhibition and the rest six showed competitive inhibition (Fig. 4.4a-h). For competitive inhibitors the Vmax value (1/Y-axis intercept) remained unchanged, whereas, for mixed inhibitors the Vmax values changed. The inhibitor constant (Ki) value was determined from the X-axis intercept of the plot. The most effective competitive inhibition was shown by cysteine hydrochloride with the lowest Ki value of 1.8 µM (Fig. 4.5d). The next effective competitive inhibitor was potassium metabisulfite with Ki value of 9 µM (Fig 4.5e).


Ascorbic acid [I]
0.01 mM
0.02 mM
No inhibitor
0.03 mM
[I]
1/[V] (absorbance/min)-1
1/[S] mM-1

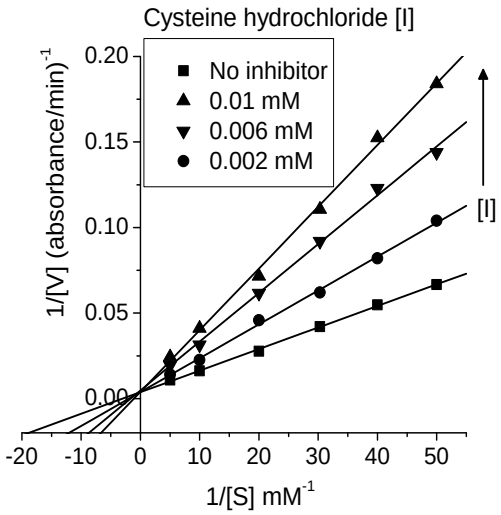
(a)


Resorcylic acid [I]
No inhibtor
50 mM
30mM
20 mM
[I]
1/[V] (absorbance/min)-1
1/[S] mM-1

(b)


Erythorbic acid [I]
No inhibitor
0.03 mM
0.02 mM
0.01 mM
[I]
1/[V] (absorbance/min)-1
1/[S] mM-1

(c)


Cysteine hydrochloride [I]
No inhibitor
0.01 mM
0.006 mM
0.002 mM
[I]
1/[V] (absorbance/min)-1
1/[S] mM-1

(d)

contd…..

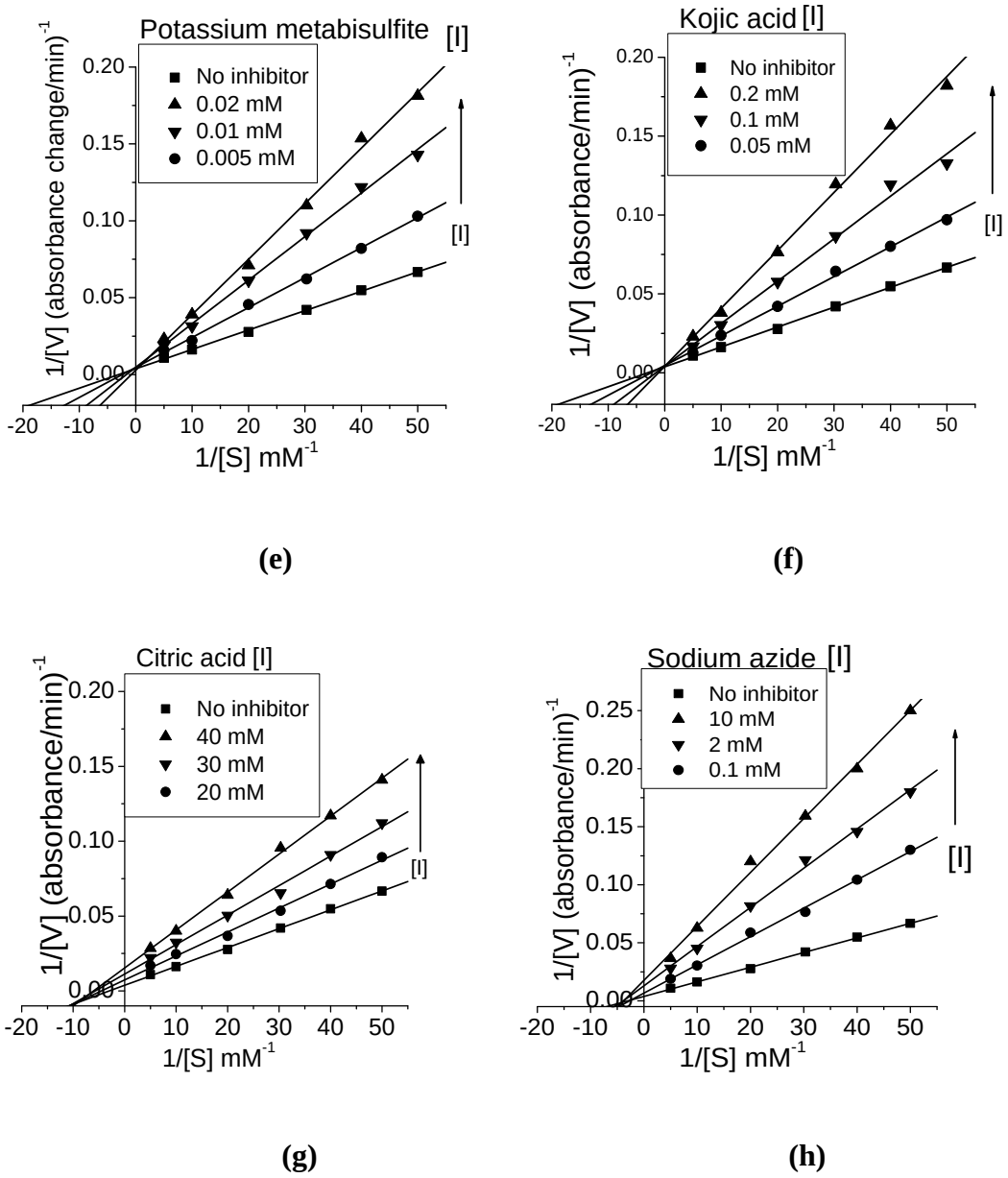
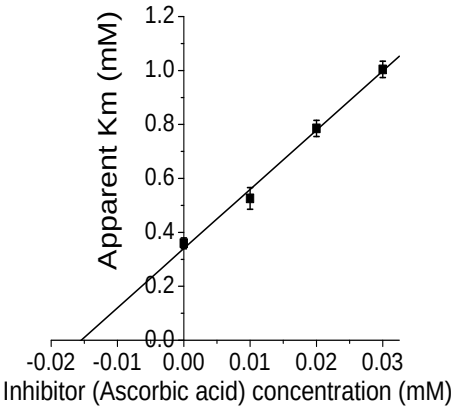
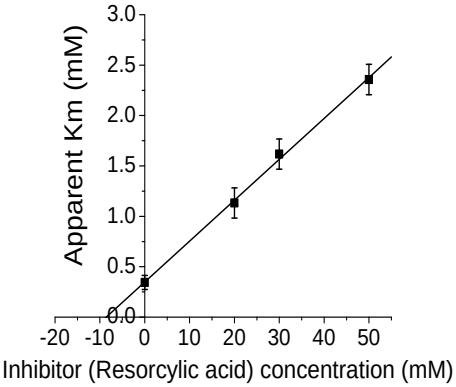


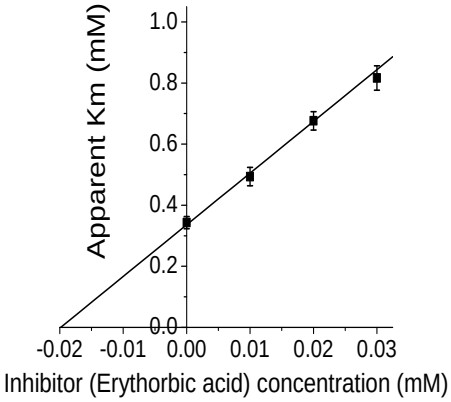
Figure 4.4. The kinetics of PPO inhibition by inhibitors analyzed using reciprocal plot of 1/[S] with 1/[V] plotted at different inhibitor concentrations. The average values of the reciprocal of the [V] were plotted. The SD values were not included for clarity.


Apparent Km (mM)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.02 -0.01 0.00 0.01 0.02 0.03
Inhibitor (Ascorbic acid) concentration (mM)

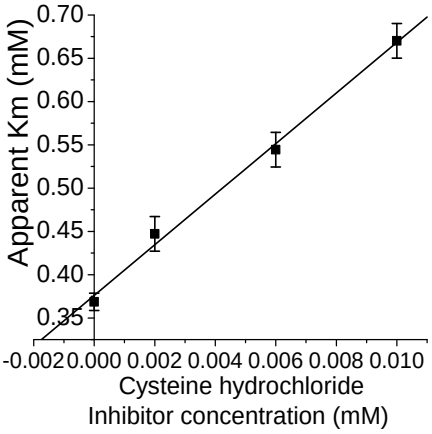
(a)


Apparent Km (mM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-20 -10 0 10 20 30 40 50
Inhibitor (Resorcylic acid) concentration (mM)

(b)

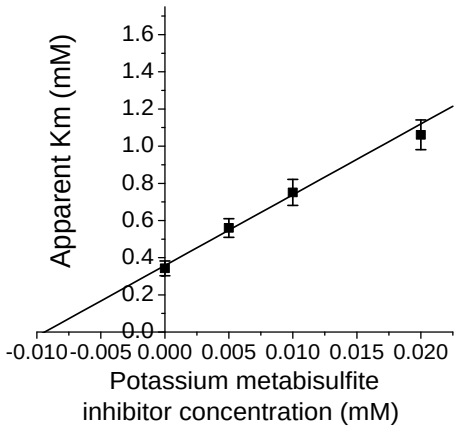

Apparent Km (mM)
1.0
0.8
0.6
0.4
0.2
0.0
-0.02 -0.01 0.00 0.01 0.02 0.03
Inhibitor (Erythorbic acid) concentration (mM)

(c)

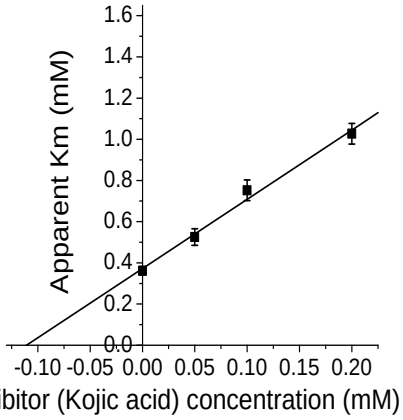

Apparent Km (mM)
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
-0.002 0.000 0.002 0.004 0.006 0.008 0.010
Cysteine hydrochloride
Inhibitor concentration (mM)

(d)

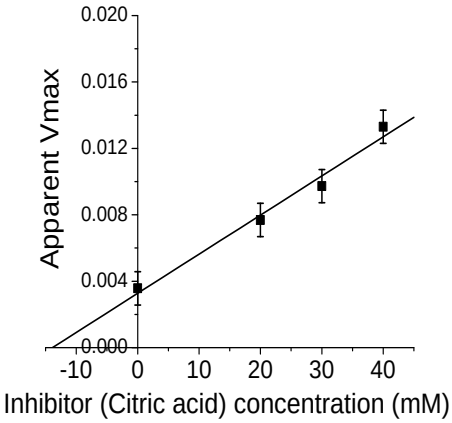
contd……..



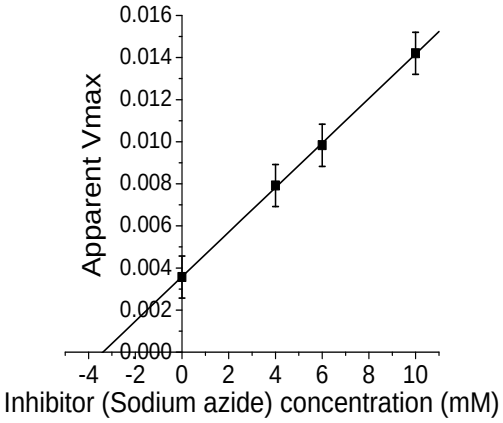
(e)



(f)



(g)



(h)

Figure 4.5. The plot of apparent Km (for competitive inhibition) and apparent Vmax (for mixed inhibition) against the inhibitor concentration for determination of inhibitor constant (Ki). The straight line in these plots crossed the X-axis at (–Ki) value.

The ascorbic and erythorbic acid showed similar competitive inhibition with Ki values of 15.2 and 19.6 µM, respectively (Fig. 4.5a and 5c). The next effective competitive inhibition was shown by kojic acid and resorcylic acid with Ki values of 0.123 and 8.2 mM, respectively (Fig. 4.5b and 5f). The mixed inhibition was shown by sodium azide and citric acid with higher Ki values of 3.3 and 13.5 mM, respectively, and the two were also less effective inhibitors of the brinjal PPO (Fig. 4.5g and 5h).

4.4. Discussion

The PPO was classified as monophenol oxidase (Tyrosinase, EC 1.14.18.1) and catechol oxidase (diphenol oxidoreductase, EC 1.10.3.2). The first one adds a –OH group to a monophenol like tyrosine in first step and then converts this diphenol to a quinone in second step. The second one which converts a diphenol to a quinone is present in brinjal. The PPO enzyme is reported to be located in thylakoid lumen of chloroplast inside plant cells either in membrane bound or soluble form (Ding et al., 1998). Membrane bound form could be weakly or strongly bound. Weakly linked enzyme can be extracted by increasing salt concentration, whereas, integral membrane bound forms could be isolated with addition of detergents in extraction solution. The latent form of this enzyme reported to be activated by treatment with SDS or with proteases (Pérez-Gilabert and Carmona, 2000) was also not detected in the current study. During homogenization process the enzyme comes in contact with substrate and oxygen resulting in excessive browning of the suspension. The inhibition of browning by PVP and PVPP is due to the binding to the substrate phenolics making them unavailable for the enzyme action (Mayer, 2006). Ascorbic acid (50 mM) inhibited PPO and browning of the extract could be controlled (Ding et al., 1998).

The fold purification achieved in the final purified PPO was as high as 259 fold with the yield of 0.02%. The specific acivity of purified PPO was found to be 4925 units/ mg protein, which is very high compared to the earlier reported values for brinjal. Concellon et al. (2004) reported a specific activity of the 11 units/ mg protein in crude extract of brinjal. Similarly, Pérez-Gilabert and Carmona (2000) have also reported a specific activity of only 2.5 units/ mg protein in ammonium sulfate precipitated fraction of brinjal. The difficulty in PPO purification from brinjal is evident from these earlier reports of a few fold specific activity and limited yield. In current study a significantly high fold (259) purification is achieved which could have resulted in a loss of yield. Though there are reports of lesser fold purification, many authors have reported higher fold purification in other vegetables and plant parts. In potato, the PPO was 5 fold purified with 18% yield, whereas, in banana peel the PPO was 460 fold purified with 2.2% yield (Sánchez-Ferrer et al., 1993; Yang et al., 2001). Similarly, in loquat fruit the PPO was 422 fold purified with about 35% yield (Ding et al., 1998). These differences could be due to differences in concentration of phenolics and their irreversible binding to the PPO protein resulting in interference with protein purification in certain fruits and vegetables (Papadopoulou and Frazier, 2004). The phenolics bound to the enzyme change its ionic and hydrophobic characteristics resulting in variation in elution behavior during purification steps (Papadopoulou and Frazier, 2004). This resulted in difficulty in purifying the wider peaks observed during elution. The collection of fractions only with maximum PPO activity for better fold purification resulted in loss of PPO protein from shoulder region of the peak, ultimately affecting the total yield of the purified protein.

The substrate specificity of brinjal PPO showed that it belonged to the catechol oxidase family (EC 1.10.3.2) as the maximum activity was observed with catechol or its derivative substrates (Table 4.2). The enzyme did not show any activity with monophenol

substrates like tyrosine, which indicated the monophenol monoxygenase (EC 1.14.18.1) activity was absent with this enzyme (data not shown). The enzyme also did not show any activity with cresol which indicated that the cresolase activity was also absent with this purified enzyme (Table 4.2). With a few exceptions, mostly the plants, animals, and fungi have been reported to have catechol oxidase, tyrosinase and cresolase activity, respectively (Mayer, 2006). In addition to this, the catechol derivatives with bigger -R group showed gradual reduction in specificity (Table 4.2). The catechol without -R group also showed lesser specificity (pyrocatechol) compared to 4-methyl catechol where the -R is a methyl group. The -R group position in the catechol ring also observed to be determinant of higher specificity. The active site of the enzyme could be interacting with these substrate molecules where -R group position and composition provided advantage for efficient catalysis.

The PPO isolated from brinjal was found to have very significant Km values with different common substrates as compared to others sources, along with better turn over number (kcat) and catalytic efficiency (kcat/Km) (Table 4.3). The potato, tobacco, and banana PPO were reported to have 0.9, 1.2, and 3.9 mM of Km value with 4-methyl catechol substrate, respectively (Sánchez-Ferrer et al., 1993; Shi et al., 2002; Yang et al., 2001). In brinjal the earlier reported value of Km for 40% ammonium sulphate precipitated PPO was 1 mM for 4 methyl catechol substrate (Pérez-Gilabert and Carmona, 2000). The lower Km value (0.34 mM) observed in the current study could be the reason for high substrate affinity of the enzyme for indigenous phenolics resulting in browning of the extract.

The inhibition of PPO is important in food industry due its role in browning. The newly studied compound cysteine hydrochloride was structurally quite similar to the

earlier studied inhibitor cysteine and showed significant inhibition of PPO activity at a concentration much below the permissible level. Cysteine hydrochloride is derived from amino acid cysteine and is highly soluble in water. It is also administered to infants in injection (7.25% in water USP) as a source of essential amino acid cysteine (Kux, 2010). It is a reducing agent and known to form adduct with intermediate quinones, which inhibit the PPO enzyme at much lower concentration. Potassium metabisulfite, another reducing agent is known to inhibit PPO by the reaction of sulfite with intermediate quinones, resulting in the formation of sulfoquinones, which irreversibly inhibits PPO, resulting in its complete inactivation (Marshall, Kim and Cheng-I, 2000). Ascorbic acid and erythorbic acid which are considered as vitamin C, inhibit PPO by reducing the intermediate quinones back to diphenols. Resorcylic acid used in the current study is a natural phenolic acid having similar structure to the known inhibitor 4-hexyl resorcinol, except having an acid group (-COOH) in place of a hexyl group ($-C_6H_{18}$). This less studied compound is having higher solubility and showed inhibition similar to 4-hexyl resorcinol with Ki value of 8.2 µM. Such compounds were reported to compete with the substrate for binding to the active site and thereby causing enzyme inhibition. Kojic acid is a fungal secondary metabolite reported to inhibit PPO weakly by chelating the copper atom in the active site of enzyme. In current study, it also showed weak inhibition against brinjal PPO. Citric acid reported to inhibit PPO by lowering pH and chelating the copper ion in active site (Marshall et al., 2000). This study provides valuable information for control of browning in fruits and vegetables processing industry using a simple GRAS compound cysteine hydrochloride.

CHAPTER 5

Genetic Polymorphism of PPO in Different Brinjal Cultivars

5.1. Introduction

Many cultivars of brinjal (*Solanum* genus) are available with varying morphological features such as colours (purple, green, purple with white and green stripes and patches), shapes (ovoid, obovate, oblong, cylindrical, club shaped), and calyx (spiny, non-spiny) (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. The PPO enzyme causes enzymatic browning in fruits and vegetables and is coded by *ppo* gene. PPO activity could vary depending upon the gene sequence or epigenetic factors. Recently, the PPO gene has been reported to exist in many isoforms and found to be expressed in different plant parts under stress conditions like wounding (Shetty et al., 2011). Similar studies were also reported in potato (Newman et al., 1993) and tomato (Thygesen et al., 1995) and many other fruits and vegetables. The study of the gene sequence and its analysis in different cultivars would help understand the unique nature of protein sequence that may have a bearing on the enzyme activity and overall browning. The molecular studies on *ppo* gene were undertaken to understand the basics, which could be helpful in exploring the possibility to inhibit this enzyme in food processing applications. There are a few reports about cloning and expression of PPO gene in bacteria. None of these studies have reported enzymatically active recombinant PPO in *E.coli* (Sullivan et al., 2004). In the current study, the PPO gene has been cloned and expressed in *E.coli* with the aim to produce the protein in high amounts for its further characterization.

5.2. Materials and methods

5.2.1. Chemicals

Chloroform, isoamyl alcohol, ethyl alcohol, agarose, agar powder, Luria broth, plate count agar were of molecular biology grade and procured from Himedia Laboratories, Mumbai, Maharashtra, India. The Taq DNA polymerase, dNTP mix, cDNA synthesis kit, TriPure RNA isolation reagent (containing guanidine thiocyanate and phenol) reagent, rapid DNA ligation kit were procured from Roche applied sciences, Mannheim, Germany. The restriction endonuclease (NdeI and XhoI) were procured from New England Biolabs, Ipswich, MA.

5.2.2. Brinjal

The six major popular cultivars of brinjal (*Solanum melongena*) 'Pusa purple long'(V1), 'Ravaiya'(V2), 'Azad kranti'(V3), 'Kalpatharu'(V5), 'Raveena'(V6), and 'Anupam'(V7) (listed in Table 2.1) were procured from a local supplier at Mumbai, India. For cloning and expression studies of PPO only the cultivar showing maximum activity ('Kalpatharu' V5) was used.

5.2.3. DNA isolation

The genomic DNA was isolated using CTAB (cetyl trimethyl ammonium bromide) method to avoid phenolics interference (Rogers and Bendich, 1994). The whole brinjal fruit was frozen in liquid N_2 and ground to fine powder. An aliquot (100 mg) was added to sterile 2 mL centrifuge tubes containing 1 mL of preheated (65 ºC) extraction buffer composed of 0.1 M Tris HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2%(w/v) CTAB and 1% PVP (polyvinyl polypyrrolidone). Tubes were held for 15 min and then equal volume

of chloroforml/isoamyl alcohol (24:1; v/v) was added, mixed thoroughly and centrifuged (5810R, Eppendorf, Hamburg, Germany) at 11000 g for 5 min. The aqueous top layer was mixed gently with 10% CTAB and equal volume of chloroform/isoamyl alcohol (24:1; v/v) and kept at -20 ºC for 30 min. The mixture was centrifuged (15000 g, 35 min) and the pellet was dissolved in high salt Tris (10 mM)-EDTA (1 mM) buffer having 1M NaCl. The precipitation was carried out with absolute ethanol followed by washing with 70% ethanol. The pellet was dried at 65 ºC and dissolved in low salt TE buffer (1 mM Tris, 0.1 mM EDTA pH 8.0).

5.2.4. RNA isolation

The RNA isolation was performed with guanidine thiocyanate-phenol-chloroform extraction method using TriPure isolation reagent (Chomczynski, P. 1993). Whole brinjal fruits procured at early stage of maturation were frozen in liquid nitrogen and ground to fine powder. An aliquot (100 mg) of this powder was transferred to a sterile 2 mL centrifuge tube containing 1 ml of readymade TriPure isolation reagent (Roche Applied Science) (containing guanidine thiocyanate and phenol), thoroughly mixed, and incubated for 5 min. Later 0.2 ml of chloroform was added and the suspension was centrifuged at 12000 g for 15 min. In a separate vial the aqueous top layer was mixed with isopropanol (0.5 ml), incubated for 10 min, and later centrifuged at 12000 g for 10 min. The pellet was resuspended in 70% ethanol (1 ml), precipitated, washed in absolute ethanol, air dried, and finally solubilized in DEPC treated RNAse free water. This was treated with DNAse to avoid any DNA contamination. The pure total RNA thus prepared was used for cDNA synthesis using a cDNA synthesis kit (Roche Applied Sciences).

Table 5.1. The oligonucleotides used for the PCR amplification of PPO gene.

Primer name*		Nucleotide sequence
P1.	For. end	5’-CACTCCTAAGCCCTCTCAACTTTTC- 3’ (25 bp)
P2.	Rev. end	5’-CCAACAGTTCCGTTATCGCC-3’ (20 bp)
P3.	For. int.	5’-GTAACTAATGCTCCATGTCCTC-3’ (22 bp)
P4.	For. int.	5’-ACCATGGCGTAACTTCAAGCC-3’ (21 bp)
P5.	Rev. int.	5’-GAGGACATGGAGCATTAGTTAC-3’ (22 bp)
P6.	Rev. int.	5’-GGCTTGAAGTTACGCCATGGT G-3’ (22 bp)
P7.	For. end	5'-ATGGCAAGCTTGTCCAATAGTAGTATACAACCC-3' (33bp)
P8.	For. int.	5'-GGGCATGCGTATGCCTGCCATGTTCGATGG -3' (30 bp)
P9.	For. int.	5'-GGACTGCCCTTTACGACCAAGTAC GTAACC-3' (31 bp)
P10.	For. int.	5'-GATTACGCACCCATGCCAAGACCGTGGCGC-3' (30 bp)
P11.	Rev. end	5'-TTAACAATCTGCAAGACTGATCGTCGCACC-3' (30 bp)
P12.	Rev. int.	5'-CATTACTGTCCACATTCAGGAACACGTCG-3' (29 bp)
P13.	Rev. int.	5'-GCAAGGTTGTACCTGGCACTGTACCAGTCC-3' (30 bp)
P14.	Rev. int.	5'-GCTGACGAATACGGAGCTTAGTCATAGAA GGG-3' (32 bp)
P15.	For. end	5'-GGAATTCCATATGGCAAGCTTGTGCAATAG-3' (30 bp)
P16.	Rev. end	5'-TACTCTCGAGTTAACAATCTGCAAGACTGAT CGTCGCACC-3' (40 bp)

*For. –Forward, Rev. –Reverse, Int. - internal

5.2.5. Amplification and sequencing of PPO DNA and cDNA

The isolated DNA and synthesized cDNA were subjected to PCR amplification using ppo gene end primers (P1 and P2, Table 5.1). The amplified fragment was reamplified with these two and additionally with a set of 4 internal primers (P3, P4, P5, and P6, Table 5.1) designed from nucleotide sequence of potato PPO gene (Genbank accession no. U22921.1). The products obtained were subjected to agarose (0.8%) gel electrophoresis along with molecular weight markers and the amplified fragments were purified by gel

elution method (Gene Elute™, Sigma-Aldrich, USA). The amplified fragments were then subjected to dideoxy nucleotide sequencing using BigDye terminator DNA sequencing kit (Applied Biosystems, Warrington, England). The sequencing reaction was performed for 25 cycles using sequence specific primers. Later, the reaction mixture was ethanol precipitated, washed and loaded on to the polyacrylamide gel in the automated sequencing machine available at Bhabha Atomic Research Centre, Mumbai, India. The sequence obtained was analysed and submitted to NCBI Genbank. The primers were again designed from the obtained sequence and were used for PPO amplification and sequencing in other brinjal cultivars. As indicated in Table 5.1 the forward oligonucleotides included were P7, P8, P9, and P10 and reverse oligonucleotides were P11, P12, P13, and P14 (Table 5.1).

5.2.6. Cloning and expression of ppo gene in *E.coli*

The *PPO* gene was PCR amplified, restriction digested and inserted in PET-28a vector plasmid downstream of *lacUV5* (IPTG) inducible promoter and transformed into *E.coli* BL21 (DE3) cells using methods as detailed in pET expression system manual (Novagen).

5.2.6.1. PCR amplification of *PPO* gene for cloning

The genomic DNA sequence of *ppo* gene was analyzed for the presence of restriction sites. Forward primers were designed to have NdeI site, whereas, reverse primer with XhoI site (P15 and P16, Table 5.1). The *ppo* gene from brinjal fruit (Kalpatharu) was isolated and PCR amplified using these primers. An aliquot (2 µl) of the amplified product was checked on agarose gel along with molecular weight markers. The remaining

amplified product was then purified from contaminating PCR reagents using a clean-up kit (GenElute™ PCR Clean-up Kit, Sigma-Aldrich, USA).

5.2.6.2. Restriction digestion and DNA isolation

The amplified and purified *ppo* gene preparation was mixed with pET-28a plasmid vector in a ratio of 3:1 and further with 40 units of NdeI and XhoI (New England Biolabs) along with the restriction buffer (4) in a 100 µl reaction mixture, which was incubated at 37 ºC in a water bath for 2 h. The restriction digested DNA was then extracted from the reaction mix using phenol: chloroform method (Chomczynski, P. 1993). The mixture was diluted with H_2O (400 µl), mixed with phenol: chloroform (500 µl), and centrifuged at 10000 g for 5 min. The supernatant was mixed with chloroform (500 µl) and centrifuged at 10000 g for 5 min. The aqueous top layer was mixed with absolute ethanol (1 ml) and 50 µl of sodium acetate (3 M, pH 5.2), and incubated at -20 ºC for 2 h. Later it was centrifuged at 15000 g for 40 min. The precipitate was then washed with 70% ethanol and centrifuged at 15000 g for 40 min. The pellet was dried and dissolved in sterile water (8 µl).

5.2.6.3. DNA ligation and transformation

The ligation reaction was performed as per the instruction in the kit (Rapid DNA ligation kit, Fermentas, Ontario, Canada). The restriction digested and purified DNA solution (10 µl) was mixed with 2X ligation buffer (10 µl), and ligase (1 µl), and then incubated at 20 ºC for 5 min. The mixture was then used for transformation of competent *E.coli* (DH5α) cells. The competent sells were prepared using $CaCl_2$ method as detailed in Sambrook et al. (1989). The fresh inoculated culture of *E.coli* (DH5α) was grown till the absorbance reached 0.2. The cells were pelleted using a centrifuge (6000g for 10 min). The pellet was mixed with chilled 10 ml of $CaCl_2$ (50 mM) and incubated on ice for 30 min. Later the

cells were centrifuged (6000g, 10 min, 4 ºC), the pellet was resuspended in chilled $CaCl_2$ (1 ml) and incubated on ice for 2 h. The competent cells (100 µl) were added to the ligated mix, kept on ice for 10 min, and subjected to heat shock at 42 ºC in a water bath for 3 min. The preheated (37 ºC) Luria broth (1 ml) was added to the transformation mixture, which was later incubated at 37 ºC on a shaker for 1 h. The cells were spread-plated on kanamycin (50 µg/ml)-Luria agar (LA) plates, and observed after 18 h of incubation at 37 ºC for transformants.

5.2.6.4. Screening of transformants

The screening of transformants was performed using colony PCR with gene specific primers. A few cells from each cfu were picked using a sterile needle and mixed with PCR reaction mix (15 µl). After 25 cycles of PCR reaction, the agarose gel electrophoresis was performed to observe the presence of amplified full size gene (>1.7 kb). Those cfus with full size gene were selected. The plasmid was isolated using a plasmid miniprep kit (Sigma-Aldrich, USA). The integrity of plasmid was observed using agarose gel electrophoresis. The plasmid was again transformed into BL 21(DE3) strain of *E. coli* using the method detailed above. The transformants were screened on kanamycin-LA plates.

5.2.6.5. Expression of cloned PPO

A transformant cell having recombinant plasmid was freshly inoculated in Luria broth having kanamycin (50 µg/ml) and incubated on a shaker (150 rpm) at 37 ºC. IPTG (1 mM) was added at the mid log phase (OD = 0.3 to 0.4). Control *E. coli* cells without recombinant *ppo* and another control without IPTG addition were cultured in parallel.

After 4 h of IPTG addition, the cells were harvested and analyzed for the presence of recombinant protein using SDS-PAGE as detailed in section 4.2.4.

5.2.7. Purification of PPO from *E.coli*

The recombinant PPO expression vector contained a histidine tag. Therefore, Ni-Sepharose column chromatography was used for affinity purification. The recombinant *E.coli* culture was grown in Luria broth (20 ml) containing kanamycin (25 µg/ml). An aliquot (2 ml) of the culture was inoculated fresh in Luria broth (200 ml) having kanamycin (25 µg/ml) and incubated at 37 ºC at180 rpm till mid log phase ($OD_{600,}$ 0.3-0.5). The IPTG (500 µM) was then added and the culture was further grown at 37 ºC at 180 rpm for 5 h. The cells were harvested by centrifugation (8000 g, 15 min) and freshly analyzed or kept at -20 ºC till further use. Upon cell lysis the protein was not found in supernatant indicating probable formation of inclusion body by the expressed protein. Hence, the pellet was suspended in 10 ml of inclusion body lysis buffer containing autoclaved Tris.cl (50 mM, pH 8.0), NaCl (100 mM), EDTA (5 mM), sodium azide (0.1%) and freshly added PMSF (0.1 mM), DTT (10 mM), Triton X100 (0.5%). The lysis was performed by sonication for 7 min with 10 sec pulse and 30 sec cooling followed by centrifugation at 5000 g for 10 min. The pellet containing inclusion body was washed by suspending in 2 ml of 2X buffer B containing NaH_2PO_4 (100 mM), Tris.cl (10 mM, pH 8.0) followed by centrifugation at 5000 g for 10 min. The pellet was then dissolved in buffer B (5 ml) containing urea (8 M) followed by centrifugation at 10000 g for 10 min. The supernatant was collected and used for Ni column affinity chromatography.

The column material (1 ml) was washed with buffer B (5 ml) containing urea (8 M). The pH of the flow-through was checked for ensuring protein binding which was found to be

8.0. The supernatant containing dissolved inclusion body was loaded on the column, the flow-through was reloaded and the final flow-through collected was stored at 4 ºC. The washing was performed with buffer C (6 ml) containing urea (8 M) and stored at 4 ºC. The buffer C composition is as that of buffer B, except the pH was 6.3 which was adjusted with HCl. The elution was performed with the decreasing pH with buffer C (6 ml) and D (6 ml) whose composition is as that of buffer B, but the pH was adjusted to 5.9 and 4.5, respectively. The eluent at each step was collected and stored at 4 ºC. The cell lysate, flow-through, washing and eluted fractions were analysed using SDS-PAGE for the presence of overexpressed and pure PPO. The fraction containing pure PPO was pooled and dialysed with 200 ml of sterile modified refolding buffer (MRB) for proper folding of the soluble protein. The MRB contained Tris.cl (0.2 M, pH 7.6), L-arginine (0.8 M), glycerol (20%), and urea (8 M). The protease inhibitor cocktail (1%) or DTT (5 mM) or PMSF (1 mM) was added fresh to the solution. The dialysis with this MRB solution (100 ml) was performed at ambient temperature overnight for equilibration. The dialysis was then performed with MRB without urea at 4 ºC for complete removal of urea. The pure PPO fraction was concentrated by centrifugation (8000 g for 40 min) using a centricon filter (10 kDa cut off, Millipore, USA) and checked by SDS PAGE and enzyme assay. For storage purpose the protein fraction was dialyzed against protein storage buffer [Tris.cl (10 mM pH 7.6), KCl (50 mM), EDTA (0.1 mM), glycerol (50%) and DTT (1 mM)] using a centricon filter for 99% removal of MRB solution.

5.3.3. Results

The complimentary DNA (cDNA) prepared from isolated RNA from fruit of ‘Kalpatharu’ (V5) cultivar was amplified with the internal and end primers designed from the potato PPO gene sequence and shown in Fig. 5.1. The amplification was observed in most of

the cases especially in lanes 2, 3, 4, 5, and 6. However, a faint band was observed with end primers showing the full length gene of about 1.7 kb (Fig. 5.1). The amplified fragment was found to be ~1 kb size with internal primers P1 and P6 (Table 5.1). The amplified fragment of ~0.4 kb size was observed with internal primers P5 and P2. The internal primers P3 and P2 yielded DNA fragments of ~0.8 kb size. The amplified fragment of ~0.9 kb size was observed with internal primers P1 and P4. Again the amplified fragment with internal primers P3 and P6 was found to be ~0.6 kb size. These fragment sizes were then compared with PPO gene sequence (Genbank accession no. U22921.1) of potato and found to be matching which indicated its similarity with the potato PPO. The genomic DNA isolated from brinjal and amplified with the same set of primers thus produced similar results. The size of amplified product of both cDNA and genomic DNA was found to be similar (~1.7 kb) as observed in agarose gel electrophoresis (Fig. 5.2). This indicated that introns were probably absent in brinjal. The full length DNA and cDNA were sequenced and submitted to Genbank (GQ149349, GQ149350). The primers were designed from this nucleotide sequence and used for amplification of the genomic DNA from other cultivars which showed substantial amplification in five cultivars and poor amplification in ‘Arka navneet’ (V4) and ‘Silki’(V8) cultivars (Fig 5.3).

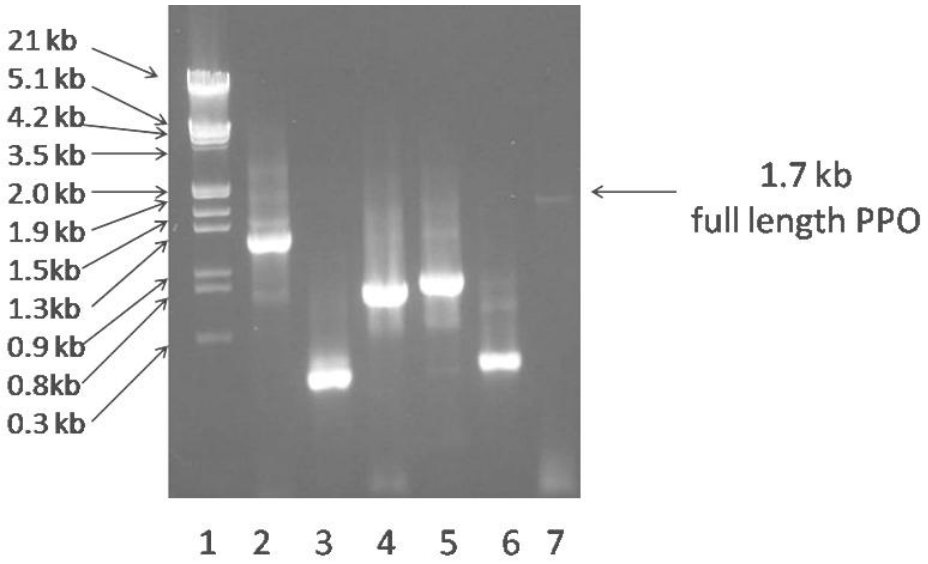


Figure 5.1. The agarose gel electrophoresis picture of PCR amplified PPO with internal and end primers. Lane 1-Moleclar weight marker (Lambda DNA/Eco R1+ Hind III double digest), Lane 2- amplified fragment of ~1 kb size with internal primers P1 and P6, Lane 3- amplified fragment of ~0.4 kb size with internal primers P5 and P2, Lane 4- amplified fragment of ~0.8 kb size with internal primers P3 and P2, Lane 5- amplified fragment of ~0.9 kb size with internal primers P1 and P4, Lane 6- amplified fragment of ~0.6 kb size with internal primers P3 and P6, Lane 7- amplified fragment of ~1.7 kb size full length PPO gene with end primers P1 and P2. The sequence of these primers is detailed in Table 5.1.

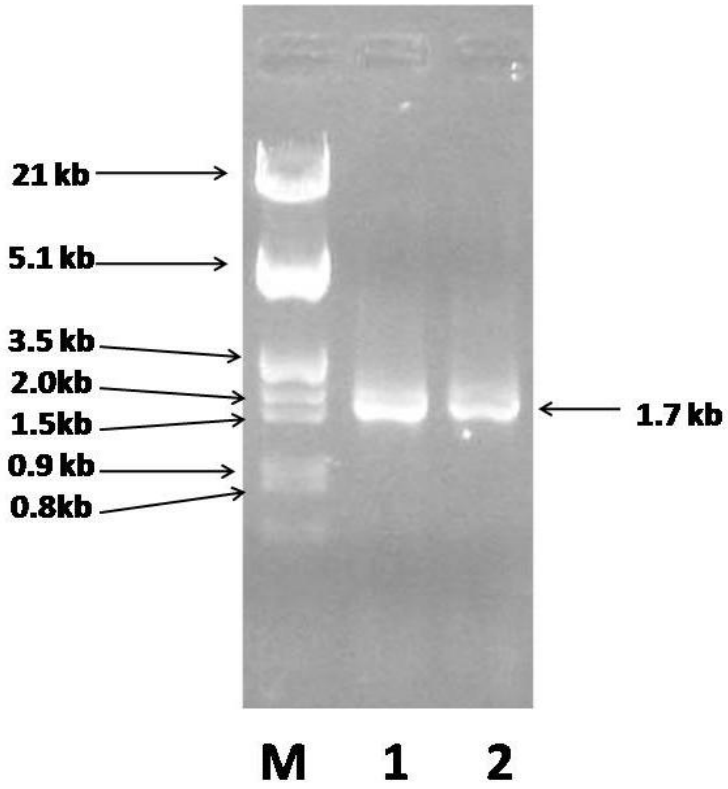


Figure 5.2. The agarose gel electrophoresis picture of PCR amplified PPO DNA and cDNA with the same set of primers. Lane M- molecular weight marker (Lambda DNA/Eco R1+ Hind III double digest), Lane 1- PCR amplified genomic DNA, lane 2- PCR amplified cDNA.

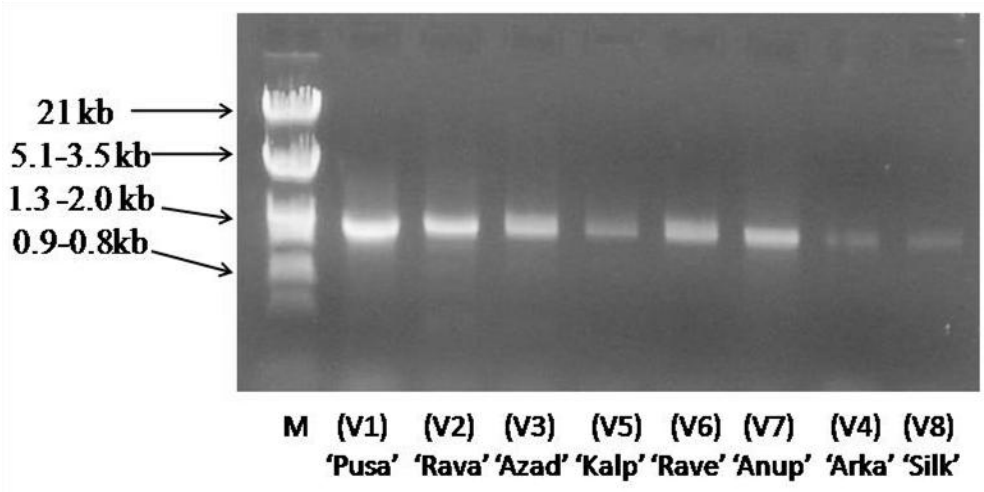


Figure 5.3. Agarose gel electrophoresis picture of PCR amplified PPO DNA from eight cultivars [‘Pusa purple long’ (V1), ‘Ravaiya’ (V2), ‘Azad kranti’ (V3), ‘Arka navneet’ (V4), ‘Kalpatharu’ (V5), ‘Raveena’ (V6), ‘Anupam’ (V7), ‘Silki’ (V8)] of brinjal detailed in Table 2.1. M- Molecular weight marker (Lambda DNA/Eco R1+ Hind III double digest). The initial four letter of cultivar name was used as their abbreviation in figures.

5.3.1. Gene sequence based conceptual translation of PPO protein

The sequence details of PPO from brinjal cultivar 'Kalpatharu' (V5) is given in Figure 5.4. The other cultivars showed similar characteristics with minor differences in amino acid positions. The presence of an 81 amino acid long transit sequence (location 1-81aa) was observed (Genbank accession no. GQ149349.1), that was also been reported in PPO from other fruits and vegetables (89 aa in apple, 86 aa in potato and 99 aa in tomato). The Chloro IP program analysis showed the higher probability of presence of a 40 amino acid long N-terminal chloroplast targeting sequence with cleavage site between serine (S40) and Cysteine (C41) residues (Fig. 5.4) (Genbank accession no. GQ149349.1). The same program also showed the cleavage site for remaining 41 amino acid long transit peptides at lysine (L81) and alanine (A82). This sequence also showed similarity with other thylakoid lumen proteins including PPO from other fruits. The protein did not contain a trans membrane or hydrophobic domain and appeared to be a soluble protein, which ruled out the possibility of being a thylakoid membrane protein. PPO was found to be a metalloprotein with active site containing two copper binding regions (A and B), each coordinated by three histidine (H) residues. One copper (copper A) binds to the approximate amino acid sequence position between 173-216 and the second one binds to the amino acid sequence between 349-403 position (Fig. 5.4) (Genbank accession no. GQ149349.1). Each of these regions contained three histidine residues, which coordinated with copper (H178, H196, H205 in copper A region and H353, H367, H389 in copper B region).

The x-ray diffraction studies in sweet potato (*Ipomoea batatas*) showed these histidine residues in two regions form a trigonal pyramidal coordination sphere. One cysteine

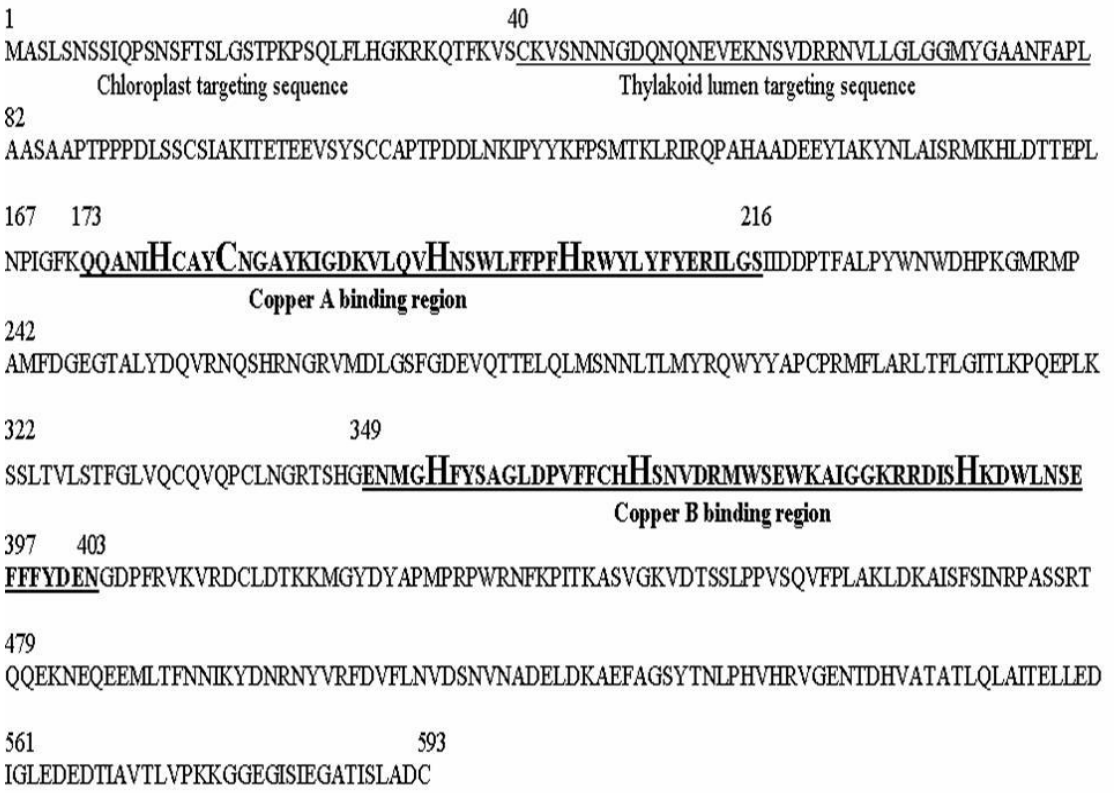
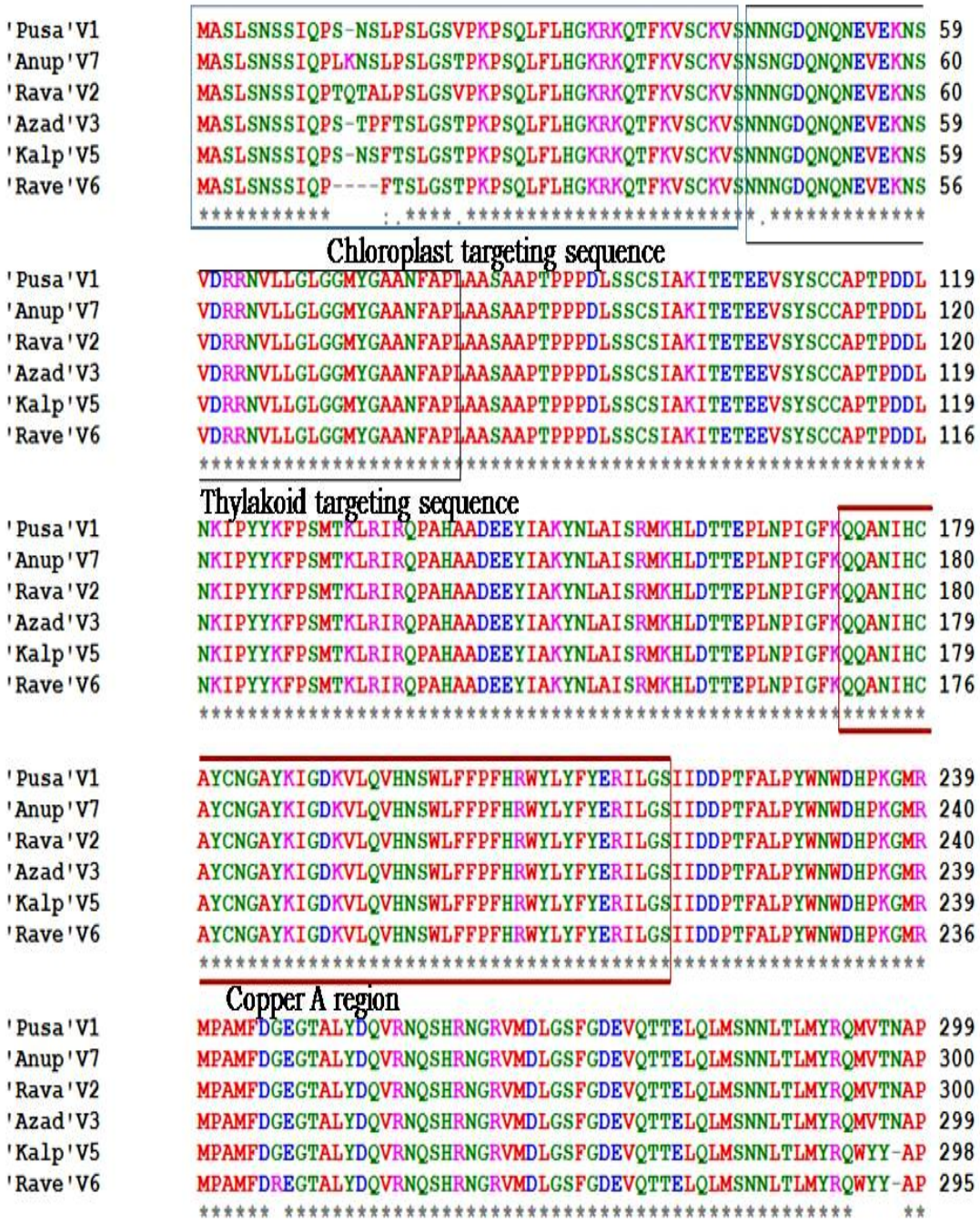


Figure 5.4. Nucleotide sequence based conceptually translated amino acid sequence of PPO from brinjal (GQ149349.1) and its sequence features. The number indicates the amino acid position from N-terminal position. The copper binding active regions are in bold font and underlined. The His residues present in active site involved in binding to copper are displayed in bigger font.

(C182) present in copper A region is also crucial for active site catalysis and forms a covalent thioether bridge with histidine (H196). The post translational modifications were also predicted in PPO. The probable amino acids undergoing phosphorylation in this protein are predicted to be as many as 13 serine, 9 threonine, and 10 tyrosine. The protein did not contain any N-myristoylation, N-acetylation, and O-glycosylation sites.

5.3.2. The cultivars with high PPO activity showed maximum sequence similarity

Out of the eight cultivars, PPO gene from only six cultivars could get PCR amplified using designed primers as discussed in materials and methods (Fig. 5.3). The PPO nucleotide sequences were submitted to gene bank (Genbank accession numbers V1 (JQ621948), V2 (JQ621949), V3 (JQ621950), V5 (GQ149349.1), (V6 (JQ621951), V7 (JQ621952)). The other two cultivars 'Arka navneet' (V4) and 'Silki' (V8) could not be PCR amplified and hence were not sequenced. These cultivars probably have a different nucleotide sequence in the primer binding site of the designed primers. The amino acid sequence of the remaining six cultivars has been compared (Fig. 5.5). The PPO nucleotide sequence was found to contain 1773-1788 bp and hence predicted conceptual protein to have 590-595 aa, as there was no intron in this gene. With minor differences the initial 81 amino acids were found to be the part of the signal sequence and were mostly identical, and the active site region was found to be conserved and identical. The differences in sequence were observed in the region between amino acid position 11 – 17. Valine was found to be present in place of threonine in V1 (at position 21) and V2 (position 22).


'Pusa'V1 MASLSNSSIQPS-NSLPSLGSVPKPSQLFLHGKRKQTFKVSCKVSNNNGDQNQNEVEKNS 59
'Anup'V7 MASLSNSSIQPLKNSLPSLGSTPKPSQLFLHGKRKQTFKVSCKVSNSNGDQNQNEVEKNS 60
'Rava'V2 MASLSNSSIQPTQTALPSLGSVPKPSQLFLHGKRKQTFKVSCKVSNNNGDQNQNEVEKNS 60
'Azad'V3 MASLSNSSIQPS-TPFTSLGSTPKPSQLFLHGKRKQTFKVSCKVSNNNGDQNQNEVEKNS 59
'Kalp'V5 MASLSNSSIQPS-NSFTSLGSTPKPSQLFLHGKRKQTFKVSCKVSNNNGDQNQNEVEKNS 59
'Rave'V6 MASLSNSSIQP----FTSLGSTPKPSQLFLHGKRKQTFKVSCKVSNNNGDQNQNEVEKNS 56
Chloroplast targeting sequence
'Pusa'V1 VDRRNVLLGLGGMYGAANFAPLAASAAPTPPPDLSSCSIAKITETEEVSYSCCAPTPDDL 119
'Anup'V7 VDRRNVLLGLGGMYGAANFAPLAASAAPTPPPDLSSCSIAKITETEEVSYSCCAPTPDDL 120
'Rava'V2 VDRRNVLLGLGGMYGAANFAPLAASAAPTPPPDLSSCSIAKITETEEVSYSCCAPTPDDL 120
'Azad'V3 VDRRNVLLGLGGMYGAANFAPLAASAAPTPPPDLSSCSIAKITETEEVSYSCCAPTPDDL 119
'Kalp'V5 VDRRNVLLGLGGMYGAANFAPLAASAAPTPPPDLSSCSIAKITETEEVSYSCCAPTPDDL 119
'Rave'V6 VDRRNVLLGLGGMYGAANFAPLAASAAPTPPPDLSSCSIAKITETEEVSYSCCAPTPDDL 116
Thylakoid targeting sequence
'Pusa'V1 NKIPYYKFPSMTKLRIRQPAHAADEEYIAKYNLAISRMKHLDTTEPLNPIGFKQQANIHC 179
'Anup'V7 NKIPYYKFPSMTKLRIRQPAHAADEEYIAKYNLAISRMKHLDTTEPLNPIGFKQQANIHC 180
'Rava'V2 NKIPYYKFPSMTKLRIRQPAHAADEEYIAKYNLAISRMKHLDTTEPLNPIGFKQQANIHC 180
'Azad'V3 NKIPYYKFPSMTKLRIRQPAHAADEEYIAKYNLAISRMKHLDTTEPLNPIGFKQQANIHC 179
'Kalp'V5 NKIPYYKFPSMTKLRIRQPAHAADEEYIAKYNLAISRMKHLDTTEPLNPIGFKQQANIHC 179
'Rave'V6 NKIPYYKFPSMTKLRIRQPAHAADEEYIAKYNLAISRMKHLDTTEPLNPIGFKQQANIHC 176
'Pusa'V1 AYCNGAYKIGDKVLQVHNSWLFFPFHRWYLYFYERILGSIIDDPTFALPYWNWDHPKGMR 239
'Anup'V7 AYCNGAYKIGDKVLQVHNSWLFFPFHRWYLYFYERILGSIIDDPTFALPYWNWDHPKGMR 240
'Rava'V2 AYCNGAYKIGDKVLQVHNSWLFFPFHRWYLYFYERILGSIIDDPTFALPYWNWDHPKGMR 240
'Azad'V3 AYCNGAYKIGDKVLQVHNSWLFFPFHRWYLYFYERILGSIIDDPTFALPYWNWDHPKGMR 239
'Kalp'V5 AYCNGAYKIGDKVLQVHNSWLFFPFHRWYLYFYERILGSIIDDPTFALPYWNWDHPKGMR 239
'Rave'V6 AYCNGAYKIGDKVLQVHNSWLFFPFHRWYLYFYERILGSIIDDPTFALPYWNWDHPKGMR 236
Copper A region
'Pusa'V1 MPAMFDGEGTALYDQVRNQSHRNGRVMDLGSFGDEVQTTELQLMSNNLTLMYRQMVTNAP 299
'Anup'V7 MPAMFDGEGTALYDQVRNQSHRNGRVMDLGSFGDEVQTTELQLMSNNLTLMYRQMVTNAP 300
'Rava'V2 MPAMFDGEGTALYDQVRNQSHRNGRVMDLGSFGDEVQTTELQLMSNNLTLMYRQMVTNAP 300
'Azad'V3 MPAMFDGEGTALYDQVRNQSHRNGRVMDLGSFGDEVQTTELQLMSNNLTLMYRQMVTNAP 299
'Kalp'V5 MPAMFDGEGTALYDQVRNQSHRNGRVMDLGSFGDEVQTTELQLMSNNLTLMYRQWYY-AP 298
'Rave'V6 MPAMFDREGTALYDQVRNQSHRNGRVMDLGSFGDEVQTTELQLMSNNLTLMYRQWYY-AP 295

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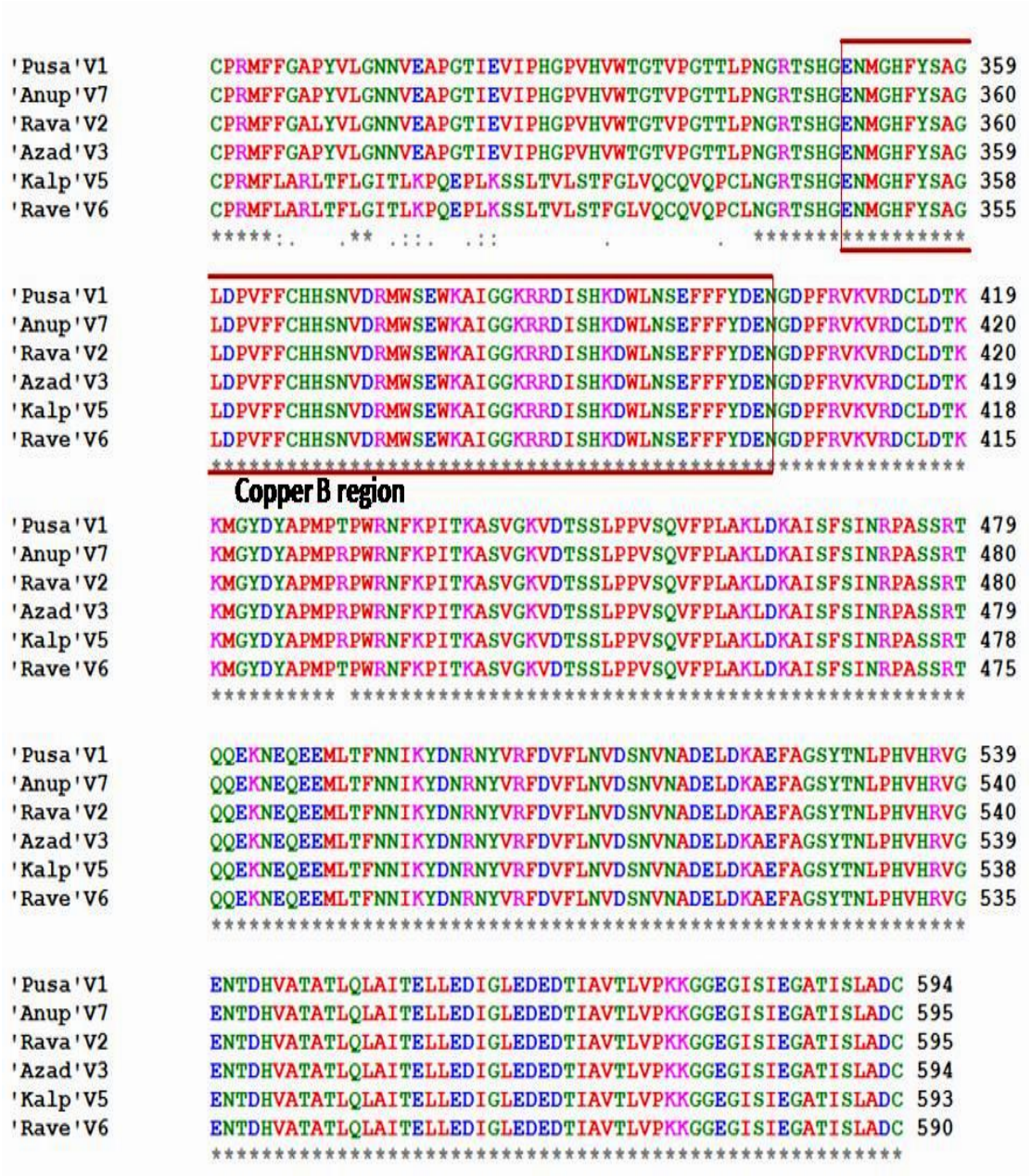

Figure 5.5. Multiple sequence alignment of PPO amino acid sequences [Genbank accession numbers V1- (JQ621948), V2- (JQ621949), V3- (JQ621950), V5- (GQ149349.1), V6- (JQ621951), V7- (JQ621952)] from six different cultivars of brinjal and their sequence features. Symbol * below the sequence showed the positions of exactly matched amino acids. Symbols ":" or "." indicate high or moderate matching at that position. The initial four letter of cultivar name was used as their abbreviation in figures.

Again there was an arginine (~243 aa) in place of glycine in cultivar V6. There was another replacement of threonine in place of arginine (R) in V1 (430) and V6 (426). A few single base differences were observed among these cultivars but these differences did not reflect any difference in the amino acid sequence. However, the region between amino acid position ~301 to 338 was found to be almost identical in two cultivars V5 (‘Kalpatharu’) and V6 (‘Raveena’) showing maximum PPO activity, whereas, this region was very different in other four cultivars ‘Pusa purple long’ (V1), ‘Ravaiya’ (V2), ‘Azad kranti’ (V3), and ‘Anupam’ (V7), which were found to have almost identical sequence in this region but moderate PPO activity. The amino acid sequence difference in this region seems to be due to a deletion of adenine base in PPO gene of V5 (at 879 bp position) and in V6 (at 870 bp position) resulting in appearance of tryptophan (W at ~291 position) in place of methonine (M) and subsequent change in the reading frame. Two subsequent deletions at 877 and 881 position in V6, and 886 and 890 position in V5 of adenine and guanine, respectively, resulted in a single amino acid deletion (asparagine at ~296 position in V5 and 293 position in V6) and the frame shift resumed for subsequent 7 amino acids. Again another deletion of thymine base (at 905 position in V6 and 914 position in V5) resulted in change in frame (from 304 to 309 in V5 and 301 to 306 in V6). From ~301 to ~338 amino acid position in these two cultivars, the change in reading frame changed the amino acid sequence till proline-cysteine-phenylalanine (PCL) (Fig. 5.5). Another insertion of thymine (at 1014 position in V5 and 1023 position in V6) compensated the frameshift and the cultivars again showed identical sequence after ~338 amino acid position (Fig. 5.5).

```
Eggplant    -MASLSNSSIQPS-------NSFTSLG----STPKPSQLFLHG---KRKQTFKVSCKVSN 45
Potato      -MASVCNSSSTTTTLKTPFISSNTSLG----STPKPSQLFLHG---KRNKTFKVSCKVSN 52
Tomato      MSSSSSITTTLPLCTNKSLSSSFTTTN--SSLLSKPSQLFLHG---RRNQSFKVSCNANN 55
Tobacco     ILASS--SSTLPLCANKTPSSSFTNTNTNSSFFAKPSQLFLHG---KRNQNFKFSCNANS 55
Sweet       -MASFTTSPCTSAAPKTP--KSLASSATISSPLPKPSQIHIAT--AKRTHHFKVSCNAPN 55
Grape       -MASLPWSLTTFTAIANT--TNIS-AFPPSPLFQRASHVPVARNRSRRFAPSKVSCNSAN 56
                :*   :             .. :          :.*:: :     :*    *.**:  .

Eggplant    NNGDQNQNE----VEKNSVDRRNVLLGLGGMYGAAN---FAPLAASAAPTPPPDLSSCSI 98
Potato      S-GDQNQN-----IETNSVDRRNVLLGLGGLYGVAN---AIPLAASATPIPSPDLKTCGR 103
Tomato      --VDKNPD---------AVDRRNVLLGLGGLYGAAN---LAPLAT-AAPIPPPDLKSCGT 100
Tobacco     GEHDKNLD---------AVDRRNVLLGLGGLYGAAN---LAPLAT-AAPIPPPDLKSCSK 102
Sweet       GD------------SQPKLDRRDVLLGLGGLAGAAS--LINNPLAFAEPIHAPEISKCVV 101
Grape       GDPNSDSTSDVRETSSGKLDRRNVLLGIGGLYGAAGGLGATKPLAFGAPIQAPDISKCGT 116
                          :***:****:**: *.*.        : . *  .*::..*

Eggplant    AKITETEEVSYS-CCAP-TPDDLNKIPYYKFP--SMTKLRIRQPAHAADEEYIAKYNLAI 154
Potato      ATISDGPLVPYT-CCPPPMPTNFDTIPYYKFP--SMTKLRIRPPAHAVDEEYIAKYNLAI 160
Tomato      AHVKEGVDVIYS-CCPP-VPDDIDSVPYYKFP--SMTKLRIRPPAHAADEEYVAKYQLAT 156
Tobacco     AHINDKEEVSYS-CCPP-IPSDMDSVPYYKFP--SMPKLRIRPAAHAADEEYIAKYQLAT 158
Sweet       PPKDLPPDAIVDSCCPP----LATNVIPSKVPKTSPSAMKIRPAIHRMDKEYIAKFEKAI 157
Grape       AT--VPDGVTPTNCCPP----VTTKIIDFQLP-SSGSPMRTRPAAHLVSKEYLAKYKKAI 169
            .       .    **.*       .:   :.*  * . :: * . *  .:**:**:: *

Eggplant    SRMKHLDTTEPLNPIGFKQQANIHCAYCNGAYKIG---DKVLQVHNSWLFFPFHRWYLYF 211
Potato      SRMKDLDKTDPLNPLGFKQQANIHCAYCNGAYIIG---GKELQVHNSWLFFPFHRWYLYF 217
Tomato      SRMRELDK-DPFDPLGFKQQANIHCAYCNGAYKVG---GKELQVHFSWLFFPFHRWYLYF 212
Tobacco     SRMRELDK-DPFDPLGFKQQANIHCAYCNGAYKIG---GKELQVHFSWLFFPFHRWYLYF 214
Sweet       RLMKELPA---DDPRNFYQQALVHCAYCNGGYVQTDYPDKEIQVHNSWLFFPFHRWYLYF 214
Grape       ELQKALPD---DDPRSFKQQANVHCTYCQGAYDQVGYTDLELQVHASWLFLPFHRYYLYF 226
               : *      :* .* *** :**:**:*.*       .  :*** ****:****:****

Eggplant    YERILGSIIDDPTFALPYWNWDHPKGMRMPAMFDGEGTALYDQVRNQSHRNGRVMDLGSF 271
Potato      YERILGKLIDDPTFALPYWNWDHPKGMRLPPMFDREGTSIYDERRNQQVRNGTVMDLGSF 277
Tomato      YERILGSLINDPTFALPYWNWDHPKGMRIPPMFDREGSSLYDEKRNQNHRNGTIIDLGHF 272
Tobacco     YERILGSLINDPTFGLPYWNWDHPKGMGIPPMFDREGSSLYDARRNQSHRNGTIIDLGFF 274
Sweet       YERILGKLINDPTFGLPFWNWDTPAGMLIPQYFRNQNSPLYDENRLQSHLP-LVMDLGYA 273
Grape       NERILAKLIDDPTIALAYWAWDNPDGMYMPTIYASSPSSLYDEKRNAKHLPPTVIDLDYD 286
             ****..:*:***:.*.:* ** * ** :*  :  . :.:**  *  .     ::**.

Eggplant    GDEVQTTELQLMSNNLTLMYRQWYY-APCPRMFLARLTFLG-ITLKP-QEPLKSSLTVLS 328
Potato      GDKVQTTQLQLMSNNLTLMYRQMVTNAPCPLLFFGAPYVLG-NNVEA-PGTIENIPHIPV 335
Tomato      GKEVDTPQLQIMTNNLTLMYRQMVTNAPCPSQFFGAAYLWV-LNPSPGQGTIENIPHTPV 331
Tobacco     GTEVQTTQLQQMTNNLTIMYRQMITNAPCPLLFFGQPYPLG-TDPSPGMGTIENIPHTPV 333
Sweet       GTDTDVTDDERISNNLALMYKSMVTNAGTAELFLGKPYKAGDDPVNKGGGSIENIPHTPV 333
Grape       GTEPTIPDDELKTDNLAIMYKQIVSGATTPKLFLGYPYRAG-DAIDPGAGTLEHVPHNIV 345
            * .   .: :  ::**::**:.    *  .  *:.          .    .::
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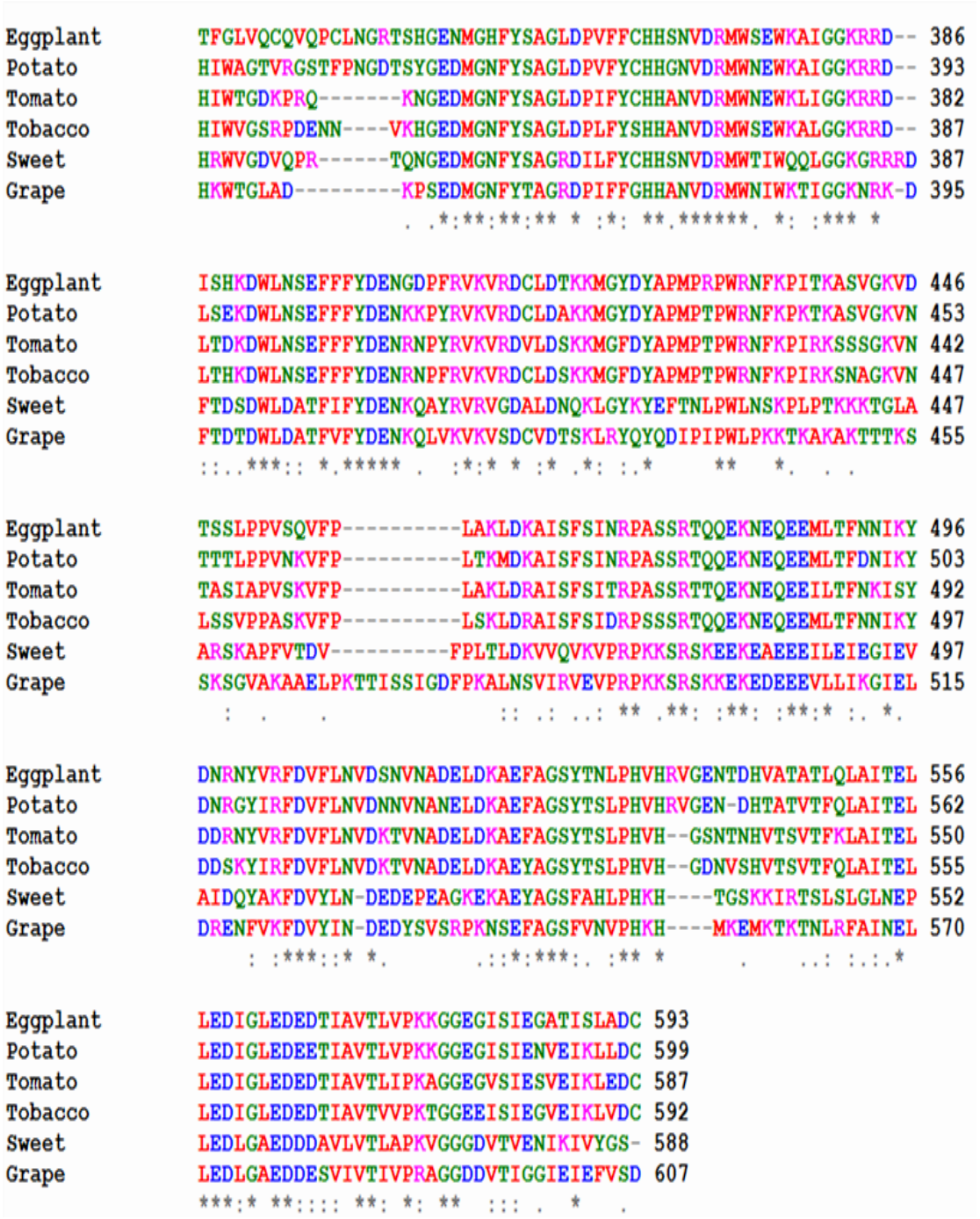

Figure 5.6. Multiple sequence alignment of PPO protein from different fruit and vegetables. The PPO sequences used were from brinjal (ACR61398.1), potato (AAA85122.1) tobacco (CAA73103.1), tomato (AAB22610.1), grape (AAB41022.1) and sweet potato (AAW78869.1). Symbol – indicate the gap introduced for optimal alignment. Symbol "*" below the sequence showed the positions of exact matching of amino acids. Symbols ":" or "." indicates high or moderate matching at that position.

5.3.3. Brinjal PPO gene sequence showed homology with that of potato and tomato

The nucleotide sequence of PCR amplified cDNA and genomic DNA of PPO gene from brinjal were found to be of similar length and sequence (NCBI Genebank accession no. GQ149349.1 for gene and GQ149350.1 for mRNA). This indicated that the introns are missing in the PPO gene of brinjal. Similar observations were also reported in apricot and other plants (Chevalier et al., 1999; Mayer, 2006). The nucleotide blast search revealed that the brinjal PPO showed high similarity with potato (86%) and tomato (84%), which are its closest relatives 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). NCBI conserved domain database (CDD) search results showed the presence of domains from three super families. The amino acid region between 168-247 and 348-380 which included two copper ion (Cu-A and Cu-B) binding regions, respectively, are similar with common central domain of Tyrosinase; pfam00264 superfamily (CDD 189478). This family includes polyphenol oxidase enzymes from other plants and hemocyanins. The amino acid region or domain between 386 and 436 showed homology with PPO1-DWL superfamily pfam12142 (CDD 192942) which was also annotated as PPO middle domain. This domain family was found in bacteria and eukaryotes and includes about 50 amino acids along with the presence of conserved DWL sequence motif which gave the family its name. Again the region between 457-590 was similar with PPO1-KFDV superfamily pfam12143 (CDD 192943). This domain found in eukaryotes particularly in plants contain 132-152 amino acids with a highly conserved sequence motif KFDV from which the name was derived.

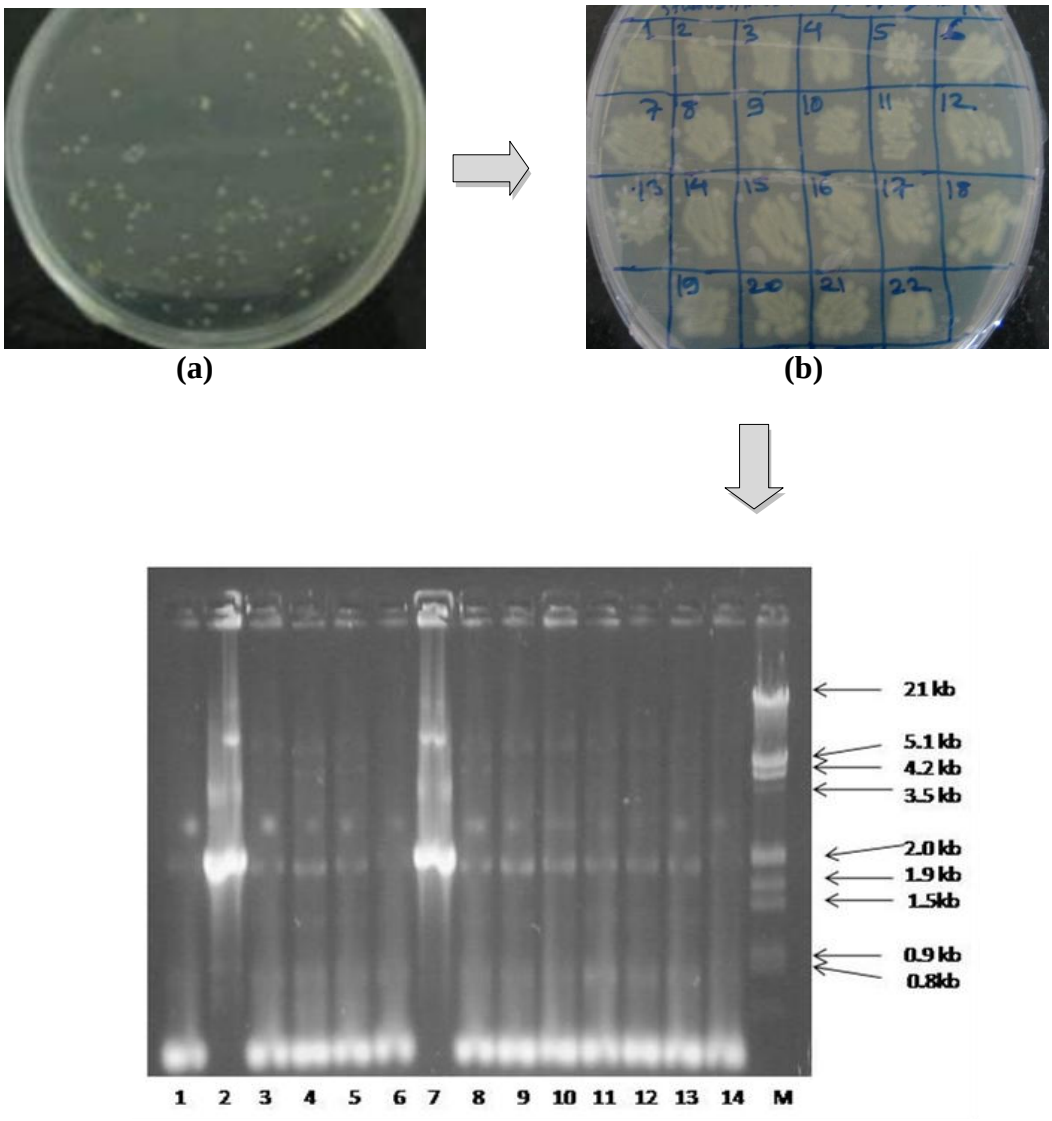

Figure 5.7. The spread plating of transformant clones **(a)**, streaked, numbered and grided colonies on a second plate **(b)**, The agarose gel electrophoresis picture of colony PCR products of transformant clones **(c)**, The plasmid vector specific primers showed amplification of about 2.0 kb (lanes 2 and 7). The lanes 1 to 14 are represent colony number from 88 to 101, respectively. The last lane (M) shows the molecular weight marker (Lambda DNA/Eco R1+ Hind III double digest).

5.3.4. Cloning and expression of polyphenol oxidase in *E.coli*

The genomic DNA sequence of *ppo* gene was analyzed and NdeI and Xho I sites were found to be absent. The PCR of genomic DNA from brinjal fruit (Kalpatharu variety) with primers having integrated restriction sites (NdeI with forward primer and XhoI site with reverse primer) resulted in amplification of a single band of about 1.8 kb size. The size matched with the earlier observed size of PPO gene and confirmed by gene sequencing. The amplified product was cloned into pET-28a expression vector downstream of *lacUV5* promoter (IPTG inducible). The recombinant plasmid thus prepared was transformed into *E.coli* (DH5α) and was selected on a Luria agar-kanamycin plate (Fig. 5.7a). Around 100 transformants were plated again on a grid plate (Fig. 5.7b) and screened using colony PCR (Fig 5.7c). Out of these, 6 were found to have *ppo* gene insert. The recombinant plasmid was further isolated and transformed to BL 21 and codon (+) strain of *E. coli*. The BL 21 (DE3) cells were selected on LB kanamycin plates. The codon (+) recombinant cells were selected on LB-plates containing kanamycin as well as chloramphenicol.

5.3.5. Overexpression of PPO

The three out of six transformants having recombinant PPO were induced using IPTG and grown under similar conditions along with *E.coli* (without PPO insert) and IPTG control (without IPTG addition). Later cells were lysed, centrifuged (12000 g, 15 min) and analysed by SDS-PAGE (Fig. 5.8). The lanes 2-8 show the supernatant fractions without any significant difference of protein expression between control and IPTG induced cells. The lanes 9-15 show the pellet fraction of protein showing significant presence of recombinant protein of 66 kDa size in IPTG induced pellet fraction (lanes 9, 11, 13),

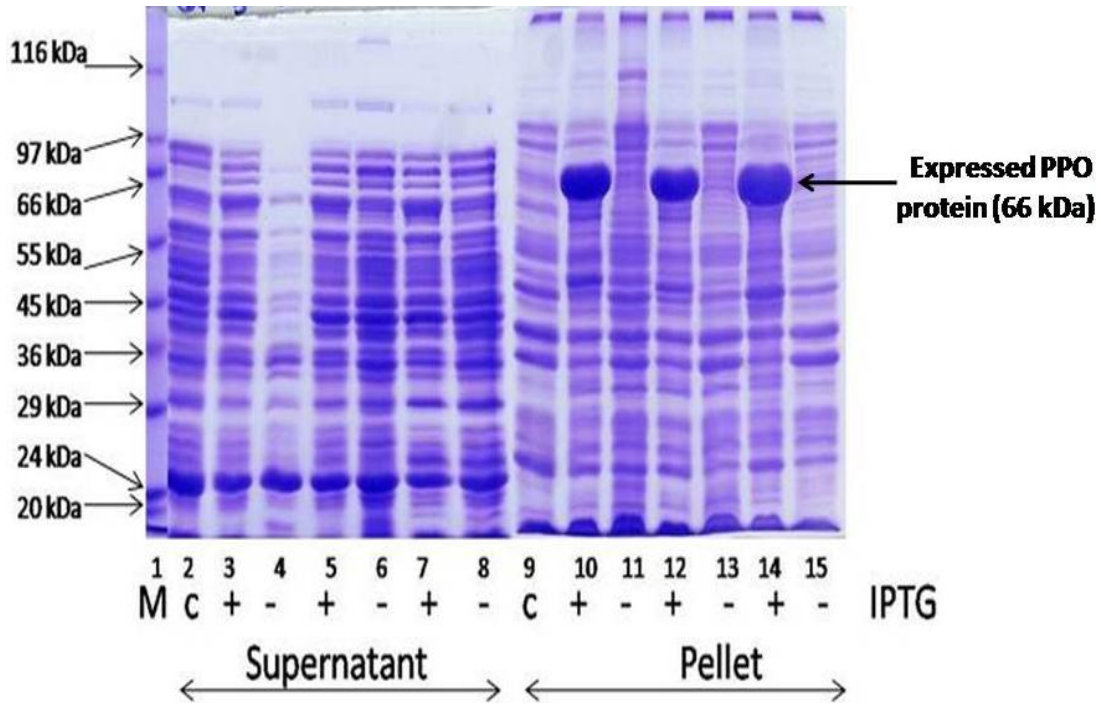

Figure 5.8. The SDS PAGE profile of expressed PPO protein in *E.coli* (BL-21) in both supernatant and pellet fractions after induction with (+) and without (-) IPTG. The lanes 2-8 shows the supernatant fractions control and IPTG induced cells. The lanes 9-15 shows the pellet fractions of control and IPTG induced cells. The over expression of ~66 protein in IPTG induced pellet fraction (lanes 9, 11, 13), which are absent in control cells (lanes 10, 12, 14). M – Molecular weight marker. C- control *E.coli* (BL-21) cells without the PPO insert.

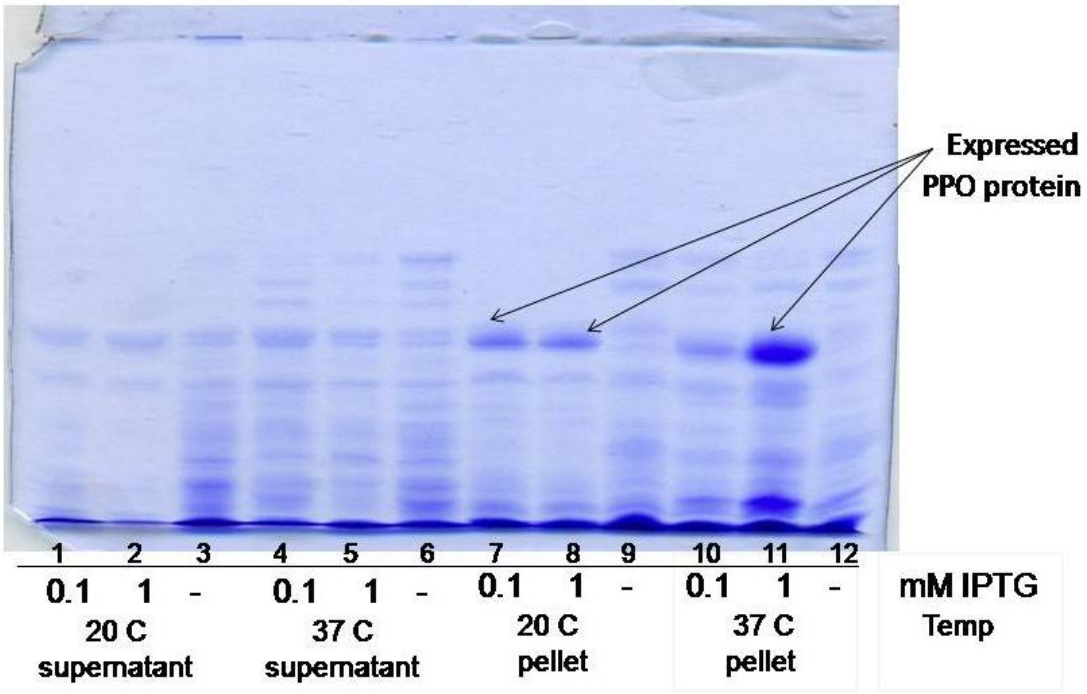

Figure 5.9. The SDS PAGE profile of the low temperature and low IPTG induction of recombinant PPO in *E.coli***.** The cells were grown at 20 ºC (lanes 1-3, 7-9) and 37 ºC (lanes 4-6 and 10-12). The IPTG concentration was 0.1 mM in lanes 1, 4, 7, and 10, and 1 mM in lanes 2, 5, 8, and 11. Lanes 1-6 supernatant fraction, lanes 7-12 pellet fraction.

which are absent in control cells (lanes 10, 12, 14) (Fig. 5.8). This has demonstrated the overexpression of inducible PPO in the pellet lysate fraction which could be due to improper folding of brinjal PPO protein in *E. coli* and its aggregation to form inclusion body. Such inclusion body proteins have been reported to lack enzyme activity and require solubilization, refolding and purification for regaining activity (Singh and Panda, 2005). The formation of inclusion bodies of recombinant proteins in *E. coli* is known to be due to rapid synthesis of recombinant protein and nonavailability of protein folding machineries (molecular chaperones) for correct folding. This resulted in mis-folded insoluble aggregates termed as inclusion bodies. In such cases low temperature or low IPTG induction is used to slow down the rate of translation or expression of the recombinant protein. The slower translation reported to cause the availability of molecular chaperones for proper folding. In current study, both low temperature (20 ºC) incubation as well as low concentration (0.1 mM) IPTG induction of recombinant *E. coli* cells was tried but both apparently did not improve the solubility of the protein (Fig. 5.9). Similar difficulty has earlier been reported in brinjal and red clover by Shetty et al. (2011) and Sullivan et al. (2004), respectively.

5.3.6. Purification of PPO from *E.coli*

The inclusion body is normally solubilized by high concentration of urea and refolded by its slow removal in the presence of oxidizing agents. Though the formation of inclusion bodies is undesirable, their formation at times becomes advantageous due to the ease of isolation and purification of the recombinant protein (Singh and Panda, 2005). This approach was used and the cell lysate was solubilized in urea (8 M), and purified using Nickel metal affinity chromatography (Fig. 5.10). The figure showed over-expression of

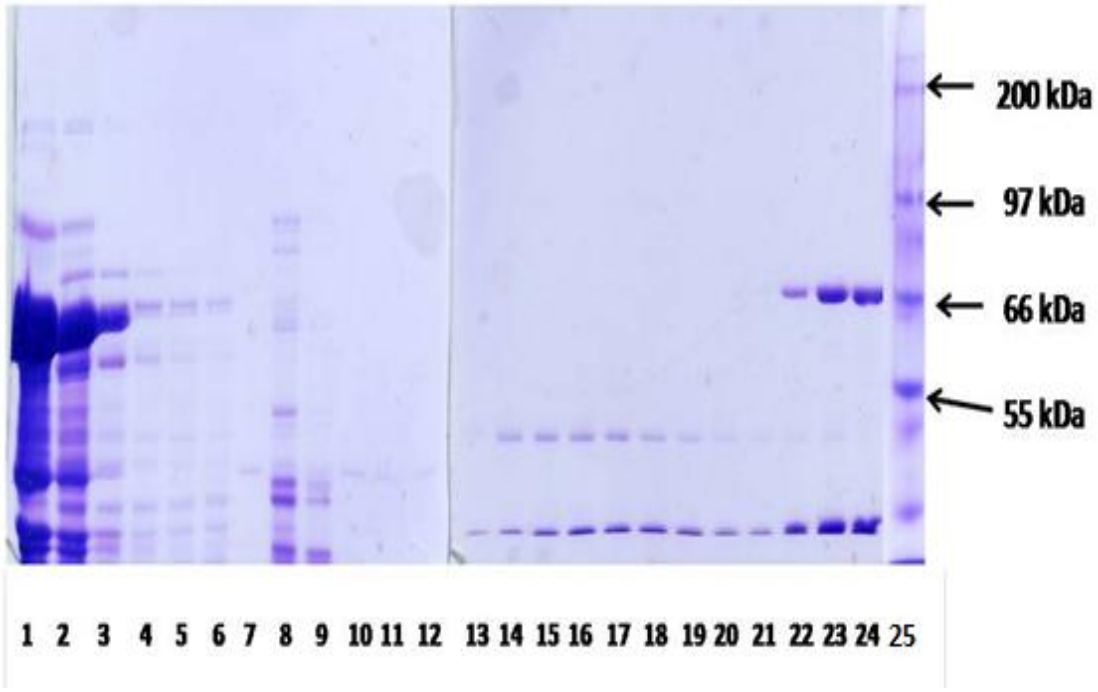

Figure 5.10. The SDS PAGE profile of eluted fractions from Ni Sepharose column showing elution of soluble PPO from *E.coli* inclusion body. Lane 1 (cell lysate), lane 2 (lysate supernatant), lanes 3-6 (flow-through fractions), lanes 7-10 (washing fractions), lanes 11-15 (pH 5.9 eluted fractions), lanes 16-24 (pH 4.5 eluted fractions), and lane 25 (molecular weight marker).

PPO (lane 1) and its solubilzation in urea (lane 2). The column binding took place as PPO band was not observed in the flow-through and washing fraction (lanes 3-10). The elution with buffer D at pH 5.9 did not elute the expressed PPO (lanes 11-15). However, the expressed PPO eluted with buffer E having pH 4.5 (lanes 22-24). The lane numbers 22-24 showed a single band at 66 kDa and no other bands observed indicated the protein to be pure. The corresponding fractions were pooled, and dialysed with modified refolding buffer for removal of urea. Later the volume of dialysed protein was concentrated to 0.5 ml with a centricon filter and the enzyme assay was performed using 4-methyl catechol as substrate (0.1 M). The purified extract (50 µl) showed very less enzyme activity of about 3 units/ml. This activity was less than 10% of the activity in the crude extract. This finding indicated that the recombinant protein was solubilized with the loss of enzyme activity. Similar reports of loss of enzyme activity after expression in *E.coli* have been earlier reported in red clover which could be due to the absence of post translational modification system required for eukaryotic proteins in *E.coli*.

5.4. Discussion

As reported in other plants, the PPO protein from brinjal too was found to contain thylakoid lumen targeting sequence and two copper atoms in its active site. The three histidine residues which interact with copper are present in each of these copper binding regions. The cysteine amino acid required for catalysis was also found to be present in copper A binding region. These features include brinjal PPO in type 3 copper proteins. The other proteins known in this class are tyrosinase and hemocyanins. The type 1 copper proteins (include proteins like azurin, plastocyanin) were known to have single copper atom linked to the protein through two histidine and one cysteine residues and are involved mostly in electron transfer reactions. The type 2 copper proteins (like superoxide

dismutase, dioxygenases) known to be linked to single copper atom and are mostly involved in oxygenation or oxidation reactions. The crystal structure of PPO has been reported in sweet potato (*Ipomoea batatas*) and grenache (V*itis vinifera*) (Eicken et al., 1999; Virador et al., 2010). The catechol oxidase enzyme of sweet potato indicated the presence of two disulfide bonds (C86-C102 and C101-C189) between cysteine residues in the secondary structure of the protein along with six α- helix and seven β- sheets (Eicken et al., 1999). The brinjal PPO showed a moderate similarity in protein sequence with them. The comparison of sequence in 301-338 amino acid resulting in grouping of these cultivars into two classes. The four cultivars 'Pusa purple long' (V1), 'Ravaiya' (V2), 'Azad kranti' (V3) and 'Anupam' (V7) showed sequence homology in this region. The 'Kalpatharu' (V5) and 'Raveena' (V6) showed high PPO activity and were found to be identical but different from the above four cultivars in this region (301-338 amino acid). Their amino acid sequence in this region was found to be unique and did not match significantly with published PPO protein in Genbank. The PPO of these two cultivars was also found to be highly active which indicated a possible role of these sequences (301-338 aa) in the activity. The total amino acid sequence showed higher similarity with other close members of the solanaceae family including potato and tomato. The similarity was more in the regions of active site. However, the overall homology was not very high indicating the uniqueness of PPO sequence in brinjal.

Though two PPO isozymes were detected in the ammonium sulfate fractionation, only a single gene could be identified and amplified. The PPO gene from brinjal was shown to contain no introns. The intron less PPO gene has recently been reported in 'Arka shirish' cultivar of brinjal (Shetty et al., 2011), as well as in potato, tomato, apricot, tobacco and few other plants (Thygesen et al., 1995; Shahar et al., 1992; Goldman et al., 1998). The gene sequence also showed homology with other members of Solanaceae family. The

protein sequence showed conserved domains of tyrosinase and PPO superfamilies. The N-terminal region showed two stretches of targeting sequence. The first sequence targets the peptide to chloroplast and the second targets to thylakoid lumen. Inside thylakoid lumen the peptide is folded to its active form. Similar localization after targeting of PPO was reported in many other plants including potato, tomato and tobacco (Thygesen et al., 1995; Shahar et al., 1992; Goldman et al., 1998). The molecular weight was reported to be 60, 58, and 55 kDa after targeting into the thylakoid lumen in potato, tomato, and tobacco (Thygesen et al., 1995, Shahar et al., 1992, Goldman et al., 1998). In some reports the PPO was reported to bind to the thylakoid membrane and have hydrophobic regions. In such cases the PPO is extracted with addition of detergents. However, brinjal PPO was analyzed to be soluble without any membrane binding region.

There are reports of cloning PPO, using partial PPO cDNA synthesized from mRNA. The cDNA was used as a probe and the complete PPO gene was fished out from the cDNA library using southern hybridization in potato and pokeweed (Hunt et al., 1993; Joy et al., 1995). In case of apple (*Malus pumila*) and apricot PPO gene was expressed in *E. coli* but the activity of the recombinant protein was not reported (Haruta et al., 1998). The eukaryotic proteins are known to undergo post-translational modifications including N-terminal and C-terminal processing along with phoshorylation, N-myristoylation, and O-glycosylation. Using bioinformatics approach, the occurrence of six potential myristoylation sites in grape PPO was predicted (Virador et al., 2010). However, in case of many plants the PPO gene sequence did not indicate the presence of sites for such modification (Mayer, 2006). In red clover grass leaves PPO cDNA was prepared, cloned and expressed in *E. coli*. However, the expressed protein in *E.coli* formed insoluble inclusion bodies. Corroborating these findings, in the current study the recombinant

protein was solubilized with the loss of enzyme activity, which was probably due to the absence of posttranslational modification of expressed eukaryotic proteins in *E.coli*.

Chapter 6

Summary

And

Conclusions

The polyphenol oxidase (PPO) catalyzed oxidation of phenolics results in instant browning upon cutting in many cut fruits and vegetables. However, the extent of browning in many of these foods differs. Brinjal (*Solanum melongena*), being a rich source of PPO, showed instant browning which negatively affected its quality upon cutting and postharvest processing. The different cultivars of brinjal also differed in the extent of browning. Eight cultivars [‘Pusa purple long’ (V1), ‘Ravaiya’ (V2), ‘Azad kranti’ (V3), Arka navneet’ (V4), ‘Kalpatharu’ (V5), ‘Raveena’ (V6), ‘Anupam’(V7), and ‘Silki’ (V8)] of brinjal were studied for their PPO activity, phenolic content, and browning index. Significant variations in post processing postharvest browning of brinjal cultivars were observed. In fresh brinjal browning was found to be dependent of both phenolic content as well as PPO activity, however, in stored brinjal a major determinant of browning was found to be total soluble phenolic content. During storage, in four of the brinjal cultivars, the level of PPO was found to gradually reduce. On the other hand the level of phenolics as well as browning index (in cut samples) increased in all the brinjal cultivars. The chlorogenic acid was found to be the major phenolic with a range of ~50-70% of total phenolics in brinjal. Each cultivar differed in chlorogenic acid content and the change in chlorogenic acid concentration was found to be independent of total soluble phenolics, PPO activity, and browning.

The mechanics of cutting and further processing were found to have very profound effect on the browning process. A method was developed for inhibition of browning in fresh cut ready-to-cook (RTC) brinjal. 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 PPO

activity and hence lesser browning. The reduced browning in sharp blade-cut sample compared to knife-cut sample was found to be due to comparative lower release of phenolics and PPO activity as reconfirmed by the inhibitor potassium metabisulfite study. For commercial acceptability of the technique, storage studies were performed with periodical determination of browning, PPO activity, phenolic content, microbial load, and orgonoleptic scores at ambient (26±2), 10 and 4 ºC storage, which indicated that fine cut samples could be stored up to 5, 12, and 16 days at these temperatures, respectively. This RTC brinjal was found to be microbiologically and organoleptically acceptable during his storage.

The brinjal cultivar 'Kalpatharu' found to be the richer source of PPO and showed maximum browning. The purification of PPO enzyme was performed using this cultivar for characterization of the enzyme including inhibitor studies. Two isoforms of PPO were found in all the studied cultivars of brinjal. One isoform precipitated at 30% ammonium sulphate, whereas, the other one precipitated at 70%. The highly active PPO which precipitated at 50-70% ammonium sulphate saturation was 259 fold purified using ion exchange (DEAE), hydrophobic interaction (phenyl Sepharose), and gel filtration (Superdex) chromatography. The specific activity of purified PPO was found to be 4925 units per mg protein. The purified PPO was characterized to be a 112 kDa homodimer. The enzyme showed very low Km (0.34 mM) and high catalytic efficiency (3.3×10^6) with 4-methyl catechol. The substrate specificity was found in order of 4-methyl catechol > tert-butylcatechol > dihydrocaffeic acid > pyrocatechol. The substrate specificity indicated its nature as a catechol oxidase (EC 1.10.3.2) with maximum activity with 4-methyl catechol. The enzyme lacked cresolase and tyrosinase activity. The catechol ring structure and smaller functional group (4-methyl) at meta position with respect to –OH group were found to be the major determinant of its activity. Cysteine hydrochloride,

potassium metabilsulfite, ascorbic acid, erythorbic acid, resorcylic acid and kojic acid showed competitive inhibition, whereas, citric acid and sodium azide showed mixed inhibition of PPO activity. Cysteine hydrochloride was found to be the most effective inhibitor with a low inhibitor constant of 1.8 µM, much less than the commonly used potassium metabisulfite (9 µM).

The PPO mRNA was isolated from cultivar 'Kalpatharu', cDNA was prepared and subjected to PCR amplification (with primers designed from potato PPO nucleotide sequence). The amplification product was confirmed to PPO by re-amplification using PPO internal primers followed by nucleotide sequencing. When genomic DNA was PCR amplified it also showed a product indicating the absence of introns in PPO gene which was confirmed by nucleotide sequencing and further bioinformatic analysis. The PPO gene was successfully amplified and sequenced in six cultivars ['Pusa purple long' (V1), 'Ravaiya' (V2), 'Azad kranti' (V3), 'Kalpatharu' (V5), 'Raveena' (V6), and 'Anupam' (V7)] and submitted to Genbank. Nucleotide sequence and conceptual protein sequence were found to be significantly conserved in these cultivars. The nucleotide sequence also showed 97% similarity with potato (AAA85122.1), 96% with tomato (AAB22610.2), 98% with tobacco (ABE96885.1), and 97% with sweet potato (AAW78869.1). The conceptual amino acid sequence showed the presence of N-terminal chloroplast targeting sequence (1-40 residue) followed by thylakoid targeting sequence (41-81). The copper A and copper B binding regions were also identified to be at approximate amino acid position from 173-216, and 349-403, respectively, from the amino terminal end. The conceptual amino acid sequence was found to be significantly conserved among these cultivars. However, two cultivars ('Kalpatharu' and 'Raveena') showing highest PPO activity and post-cut browning in fresh samples were found to differ in 38 amino acid

long sequence in the region close to 301 to 338 of total ~593 residues in comparison with the other cultivars.

The PPO gene from cultivar ‘Kalpatharu’ was cloned using PET 28a expression vector downstream of *lac I* promoter (IPTG inducible) in *E.coli* (BL-21). The IPTG induction showed overexpression of a protein of 66 kDa size, observed to form inclusion body. This insoluble PPO was solubilized and purified but showed very poor enzyme activity, which could be attributed to the absence of post translational modifications in *E. coli*.

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1. **Mishra, B. B**., Mukhopadhyaya, R. Sharma, A. (2008). *Solanum melongena* polyphenol oxidase mRNA, partial cds. NCBI Accession no. GQ149350.
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Free phenolics and polyphenol oxidase (PPO): The factors affecting post-cut browning in eggplant (*Solanum melongena*)

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ABSTRACT

Polyphenol oxidase (PPO) catalyses oxidation of phenolics, which results in instant but differential browning in many cut fruits and vegetables, including eggplant. Eight cultivars of eggplant were characterised by their PPO specific activity, phenolic content, browning index, and PPO polymorphism. In fresh eggplant, browning was found to be dependent on both the phenolic content and PPO specific activity, whereas, total phenolic content played a major role in browning of stored fruits. Interestingly, although browning index increased in stored eggplant fruits, PPO activity reduced in four out of eight cultivars studied. Phenolic level was found to increase in all these cultivars during storage. Although a significant level of homology was observed in PPO nucleotide and conceptually translated protein sequence, two cultivars, which displayed highest PPO specific activity, differed in the 38 amino acid stretch in the peptide region 301–338.

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1. Introduction

Polyphenol oxidase (PPO) oxidises phenolics in the presence of oxygen on the cut surface of fruits and vegetables, producing *o*-quinones, which autopolymerise to form brown-coloured pigments (Madinez & Whitaker, 1995). Upon cutting, disruption of cellular structures leads to release of PPO enzyme and its phenolic substrates, which has been demonstrated using electron and fluorescence microscopy (Mishra, Gautam, & Sharma, 2012a). The peroxidase (POD) activity can also cause browning in the presence of hydrogen peroxide. Hydrogen peroxide concentration is low in fruits and vegetables, so the contribution of POD in the post-cut browning is considered to be lesser than that of PPO (Mayer, 2006). Many cultivars of eggplant (*Solanum melongena*) are available, with varying morphological features, such as colours (purple, green, purple with white and green stripes and patches), shapes (ovoid, obovate, oblong, cylindrical, club shaped), and calyx (spiny, non-spiny) (Raigón, Prohens, Muñoz-Falcón, & Nuez, 2008). These cultivars differ in their extents of post-cut browning which could be due to variations in the PPO activity or level of soluble phenolics. PPO activity could vary, due to changes in gene sequence or epigenetics. Recently, the PPO gene has been reported to exist in many isoforms and found to be expressed under stress, e.g. wounding in different parts of the plant (Shetty, Chandrashekar, & Venkatesh, 2011). There are very few reports on characterisation of PPO from eggplant. Roudsaria, Signoreta, and Crouzeta (1981) reported purification of PPO using chromatography. Pérez-Gilabert and Carmona (2000) and Doğan, Arslan, and Doğan (2002) have characterised the ammonium sulfate-precipitated PPO from eggplant. Concellón, Añón, and Chaves (2004) have also reported the PPO activity of crude extract during low temperature storage of eggplant. Todaro, Cavallaro, Argento, Branca, and Spagna (2011) have reported browning inhibition and kinetics of PPO activity in crude extract of eggplant. There are also reports of the various phenolic compounds present in eggplant (Luthria et al., 2010). The study of the gene sequence and its analysis in different cultivars would help us to understand the uniqueness of protein sequence that may have a bearing on the enzyme activity and overall browning. In the current study, a comparative evaluation of the nucleotide sequence, enzyme activity, substrate status, and the overall browning index, in different eggplant cultivars, has been performed. The findings will help in understanding the contribution of these factors, in the post-cut browning process, in stored raw eggplant. Further studies with the ambient-as well as low temperature-stored eggplant were also performed to understand the role of storage temperature in post-cut browning of stored eggplant.

2. Materials and methods

2.1. Chemicals

Agarose, ammonium sulfate, ascorbic acid, Bradford reagent, cetyl trimethyl ammonium bromide, chloroform, chlorogenic acid, disodium hydrogen phosphate, Folin–Ciocalteu reagent, gallic acid,

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isoamyl alcohol, 4-methyl catechol, sodium dihydrogen phosphate, polyvinyl pyrrolidone (PVP), polyvinyl polypyrrolidone (PVPP), sodium carbonate, and triton X-100 were procured from Sigma–Aldrich Inc., St. Louis, MO.

2.2. Eggplant

The eight popular cultivars of eggplant (*S. melongena*), namely pusa purple long (V1), ravaiya (V2), azad kranti (V3), arka navneet (V4), kalpatharu (V5), raveena (V6), anupam (V7), and silki (V8), were procured from a local supplier (4 kg of each variety). The vegetables were cleaned in potable water and stored at ambient (26 ± 2 °C) temperature, as well as 10 °C and 70 ± 5% relative humidity. The samples were analysed periodically for browning index, PPO activity, and total soluble phenolics.

2.3. Determination of the extent of browning

For measurement of browning in cut eggplant, the reflectance in the visible spectrum region (360–780 nm) was recorded at 10 nm wavelength intervals, using a Minolta CM-3600D spectrophotometer (Konica Minolta Sensing, Inc., Osaka, Japan). A D65 lamp was used as reference light source and the detector was fixed at an angle of 10° with respect to the light source (Ramírez-Jiménez, Guerra-Hernández, & García-Villanova, 2000). The equipment was calibrated prior to analysis with a standard white tile and a black box for 100 and 0% reflectance, respectively. The colour parameter used was L (Lightness) which denotes the amount of light or luminance reflected from the sample. The browning/darkening was calculated as (100-L), which is the opposite of lightness (Ramírez-Jiménez et al., 2000). For measurement of browning in eggplant, the slices were cut and observed at the saturation point where maximum browning was achieved in most of the samples (Mishra et al., 2012a). During the current study this occurred within 30 min of cutting.

2.4. Extraction of polyphenol oxidase (PPO)

The cut eggplant (30 g) was frozen in liquid N_2, ground to fine powder and homogenised in 100 ml of extraction solution for better extractability, using a polytron homogenizer (Model PT3100, Kinematica AG, Switzerland). The extraction solution contained sodium phosphate buffer (pH 6.8, 0.05 M) with polyvinyl pyrrolidone (PVP) (1%), polyvinyl polypyrrolidone (PVPP) (2%), triton X-100 (1%) and ascorbic acid (30 mM). The concentration of ascorbic acid was optimised in this extraction solution to be 30 mM (Mishra, Gautam, & Sharma, 2012b). Its concentration above 50 mM affected the enzyme activity. Again, the concentration of ascorbic acid below 15 mM did not inhibit browning. Similar findings have also been reported in loquat fruit (Ding, Chachin, Ueda, & Imahori, 1998). The extract was stirred for 30 min, filtered using muslin cloth, and centrifuged (5810R, Eppendorf, Hamburg, Germany) at 10000×*g* for 15 min at 4 °C. The supernatant was collected and fractionated using ammonium sulfate precipitation (up to 80%). Initially, to remove most hydrophobic proteins, 20% ammonium sulfate precipitation was performed (Englard & Seifter, 1990). Later, supernatant of this fraction was saturated up to 80% ammonium sulfate and precipitated. This precipitate represented the total PPO. Based on earlier findings, the supernatant of the 20% fraction was also parallelly fractionated using 10% sequential increase of ammonium sulfate, which resulted in precipitation of two isoforms of PPO called PPO 1 (precipitated at 30% ammonium sulfate fractionation) and PPO2 (precipitated at 70% ammonium sulfate fractionation) (Mishra et al., 2012b). The individual fractions were solubilised in 10 ml of phosphate buffer (pH 6.8, 20 mM) and dialysed using 10 kDa cut off membrane in 3 l of phosphate buffer (pH 6.8, 2 mM) at 4 °C with three buffer changes at 4 h intervals for removal of salt.

2.5. Assay of protein and polyphenol oxidase (PPO) specific activity

The polyphenol oxidase (PPO) activity was determined spectrophotometrically, using 4-methyl catechol as substrate (Concellón et al., 2004). The enzyme assay was carried out by taking 0.88 ml of phosphate buffer (pH 6.8, 50 mM), 0.1 ml of substrate (0.1 M) and 0.02 ml of enzyme extract (prepared as above). The increase in absorbance at 420 nm was monitored at 30 s intervals for 3 min, using a spectrophotometer (Model UV 4-100, Unicam, Cambridge, UK), and the average change in absorbance per min, were calculated. One unit of enzymatic activity was defined as the amount of enzyme which caused a change in absorbance of 0.1/min. The PPO activity was expressed as U/g of eggplant weight. The PPO specific activity was determined by expressing PPO activity/mg protein (U/mg protein). Protein content of the eggplant extract was determined by the Bradford method (1976), using bovine serum albumin (BSA, Sigma Chemical, St. Louis, USA) as standard.

2.6. Estimation of total soluble phenolics

Eggplant (7.5 g) was homogenised in 80% methanol (50 ml), using a polytron homogenizer (Model PT3100, Kinematica AG, Switzerland). The suspension was centrifuged at 10,000×*g* for 20 min and the supernatant was collected. A 25 μl aliquot of the supernatant was mixed with an equal volume of milli Q water and further mixed with 50 μl of 0.2 N Folin–Ciocalteu reagent. The suspension was incubated at ambient temperature (26 ± 2 °C) for 15 min and later mixed with 0.15 ml of sodium carbonate solution (0.2 g/ml). The reaction mixture was incubated in a water bath at 40 °C for 20 min, placed on ice for 5 min and then the absorbance was measured at 755 nm, using a spectrophotometer (Model UV 4-100, Unicam, Cambridge, UK). The total phenolics were calculated, using gallic acid as a standard, and expressed as mg GAE equivalents/g of eggplant (Luthria et al., 2010).

2.7. DNA and RNA isolation

The cut eggplant was frozen in Liquid N_2, ground to fine powder and the genomic DNA was isolated using the CTAB (cetyl trimethyl ammonium bromide) method to avoid phenolics interference (Rogers & Bendich, 1994). RNA isolation was performed by the guanidinium thiocyanate–phenol–chloroform extraction method, using an isolation reagent (TriPure, Roche Applied Sciences, Mannheim, Germany) (Chomczynski & Sacchi, 1987). Eggplant fruits were frozen in liquid nitrogen, ground to fine powder, and total RNA was isolated according to manufacturer's guidelines of the kit (TriPure). Isolated RNA was solubilised in DEPC (diethylpyrocarbonate)-treated RNAse-free water and also treated with DNAse to avoid any DNA contamination. The pure total RNA thus prepared was used for cDNA synthesis, using a commercial kit (Roche Applied Sciences, Mannheim, Germany).

2.8. Amplification of DNA and nucleotide sequencing

The genomic DNA and cDNA were subjected to PCR amplification, using ppo gene-specific end primers (Forward primer 5′-GGAATTCCATATGGCAAGCTTGTGCA ATAG-3′ and Reverse primer 5′-TACTCTCGAGTTAACAATCTGCAAGACTGAT CGTCGCACC-3′). The amplified fragment was re-amplified with an additional set of internal primers (Forward 5′-GTAACTAATGCTCCATGTCCTC-3′, and reverse, 5′-GGCTTGAAGTTACGC CATGGTG-3′). These primers were initially designed from the coding sequence of PPO gene from

potato (Genbank accession No. U22921.1) due to unavailability of the eggplant PPO gene sequence. After completion of sequencing of PPO gene from 'Kalpatharu' (V5), the primers were later designed from this eggplant cultivar (Forward 5′-ATGG-CAAGCTTGTCCAATAGTAGTATACAACCC-3′, 5′-G GGCATGCG-TATGCCTGCCATGTTCGATGG-3′ and reverse 5′-TTAACAATCT GCAA GACTGATCGTCGCACC-3′, 5′-TTAACAATCTGCAAGACTGATCGT CG CACC-3′) and used for amplification of DNA from remaining cultivars. The amplified DNA was subjected to agarose (0.8%) gel electrophoresis, along with a molecular weight marker. Later, the DNA band was gel-purified, using a gel elution kit (Gene Elute, Sigma, MO), and subjected to dideoxy nucleotide sequencing, followed by nucleotide homology analysis, using NCBI blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The location of the protein inside the cell was determined using a Target P and ChloroIP programme and the signal peptide cleavage site was determined, using a Signal IP programme (Emanuelsson, Brunak, Heijne, & Nielsen, 2007). The presence of post-translational modifications, if any, was verified using online tools available at http://expasy.org/tools/.

2.9. Senescence measurement

The senescence of eggplant vegetables was determined in terms of % weight loss (PWL), as well as firmness measurement, using 10 replicates of eggplant from each cultivar stored at 26 ± 2 °C or low temperature (10 ± 2 °C). The firmness was measured, using a texture analyzer (TA.XT2, Stable Micro Systems Texture Technologies, Scarsdale, NY, USA) fitted with a P/2 N needle probe. Each sample was compressed for a 5 mm distance at the central position, at the rate of 0.5 mm/s, and the maximum force (N) in terms of resistance experienced during the test was instrumentally recorded and analysed.

2.10. Statistical analysis

In general, experiments were repeated in three sets independently, each set having 3 replicates. The means and standard deviations were calculated, taking all the readings into consideration. Two-way ANOVA was performed to ascertain the significance of difference of the mean, using Tukey's test. Statistical analysis was performed using BioStat 2009 Version Professional 5.8.0.0 (AnalystSoft Inc., Canada).

3. Results and discussion

3.1. General

Different cultivars of eggplant displayed a wide range of shapes and colours, such as pure white, purple, black, green, and variegated, in different shades. The photographs of different eggplant cultivars used in this study are shown in Figs. 1 and 3. The eggplant cultivars displayed variation in PPO activity, phenolic content and post-cut browning, which was independent of its outer skin colour. The 'Kalpatharu' (V5) and 'Raveena' (V6) are purple and green in colour, respectively, but showed higher browning, whereas, highly purple 'Ravaiya' (V2) eggplant showed comparatively less browning after cutting. The green and purple skin colour is due to pigments such as chlorophyll and anthocyanins, respectively (Raigón et al., 2008).

3.2. Extent of browning in fresh cut samples varied among cultivars

Browning started immediately after cutting and increased with time. The extent of browning, within 30 min of cutting, was found to be suitable for comparison among the cultivars on day 1 and findings are shown in Fig. 1a. The 'Kalpatharu' (V5) showed the maximum browning, followed by other cultivars (V6 > V8 > V3 > V4). The browning in 'Ravaiya' (V2) and 'Anupam' (V7) was found to be even less and the differences among them were significant ($P \leqslant 0.05$). These two cultivars showed minimum browning and were about 40 and 28% less, respectively, compared to the cultivar (V5), showing maximum browning. As PPO and its phenolic substrates have been reported as major factors responsible for post-cut browning in various fruits and vegetables, it was interesting to know their status in these eggplant cultivars.

3.3. Cultivars showing maximum browning in fresh cut samples exhibited higher PPO specific activity

The maximum PPO specific activity was observed in cultivar 'Kalpatharu' (V5) which was marginally higher than 'Raveena' (V6) in the day 1 sample. The PPO sp. activity in both 'Anupam' (V7) and 'Ravaiya' (V2) was about 66% less than that in V5 (Fig. 1b and c). The cultivar 'Silki' (V8) showed 43% less PPO sp. activity than did V5. The cultivar 'Pusa purple long' (V1) showed the least PPO specific activity and the differences among other cultivars (V3 > V4) were found to be insignificant ($P \leqslant 0.05$). In all these cultivars, PPO2 was found to be predominant, except 'Ravaiya' (V2). Comparatively, the activity of PPO2 was significantly lower in 5 cultivars (V1, V2, V3, V4, and V7). Total PPO specific activity has been observed to be more than the individual specific activities of PPO1 and PPO2 in all the cultivars (Fig. 2). This also indicated that total specific PPO activity possibly represents both PPO1 and PPO2 specific activities together. Besides, PPO2 specific activity was earlier reported to be about 62% higher than that of PPO1 (Mishra et al., 2012b). These two isoforms differ in their hydrophobic characteristics which resulted in precipitation at two different ammonium sulfate concentrations. Similarly, PPO1 (fractionated at 40% ammonium sulfate) has been characterised to have the presence of cresolase and catecholase activities by Pérez-Gilabert and Carmona (2000). In our earlier study, PPO2 has been shown to have a prominent catecholase activity. However, it lacked the cresolase activity (Mishra et al., 2012b). The varietal difference in PPO activity has been reported in other fruits and vegetables, including litchi, which may be due to variations in its levels of expression or bioactivity (Mishra et al., 2012). Existence of isozymes of PPO has been reported earlier in other fruits and vegetables, including potato (Thygesen, Dry, & Robinson, 1995). In eggplant, two different alleles of PPO have been reported in root, leaf and fruit (Shetty et al., 2011).

3.4. Phenolic concentration varied significantly among fresh cut samples of eggplant cultivars

The phenolic content in eggplant cultivars is shown in Fig. 1d. In the case of fresh samples (day 1) it was found to be highest in cultivar 'Arka navneet' (V4). In the case of cultivars 'Pusa purple long' (V1), 'Azad kranti' (V3), and 'Silki' (V8), the phenolic contents were similar. The cultivars V3, V8 showed about 37% less, whereas, V6 and V7 showed about 45% less phenolic content than did V4. The phenolic content has been reported to vary in different cultivars of fruits and vegetables (Mishra et al., 2012).

The comparison among cultivars also indicated a major role for both PPO and phenolics in browning, particularly in the case of fresh-cut eggplant. For example, in cultivars 'Raveena' (V6) and 'Anupam' (V7), the phenolic concentrations were similar, but 'Raveena' (V6) showed higher browning, which could be due to the observed higher PPO specific activity. Similarly, in V5 and V6, which had a comparatively lower concentration of phenolics,

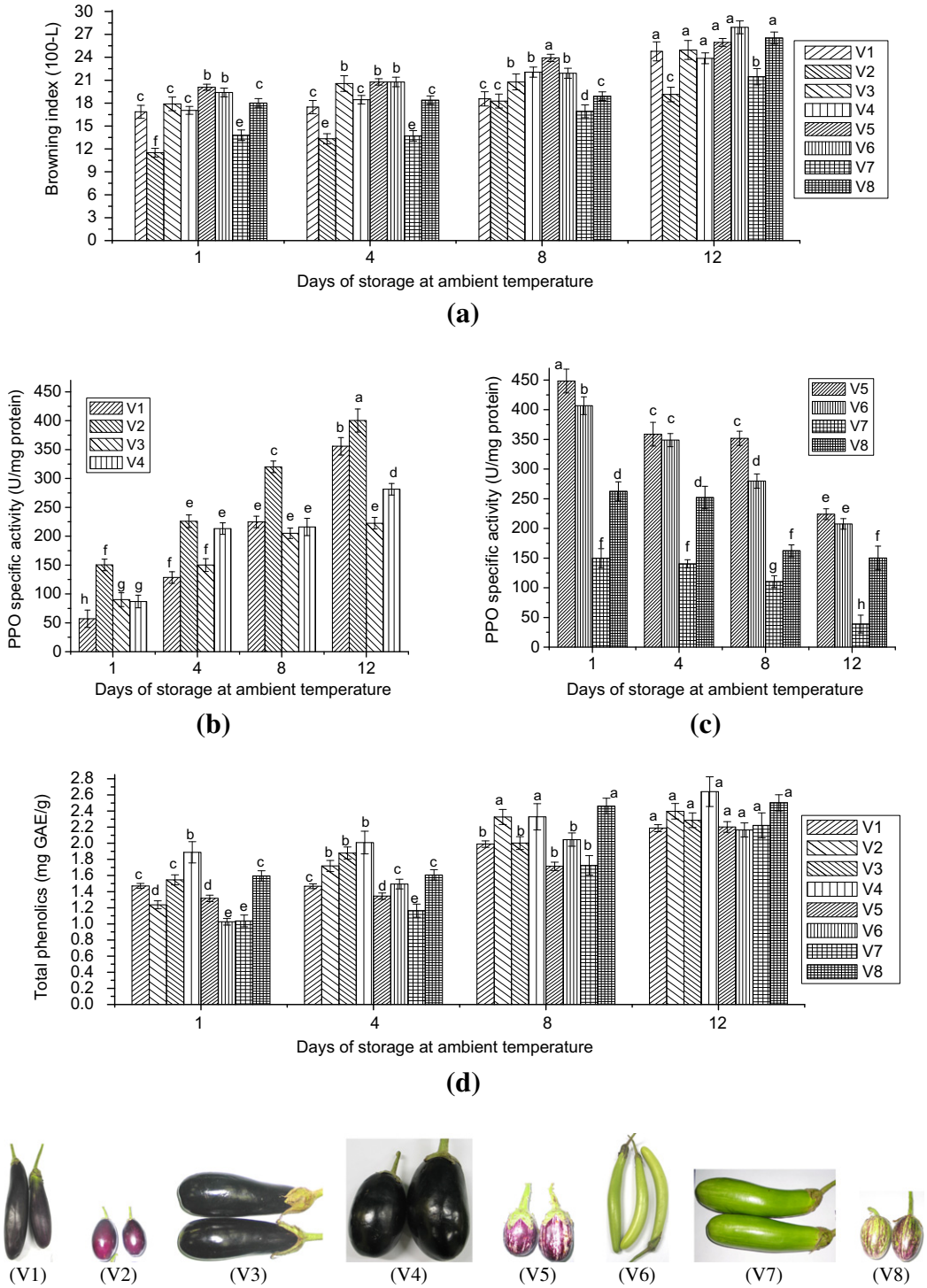

Fig. 1. Comparative profile of browning index (a), polyphenol oxidase (PPO) specific activity (b, c), and total soluble phenolic content (d) in different eggplant cultivars stored at ambient temperature (26 ± 2) °C. For each parameter, the columns with different letter superscripts are significantly different ($P \leqslant 0.05$). GAE – Gallic acid equivalents. U – unit of PPO activity (change in 0.1 absorbance/min). V1, Pusa purple long; V2, Ravaiya; V3, Azad kranti, V4, Arka navneet; V5, Kalpatharu; V6, Raveena; V7, Anupam; and V8, Silki. V1–V4 are purple, V5 and V8 are variegated, V6 and V7 are green in colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

higher browning correlated with higher PPO specific activity. However, in 'Azad kranti' (V3) and 'Arka navneet' (V4), although PPO specific activity was less, browning was found to be higher, which could be due to the high concentration of phenolics (Fig. 1). Thus, in fresh (Day 1) cut eggplant, both of these factors (phenolics and PPO) were found to play a role in browning and complemented each other.

3.5. Kinetics of changes in browning index, PPO specific activity and phenolic content in stored eggplant

3.5.1. General

The change in browning, phenolic content and PPO specific activity was evaluated for different cultivars during ambient temperature (26 ± 2 °C) storage up to 12 days (Fig. 1). The profile of

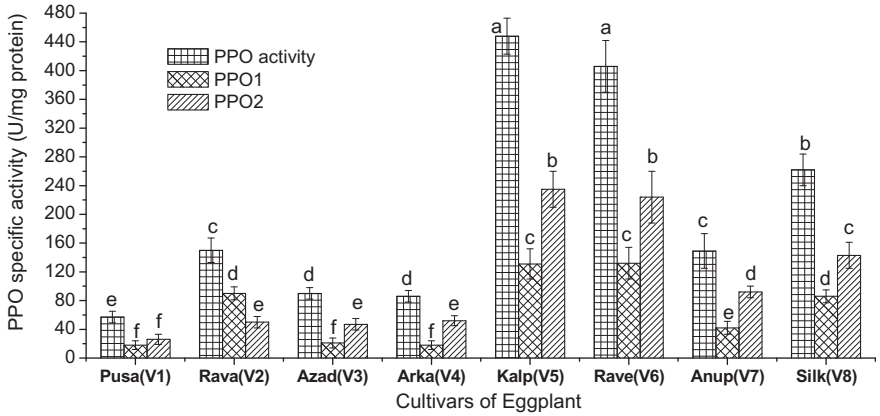

Fig. 2. The specific activity of total PPO, PPO1 and PPO2 from fresh eggplant. U – unit of PPO activity (change in 0.1 absorbance/min). The different superscripts indicated significance of difference ($P \leqslant 0.05$). The initial four letters of the cultivar name were used as their abbreviation in Figure.

these parameters were found to change significantly during storage and also profound cultivar based variations were noticed.

3.5.2. Browning index and soluble phenolics increased during storage but PPO specific activity varied among cultivars

Browning index and phenolic content increased during storage in all the eight cultivars (Fig. 1a and d). However, PPO specific activity was found to increase in four cultivars, namely 'Pusa purple long' (V1), 'Ravaiya' (V2), 'Azad kranti' (V3), and 'Arka navneet' (V4) and decrease in other cultivars studied, namely 'Kalpatharu' (V5), 'Raveena' (V6), 'Anupam' (V7), and 'Silki' (V8). Based upon the change observed in PPO activity during storage, eggplant cultivars were categorised into two groups, V1, V2, V3, and V4 in group A (Fig. 1c) and V5, V6, V7, and V8 in group B (Fig. 1d). Cultivar 'Pusa purple long' (V1) did not show any significant increase in browning until day 8 with respect to fresh eggplant samples and then displayed a significant increase of about 25% on day 12 (Fig 1a). The PPO sp. activity and soluble phenolics during the same period increased by 700 and 25%, respectively (Fig. 1b and d). Among these cultivars, the increase in PPO sp. activity was found to be highest in V1. The increase in PPO activity was comparatively very much higher than the phenolic content and browning index. In the case of 'Ravaiya' (V2) too, an increase in these parameters was observed during storage. The browning and PPO activity steadily increased to about 64 and 160% during the 12 day storage (Fig. 1a and b). The increase in browning was highest in this cultivar. The phenolics level increased by about 100% on day 12 (Fig. 1d). In eggplant 'Azad kranti' (V3), the browning and PPO sp. activity increased by 38 and 150%, respectively (Fig. 1a and b). The phenolic content increased by about 53% during 12 days of storage (Fig. 1d). In cultivar 'Arka navneet' (V4), the browning, PPO sp. activity and phenolics increased by 41, 200, and 100%, respectively (Fig. 1a and b). The activity of oxidative enzymes, e.g. PPO, is known to increase during storage in fruits (Jiang, Duan, Joyce, Zhang, & Li, 2004). Such an increase in PPO sp. activity, phenolics and browning during storage was earlier reported in fruits of two litchi cultivars (Mishra et al., 2012). The change in total phenolics during storage could be due the physiological changes associated with senescence of eggplant. The increase in PAL (phenylalanine ammonia lyase) activity, which is a regulatory enzyme in phenolic biosynthesis, could also be the reason for this increase in total phenolics (Camm & Towers, 1973).

Upon 12 days of storage, the browning in 'Kalpatharu' (V5), 'Raveena' (V6), 'Anupam' (V7), and 'Silki' (V8), increased by 30%, 47%, 50%, and 45%, respectively, and the phenolic content increased by 61%, 107%, 120%, and 56%, respectively (Fig. 1a and d). Among these cultivars, the increase in phenolic content was highest in 'Anupam' (V7). However, during the same period, PPO sp. activity showed a decrease of 50% in V5, 49% in V6, 78% in V7 and 43% in V8 (Fig. 1c). Such a decrease in PPO activity has been reported earlier in low temperature stored eggplant cultivars (Concellón et al., 2004). The increase in browning index, even after the decrease in PPO activity, signifies the role of free phenolics in post-cut browning in stored eggplant. A similar observation with respect to PPO activity has also been reported earlier in the case of rambutan (Yingsanga, Srilaonga, Kanlayanarata, Noichindab, & Glassonc, 2008) and longkong (*Aglaia dookkoo*) fruit (Lichanporn, Srilaong, Wongs-Aree, & Kanlayanarat, 2009), where increase in browning was reported due to an increase in phenolic content but not PPO activity.

3.5.3. Cultivar-based variation in PPO specific activity, phenolics and browning was independent of storage temperature

During ambient temperature storage, all these cultivars underwent senescence, which was determined in terms of loss of weight and firmness. The percentages of weight loss and firmness in these cultivars, after 12 days, were found to be in the range of 12–23% and 18–31%, respectively. Similar percentages of weight loss and firmness were observed after 21 days of storage at 10 °C. This indicated that, under low temperature storage, eggplant senescence was delayed and an extension of shelf life up to 21 days was observed. The variation in PPO sp. activity, phenolic content, and browning was also analysed in eggplant fruit stored at 10 °C, to assess if these changes were similar to those observed in the case of ambient temperature-stored samples. As observed for ambient temperature-stored sample in group 1 cultivars (V1–V4), the browning, soluble phenolic content, and PPO sp. activity increased during storage (Fig. 3b). However, in group 2 cultivars (V5–V8), the browning and soluble phenolics increased but PPO sp. activity decreased during storage. These findings indicated that the change in these parameters was senescence-independent and could be characteristic features of the specific cultivars. The decrease in storage temperature did not affect the enzyme activity and thus indicated a variety-specific variation in the kinetics of PPO activity during storage.

3.5.4. Correlation analysis of browning with PPO activity and phenolics

In fresh samples of most of the cultivars, PPO activity correlated well with the browning index. However, in stored samples, phenolics were found to be the major contributors to the browning. In four cultivars (V1–V4; Group A) the PPO activity showed positive correlation (r) in the range of 0.86–0.88, whereas phenolics showed marginally higher correlation (r) (0.85–0.97) with

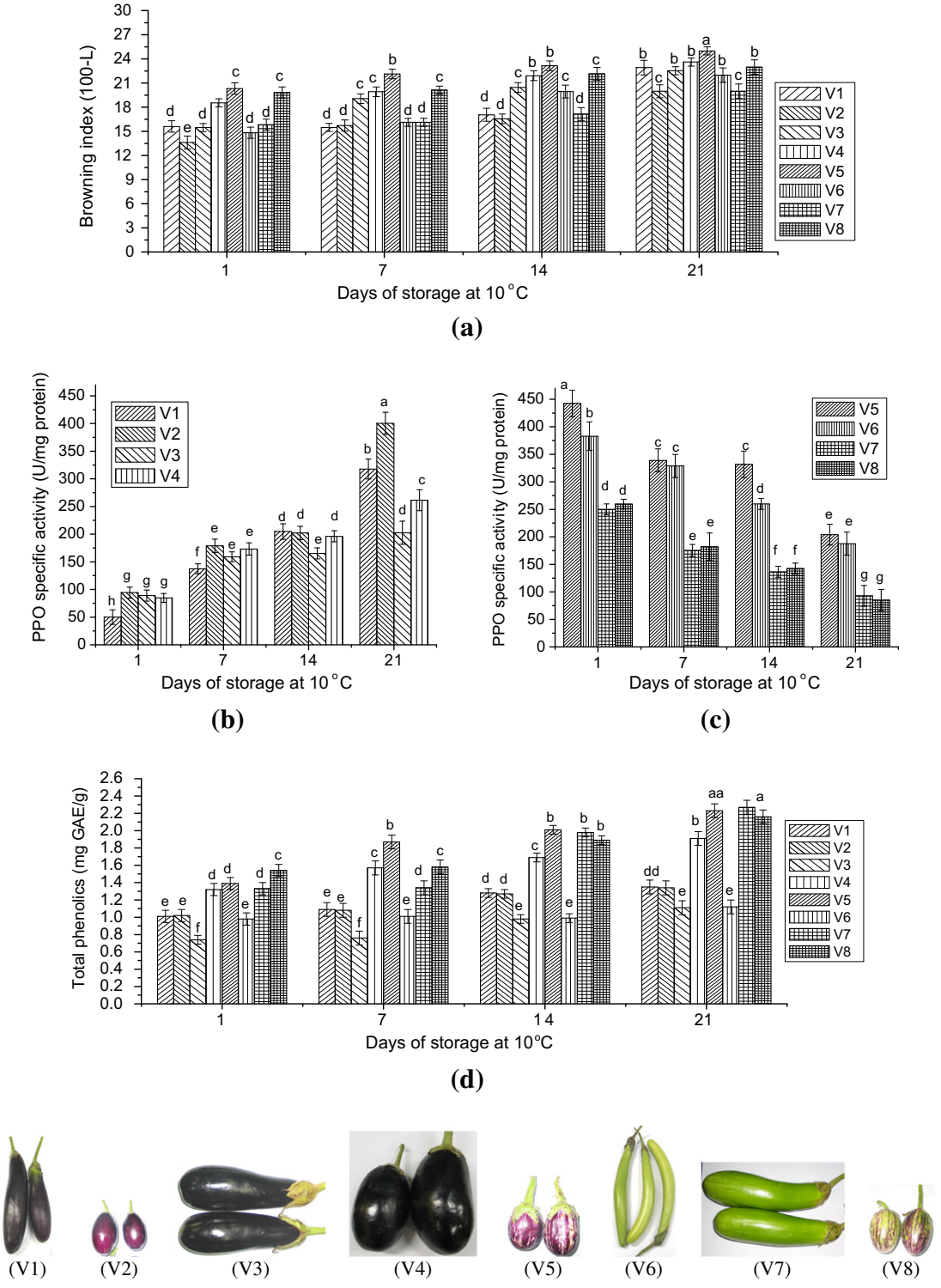

Fig. 3. Comparative profile of browning index (a), polyphenol oxidase (PPO) specific activity (b, c), and total soluble phenolics (d) in different eggplant cultivars during storage at 10 (±2)°C. For each parameter, data points with different superscripts are significantly different ($P \leqslant 0.05$). GAE – Gallic acid equivalents. U – unit of PPO activity (change in 0.1 absorbance/ min). V1, Pusa purple long; V2, Ravaiya; V3, Azad kranti, V4, Arka navneet; V5, Kalpatharu; V6, Raveena; V7, Anupam; and V8, Silki. V1–V4 are purple, V5 and V8 are variegated, V6 and V7 are green in colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

browning. Contrary to this, in four other cultivars (V5–8; Group B), a strong negative correlation (r) (−0.80 to −0.91) between PPO activity and browning was observed during storage. This indicated that, in addition to PPO activity, other factors may also be important for browning, particularly in the case of stored eggplant. The higher concentration of phenolics could cause higher browning with even a moderate level of PPO activity (Lichanporn et al., 2009). It has also been previously reported that the browning of fruit and vegetables is related to increase in PAL activity and phenolic compounds (Lichanporn et al., 2009). Moreover, in intact tissue, the enzyme and its phenolic substrates are compartmentalised in chloroplast and vacuoles, respectively. During storage,

senescence-related loss of tissue integrity, could result in physical contact of enzyme and substrate, causing enzymatic reaction and contributing to browning (Concellón et al., 2004).

Among the factors associated with browning in post-cut eggplant, PPO and phenolics played a very prominent role. In addition, the peroxidase enzyme has also been reported to be associated with browning. The 80% ammonium sulfate-precipitated protein fraction has been previously shown to contain high peroxidase activity in eggplant fruits (Pérez-Gilabert & Carmona, 2000). However, the concentration of peroxidase in eggplant was reported to be small compared to PPO activity (Massolo, Concellón, Chaves, & Vicente, 2011). The peroxidase activity requires the presence of hydrogen peroxide as substrate, which is reported to be low (~0.03%) in fruits and vegetables. The eggplant is also rich in antioxidants, including ascorbic acid (~0.1%), with high superoxide-scavenging activity (Hanson et al., 2006). The phenolics present in eggplant also contribute to the antioxidant status of the vegetable. For these reasons, peroxidase could be playing a comparatively smaller role in post-cut browning in eggplant.

3.6. Gene sequence-based conceptual translation of PPO protein

The sequence details of PPO from representative eggplant cultivar V5 ('Kalpatharu') are given in Fig. 4. In comparison with related plants, the eggplant PPO amino acids sequence (deduced from gene sequence), showed higher similarity in the regions of the targetting sequence, active site and partial similarity in other regions. The presence of a N-terminal 81 amino acid (aa) long transit sequence was observed (Genbank accession No. GQ149349.1), whose presence has been reported in PPO from other fruits and vegetables (89 in apple, 86 in potato and 99 in tomato) (Emanuelsson et al., 2007). The Chloro IP programme analysis showed the higher probability of presence of a 40 amino acid long N-terminal chloroplast targetting sequence with cleavage site between serine (S40) and cysteine (C41) residues (Fig. 4) (Genbank accession No. GQ149349.1) (Emanuelsson et al., 2007). The same algorithm also showed the existence of a cleavage site for the remaining 41 amino acid long transit peptides between lysine (L81) and alanine (A82).

In some reports, PPO was found to be bound to the thylakoid membrane (Mayer, 2006). However, the eggplant PPO sequence showed similarity to other thylakoid lumen proteins, including PPO from other fruits. This could be due to the higher number of hydrophilic amino acids on the surface of the protein (Emanuelsson et al., 2007). The molecular weight was calculated to be approximately 56 kDa (Mishra et al., 2012b). The molecular weight of the protein was reported to be 60, 58, and 55 kDa in potato, tomato, and tobacco (Goldman, Seurinck, Marins, Goldman, & Mariani, 1998; Shahar, Hennig, Gutfinger, Hareven, & Lifschitz, 1992; Thygesen et al., 1995). This observed minor difference in molecular weight could be due to difference in amino acid composition and corresponding gene sequence. PPO was found to be a metalloprotein with the active site containing two copper-binding regions (A and B), each coordinated by three histidine (H) residues. One copper (Copper A) binds to the amino acids located within 173–216 and the second one binds to the amino acid residues in the region 349–403 (Fig. 4) (Genbank accession No. GQ149349.1). Each of these regions contained three histidines, which coordinated with copper [(H178, H196, H205 in the copper A region] and (H353, H367, H389 in the copper B region)]. The greatest sequence conservation, within and between species, was observed around these His residues. The post-translational modifications were also predicted in the PPO sequence. The probable amino acid residues undergoing phosphorylation are predicted to be thirteen serine residues, nine threonine residues, and ten tyrosine residues. The protein did not contain any *N*-myristoylation, *N*-acetylation, or *O*-glycosylation sites.

3.7. The cultivars with high PPO specific activity showed maximum sequence similarity

PPO genes from six cultivars were sequenced. The PPO nucleotide sequences were submitted to Genbank (Genbank accession numbers V1 (JQ621948), V2 (JQ621949), V3 (JQ621950), V5 (GQ149349.1), V6 (JQ621951), V7 (JQ621952)). The other two cultivars 'Arka navneet' (V4) and 'Silki' (V8) could not be PCR-amplified and hence were not sequenced. These two cultivars may have different nucleotide sequences in the primer binding site. The longest PPO nucleotide sequence (V2, V7) was of 1788 base pairs while the smallest one (V6) was of 1773 bp. As there was no intron reported in this gene, the conceptually translated protein

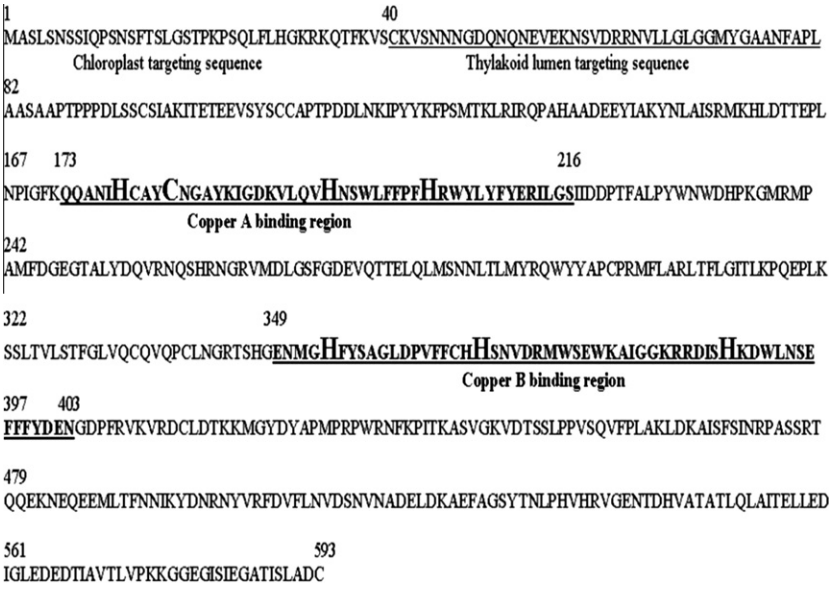

Fig. 4. Nucleotide sequence-based conceptually-translated amino acid sequence of PPO from egg plant (GQ149349.1). The number indicates the amino acid position from N-terminal position. The copper-binding active site regions are in bold font and underlined. The His residues in active site involved in binding to copper are displayed in bigger font.

contains 590–595 amino acids. Amino acid sequences of the six cultivars were compared with each other (Fig. 5). The active site region was found to be conserved and identical among all the six cultivars. The initial 81 amino acids of the signal sequence were also mostly identical, with minor differences in the region within 11–17 residues. The amino acid sequence comparison among these cultivars suggested the possible role of a stretch of amino acid residues in PPO activity. Two cultivars ('V5' and 'V6') showing the highest PPO activity and post-cut browning (in fresh samples) were found to differ in amino acids sequence in the region close to 301–338, in comparison to other (V1, V2, V3, and V7) cultivars (Fig. 5). This region of 38 amino acids was found to be unique and did not match significantly with the published PPO nucleotide sequence submitted in Genbank. However, this region (~301 to ~338) from V1, V2, V3, and V7 cultivars showed considerable match with PPO proteins from other plants. A change in gene

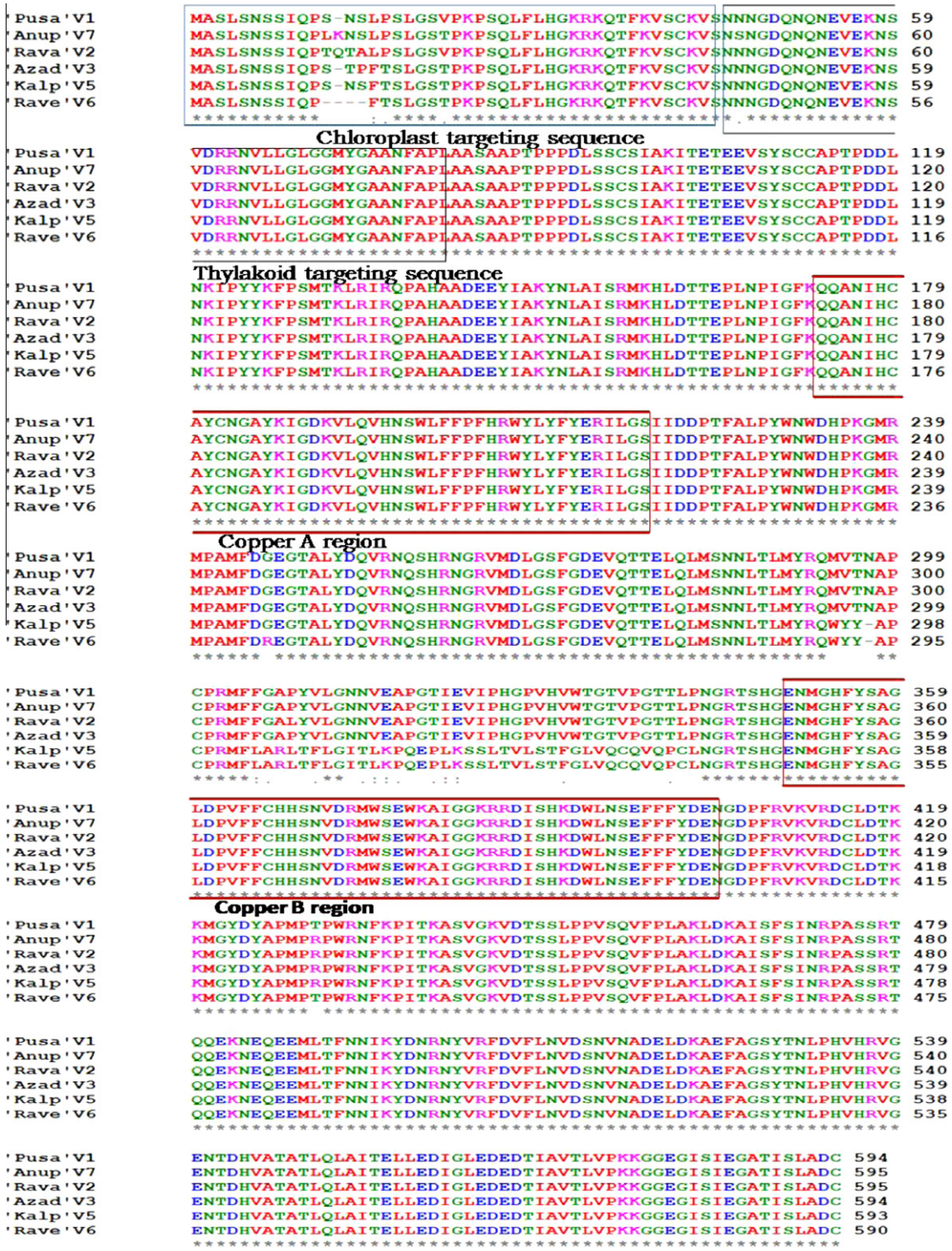

Fig. 5. The Multiple alignment of PPO protein sequences [Genbank accession numbers V1 (JQ621948), V2 (JQ621949), V3 (JQ621950), V5 (GQ149349.1), V6 (JQ621951), V7 (JQ621952)] from six different cultivars of eggplant. Symbol (*) below the sequence showed the positions of exactly matched amino acids. Symbols (: or .) indicate high or moderate matching at that position. The initial four letter of cultivar name were used as their abbreviation in Figure.

sequence, due to insertion or deletion of a base, resulted in change of reading frame and hence amino acid sequence (Shetty et al., 2011), whereas a substitution of base may result in change of a single amino acid of the protein. These changes could affect the structure and activity of enzyme (Thygesen et al., 1995). The differences in amino acid residues in this region (~301 to ~338) among eggplant cultivars could be due to a few deletions of bases in the PPO gene of V5 and V6, resulting in change in the reading frame in this region up to proline–cysteine–phenylalanine (PCL; 336–38) residues (Fig. 5). There are reports about the varietal differences resulting in differential expressions of enzymes, as well as tissue-specific expressions, due to existence of multiple isoforms of protein in plants (Shahar et al., 1992; Thygesen et al., 1995). In eggplant too, the existence of the PPO multigene family has been

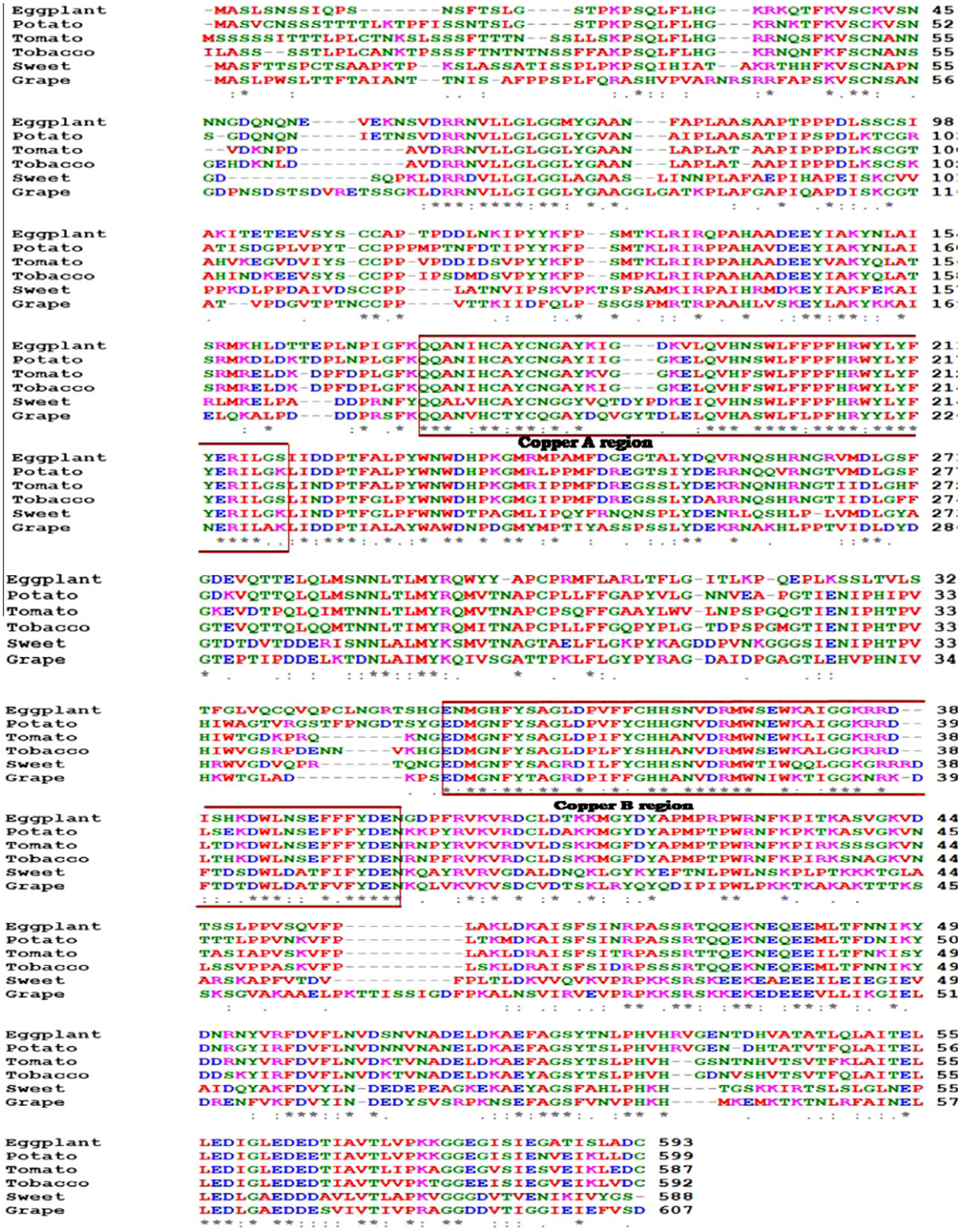

Fig. 6. Multiple alignment of PPO protein sequence from different fruits and vegetables. The PPO sequences used were from eggplant (ACR61398.1), potato (AAA85122.1) tobacco (CAA73103.1), tomato (AAB22610.1), grape (AAB41022.1) and sweet potato (AAW78869.1). Symbol – indicates the gap introduced for optimal alignment. Symbol (*) below the sequence shows the positions of exact matching of amino acids. Symbols (: or .) indicate high or moderate matching at that position.

reported, with many isoforms of PPO (Shetty et al., 2011). Besides, some silent single base differences were observed among these cultivars which did not affect the amino acid sequence of the protein.

3.8. Eggplant PPO gene sequence showed homology with those of potato and tomato

The gene sequence of PPO showed homology with other members of the solanaceae family (Fig. 6). The nucleotide sequences of PCR-amplified cDNA and genomic DNA of the PPO gene from eggplant were found to be of similar length and sequence {(NCBI Genbank accession No. GQ149349.1 (gene) and GQ149350.1 (mRNA)}. This indicated that there are no introns in the PPO gene of eggplant, which is unusual compared to other genes. However, the intronless PPO gene has been reported in the 'Arka shirish' cultivar of eggplant, as well as in potato, tomato, tobacco and a few other plants (Goldman et al., 1998; Shahar et al., 1992; Shetty et al., 2011; Thygesen et al., 1995). The eggplant PPO nucleotide blast search revealed high similarity to potato (86%) and tomato (84%), which are its closest members in the Solanaceae family and about 80% similarity to tobacco PPO (Fig. 6). The NCBI protein blast results showed about 97% similarity to potato (AAA85122.1), 96% to tomato (AAB22610.2), 98% to tobacco (ABE96885.1), and 97% to sweet potato (AAW78869.1).

A NCBI Conserved Domain Database (CDD) search showed the presence of domains from three super families. The regions within 168–247 and 348–380 amino acids, which include two copper ion (Cu-A and Cu-B binding regions, respectively), are similar to a common central domain of tyrosinase: pfam00264 superfamily (CDD 189478). This family includes polyphenol oxidase enzymes from other plants and hemocyanins. The region or domain between 386 and 436 showed homology with the PPO1-DWL superfamily, pfam12142 (CDD 192942), which was also annotated as the PPO middle domain. This domain family was found in bacteria and eukaryotes and includes about 50 amino acid residues, along with the presence of a conserved DWL sequence motif, which gave the family its name. Again, the region within 457–590 amino acids was similar to the PPO1-KFDV superfamily, pfam12143 (CDD 192943). This domain is found in eukaryotes, particularly in plants with the highly conserved sequence motif KFDV and contains about 132–152 amino acids. The eggplant PPO showed moderate similarity in protein sequence, including the above-discussed typical structural elements to these closely related plants; however, overall, homology was not very high, indicating the uniqueness of the eggplant PPO sequence.

4. Conclusion

Significant variations in the postharvest browning of cut fruit from different eggplant cultivars were observed. The extent of browning in raw fresh eggplant was found to depend both on PPO specific activity and total soluble phenolics. However, during a 12 day storage at ambient temperature, the phenolic content was found to be the major determinant of browning. Nucleotide sequence analysis of the PPO gene of different cultivars of eggplant indicated homology, but two of the cultivars, showing high PPO specific activity and browning index, were found to have unique amino acids in the region 301–338 from the N-terminal end.

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Browning of fresh-cut eggplant: Impact of cutting and storage

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ABSTRACT

Browning is a major postharvest problem in fresh-cut fruit and vegetables. This phenomenon is markedly observed in eggplant which immediately turns brown after cutting. In the current study, mechanics of cutting and further processing were found to have profound effects on the browning process. 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. Scanning electron and fluorescence microscopic examinations showed that sharp blade cutting caused less physical injury and cell 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.

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1. Introduction

The post-processing enzymatic browning of eggplant is a major problem for industries dealing with cut vegetables. Browning results in loss of eye appeal for consumers and may adversely affect nutritional and sensory properties of fresh-cut eggplant (Oms-Oliu et al., 2010). This has also negatively affected the commercial availability of shelf-stable cut eggplant. The level of phenolics and their oxidizing enzyme polyphenol oxidase (PPO) are known to be key players in the browning process of various raw and cut fruit and vegetables (Mayer, 2006; Hodges and Toivonen, 2008; Mishra et al., 2011). Several studies have suggested measures to control browning and these include chemical dip treatments and modified atmosphere packaging, excluding oxygen from the environment of the product (Kang and Saltveit, 2003; Saxena et al., 2009; Oms-Oliu et al., 2010). In the present study a novel but simple approach was used, where cutting of eggplant was performed using a sharp blade which caused lesser physical injury to the tissues. This was followed by immediate dipping in water that helped wash away the leached phenolics and enzymes from the cut surface. Air-drying followed by packaging in commercial styrofoam trays wrapped with stretchable film and storage at ambient temperature (26 ± 2 °C), 10 ± 2 °C and 4 ± 2 °C was further explored to achieve maximum shelf life and improved microbiological quality of this product.

2. Materials and methods

2.1. Chemicals

Chlorogenic acid, disodium hydrogen phosphate, Folin-Ciocalteu reagent, gallic acid, 4-methyl catechol, polyvinyl pyrrolidone (PVP), polyvinyl polypyrrolidone (PVPP), potassium metabisulfite (PMS), sodium carbonate, sodium dihydrogen phosphate, and triton X-100 were procured from Sigma–Aldrich Inc., St. Louis, MO, USA. The plate-count agar and potato dextrose agar were procured from Himedia Laboratories, Mumbai, Maharashtra, India.

2.2. Sample preparation

Eggplant (*Solanum melogena* L.) cv. Kalpatharu (12 kg) was procured from a local market and cleaned in potable water. The fruit was cut using a sharp blade into cubes of approximately 3 g each. To assess the effect of cutting procedure on the browning of eggplant, a sharp blade (thickness about 0.04 mm) and conventional vegetable cutting knife (blade thickness about 0.25 mm) were separately used. The blade thickness was measured using a digital caliper (Digimatic caliper, Mitutoyo, Kanagawa, Japan). The pieces were immediately submerged in potable water in a plastic bowl for 10 min, air-dried on a clean surface, packaged in styrofoam trays and wrapped with stretchable film. The samples were then stored at ambient (26 ± 2 °C), 10 ± 2 °C and 4 ± 2 °C and periodically assessed for browning parameters and related biochemistry.

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2.3. Scanning electron microscopy (SEM)

The sample preparation for SEM was carried out using a modified method by Dwivedi and Ahmad (1985). The thin sections from the mesocarp portion of the eggplant were immediately fixed in cold 2% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.0) for 48 h, washed twice in cold phosphate buffer, and then stained in 1% osmium tetroxide (w/v) in 0.1 M phosphate buffer (pH 7.0) overnight. Sections were then washed in deionized water for 20 min and sequentially dehydrated with increasing concentrations (40, 60, and 80%) of ethanol for 2 h each and finally in 100% ethanol overnight. The sections were then air-dried at ambient temperature and stored in a desiccator until use. SEM was carried out using a facility (SEM Model Quanta 200 SEM, FEI Company, Oregon, USA) available from Icon Analytical Equipments Pvt. Ltd., Mumbai, India. The sample was fixed on a carbon tape and observed at different magnifications using a high vacuum mode at 90 Pa and 20 kV voltage.

2.4. Fluorescence microscopy

The thin sections of mesocarp portions of eggplant were stained with propidium iodide solution and fluorescein diacetate solution for 10 min following the procedure described by Regan and Moffatt (1990). A stock solution of fluorescein diacetate (2 mg mL^{-1}) was made in acetone and diluted to 0.01 mg mL^{-1} with 70 mg mL^{-1} sucrose. The propidium iodide stock solution (1 mg mL^{-1}) was prepared in milli Q water and diluted to 0.1 mg mL^{-1} solution with 70 mg mL^{-1} sucrose. The eggplant sections were stained for 10 min, later rinsed in 70 mg mL^{-1} sucrose, transferred to a glass slide, and gently covered with a coverslip. The stained sections were then observed under a fluorescence microscope (model Axiolab, Carl Zeiss, Germany) equipped with HBO 50 mercury lamp, a camera (Axiocam MRc, Carl Zeiss, Germany) and filter sets no. 9 and 15 (Carl Zeiss, Germany) for fluorescein diacetate and propidium iodide, respectively. The images were captured and processed using Axiovision AC.4.1. Software (Zeiss, Haller-Bergmoos, Germany).

2.5. Determination of the extent browning

For browning measurements of cut eggplant, reflectance in the visible spectrum region (360–780 nm) was recorded at 10 nm wavelength intervals using a Minolta CM-3600D spectrophotometer (Konica Minolta Sensing, Inc., Osaka, Japan). A D65 lamp was used as the reference light source and the detector was fixed at an angle of 10° with respect to the light source (Ramírez-Jiménez et al., 2000). The equipment was calibrated prior to analysis with a standard white tile and a black box for 100 and 0% reflectance, respectively. The color parameter used was CIE L (Lightness) which denotes the amount of light or luminance reflected from the sample. The extent of browning/darkening was calculated as (100-L), which is the opposite of lightness (Ramírez-Jiménez et al., 2000).

2.6. Determination of polyphenol oxidase activity

The polyphenol oxidase (PPO) enzyme activity was determined spectrophotometrically using 4-methyl catechol as substrate (Concellón et al., 2004). The eggplant material (30 g) was frozen in liquid N_2, grounded to a fine powder and homogenized in 100 mL of extraction solution using a polytron homogenizer (Model PT3100, Kinematica AG, Switzerland). The extraction solution contained sodium phosphate buffer (pH 6.8, 0.05 M) with polyvinyl pyrrolidone (10 mg mL^{-1}), polyvinyl polypyrrolidone (10 mg mL^{-1}), triton X-100 (1 mg mL^{-1}) and ascorbic acid (30 mM). The extract was stirred for 30 min, filtered using muslin cloth, and centrifuged (CMF 15 KR, Tigra, Poland) at 10,000 × *g* for 15 min at 4 °C. The supernatant was collected and ammonium sulfate precipitated. The 20–80% saturation fraction showing PPO activity was solubilized in 8 mL of phosphate buffer (pH 6.8, 20 mM) and dialyzed for removal of salt. The enzyme assay was carried out taking 0.85 mL of phosphate buffer (pH 6.8, 50 mM), 0.1 mL substrate (0.1 M) and 0.02 mL of enzyme extract. The increase in absorbance at 420 nm was monitored at 1 min intervals for 3 min using a spectrophotometer (Model UV 4–100, Unicam, Cambridge, UK) and the average change in absorbance per min was calculated. One unit of enzyme activity was defined as the amount of enzyme which caused a change of 0.1 in absorbance/min. The PPO activity was expressed as U/g of eggplant weight. The specific activity was determined by expressing PPO activity/mg protein. Protein content of the eggplant extract was determined by the Bradford (1976) method using bovine serum albumin (BSA; Sigma Chemical, St. Louis, USA) as a standard.

2.7. Determination of leached PPO activity from the cut surface of eggplant

The leached PPO activity from the cut surface of eggplant was determined spectrophotometrically using 4-methyl catechol as substrate. The sample (20 g) was dipped in 26 mL of phosphate buffer (50 mM, pH, 6.8) containing 4 mL substrate (0.1 M) for 4 min. The increase in absorbance at 420 nm of buffer component due to leached enzymes from the cut surface of the eggplant was monitored at 1 min intervals for 3 min using a spectrophotometer (Model UV 4-100, Unicam, Cambridge, UK) and the average change in absorbance per min was calculated. The PPO activity was expressed as described above.

2.8. Estimation of total soluble phenolics

Eggplant material (7.5 g) was soaked in 50 mL of 80% methanol and homogenized using a polytron homogenizer (Model PT3100, Kinematica AG, Switzerland). The suspension was centrifuged at 10,000 × *g* for 20 min and the supernatant was collected. A 25 μL of aliquot of the supernatant was mixed with equal volume of milli Q water and further mixed with 50 μL of 0.2 N Folin-Ciocalteu reagent. The suspension was incubated at ambient temperature (26 ± 2 °C) for 15 min and later mixed with 0.15 mL of sodium carbonate solution (0.2 g mL^{-1}). The reaction mixture was incubated in a water bath at 40 °C for 20 min. It was then placed on ice for 5 min and the absorbance was measured at 755 nm using a spectrophotometer (Model UV 4-100, Unicam, Cambridge, UK). The total phenolics were calculated using gallic acid as a standard and expressed as mgGAE equivalent/g of eggplant (Luthria et al., 2010).

2.9. Estimation of chlorogenic acid

Chlorogenic acid was estimated using HPLC by the method of Luthria et al. (2010). Eggplant material (7.5 g) was soaked in 50 mL of 80% methanol and homogenized using a polytron homogenizer. The suspension was centrifuged at 10,000 × *g* for 20 min, the supernatant was collected and membrane (0.2 μm) filtered. A 60 μL aliquot was loaded on a reverse phase C18 column. Elution was carried out using a gradient of formic acid (0.1%) from 90 to 55% over methanol for 26 min at a flow rate of 1 mL/min. Detection was performed at 350 nm using an UV detector. The chlorogenic acid concentration was calculated using pure commercially available chlorogenic acid (retention time 16 min in specified mobile phase) as standard.

2.10. Microbiological studies

The microbiological analyzes were performed at regular intervals following the methods detailed in ICMSF (International

Commission for the Microbiological Specifications of Foods) (2002). The total bacterial count (TBC) and yeast and mold count (YMC) of fresh-cut eggplant was determined using the standard pour plate method. The 10 g of eggplant material was suspended in 90 mL sterile saline (8.5 mg mL^{-1} NaCl) and homogenized using a stomacher lab blender (model 400, Seward, UK). An aliquot (1 mL) of the suspension was withdrawn, serially diluted using sterile saline and pour plated in duplicate on plate count agar and potato dextrose agar plates (Himedia Laboratories, Mumbai, India) to determine TBC and YMC as colony forming units (cfu)/g, respectively.

2.11. Organoleptic evaluation

Sensory evaluation of fresh-cut eggplant was performed by a panel of 15 trained and experienced panelists from the Food Technology Division, Bhabha Atomic Research Centre, Mumbai, India in a taste panel laboratory in individually partitioned compartments of a controlled environment room (Meilgaard et al., 1999). The sensory panel scored different quality attributes such as appearance, color and browning for fresh-cut eggplant. Besides these features, texture, taste, and overall acceptability were also evaluated for cooked samples. Scoring was done based on a 9-point hedonic scale (9 – like extremely; 8 – like strongly; 7 –like very well; 6 – like fairly well; 5 – like moderately; 4 – like slightly; 3 – dislike slightly; 2 – dislike moderately; 1 – dislike extremely). Sensory analysis was performed until the samples were visibly acceptable during the storage period. Visible fungal growth was considered as the indicator of spoilage and hence, no further sensory evaluation was carried out with such samples.

2.12. Statistical analysis

Experiments including PPO activity, phenolics estimation, and browning measurements were repeated in three sets independently, each set having 3 replicates. Chlorogenic acid was estimated using HPLC in three replicates. Organoleptic evaluation was performed in three independent experiments. Microbiological analysis was performed in two sets of experiments each having three replicates. The means and standard deviations were calculated taking all the readings in consideration. As two factors (temperature and storage days) were used in this study, two way ANOVA (analysis of variance) at the level of significance $P < 0.05$ using Tukey test was performed for comparison of means. For other experiments, one way ANOVA was performed to ascertain the significance of the means. Statistical analysis was performed using BioStat 2009 Version Professional 5.8.0.0 (AnalystSoft Inc., Canada).

3. Results and discussion

3.1. Effect of cutting on physical injury of eggplant

The thickness of the thin blade used for cutting was about 0.04 mm and that of conventional knife was 0.25 mm, the latter being more than 6-fold thicker. More physical damage is expected with a thicker knife due to an increase in contact area, whereas, fine cutting is expected to do less physical injury, possibly limited to the plane of cutting (Watada and Qi, 1999). The sharp blade cut and water-dipped eggplant had markedly less browning, with the eggplant cut with the conventional knife showing significant browning, shown in Fig. 1(a) and (b), respectively.

3.1.1. SEM analysis showed less abrasive injury due to thin blade cutting

The scanning electron microscopy images of the surface of eggplant cut with the sharp blade and the knife are shown in Fig. 1(c)–(f). There was comparatively higher uneven and corrugated features in the knife cut sample (Fig. 1(d)) than in blade cut material (Fig. 1(c)) when analyzed using 100× magnification. Such higher abrasive impact indicated increased physical injury. The 10,000× magnification of the same blade and knife cut samples are shown in Fig. 1(e) and (f), showing that abrasiveness and wrinkle formation due to knife cutting was much greater than in the blade cut sample. Thus the knife cutting of eggplant seems to be quite harsh and rough, eventually resulting in greater physical injury.

3.1.2. Fluorescence microscopy indicates less cell death due to thin blade cutting

A profile of the dead cell population in eggplant cut by these two different methods was analyzed using propidium iodide (PI) staining (Fig. 2(a) and (b)). The dead cells with damaged membranes allowed entry of the fluorescent dye into cells, which stained the nucleus red making them visible in the knife cut sample (Fig. 2(b)) under a fluorescence microscope. The fine blade cut sample showed fewer stained nuclei due to comparatively less cell injury and death (Fig. 2(a)). The fluorescein diacetate (FDA) staining of eggplant cut with the sharp blade and the knife is shown in Fig. 2(c) and (d), respectively. The non-fluorescent FDA stain is lipid soluble and hence easily enters cells. In metabolically active viable cells, reactive oxygen species are frequently produced, which activate cytoplasmic esterase enzymes, which in turn are reported to cleave FDA to yield a fluorescent product. The product is retained within the cell if membrane function is intact. Hence, live cells fluoresce bright green and non-viable cells are dim or less fluorescent (Coder, 1997). The fine blade cut sample showed higher fluorescence upon FDA staining than the knife cut sample, indicating higher numbers of viable cells in the fine blade cut sample. The greater physical injury in the knife cut sample might have resulted in a higher loss of cellular viability due to excessive membrane damage.

3.1.3. Effect of cutting and water dip on browning

The extent of browning in differently cut and water dipped samples is shown in Table 1. The thin blade cut samples had 39% less browning compared to knife (thick blade) cut samples. The noticeable difference in browning was after 2 h of cutting. The blade cut and water dipped sample showed 56% lesser browning compared to knife cut samples. When blade and knife cut samples were both dipped in water, the former showed 45% less browning. Thus it was evident from the data that blade cut and water dipped samples had significantly less browning compared to the other combinations ($P < 0.05$).

3.1.4. Higher polyphenol oxidase (PPO) activity in knife cut samples

The PPO enzyme activity and specific activity in eggplant were found to be significantly influenced by the method of cutting (Table 1). PPO activity increased by more than 57 and 25% in knife and blade cut samples, respectively. Browning has been reported to be caused due to activity of the enzyme polyphenol oxidase (PPO) (Mayer, 2006). In eggplant, PPO and phenolics are reported to be present in chloroplast and vacuoles, respectively (Thipyapong et al., 1995). During physical cutting, the cells are damaged along the line of cutting and adjacent cells could also become mechanically injured, resulting in disrupted cellular structure. This eventually leads to release of PPO and its phenolics substrate, allowing physical contact. In the presence of oxygen, this enzymatic reaction takes place, leading to the formation of melanin-like brown colored pigment (Thipyapong et al., 1995). The pigment turns the color of cut surfaces brown, and thereby, the original appearance is lost. The specific activity of PPO was also found to increase by 94 and 41% with knife and blade cutting, respectively, which suggests an

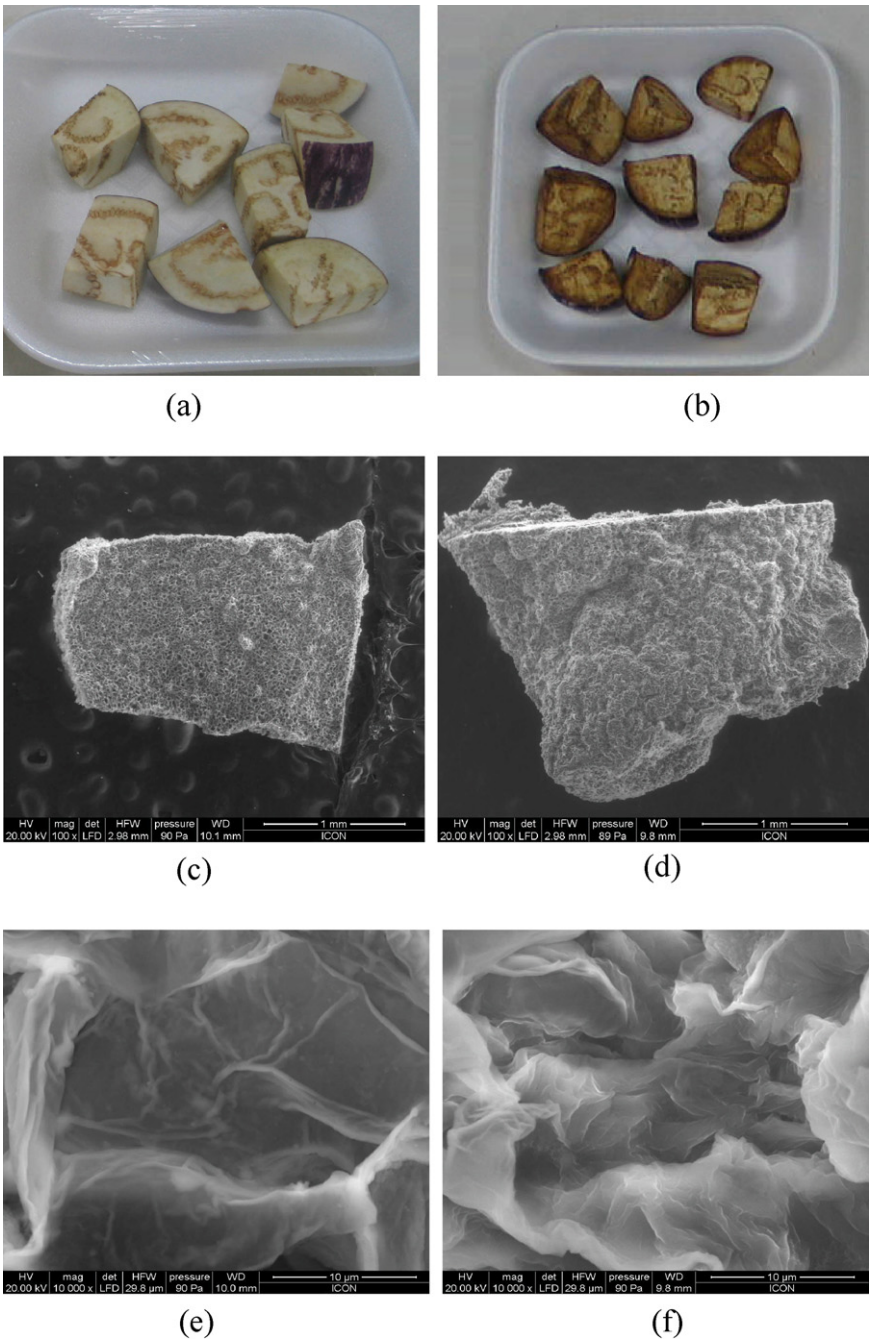

Fig. 1. Photograph of cut eggplant showing (a) reduced browning in a blade cut water dipped sample and (b) increased browning in a knife cut but not water dipped sample. Scanning electron microscopy image of bladecut (c and e) and knifecut (d and f) pieces of eggplant. Figures (c) and (d) are with 100× magnification whereas, figures (e) and (f) are with 10,000× magnification.

Table 1
Browning-related parameters of fresh-cut eggplant prepared using different cutting methods.

	Browning index (100-L)	PPO activity (U/g)	PPO sp. activity (U/mg protein)	Leached PPO activity	Total soluble phenolics (mg GAE/g)	Leached soluble phenolics (mg GAE/g)	Chlorogenic acid (mg/g)
Whole eggplant	–	$145^{c} \pm 11$	$17^{c} \pm 1.6$	–	$1.23^{d} \pm 0.05$	–	$0.74^{c} \pm 0.02$
Knifecut	$36^{a} \pm 2$	$229^{a} \pm 19$	$33^{a} \pm 1.8$	$7.51^{a} \pm 0.38$	$1.57^{a} \pm 0.06$	$0.35^{a} \pm 0.02$	$0.95^{a} \pm 0.03$
Knifecut water dip	$35^{a} \pm 2$	$218^{a} \pm 17$	$30^{a} \pm 1.5$	$5.93^{b} \pm 0.29$	$1.45^{b} \pm 0.04$	$0.24^{c} \pm 0.02$	$0.84^{b} \pm 0.02$
Bladecut	$22^{b} \pm 1$	$182^{b} \pm 14$	$24^{b} \pm 1.6$	$6.65^{c} \pm 0.31$	$1.52^{a} \pm 0.03$	$0.28^{b} \pm 0.01$	$0.94^{a} \pm 0.03$
Bladecut water dip	$16^{c} \pm 2$	$172^{b} \pm 16$	$22^{b} \pm 1.8$	$4.99^{d} \pm 0.25$	$1.39^{b} \pm 0.04$	$0.22^{c} \pm 0.01$	$0.81^{b} \pm 0.02$
Knife cut PMS dip	$14^{c} \pm 1$	$108^{d} \pm 14$	$12^{d} \pm 1.6$	$1.98^{e} \pm 0.15$	$1.43^{c} \pm 0.04$	$0.24^{c} \pm 0.02$	$0.82^{b} \pm 0.03$
Blade cut PMS dip	$14^{c} \pm 1$	$102^{d} \pm 12$	$11^{d} \pm 1.6$	$1.79^{e} \pm 0.18$	$1.38^{c} \pm 0.04$	$0.22^{c} \pm 0.01$	$0.80^{b} \pm 0.03$

Mean values in a column with different letter superscripts are significantly different ($P < 0.05$).
(–) Denotes unavailable sample. U, Units; L, Lightness; GAE, Gallic acid equivalent. PMS, potassium metabisulfite.

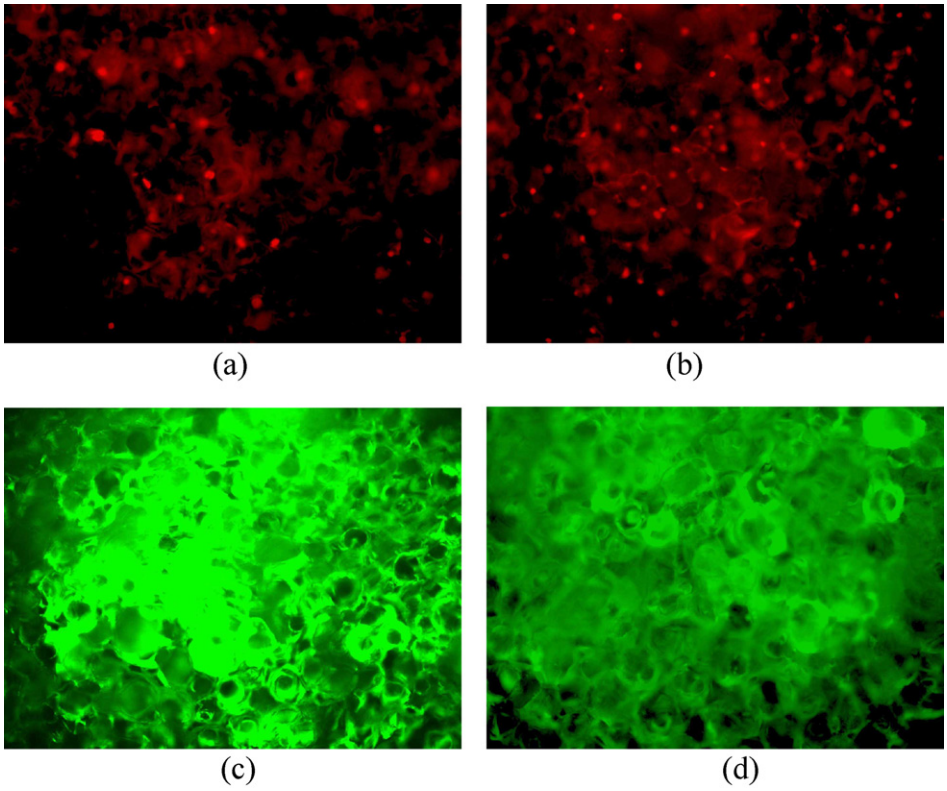

Fig. 2. Propidium iodide (red) and fluorescein diacetate (green) staining of blade cut (a, c) and knife cut (b, d) eggplant. Propidium iodide, a nuclear dye indicates increased dead cell populations (b), fluoroscein diacetate staining indicates physiologically live cells showing intense bright green fluorescence (c). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

increase in the ratio of active PPO protein to the total protein. This could be due to cutting-induced increase in synthesis or activity of PPO. An injury-induced increase in PPO activity has been reported in potato and other plants (Thipyapong et al., 1995).

The analysis of leached PPO activity from the cut surface of eggplant also showed similar results, where 12% higher PPO activity was observed in knife cut compared to blade cut samples (Table 1). Further, the water dipping reduced leached PPO activity by 22 and 25% in knife and blade cut samples compared to non-dip samples, respectively, and resulted in significantly reduced browning ($P < 0.05$). These results reconfirmed that dipping in water contributed to decreased browning.

3.1.5. Higher phenolics levels in knife cut samples

The total soluble phenolics in differently cut eggplant samples are shown in Table 1. In knife cut eggplant, a significant increase of more than 27% was observed in soluble phenolics compared to whole eggplants. This is probably due to mechanical injury leading to decompartmentaliztion of phenolics and wound-induced increased synthesis of phenolics by the key regulatory enzyme phenylalanine ammonia lyase (PAL) (Kang and Saltveit, 2003). The post-cutting dipping in water marginally lowered the phenolics levels by nearly 8% both in knife and blade cut samples. This could be due to the diffusion or leaching of soluble phenolics to water through the cut surface. This was confirmed by measuring the leached out phenolics in the leftover water after the dipping step. The leached phenolics levels were about 32 and 22% higher in knife and blade cut samples, respectively compared to the respective water dipped samples. The results showed that significant amounts of phenolics leached out into the water during the dipping step (Table 1). The leaching of phenolics was found to be significantly higher (20%) in knife cut samples compared to blade cut samples which could be due to higher tissue damage ($P < 0.05$).

3.1.6. Higher level of chlorogenic acid in knife cut samples

The increase in chlorogenic acid was observed in cut eggplant compared to the whole vegetable (Table 1). The major phenolic compound present in eggplant is chlorogenic acid (5-caffeoylquinic acid) and contributes to about 60% of total soluble phenolics (Luthria et al., 2010). The 80% methanolic extract of eggplant when analyzed by HPLC showed a single major peak of this compound with retention time of 16 min in the mobile phase at 350 nm. The chlorogenic acid in fresh-cut eggplant was found to be nearly 27% higher than in whole eggplant. This could be due to physical injury or wound-induced increased synthesis of chlorogenic acid by PAL as discussed above (Kang and Saltveit, 2003). A significant decrease of 12 and 14% in chlorogenic acid content was observed in knife and blade cut water dipped samples, which could be due to its leaching in water. The difference in its content between blade cut water dipped and knife cut water dipped samples was not found to be significant ($P < 0.05$).

3.1.7. Inhibition of browning in cut eggplant by a PPO inhibitor

The knife and blade cut samples were dipped in potassium metabisulfite (2 mg mL^{-1}, 10 min; PMS), an inhibitor of PPO, to study the comparative contribution of phenolics and PPO activity on browning (Table 1). The knife and blade cut samples dipped in the inhibitor were compared with respective water dipped control samples. Due to the inhibitor dip treatment, the browning was inhibited by 60 and 13% in knife and blade cut samples, respectively compared to water dip control samples. Similarly, PPO activity was inhibited by 51 and 41% in knife and blade cut samples, respectively. The leached PPO activity was also significantly reduced in PMS dipped samples by 67 and 65% in knife and blade cut

samples compared to water dip controls, respectively. However, the change in phenolics and chlorogenic acid concentrations, both in knife and blade cut samples, due to the PMS dip was found to be only about 2% and was insignificant ($P < 0.05$). Thus browning had a high correlation with PPO activity and little correlation with phenolics, including chlorogenic acid levels, which justified the observation that PPO enzyme is a greater contributor to browning than its phenolics substrate.

3.2. Status of browning parameters during storage

Since fine blade cutting was found to significantly inhibit browning in cut eggplant, further studies were performed with these samples where analysis of browning related parameters, as well as microbiological and organoleptic status, were performed during storage at different temperatures to assess the commercial acceptability of the process and product.

3.2.1. Browning index

The change in browning status of sharp blade cut eggplant during storage at different temperatures is shown in Table 2. The increase in browning was found to be insignificant until day 4 of storage. However, a significant increase of about 25% was observed on day 6 in ambient temperature storage ($P < 0.05$). Such an increase in browning in other fresh-cut vegetables has also been reported even earlier in low temperature storage (Aguila et al., 2010). Physical injury is known to induce physiological responses including browning, which is further manifested if storage temperature is high. However, the kinetics of browning was found to be slower at the lower storage temperatures of 10 and 4 °C (Table 2). In these samples, the increase in browning was found to be insignificant until day 8 and 10 at 10 °C and 4 °C, respectively. Although, browning was found to be increased significantly by 25% at the end of low temperature storage, this was still 44% less than that observed in knife cut samples on day 1 ($P < 0.05$). The low temperature storage inhibited biochemical reactions involved in browning and is the most general approach for shelf life improvement of many fresh-cut vegetables. The temperature of about 5 °C has also been recommended earlier for storage of fresh-cut produce (Aguila et al., 2010).

3.2.2. PPO activity

The change in PPO activity of fine blade cut eggplant during storage at different temperatures is shown in Table 2. A gradual increase in PPO activity was observed during ambient storage until day 4 which was found to be insignificant ($P < 0.05$). But on day 6 a significant increase of 33% was observed. This change could be due to increase in activity of oxidative enzymes observed during senescence in fruit and vegetables which accelerates in ambient temperature storage (Concellón et al., 2004). Similarly, a 41% increase in the specific activity of PPO was also observed on day 6. In whole (uncut) eggplant, a increase in PPO activity has also been reported in low temperature storage, which could be due to senescence (Concellón et al., 2004). The PPO activity and specific activity did not increase significantly until day 8 and 10 of storage at 10 °C and 4 °C, respectively (Table 2). However, a significant ($P < 0.05$) increase was observed at the end of the storage period on day 12 and 16 for low temperature (10 °C and 4 °C) stored samples. Though such an increase in PPO activity was also observed in ambient temperature stored samples, lower temperature storage has slowed down the process (Concellón et al., 2004).

3.2.3. Total soluble phenolics

The change in total soluble phenolic contents in blade cut stored eggplant is shown in Table 2. There was no significant decrease in phenolics observed in ambient temperature stored samples until day 4. However, on day 6, a significant 17% decrease was observed ($P < 0.05$). This could be due to the oxidation of phenolics to insoluble forms due to increased PPO activity. A similar decrease in phenolics has also been reported in fresh-cut jack fruit during storage (Saxena et al., 2009). The phenolics levels did not change significantly until day 8 and 10 of storage at 10 °C and 4 °C, respectively (Table 2). The insignificant change in PPO activity at low temperature storage could be the reason, associated with unchanged levels of total soluble phenolics during this storage period. However, a significant ($P < 0.05$) decrease of 28 and 23% in soluble phenolics contents was observed during the end of the storage period at 10 °C and 4 °C, respectively, which was well correlated with an increase in PPO activity (Table 2).

3.2.4. Chlorogenic acid

The chlorogenic acid content gradually decreased in blade cut eggplant during 6 days of shelf life at ambient temperature (Table 2). On days 4 and 6, a significant decrease of 17 and 30% in chlorogenic acid concentrations was observed ($P < 0.05$). Such a decrease could be due to increases in PPO activity, which leads to oxidation of chlorogenic acid to insoluble phenolics. The decrease in chlorogenic acid content was found to be 28% during a shelf life of 12 days at 10 °C. Again, at 4 °C the decrease was found to be 32% during 16 days of shelf life (Table 2). As discussed above, this could be due to the biochemical changes associated with senescence of eggplant involving an increase in activity of oxidative enzymes (Saxena et al., 2009).

3.3. Microbiological analysis

The analyzes were periodically performed during storage to determine the microbial load in blade cut and stored eggplant. Visible mold growth was considered as the indicator of spoilage making the product unusable. The total bacterial count (TBC) and yeast and mold count (YMC) were found to be in range of 3–4 log cfu/g on day 1 of storage at ambient temperature. Bacteria, yeast and mold are important causes of spoilage in fresh-cut vegetables. During the preparatory steps of minimal processing, the natural protection of the vegetable is compromised and hence, they become highly susceptible to microbial spoilage. In addition, microbial contamination also occurs during cutting. The high water activity and near neutral pH of eggplant make it a suitable host material for many types of microorganisms (Oms-Oliu et al., 2010). During 6 days of ambient storage the bacterial and yeast mold counts were found to increase by more than 2 log cycles (Table 2). However, the increase in counts was found to be within the permissible limit and may be acceptable. Legislation in different countries has set the limits of microbial load as 4.7 log cfu/g at the production stage and 7.7 log cfu/g at the consumption stage for fresh cut vegetables (Erturk and Picha, 2006). However, beyond 6 days of storage, a few samples started showing visible mold growth and hence could not be accepted. On day 1 of storage the counts in the 10 °C and 4 °C stored samples were found to be less than those of ambient temperature stored samples. This could be due to inhibition of microbial growth at low temperature (Oms-Oliu et al., 2010). During 12 days of shelf life at 10 °C the bacterial and yeast mold counts were found to increase by 2 and 3 log cycles, respectively. During 16 days of shelf life at 4 °C the counts showed nearly similar increases (Table 2). The increase in bacterial counts was higher than that of yeast and mold counts. However, towards the end of storage the mold growth became prominent, rendering the product unacceptable. In general, the cutting process was not found to have any profound effect on microbiological status of eggplant material.

Table 2
Status of browning-related parameters and microbial load of sharp blade cut and water dipped eggplant during storage at different temperatures.

Storage days	Browning (100-L)	PPO activity (U/g)	Specific activity (U/mg protein)	Total soluble phenolics (mg GAE/g)	Chlorogenic acid (mg/g)	Microbiology	
						TBC (cfu/g)	YMC (cfu/g)
26 ± 2 °C							
Day 1	$16^{b} \pm 0.9$	$172^{b} \pm 10.2$	$24^{b} \pm 1.7$	$1.39^{a} \pm 0.06$	$0.82^{a} \pm 0.04$	$3.7^{c} \pm 0.2$	$3.4^{b} \pm 0.4$
Day 2	$17^{b} \pm 0.8$	$182^{b} \pm 13.4$	$25^{b} \pm 1.8$	$1.33^{a} \pm 0.05$	$0.77^{a} \pm 0.03$	$3.4^{c} \pm 0.2$	$3.5^{b} \pm 0.4$
Day 4	$18^{b} \pm 0.7$	$192^{b} \pm 12.4$	$27^{b} \pm 1.4$	$1.26^{a} \pm 0.07$	$0.68^{b} \pm 0.03$	$4.4^{b} \pm 0.3$	$3.7^{b} \pm 0.5$
Day 6	$20^{a} \pm 0.9$	$229^{a} \pm 11.2$	$34^{a} \pm 1.6$	$1.06^{b} \pm 0.06$	$0.57^{c} \pm 0.02$	$6.0^{a} \pm 0.4$	$5.8^{a} \pm 0.4$
10 ± 2 °C							
Day 1	$16^{b} \pm 0.8$	$174^{b} \pm 15$	$26^{b} \pm 1.7$	$1.40^{a} \pm 0.03$	$0.82^{a} \pm 0.03$	$3.5^{c} \pm 0.4$	$2.0^{d} \pm 0.2$
Day 4	$17^{b} \pm 0.5$	$184^{b} \pm 14$	$28^{b} \pm 1.4$	$1.36^{a} \pm 0.03$	$0.78^{a} \pm 0.04$	$4.5^{b} \pm 0.5$	$2.5^{c} \pm 0.2$
Day 8	$18^{b} \pm 0.6$	$189^{b} \pm 11$	$29^{b} \pm 1.3$	$1.31^{a} \pm 0.02$	$0.64^{b} \pm 0.03$	$4.9^{b} \pm 0.3$	$3.8^{b} \pm 0.2$
Day 12	$20^{a} \pm 0.8$	$218^{a} \pm 12$	$33^{a} \pm 1.6$	$1.01^{b} \pm 0.06$	$0.59^{c} \pm 0.04$	$5.7^{a} \pm 0.3$	$5.5^{a} \pm 0.2$
4 ± 2 °C							
Day 1	$16^{b} \pm 0.8$	$164^{b} \pm 14$	$25^{b} \pm 1.9$	$1.38^{a} \pm 0.05$	$0.81^{a} \pm 0.04$	$2.4^{d} \pm 0.2$	$1.9^{d} \pm 0.2$
Day 5	$17^{b} \pm 0.7$	$172^{b} \pm 15$	$27^{b} \pm 1.7$	$1.27^{a} \pm 0.06$	$0.76^{a} \pm 0.03$	$3.4^{c} \pm 0.3$	$2.4^{c} \pm 0.1$
Day 10	$18^{b} \pm 0.6$	$183^{b} \pm 12$	$28^{b} \pm 1.3$	$1.21^{a} \pm 0.05$	$0.66^{b} \pm 0.04$	$4.5^{b} \pm 0.4$	$2.6^{c} \pm 0.1$
Day 16	$20^{a} \pm 0.7$	$215^{a} \pm 15$	$34^{a} \pm 1.8$	$1.06^{b} \pm 0.06$	$0.55^{c} \pm 0.03$	$5.5^{a} \pm 0.2$	$3.7^{b} \pm 0.2$

Mean values in a column with different letter superscripts are significantly different ($P < 0.05$). U, Units; L, Hunter's Lightness color parameter; GAE, Gallic acid equivalent; TBC, total bacterial count; YMC, Yeast and mold count; cfu, colony forming unit.

Table 3
Organoleptic evaluation of knife cut and blade cut water dipped eggplant during storage at different temperatures.

	Knife cut control		Blade cut								
			26 ± 2 °C			10 ± 2 °C			4 ± 2 °C		
	Day 0*	Day 1#	Day 1	Day 3	Day 5	Day 1	Day 8	Day 12	Day 1	Day 8	Day 16
Raw											
Appearance	$6.8^{a} \pm 0.6$	$4.2^{c} \pm 0.3$	$8.0^{a} \pm 0.8$	$7.7^{a} \pm 0.7$	$7.1^{a} \pm 0.4$	$8.0^{a} \pm 0.8$	$7.7^{a} \pm 0.8$	$7.2^{a} \pm 0.5$	$8.2^{a} \pm 0.7$	$7.6^{a} \pm 0.7$	$7.1^{a} \pm 0.7$
Color	$6.7^{a} \pm 0.5$	$4.1^{c} \pm 0.3$	$7.8^{a} \pm 0.8$	$7.7^{a} \pm 0.8$	$7.0^{a} \pm 0.6$	$8.0^{a} \pm 0.7$	$7.9^{a} \pm 0.8$	$7.0^{a} \pm 0.6$	$8.2^{a} \pm 0.8$	$7.7^{a} \pm 0.6$	$7.2^{a} \pm 0.7$
Browning	$6.7^{a} \pm 0.5$	$2.9^{c} \pm 0.3$	$7.7^{a} \pm 0.8$	$7.8^{a} \pm 0.7$	$6.9^{a} \pm 0.5$	$7.8^{a} \pm 0.7$	$7.5^{a} \pm 0.8$	$7.1^{a} \pm 0.5$	$7.8^{a} \pm 0.8$	$7.6^{a} \pm 0.7$	$7.2^{a} \pm 0.6$
Cooked											
Appearance	$6.9^{a} \pm 0.6$	$6.7^{a} \pm 0.6$	$7.1^{a} \pm 0.6$	$7.3^{a} \pm 0.6$	$6.7^{a} \pm 0.5$	$7.1^{a} \pm 0.6$	$6.7^{a} \pm 0.5$	$6.9^{a} \pm 0.5$	$7.1^{a} \pm 0.7$	$7.1^{a} \pm 0.7$	$6.7^{a} \pm 0.4$
Color	$6.9^{a} \pm 0.7$	$6.7^{a} \pm 0.5$	$7.3^{a} \pm 0.7$	$7.3^{a} \pm 0.7$	$6.8^{a} \pm 0.6$	$7.4^{a} \pm 0.8$	$6.8^{a} \pm 0.4$	$6.6^{a} \pm 0.6$	$7.4^{a} \pm 0.8$	$7.2^{a} \pm 0.6$	$7.1^{a} \pm 0.6$
Browning	$7.1^{a} \pm 0.7$	$6.6^{a} \pm 0.6$	$7.5^{a} \pm 0.8$	$7.8^{a} \pm 0.8$	$7.3^{a} \pm 0.7$	$7.5^{a} \pm 0.7$	$6.7^{a} \pm 0.5$	$6.6^{a} \pm 0.6$	$7.3^{a} \pm 0.6$	$6.6^{a} \pm 0.5$	$6.7^{a} \pm 0.5$
Aroma	$6.7^{a} \pm 0.5$	$6.6^{a} \pm 0.6$	$6.7^{a} \pm 0.5$	$6.8^{a} \pm 0.5$	$7.0^{a} \pm 0.6$	$6.9^{a} \pm 0.7$	$7.2^{a} \pm 0.6$	$7.1^{a} \pm 0.6$	$7.2^{a} \pm 0.5$	$7.2^{a} \pm 0.4$	$6.9^{a} \pm 0.6$
Taste	$7.1^{a} \pm 0.3$	$4.1^{c} \pm 0.4$	$7.3^{a} \pm 0.6$	$7.2^{a} \pm 0.6$	$6.6^{a} \pm 0.6$	$7.3^{a} \pm 0.4$	$7.2^{a} \pm 0.7$	$6.8^{a} \pm 0.5$	$7.4^{a} \pm 0.6$	$7.1^{a} \pm 0.6$	$6.6^{a} \pm 0.6$
Texture	$5.2^{b} \pm 0.4$	$5.2^{b} \pm 0.4$	$5.3^{b} \pm 0.4$	$5.4^{b} \pm 0.4$	$5.2^{b} \pm 0.5$	$5.1^{b} \pm 0.4$	$5.2^{b} \pm 0.4$	$5.1^{b} \pm 0.4$	$5.1^{b} \pm 0.4$	$5.3^{b} \pm 0.4$	$5.2^{b} \pm 0.4$
Overall acceptability	$6.9^{a} \pm 0.6$	$3.6^{c} \pm 0.4$	$7.3^{a} \pm 0.7$	$7.3^{a} \pm 0.7$	$6.6^{a} \pm 0.6$	$7.3^{a} \pm 0.7$	$7.1^{a} \pm 0.6$	$6.9^{a} \pm 0.8$	$7.2^{a} \pm 0.7$	$7.2^{a} \pm 0.7$	$6.8^{a} \pm 0.6$

The data are mean ± SD of scores given by 15 panelists. Mean values in a row with different letter superscripts are significantly different ($P < 0.05$).
* Samples were analyzed within 1 h after cutting.
# The knife cut samples stored at 26 ± 2, 10 ± 2, 4 ± 2 °C were not appreciated (overall acceptability score <4) even on day 1.

3.4. Organoleptic analysis

The results of organoleptic evaluation of fresh knife cut and blade cut stored samples (at different temperatures) after cooking are shown in Table 3. The fresh knife cut samples analyzed within 1 h of cutting (day 0) did not show any significant difference ($P < 0.05$) in surface browning compared to day 1 blade cut water dipped samples. However, the fresh knife cut cooked samples did not show any significant difference with day 1 blade cut water dipped samples during the remaining storage days. The day 1 knife cut samples were scored as 'like slightly,' whereas, blade cut eggplant rated 'like strongly' when analyzed for visual observations such as appearance, color and browning on day 1 of storage. The knife cut samples stored at 26 ± 2, 10 ± 2, 4 ± 2 °C were not appreciated (overall acceptability score < 4) even on day 1, and hence were not studied further due to poor acceptability. During storage, only a marginal decrease in these ratings was observed in blade cut water dipped samples. This could be due to the physiological changes associated with senescence and water loss during storage. Most vegetables are known to loose water during storage, resulting in dryness on their skin (Toivonen and Brummell, 2008). However, these changes are reduced with low temperature. Eggplant with bruising and scars is known to taste bitter and astringent. Such physical injury is known to cause an increase in phenolic biosynthesis and oxidation, which leads to astringency and bitterness in cooked eggplant (Es-Safi et al., 2003). Since cutting involves physical injury, the cooked vegetable was also organoleptically evaluated to judge its effect on taste. The cooked blade cut eggplant scored little better than the knife cut samples in appearance color and browning. This is because during cooking, eggplant looses its whitish color and turns brown. The differences in texture of knife cut and blade cut cooked eggplant was found to be insignificant. However, the taste scores in knife cut samples were significantly less than the blade cut samples ($P < 0.05$). The knife cut sample was bitter in taste compared to the blade cut samples. This could be due to greater physical injury induced oxidation of phenolics, resulting in bitterness of cooked eggplant (Es-Safi et al., 2003). In overall acceptability category, the knife cut samples had about 51% lesser acceptability than the blade cut samples on day 1 of storage. Blade cut water dipped eggplant samples were found to have a shelf life of 6 days at 26 °C, 12 days at 10 °C, and 16 days at 4 °C. All these samples scored about 7 ('like very well') during organoleptic analysis.

4. Conclusions

Browning in fresh cut ready-to-cook eggplant was found to be markedly low when a sharp blade was used for cutting followed by immediate dipping in water for 10 min, ambient air drying, and packaging. The reduced browning in the sharp blade cut samples compared to knife cut samples was due to comparative lower release of phenolics and polyphenol oxidase activity, as reconfirmed by the inhibitor (PMS) study. The shelf life of the sharp blade cut eggplant was extended up to 16 days at 4 °C, 12 days at 10 °C, and 5 days at 26 °C storage temperatures. This eggplant material was found to be microbiologically and organoleptically acceptable.

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Purification and characterization of polyphenol oxidase (PPO) from eggplant (*Solanum melongena*)

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ABSTRACT

Eggplant (*Solanum melongena*) is a very rich source of polyphenol oxidase (PPO), which negatively affects its quality upon cutting and postharvest processing due to enzymatic browning. PPO inhibitors, from natural or synthetic sources, are used to tackle this problem. One isoform of PPO was 259-fold purified using standard chromatographic procedures. The PPO was found to be a 112 kDa homodimer. The enzyme showed very low K_m (0.34 mM) and high catalytic efficiency (3.3×10^6) with 4-methyl catechol. The substrate specificity was in the order: 4-methyl catechol > tert-butylcatechol > dihydrocaffeic acid > pyrocatechol. Cysteine hydrochloride, potassium metabilsulfite, ascorbic acid, erythorbic acid, resorcylic acid and kojic acid showed competitive inhibition, whereas, citric acid and sodium azide showed mixed inhibition of PPO activity. Cysteine hydrochloride was found to be an excellent inhibitor with the low inhibitor constant of 1.8 μM.

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1. Introduction

The postharvest processing of eggplant (*Solanum melongena*) is one of the major problems for industries dealing with cut vegetables (Madinez & Whitaker, 1995). Polyphenol oxidase has been reported to be one of the important factors contributing to browning during postharvest processing. In eggplant, the PPO and phenolics are reported to be present in chloroplast and vacuoles, respectively (Güllçin, Küfrevioğlu, & Oktay, 2005; Mayer & Harel, 1979). Disruption of cellular structures during physical cutting, leading to release of PPO and its phenolic substrate, has been demonstrated using electron and fluorescence microscopy (Mishra, Gautam, & Sharma, 2012). PPO catalyzes the hydroxylation of monophenols to o-diphenols (EC 1.14.18.1) through a monophenolase activity and a subsequent oxidation of these o-diphenols to the corresponding o-quinones (EC 1.10.3.1) by a catecholase/diphenolase activity (Mayer, 2006). In the presence of oxygen, this enzymatic reaction takes place, leading to the formation of melanin-like brown-coloured pigment (Madinez & Whitaker, 1995). The pigment turns the colour of a cut surface brown, and thereby, the original appearance is lost. The inhibition of this enzyme activity and inhibition of browning have remained a challenge for the processed fruits and vegetables industry (Mayer, 2006). The purification and characterization of the enzyme in eggplant could help find suitable methods for controlling its activity. The purification of PPO to homogeneity has remained a difficulty in eggplant, due to the high phenolics content and their irreversible binding to PPO during the purification process. Therefore, there are few reports on partial purification and characterization of PPO from eggplant (Concellón, Añón, & Chaves, 2004; Doğan, Arslan, & Doğan, 2002; Pérez-Gilabert & Carmona, 2000; Roudsaria, Signoreta, & Crouzeta, 1981). Roudsaria et al. (1981) reported 15-fold partial purification using ion-exchange and gel filtration chromatography. Pérez-Gilabert and Carmona (2000) characterized the 40% ammonium sulphate precipitated fraction without the use of chromatographic purification methods. Doğan et al. (2002) have characterized the PPO activity of an ammonium sulphate-precipitated fraction. Concellón et al. (2004) have also reported the PPO activity of crude extract during low temperature storage of eggplant without any purification process. In the current study, PPO enzyme from eggplant was purified to homogeneity and the kinetic parameters were determined using the purified PPO. The kinetics of enzyme inhibition of the purified PPO were also studied, using natural and synthetic inhibitors.

2. Materials and methods

2.1. Chemicals

Ammonium sulphate, ascorbic acid, Bradford reagent, bovine serum albumin, caffeic acid, chlorogenic acid, citric acid, cysteine hydrochloride, dihydrocaffeic acid, 3,4-dihydroxybenzaldehyde, disodium hydrogen phosphate, D and L-DOPA (dihydroxyphenylalanine), EDTA (Ethylenediaminetetraacetic acid), erythorbic acid,

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gallic acid, glycine, kojic acid, 4-methyl catechol, p-cresol, polyvinyl pyrrolidone (PVP), polyvinyl polypyrrolidone (PVPP), potassium metabisulfite, pyrocatachol, pyrogallol, α-resorcylic acid, tert-butylcatechol, tris buffer, sodium azide, sodium dihydrogen phosphate, sodium chloride, and triton X-100 were procured from Sigma–Aldrich Inc., St. Louis, MO. The DEAE Sepharose CL6B, phenyl Sepharose CL4B, and Sepharose 6B were procured from GE Healthcare Bio-Sciences Uppsala, Sweden.

2.2. Assay of protein and polyphenol oxidase (PPO) activity

The polyphenol oxidase (PPO) activity was determined spectrophotometrically, using 4-methyl catechol as substrate (Concellón et al., 2004). The enzyme assay was carried out by taking 0.88 ml of phosphate buffer (pH 6.8, 50 mM), 0.1 ml of substrate (0.1 M) and 0.02 ml of enzyme extract (prepared as discussed later). The increase in absorbance at 420 nm was monitored at 30 s intervals, for 3 min, using a spectrophotometer (Model UV 4–100, Unicam, Cambridge, UK) and the average change in absorbance per min was calculated. One unit of enzymatic activity was defined as the amount of enzyme which caused a change of 0.1 in absorbance/min. The PPO activity was expressed as U/g of eggplant weight. The specific activity was determined by expressing PPO activity/mg protein. Protein content of the eggplant extract was determined by the Bradford method (1976), using bovine serum albumin (BSA, Sigma Chemical, St. Louis, USA) as standard.

2.3. Purification of native PPO

2.3.1. General

The popular bangi kotary eggplant variety (1 kg) procured from a local vegetable market was frozen in liquid N_2, ground to fine powder and homogenized in 3 l of extraction solution for better extractability using a polytron homogenizer (Model PT3100, Kinematica AG, Switzerland). The extraction solution contained sodium phosphate buffer (pH 6.8, 0.05 M) with polyvinyl pyrrolidone (PVP) (1%), polyvinyl polypyrrolidone (PVPP) (2%), triton X-100 (1%) and ascorbic acid (30 mM). The extract was stirred for 30 min, filtered using muslin cloth, and centrifuged (5810R, Eppendorf, Hamburg, Germany) at 10,000*g* for 15 min at 4 °C. The supernatant was collected and its total activity was considered as 100% for ease of enzyme yield calculation. The yield of each purification step was calculated as its percentage. The specific activity of this crude extract was considered as basal and the fold increase in each step of purification was considered as fold purification. The fractionation of crude extract was performed using the ammonium sulphate precipitation method, with 10% concentration increase at each step (Englard & Seifter, 1990). The individual fractions were solubilized in 50 ml of phosphate buffer (pH 6.8, 20 mM) and dialyzed, using a 10 kDa cut off membrane in 5 l of phosphate buffer (pH 6.8, 2 mM) at 4 °C with three buffer changes at 4 h intervals for removal of salt.

2.3.2. DEAE anion-exchange chromatography

The DEAE (diethylaminoethyl) Cl-6B Sepharose column material (40 ml) was prepared by washing initially with distilled water (250 ml) and then with phosphate buffer (pH 8.0, 2 mM). The dialyzed enzyme extract (70 ml) was diluted to 700 ml (pH adjusted to 8.0) and mixed with DEAE column material, kept at slow stirring for 30 min at 4 °C. This slurry was then washed twice with 500 ml of phosphate buffer (pH 8.0, 2 mM) and loaded onto the column. The elution was carried out using an increasing gradient of NaCl from 0 to 0.5 M in 150 ml of phosphate buffer (pH 8.0, 2 mM). The fractions (5 ml each) were tested for enzyme activity and the protein was estimated. The fractions having maximum activity were pooled.

2.3.3. Phenyl Sepharose hydrophobic interaction chromatography

The phenyl Sepharose column material (10 ml) was prepared by washing initially with distilled water (50 ml) and then with phosphate buffer (pH 6.8, 20 mM). The enzyme extract (15 ml) was saturated with 18% ammonium sulphate and centrifuged. The supernatant was loaded onto a phenyl Sepharose column, washed with 25 ml of 18% ammonium sulphate in 20 mM phosphate buffer. The elution was carried out with a decreasing gradient of ammonium sulphate from 18% to 0% in phosphate buffer (50 ml, pH 6.8, 20 mM). The enzyme assay and protein estimation (Bradford reagent) of each fraction (2 ml) were carried out. The fractions with the peak of PPO activity were pooled.

2.3.4. Superdex gel filtration chromatography

Further purification of PPO and molecular weight determination were carried out using gel filtration chromatography in a Superdex™ 200 GE healthcare column using HPLC equipped with a quaternary pump (Model PU-2089, Jasco, Tokyo, Japan) and a UV detector (Model 2500, Knauer, Berlin, Germany). The protein was loaded onto an HPLC column and the elution was carried out in phosphate buffer with 150 mM NaCl. The flow rate was maintained at 0.5 ml/min and the detection was carried out using an UV detector set at 280 nm. The molecular weight was determined, using a calibration curve prepared from the retention time of proteins of known molecular weight. The PPO activity and protein estimation were also performed for different fractions collected at 30 s intervals. The molecular weight was determined for the single peak showing PPO activity, based on its retention time.

2.4. SDS and native PAGE

The protein samples for native and SDS–PAGE (10%) were prepared, electrophoresed and visualized as described by Davis (1964) and Laemmli (1970). The protein samples were prepared by mixing 2× (double strength) gel loading buffer (0.1 M Tris-C1, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and β-mercaptoethanol (0.2 M) mixed in the proportion of 1:1, boiled for 5 min, centrifuged (10,000*g*) for 2 min, and then loaded onto gel, along with molecular weight marker mix. The total protein for each well was calculated to be approximately 6–20 μg. After electrophoresis, the gel was fixed in acidified methanol water (40% methanol and 10% acetic acid) for 1 h and then stained for 3 h with 0.1% Coomassie brilliant blue R-250 on a rocker (Neolab, Mumbai, India). Later, the gel was de-stained with the same acidified methanol–water until the protein bands became distinguishable and prominent. The native PAGE (10%) was performed as above, except for the use of SDS and β-mercaptoethanol. The substrate staining of the gel was performed with 4-methyl catechol (0.2 M) substrate in phosphate buffer (pH 6.8, 50 mM) for up to 1 h, until the bands became prominent.

2.5. Determination of substrate specificity

The enzyme assay was performed by taking 0.9 ml of phosphate buffer (pH 6.8, 50 mM), 0.1 ml of substrate (0.1 M) and 5 μl of purified enzyme. The changes in absorbance for substrates 4-methyl catechol, tert-butylcatechol, pyrocatechol, chlorogenic acid, L-DOPA (dihydroxyphenylalanine), and cresol were monitored at 420 nm, using a spectrophotometer (Pérez-Gilabert & Carmona, 2000). For dihydrocaffeic acid, caffeic acid, and dihydroxybenzaldehyde, the absorbance was monitored at 400 nm (Ding, Chachin, Ueda, & Imahori, 1998). The absorbances for pyrogallol, D-DOPA, and gallic acid were monitored at 334, 480, and 350 nm, respectively. The absorbance was recorded at 30 s intervals for 4 min and the average change in absorbance per min was calculated and termed as activity. The relative activities of these substrates

were expressed as percentages with respect to that of 4-methyl catechol substrate (considered 100%).

2.6. Determination of kinetic parameters of PPO

The enzymatic activity was monitored with a fixed purified enzyme concentration (10 μl containing ~4 units) and increasing substrate [S] concentration (0.04–0.2 mM) in the reaction mixture. The reaction velocity [V] was determined by monitoring change in absorbance per min. The reciprocal plot (1/[V] vs. 1/[S]) was plotted and the K_m and V_{max} were determined from the slope and intercept of the straight line (Yang et al., 2001). The total enzyme molecule concentration was determined by dividing estimated total protein by molecular weight of PPO. The turnover number (K_{cat}) was calculated by dividing total substrate molecules changed per min by total molecules of PPO in the reaction mix. The catalytic efficiency (K_{cat}/K_m) was calculated by dividing K_{cat} by K_m.

2.7. Inhibitor studies

The inhibitor studies were carried out with ascorbic acid (10–30 μM), α-resorcylic acid (20–50 mM), erythorbic acid (10–30 μM), cysteine hydrochloride (6–10 μM), potassium metabisulfite (5–20 μM), kojic acid (0.05–0.2 mM), citric acid (20–40 mM), and sodium azide (0.1–10 mM), using the method described by Janovitz-Klapp, Richard, Goupy, and Nicolas (1990). The enzymatic reactions were monitored with a fixed amount of purified enzyme (10 μl containing ~4 units) and increasing concentrations of 4-methyl catechol substrate (0.2–0.02 mM), along with different concentrations of the inhibitors under test. The reciprocal plot of 1/[S] on the *X*-axis was plotted against 1/[V] on the *Y*-axis for each concentration of inhibitor. The inhibition mechanism, either competitive or mixed, was determined from the nature of the graphs. Compounds with competitive inhibition mechanisms had similar V_{max} values. For each straight line graph the apparent K_m was calculated from the *X*-axis intercept ($-1/K_m$). For determination of inhibitor constant (Ki), inhibitor concentration [I] on the *X*-axis was again plotted against apparent K_m value on the *Y*-axis. The Ki value was calculated from the value of the *X*-axis intercept of this straight line (-Ki). Similarly, for compounds with mixed inhibition mechanism, the Ki value was calculated from the plot of inhibitor concentration on the *X*-axis against apparent V_{max} on the *Y*-axis.

2.8. Statistical analysis

Experiments were repeated in three sets, independently, each set having three replicates. The means and standard deviations were calculated by taking all the readings into consideration. One way ANOVA (at the level of significance $P \leqslant 0.05$) was performed to ascertain the significance of the means. Statistical analysis was performed using BioStat 2009 Version Professional 5.8.0.0 (AnalystSoft Inc., Canada).

3. Results

3.1. Optimization of parameters for extraction of PPO

Although PPO has been purified from many plant sources, there is a scarcity of reports describing purification of PPO from eggplant. Plant sources rich in phenolics pose problems during purification due to irreversible binding of PPO with its phenolic substrates that affects the ionic and hydrophobic characteristics of the enzyme; that in turn affects the elution pattern during chromatographic purification (Mayer, 2006). In the current study, the problem was sorted out by using a combination of polyvinyl pyrrolidone (PVP) (1%), polyvinyl polypyrrolidone (PVPP) (2%), and ascorbic acid (30 mM) in the extraction solution. This significantly inhibited the binding of phenolics to PPO. However, addition of excess ascorbic acid had a negative effect on the enzyme activity. The extraction was performed at 4 °C.

The crude sodium phosphate buffer extract from eggplant showed extensive browning during the extraction process. The extract showed 90% of activity in the supernatant. Addition of detergent (triton X-100, 1%), salt (NaCl, 0.5 M), or SDS (0.5%) during extraction did not increase PPO activity, which indicated the absence of strongly membrane-bound, weakly ionic-bound, or latent forms of PPO in eggplant, respectively.

In most of the reported works, a 20–80% ammonium sulphate precipitation fraction was chosen for purification purposes. But, in the current study, the ammonium sulphate precipitation was performed with gradual increase of 10–20% at each step. High PPO activity was observed in two discrete fractions (20–30% and 50–70%), which were significantly high compared to other fractions ($P \leqslant 0.05$) and this could be due to the presence of two isoforms of PPO (Fig. 1a). These two fractions were tested with the PPO inhibitor potassium metabisulfite (PMS) for ruling out the enzyme activity by any other proteins, including peroxidase (Mayer, 2006). However, PPO activity was completely inhibited by PMS in both fractions. This indicated that PPO in these two fractions may differ in its hydrophobic characteristics, resulting in precipitation at two different ammonium sulphate concentrations. Pérez-Gilabert and Carmona (2000) characterized the crude 20–30% ammonium sulphate-precipitated fraction and showed the presence of cresolase and catacholase activities. During the current study, basal PPO activity was also observed in other remaining fractions, which indicated the probable nonspecific precipitation of this protein due to binding of phenolic compounds, as reported earlier by Papadopoulou and Frazier (2004). However, the specific activity in the 50–70% fraction was highest and was about 62% higher than that of the 20–30% fraction and hence was used for further purification and characterization (Fig. 1a).

3.2. Purification of PPO

The PPO was observed to bind DEAE column material at pH 8.0 with low buffer concentration (2 mM). During gradient elution (0–0.5 M NaCl), a high PPO activity in fraction Nos. 25 to 35 (NaCl concentration about 0.15 M) was observed with peak activity in fraction No. 32 (Fig. 1b). The protein content increased gradually with a peak at about fraction number 40 and a gradual decrease towards the end of the gradient elution. The peak region was therefore pooled and used for phenyl Sepharose chromatography. The PPO binding to phenyl Sepharose was observed at 18% ammonium sulphate saturation in phosphate buffer (20 mM, pH 6.8). The active PPO was eluted early in the gradient at about 15% of ammonium sulphate concentration in the eluent buffer, with peak activity in fraction No. 10 (Fig. 1c). The protein content in the eluted fraction showed two peaks. The first sharp peak coincided with the PPO activity peak and the second broad one appeared in fractions 20–25 with an ammonium sulphate concentration of about 7–8%. This peak could be due to other interfering proteins. The fractions with peak PPO activity were pooled and were used for gel filtration chromatography for further purification and approximate molecular weight determination. The gel filtration showed a single peak eluted at 27 min, whereas, the protein content peak was observed at 32 min (Fig. 1d). The eluent at 27 min on SDS PAGE showed multiple peaks (data not shown); therefore, the peak PPO activity was collected, concentrated and injected again, which helped in achieving further improvement in purity.

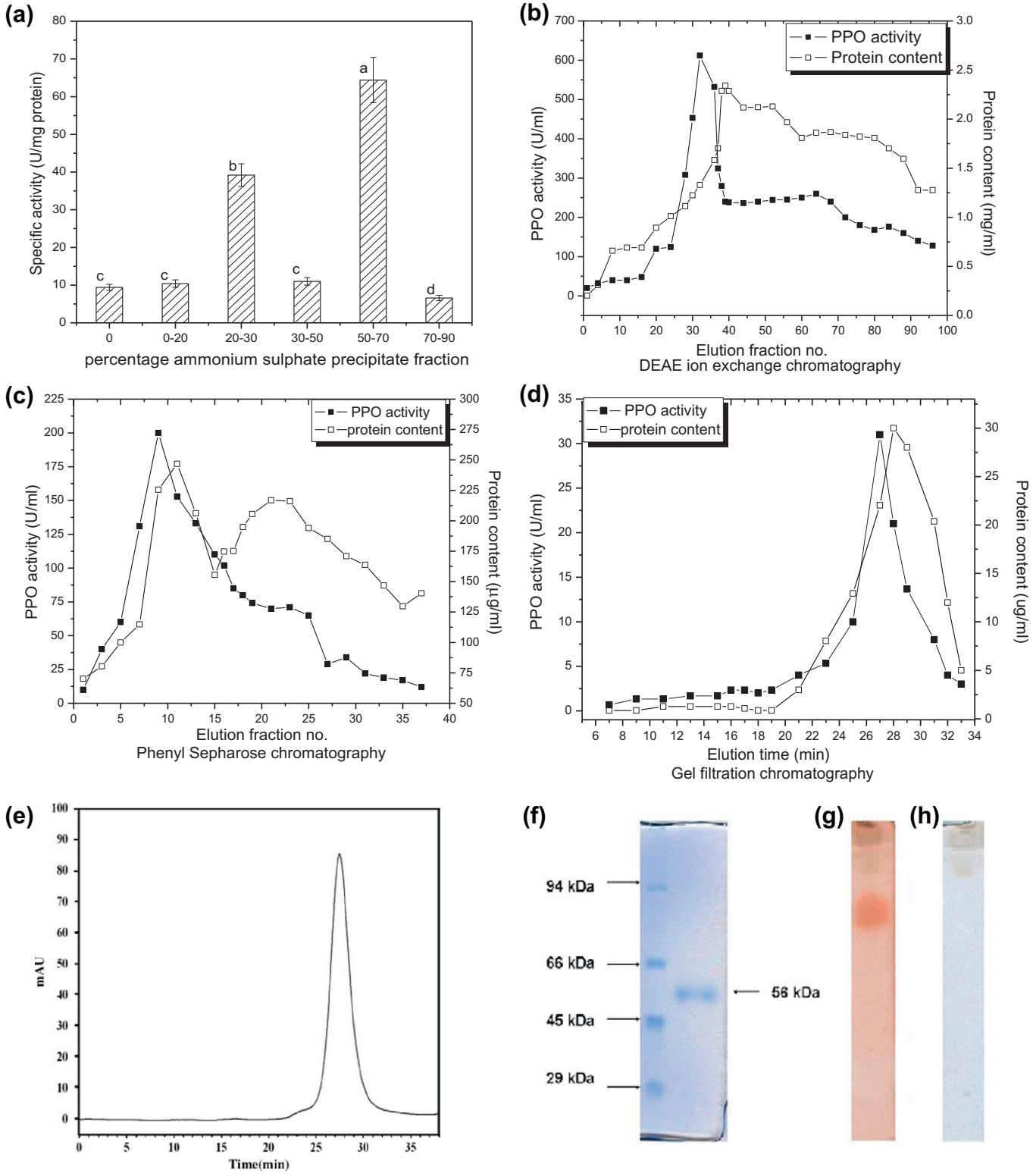


Fig. 1. The different steps of purification and profile of PPO from eggplant. (a) Ammonium sulphate precipitation (different letters in the superscript indicate significance of difference in the mean), (b) DEAE ion-exchange chromatography, (c) phenyl Sepharose hydrophobic interaction chromatography, (d) Superdex gel filtration chromatography, (e) HPLC profile, indicating gel filtration-purified PPO, (f) SDS–PAGE, exhibiting purified PPO with molecular weight markers, (g) native PAGE, indicating PPO activity in eggplant crude extract while incubated with buffer and substrate (4-methyl catechol) without (g) and with (h) potassium metabisulfite, a PPO inhibitor.

3.3. PPO is a dimer of 112 kDa molecular weight

The molecular weight of purified PPO was observed to be about 112 kDa, based on a calibration curve prepared using proteins of known molecular weight (Fig. 1e). The SDS–PAGE profile of the finally purified protein showed a single band of about 56 kDa (Fig. 1f). This indicated the active protein to be a homodimer of 56 kDa.

3.4. Native PAGE showed presence of a single band

The 20–80% ammonium sulphate-precipitated extract, which practically included most of the proteins, were analysed for presence of different isoforms of PPO on a native PAGE without addition of denaturing (SDS) and reducing (β-mercaptoethanol) agents. The substrate staining with 4-methyl catechol (0.1 M), immediately after electrophoresis, showed the presence of a single band (Fig. 1g). The substrate staining of a gel in the presence of potassium metabisulfite, a PPO inhibitor, showed no visible band, confirming the identity of a PPO (Fig. 1h). The isoforms of PPO observed in the ammonium sulphate precipitation, did not resolve in native PAGE, indicating that these isoforms could be of similar structure.

3.5. Purification and yield of PPO

At subsequent steps of purification, the PPO activity/ml increased and, as expected, the protein (PPO) content decreased. This resulted in increase in specific activity to 259-fold after gel filtration chromatography (Table 1). However, at each step of purification, a loss in total activity was noticed because only peak fractions displaying PPO activity were purposely collected. This significantly increased the purification-fold; however, the total yield was significantly reduced to about 0.02%. Out of 1 kg of eggplant, the finally purified PPO fraction contained 0.37 mg of active PPO protein (Table 1).

3.6. PPO substrate specificity showed higher catechol oxidase activity

Purified PPO was characterized for its substrate specificity, using eleven well known substrates, namely, 4-methyl catechol, tert-butylcatechol, pyrocatechol, chlorogenic acid, D and L-DOPA, cresol, dihydrocaffeic acid, caffeic acid, dihydroxybenzaldehyde, pyrogallol and gallic acid. Out of these, 4-methyl catechol showed maximum activity and this was considered as 100% to compare its activity with other substrates (Table 2). The specificities of each substrate were found to be significantly different ($P \leqslant 0.05$). Higher enzyme activity was observed with substrates having catechol ring structures and a functional group (−R) at meta position with respect to the —OH group. A smaller functional group (—CH_3), present in 4-methyl catechol, probably conferred maximum activity. With increase in size of this functional group the activity decreased as was observed in the case of tert-butylcatechol, chlorogenic acid, and DL-DOPA (Table 2). By contrast, the absence of this functional group also decreased activity by 30%, as observed in the case of pyrocatechol. These observations indicated the important role of the —CH_3 group, and its location in the catechol structure, in determining its specificity. Most of the substrates with a catechol ring structure and a functional group at other positions of the ring (—OH at *ortho* position as in pyrogallol, gallic acid, and —CH_3 at *para* position, as in p-cresol) showed very much less or absence of activity. The enzyme did not show any activity against monophenols such as tyrosine (data not shown).

3.7. Kinetic characteristics of PPO

The K_m and V_{max} values were determined for substrates having higher activity ($\geqslant$70%), using Lineweaver–Burk plots (1/S and 1/V). The lowest K_m was observed for 4-methyl catechol (0.34 mM) and substrate concentration at V_{max} was about 212 μM, with absorbance change of about 280/min (Table 3). The K_m value increased with less specific substrates. However, the V_{max} remained almost the same. The tert-butyl catechol, dihydrocaffeic acid, and pyrocatechol showed 29, 41, and 58% higher K_m values compared to 4-methyl catechol and the difference was found to be significant ($P \leqslant 0.05$). The turnover number (K_{cat}) and catalytic efficiency (K_{cat}/K_m) were also found to be maximum for 4-methyl catechol and less for pyrocatechol (Table 3).

3.8. Inhibitor studies

The optimum concentration range for each inhibitor, to perform a kinetic study, was determined by preliminary experiments (data not shown) and the values were found to vary significantly among different inhibitors. Eight different inhibitors, namely ascorbic acid, α-resorcylic acid, erythorbic acid, cysteine hydrochloride, potassium metabisulfite, kojic acid, citric acid, and sodium azide, were studied, out of which, citric acid and sodium azide showed mixed inhibition and the rest showed competitive inhibition. The competitive inhibitors showed the same V_{max} value, with the same *Y*-axis intercept, whereas, the mixed inhibitors had different V_{max} values. The inhibitor constant (Ki) value was determined from the *X*-axis intercept of the plot. The most effective competitive inhibition was shown by cysteine hydrochloride with the lowest Ki value of 1.8 μM. The next effective competitive inhibitor was potassium metabisulfite with a Ki value of 9 μM. Ascorbic acid and erythorbic acid showed similar competitive inhibitions with Ki values of 15.2 and 19.6 μM, respectively. The next effective competitive inhibitions were shown by kojic acid and resorcylic acid with Ki values of 0.123 and 8.2 mM, respectively. Mixed inhibition was shown by sodium azide and citric acid, with higher Ki values of 3.3 and 13.5 mM, respectively, and these were observed to be less effective.

4. Discussion

The PPO was classified as a monophenol oxidase (Tyrosinase, EC 1.14.18.1) and catechol oxidase (diphenol oxidoreductase, EC 1.10.3.1). The first one adds an —OH group to a monophenol (e.g. tyrosine) at the first step and then converts this diphenol to a quinone at the second step. The second one, which converts a diphenol to a quinone, is present in eggplant. The PPO is reported to be located in the thylakoid lumen of the chloroplast inside plant cells, in either membrane-bound or soluble form (Ding et al., 1998). The membrane-bound form could be weakly or strongly bound. Weakly linked enzyme can be extracted by increasing salt concentration, whereas, integral membrane-bound forms could be isolated with addition of detergents to the extraction solution. The latent form of this enzyme, reported to be activated by treatment with SDS or with proteases (Pérez-Gilabert & Carmona, 2000), was also not detected in the current study. During the homogenization process, the enzyme comes into contact with substrate and oxygen, resulting in excess browning of the suspension. The inhibition of browning, by PVP and PVPP, was due to binding to the substrate phenolics, making them unavailable for the enzyme (Mayer, 2006). Ascorbic acid (up to 50 mM concentration) inhibited PPO and thus browning of extract was suppressed (Ding et al., 1998).

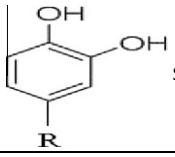
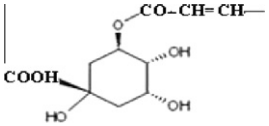
The purification achieved in the final purified PPO was as high as 259-fold with yield as low as 0.02%. Though there are reports of lesser-fold purification, many authors have reported higher-fold purifications in other vegetable and plant parts. In potato, the PPO was 5-fold purified with 18% yield whereas in, banana peel, the PPO was 460-fold purified with 2.2% yield (Sánchez-Ferrer, Laveda, & Garcia-Carmona, 1993; Yang et al., 2001). Similarly, in loquat fruit, the PPO was 422-fold purified, with about 35% yield (Ding et al., 1998). These differences could be due to differences in concentrations of phenolics and their irreversible binding to the PPO protein, resulting in interference with protein purification in certain fruits and vegetables (Papadopoulou & Frazier, 2004). The phenolics bound to the enzyme change its ionic and hydrophobic

Table 1
The purification of PPO from eggplant.

Purification step	Volume (ml)	Activity (Units/ml)	Total activity (Units)	Protein (mg/ml)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
Crude	$3000^{a} \pm 50$	$28^{d} \pm 5$	$(8.4^{a} \pm 0.6) \times 10^{4}$	$1.47^{a} \pm 0.1$	$19^{e} \pm 5$	$100^{a} \pm 0^{*}$	$1^{e} \pm 0$
50–70% Ammonium sulphate saturation	$400^{b} \pm 20$	$133^{c} \pm 12$	$(3.8^{b} \pm 0.3) \times 10^{4}$	$2.05^{b} \pm 0.2$	$65^{d} \pm 5$	$45^{b} \pm 5$	$3.42^{d} \pm 0.3$
Ion exchange (DEAE)	$43^{c} \pm 5$	$256^{b} \pm 30$	$(1.1^{c} \pm 0.2) \times 10^{4}$	$1.5^{a} \pm 0.1$	$170^{c} \pm 16$	$13^{c} \pm 1.1$	$8.94^{c} \pm 0.7$
Hydrophobic (phenyl Sepharose)	$6^{d} \pm 1$	$378^{a} \pm 35$	$2268^{d} \pm 226$	$0.45^{c} \pm 0.04$	$840^{b} \pm 55$	$2.7^{d} \pm 0.2$	$44^{b} \pm 3$
Gel filtration (Superdex)	$4.7^{d} \pm 0.5$	$394^{a} \pm 32$	$1851^{e} \pm 154$	$0.08^{d} \pm 0.01$	$4925^{a} \pm 460$	$0.02^{e} \pm 0.002$	$259^{a} \pm 23$

* Represents activity of extract prepared by homogenizing 1 kg of eggplant in 3 l of extraction solution. The values are expressed as means ± SD of three parallel experiments. The values in column with different superscripts are significantly different ($P \leqslant 0.05$).

Table 2
Substrate specificity of native PPO purified from eggplant.

 Substrate	Functional group (R) at meta position of —OH group	Relative Activity* (%)
4-Methylcatechol	$-CH_3$	$100^{a} \pm 0$
Tert-butylcatechol	$-C(CH_3)$	$83^{b} \pm 4$
Dihydrocaffeic acid	$-CH_2-CH_2-COOH$	$82^{b} \pm 3$
Pyrocatachol	Nil	$70^{c} \pm 3$
D-DOPA, L-DOPA	$-CH_2-CH(NH_2)-COOH$	$40^{d} \pm 2$, $22^{f} \pm 2$
Caffeic acid	—CH=CH—COOH	$32^{e} \pm 2$
Chlorogenic acid		$31^{e} \pm 2$
Pyrogallol	—OH at *ortho* position	$17^{g} \pm 2$
3,4-Dihydroxybenzaldehyde	—CHO	$5^{h} \pm 1$
Gallic acid	—COOH (and —OH at *ortho*)	$1.5^{h} \pm 0.3$
p-Cresol	Nil, ($-CH_3$ at *para* position)	0

* Relative activity is expressed with respect to 4-methyl catechol, considering it as 100%. The values are expressed as means ± SD of three parallel experiments. The values in column with different superscripts are significantly different ($P \leqslant 0.05$).

Table 3
The kinetic characteristics* of purified eggplant PPO.

Substrate	K_m (mM)	V_{max} (ΔOD/min)	Substrate concentration (μM) at V_{max}	K_{cat}	K_{cat}/K_m (mM^{-1})
4-Methyl catechol	$0.34^{d} \pm 0.05$	$280^{a} \pm 26$	$212^{a} \pm 10$	$(1.14^{d} \pm 0.1) \times 10^{6}$	$(3.3^{c} \pm 0.2) \times 10^{6}$
Tert-butyl catechol	$0.44^{c} \pm 0.02$	$277^{a} \pm 24$	$209^{a} \pm 12$	$(3.8^{a} \pm 0.2) \times 10^{5}$	$(8.6^{a} \pm 0.5) \times 10^{5}$
Dihydrocaffeic acid	$0.48^{b} \pm 0.01$	$280^{a} \pm 25$	$205^{a} \pm 11$	$(2.3^{b} \pm 0.2) \times 10^{5}$	$(4.7^{b} \pm 0.4) \times 10^{5}$
Pyrocatechol	$0.54^{a} \pm 0.04$	$267^{a} \pm 21$	$203^{a} \pm 10$	$(1.6^{c} \pm 0.1) \times 10^{5}$	$(2.9^{d} \pm 0.1) \times 10^{5}$

* K_m – Michaelis–Menten constant, V_{max} – maximum reaction velocity, K_{cat} – turn over number of enzyme, K_{cat}/K_m – catalytic efficiency. The values are expressed as means ± SD of three parallel experiments. The values in column with different superscripts are significantly different ($P \leqslant 0.05$).

characteristics, resulting in variation in elution behaviour during purification steps (Papadopoulou & Frazier, 2004). This resulted in difficulty in purifying the wider peaks observed during elution. The collection of fractions only with maximum PPO activity for better-fold purification resulted in loss of PPO protein from the shoulder region of the peak, ultimately affecting the total yield of purified protein.

The substrate specificity of eggplant PPO showed that it belongs to the catechol oxidase family (EC 1.10.3.1) as the maximum activity was observed with catechol or its derivative substrates (Table 2). The enzyme did not show any activity with monophenol substrates, e.g. tyrosine, which indicated that the monophenol monoxygenase (EC 1.14.18.1) activity was absent with this enzyme (data not shown). The enzyme also did not show any activity with cresol, which indicated that the cresolase activity was also absent with this purified enzyme (Table 2). With a few exceptions, most plants, animals and fungi have been reported to have catechol oxidase, tyrosinase and cresolase activities, respectively (Mayer, 2006). In addition to this, the catechol derivatives with bigger −R group showed gradual reduction in specificity (Table 2). The catechol without −R group also showed less specificity (pyrocatechol) compared to 4-methyl catechol where the −R is a methyl group. The −R group position in the catechol ring was also observed to be a determinant of higher specificity. The active site of the enzyme could interact with these substrate molecules where the −R group position and composition provide advantage for efficient catalysis.

The PPO isolated from eggplant was found to have very significant K_m values with different common substrates (as compared to other sources), along with better turn over number (K_{cat}) and catalytic efficiency (K_{cat}/K_m) (Table 3). Potato, tobacco, and banana PPOs were reported to have 0.9, 1.2, and 3.9 mM K_m values with 4-methyl catechol substrate, respectively (Shi, Dai, Xu, Xie, & Liu, 2002; Sánchez-Ferrer et al., 1993; Yang et al., 2001). In eggplant, the earlier reported value of K_m for 40% ammonium sulphate-

precipitated PPO was 1 mM for 4-methyl catechol substrate (Pérez-Gilabert & Carmona, 2000). The lower K_m value (0.34 mM) observed in the current study could be the reason for the high substrate affinity of the enzyme for indigenous phenolics, resulting in extract browning.

The inhibition of PPO is important in the food industry, due its role in browning. The newly studied compound, cysteine hydrochloride, was structurally quite similar to the earlier-studied inhibitor, cysteine, and showed significant inhibition of PPO activity at a concentration much below the permissible level. Cysteine hydrochloride is derived from the amino acid cysteine and is highly soluble in water. It is also administered to infants in injection (7.25% in water USP) as a source of the essential amino acid cysteine (Kux, 2010). It is a reducing agent and known to form an adduct with intermediate quinones, which inhibits the PPO at a much lower concentration. Potassium metabisulfite, another reducing agent, is known to inhibit PPO by the reaction of sulfite with intermediate quinones, resulting in the formation of sulphoquinones, which irreversibly inhibit PPO, resulting in its complete inactivation (Marshall, Kim, & Cheng-I, 2000). Ascorbic acid and erythorbic acid which are considered as vitamin C, inhibit PPO by reducing the intermediate quinones back to diphenols. Resorcylic acid, used in the current study, is a natural phenolic acid having a structure similar to the known inhibitor 4-hexyl resorcinol, except having an acid group (—COOH) in place of a hexyl group ($—C_6H_{18}$). This less studied compound has higher solubility and shows inhibition similar to 4-hexyl resorcinol with a Ki value of 8.2 μM. Such compounds were reported to compete with the substrate for binding to the active site and thereby cause enzyme inhibition. Kojic acid is a fungal secondary metabolite reported to inhibit PPO weakly by chelating the copper atom from the active site of the enzyme. In the current study, it also showed weak inhibition against eggplant PPO. Citric acid is reported to inhibit PPO by lowering pH and chelating the copper ion from the active site (Marshall et al., 2000). This study provides valuable information for control of browning in fruit and vegetable processing, using a simple GRAS compound, cysteine hydrochloride.

5. Conclusions

A highly active PPO from eggplant, which is precipitated by 50–70% ammonium sulphate saturation, was 259-fold purified, and characterized to be a homodimer of 56 kDa. The substrate specificity indicated its nature as a catechol oxidase (EC 1.10.3.2), showing maximum activity with 4-methyl catechol. The enzyme lacked cresolase and tyrosinase activities. Catechol ring structure and a smaller functional group (4-methyl) at the meta position, with respect to the —OH group, were found to be major determinants of its activity. It showed significant competitive inhibition by a GRAS compound, cysteine hydrochloride, which has higher efficiency than the conventionally used potassium metabisulfite. Potassium metabisulfite is also associated with some health risks to consumers.

Acknowledgments

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The information on this web site remains accessible; but, due to the lapse in government funding, the information may not be up to date, and the agency may not be able to respond to inquiries until appropriations are enacted. For updates regarding government operating status see USA.gov.

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The information on this web site remains accessible; but, due to the lapse in government funding, the information may not be up to date, and the agency may not be able to respond to inquiries until appropriations are enacted. For updates regarding government operating status see USA.gov.

Display Settings: GenBank

Solanum melongena cultivar Azad kranti chloroplast polyphenol oxidase gene, complete cds; nuclear gene for chloroplast product

GenBank: JQ621950.1

FASTA Graphics PopSet

Go to:

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            oxidase gene, complete cds; nuclear gene for chloroplast product.
ACCESSION   JQ621950
VERSION     JQ621950.1  GI:387538850
KEYWORDS    .
SOURCE      Solanum melongena (eggplant)
  ORGANISM  Solanum melongena
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            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            asterids; lamiids; Solanales; Solanaceae; Solanoideae; Solaneae;
            Solanum.
REFERENCE   1  (bases 1 to 1785)
  AUTHORS   Mishra,B.B., Gautam,S. and Sharma,A.
  TITLE     Soluble phenolics and active polyphenol oxidase (PPO): Two major
            factors determining post processing browning in eggplant (Solanum
            melongena)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1785)
  AUTHORS   Mishra,B.B., Gautam,S. and Sharma,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-FEB-2012) Food Technology Division, Bhabha Atomic
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Nucleotide

The information on this web site remains accessible; but, due to the lapse in government funding, the information may not be up to date, and the agency may not be able to respond to inquiries until appropriations are enacted. For updates regarding government operating status see USA.gov.

Display Settings: GenBank

Solanum melongena cultivar raveena chloroplast polyphenol oxidase gene, complete cds; nuclear gene for chloroplast product

GenBank: JQ621951.1

FASTA Graphics PopSet

Go to:

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DEFINITION  Solanum melongena cultivar raveena chloroplast polyphenol oxidase
            gene, complete cds; nuclear gene for chloroplast product.
ACCESSION   JQ621951
VERSION     JQ621951.1  GI:387538852
KEYWORDS    .
SOURCE      Solanum melongena (eggplant)
  ORGANISM  Solanum melongena
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            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            asterids; lamiids; Solanales; Solanaceae; Solanoideae; Solaneae;
            Solanum.
REFERENCE   1  (bases 1 to 1773)
  AUTHORS   Mishra,B.B., Gautam,S. and Sharma,A.
  TITLE     Soluble phenolics and active polyphenol oxidase (PPO): Two major
            factors determining post processing browning in eggplant (Solanum
            melongena)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1773)
  AUTHORS   Mishra,B.B., Gautam,S. and Sharma,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-FEB-2012) Food Technology Division, Bhabha Atomic
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Nucleotide

The information on this web site remains accessible; but, due to the lapse in government funding, the information may not be up to date, and the agency may not be able to respond to inquiries until appropriations are enacted. For updates regarding government operating status see USA.gov.

Display Settings: GenBank

Solanum melongena cultivar anupam chloroplast polyphenol oxidase gene, complete cds; nuclear gene for chloroplast product

GenBank: JQ621952.1

FASTA Graphics PopSet

Go to:

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DEFINITION  Solanum melongena cultivar anupam chloroplast polyphenol oxidase
            gene, complete cds; nuclear gene for chloroplast product.
ACCESSION   JQ621952
VERSION     JQ621952.1  GI:387538854
KEYWORDS    .
SOURCE      Solanum melongena (eggplant)
  ORGANISM  Solanum melongena
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            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            asterids; lamiids; Solanales; Solanaceae; Solanoideae; Solaneae;
            Solanum.
REFERENCE   1  (bases 1 to 1788)
  AUTHORS   Mishra,B.B., Gautam,S. and Sharma,A.
  TITLE     Soluble phenolics and active polyphenol oxidase (PPO): Two major
            factors determining post processing browning in eggplant (Solanum
            melongena)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1788)
  AUTHORS   Mishra,B.B., Gautam,S. and Sharma,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-FEB-2012) Food Technology Division, Bhabha Atomic
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Nucleotide

The information on this web site remains accessible; but, due to the lapse in government funding, the information may not be up to date, and the agency may not be able to respond to inquiries until appropriations are enacted. For updates regarding government operating status see USA.gov.

Display Settings: GenBank

Solanum melongena polyphenol oxidase gene, complete cds

GenBank: GQ149349.1

FASTA Graphics

Go to:

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DEFINITION  Solanum melongena polyphenol oxidase gene, complete cds.
ACCESSION   GQ149349
VERSION     GQ149349.1  GI:238836379
KEYWORDS    .
SOURCE      Solanum melongena (eggplant)
  ORGANISM  Solanum melongena
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REFERENCE   1  (bases 1 to 1782)
  AUTHORS   Mishra,B.B. and Sharma,A.
  TITLE     Polyphenol oxidase gene isolated from Solanum melongena
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1782)
  AUTHORS   Mishra,B.B. and Sharma,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-MAY-2009) Food Technology Division, Bhabha Atomic
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Nucleotide

The information on this web site remains accessible; but, due to the lapse in government funding, the information may not be up to date, and the agency may not be able to respond to inquiries until appropriations are enacted. For updates regarding government operating status see USA.gov.

Display Settings: GenBank

Solanum melongena polyphenol oxidase mRNA, partial cds

GenBank: GQ149350.1

FASTA Graphics

Go to:

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  AUTHORS   Mishra,B.B., Mukhopadhyaya,R. and Sharma,A.
  TITLE     Polyphenol oxidase cDNA from Solanum melongena
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1640)
  AUTHORS   Mishra,B.B., Mukhopadhyaya,R. and Sharma,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-MAY-2009) Food Technology Division, Bhabha Atomic
            Research Centre, Mumbai, Maharashtra 400085, India
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DAE-BRNS Life Sciences Symposium 2012 (LSS-2012)
on
Trends in Plant, Agriculture and Food Sciences (TIPAFS)
December 17-19, 2012

Certificate

This is to certify that

~~Prof./~~Dr./~~Mr./Ms.~~ Bibhuti Bhusan Mishra

participated in the DAE-BRNS Life Sciences Symposium 2012 (LSS-2012) On Trends in Plant, Agriculture and Food Sciences (TIPAFS) December 17-19, 2012 organized by Bio-Medical Group, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085 held at Anushaktinagar, Mumbai and has delivered an ~~Invited lecture~~ /~~Oral~~/ Poster Presentation /~~as an observer~~. He/She has won the Best Poster Award.

Dr. S.F. D'Souza
Chairman, LSS-2012

Dr. P. Suprasanna
Convenor, LSS-2012

POS-88

Characterization of polyphenol oxidase (PPO) from brinjal (*Solanum melongena*)

Bibhuti Bhusan Mishra, Satyendra Gautam and Arun Sharma

Food Technology Division, Bhabha Atomic Research Centre, Mumbai-400085, Maharashtra, India

Brinjal is a very rich source of polyphenol oxidase (PPO), which negatively affects its quality upon cutting and postharvest processing due to enzymatic browning. PPO inhibitors from natural or synthetic sources are used to tackle this problem. With this aim, PPO enzyme purification from brinjal and its characterization including inhibitor studies were undertaken. Brinjal was found to contain two isoforms of PPO (PPO1 and PPO2) which were resolved during differential ammonium sulphate precipitation and native PAGE. PPO1 was precipitated at 30%, whereas, PPO2 was precipitated at 70% ammonium sulphate saturation. The PPO2 showed higher specific activity and was further purified to about 259 fold using ion exchange, hydrophobic interaction, and gel filtration chromatography. The molecular size of purified brinjal PPO2 was found to be a 112 kDa, a homodimer of 56 kDa each. This purified enzyme showed very low Km (0.34 mM) and high catalytic efficiency (3.3×10^6) with 4-methyl catechol. The substrate specificity was found of the order 4-methyl catechol > tert-butylcatechol > dihydrocaffeic acid > pyrocatechol. Cysteine hydrochloride, potassium metabilsulfite, ascorbic acid, erythorbic acid, resorcylic acid and kojic acid showed competitive inhibition, whereas, citric acid and sodium azide showed mixed inhibition of PPO activity. Cysteine hydrochloride was found to be an excellent inhibitor with low inhibitor constant of 1.8 µM.

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FV-02

Inhibition of browning and PPO activity in ready-to-cook eggplant

Bibhuti Bhusan Mishra*, Satyendra Gautam and Arun Sharma

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Bhabha Atomic Research Centre
Mumbai - 400085

*Email: bimisra@barc.gov.in

Browning is a major postharvest problem in fresh-cut fruits and vegetables which is markedly observed in eggplant upon processing. In the current study, mechanics of cutting and further processing were found to have very significant effect on the extent of browning. Interestingly, browning was found to be significantly inhibited in the samples cut by sharp blade (thickness, 0.04 mm), immediately dipped in water for 10 min, ambient air drying, and packaged in styrofoam trays wrapped with stretchable film. The scanning electron and fluorescence microscopic examinations indicated that sharp blade cutting caused lesser physical injury and cellular death, resulting in reduced leaching of phenolics and polyphenol oxidase activity. This resulted in lesser browning measured in terms of L (Lightness) values. For commercial acceptability of the technique, storage studies were performed at 4, 10, and ambient (26 ±2 °C) storage temperature and the findings indicated that fine cut samples could be stored up to 16, 12, and 5 days at these temperatures, respectively with organoleptically and visually acceptable scores.