Chapter 10

# Enzymatic Browning

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## Chapter Outline



## I. INTRODUCTION

Enzymatic browning is a phenomenon which occurs in many fruits and vegetables, such as potatoes, mushrooms, apples, and bananas. When the tissue is bruised, cut, peeled, diseased, or exposed to any abnormal conditions, it rapidly darkens on exposure to air as a result of the conversion of phenolic compounds to brown melanins (Figure 10.1).

The international nomenclature for the enzymes involved in the browning reaction has changed. The first enzyme, monophenol monoxygenase or tyrosinase (EC 1.14.18.1), initiates the browning reaction, which later involves diphenol oxidase or catechol oxidase (EC 1.10.3.2) and laccase (EC 1.10.3.1). Catecholase oxidase will be referred to as 'polyphenol oxidase' (PPO) in this chapter. This enzyme requires the presence of both a copper prosthetic group and oxygen. The copper is believed to be monovalent in the case of the mushroom PPO and divalent in the case of the potato enzyme (Bendall and Gregory, 1963). PPO is classified as an oxidoreductase, with oxygen functioning as the hydrogen acceptor. The enzyme is widely distributed in higher plants and fungal and animal tissue and was reviewed by Swain (1962), Mathew and Parpia (1971), Mayer and Harel (1979), Vamos-Vigyazo (1981), Mayer (1987), and (2006).

### A. Historical Aspects of Polyphenol Oxidase

The earliest work is attributed to Lindet, who in 1895 recognized the enzymatic nature of browning while working on cider. At the same time Bourquelot and Bertrand commenced studies on mushroom tyrosine oxidase. Subsequently, Onslow in 1920 showed that enzymatic browning of plant tissue in air was attributed to the presence of *o*-diphenolic

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**FIGURE 10.1 Browning disorder in 'Conference' pears after 4 months in browning-inducing storage conditions (no cooling period, 1% $O_2$, 10% $CO_2$, −1°C)** *(Reprinted from Franck* et al.*, 2007). © 2007 with permission form Elsevier.*

compounds, such as catechol, protocatechuic acid, and caffeic acid, plus the appropriate enzymes (oxygenases). The product of this reaction was thought to be a peroxide which reacted with a 'chromogen' to form a brown pigment. Many fruits and vegetables, including apples, pears, apricots, and potatoes, were all found to be rich in phenolic compounds and oxygenases. Other fruits, such as citrus, pineapples, and redcurrants, lacked these substances and were thus termed 'peroxidase plants'.

This distinction was eliminated when it was shown that peroxidase and catalase were present in both groups of plants and in fact were ubiquitous in plant tissues. The term 'oxygenase' was subsequently replaced by 'phenolase' or 'polyphenol oxidase'. In 1937, Kubowitz demonstrated that PPO was a copper-containing enzyme.

## II. CHARACTERISTICS OF POLYPHENOL OXIDASE

### A. Structure and Sequence

In order to understand the physiological role of PPO, much research has been conducted to identify gene expression and protein sequences of the PPO of different plants. It is known that plant PPOs are synthesized as preproteins and contain putative plastid transit peptides at the N-terminal region, which target the enzyme into chloroplasts and thykaloid lumen (Marusek *et al.*, 2006). Molecular studies have described PPO as a single genome or as a multiple gene family. For example, the cherimoya PPO gene was shown to be present in one copy of the genome and had a very different nucleotide sequence from other published sequences. Despite these divergences, it presented conserved proteins in the proposed active site and also basic and strategic amino acids related to active site accessibility, protein structure, and stability (Prieto *et al.*, 2007). In Fuji apple plants the expression of two PPO genes (APO5 and MD-PPO2) was observed during vegetative and reproductive development and in response to wounding. However, they were not expressed at the same moment or by the same stress stimulus. The maximum level of APO5 messenger RNA (mRNA) was observed in damaged fruits after 24 hours, while the expression of MD-PPO2 was not increased, suggesting that the selective activation of individual genes under different conditions may reflect the existence of distinct signal transduction pathways to turn on the different PPO genes (J. Y. Kim *et al.*, 2001). In addition, seven genes were identified from tomato (Newmann *et al.*, 1993) and five distinct PPO cDNAs were isolated from potato plants (Hunt *et al.*, 1993).

The existence of multiple genes leads to the presence of PPO isozymes and the variability on protein sequences observed in the studies explains the differences among enzymes extracted from distinct sources. Two izoenzymes were present in persimmon, Fuji apple (J. Y. Kim *et al.*, 2001), coffee (Mazzafera and Robinson, 2000), and artichoke (Aydemir, 2004), while medlar PPO showed four isoforms (Dincer *et al.*, 2002). PPO purified from field bean is a tetramer with 120 kDa and exists as a single isoform in the seed (Paul and Gowda, 2000), while pineapple PPO has three isoenzymes, with the major isoform being a tetramer of identical subunits of 25 kDa (Das *et al.*, 1997).

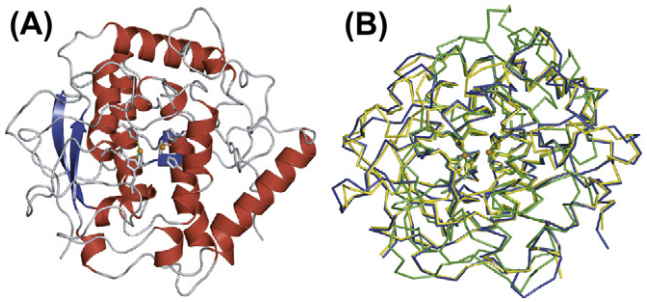



**FIGURE 10.2 X-ray structure of polyphenol oxidase (PPO) from *Vitis vinifera*.** (A) Ribbon model showing the overall ellipsoidal shape, two β-sheets, and the dicopper center within four-helix bundle. (B) Cα representation of *V. vinifera* PPO (blue) overlapping with those PPO from sweet potato (yellow) and *Streptomyces castaneoglobisporus* (green) See online for a colour version of this figure *(Virador* et al., *2010). Reprinted with permission, © 2010 American Chemical Society.*

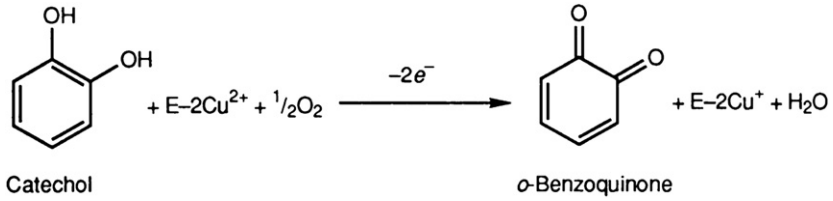
The molecular mass of PPO from other species has been reported as follows: mulberry, 65 kDa (Arslan *et al.*, 2004); Henry chestnuts, 69 kDa (Xu *et al.*, 2004); and pulp pine, 90 kDa (Lima *et al.*, 2001).

The crystal structure of PPO has been elicited for sweet potato, grape, and *Neurospora crassa*. The overall structure of the PPO from *Vitis vinifera* is a monomeric protein of 38.4 kDa. It is an ellipsoid with dimensions $56.7 \times 48.0 \times 48.3$ Å$^3$ (Figure 10.2A, B). The secondary structure is primarily R-helical with the core of the protein formed by a four-helix bundle composed of R-helices R4, R5, R12, and R14 (Figure 10.2A). Figure 10.2(B) shows overlapping images of the three existing PPO structures. There are a few areas where the folding is slightly different. For the most part, these differences occur on the surface and do not involve changes in R-helices or β-strands (Virador *et al.*, 2010).

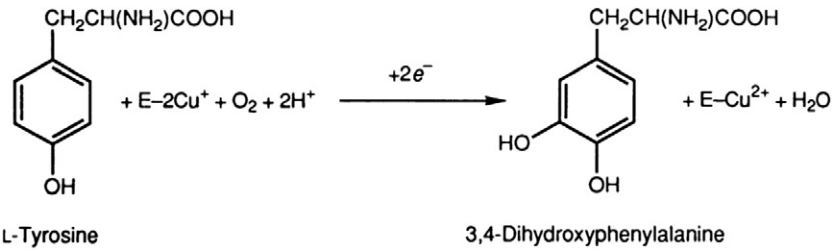
## B. Mechanism of Reaction

The mechanism of action proposed for PPO is based on its capacity to oxidize phenolic compounds. When the tissue is damaged the rupture of plastids, the cellular compartment where PPO is located, leads to the enzyme coming into contact with the phenolic compounds released by rupture of the vacuole, the main storage organelle of these compounds (Mayer and Harel, 1979).

PPO catalyzes two types of reaction: cresolase activity, in which monophenols are hydroxylated to *o*-diphenols, and catecholase activity. The catecholase or diphenolase type of reaction is best illustrated by the oxidation of catechol, an *o*-diphenol which is a commonly used laboratory substrate:




The cresolase or monophenolase activity involves hydroxylation of monophenols to *o*-diphenols, as shown by the oxidation of L-tyrosine to 3,4-dihydroxyphenylalanine, which occurs in potatoes (Schwimmer and Burr, 1967):




The active site of PPO consists of two copper atoms coordinated with six histidines, and exists in three oxidation states: *deoxy* ($E_d$), *met* ($E_m$), and *oxy* ($E_o$) (Figure 10.3). One of the $Cu^{2+}$ atoms is bound to monophenols while

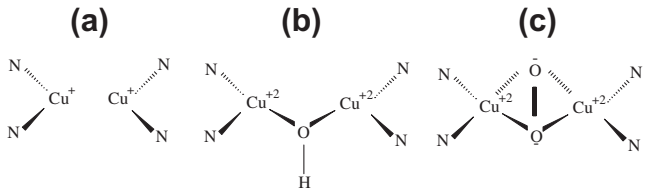
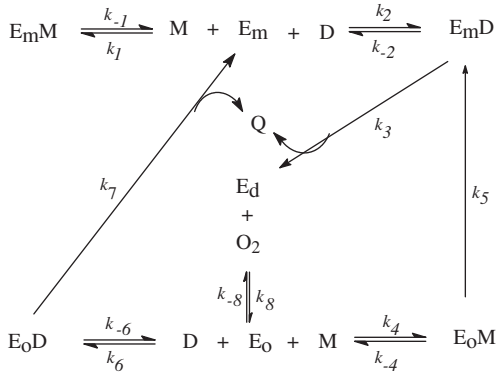



**FIGURE 10.3** **Polyphenol oxidase structure: (a) *deoxy*PPO, (b) *met*PPO, and (c) *oxy*PPO.** *(Adapted with permission from Espín* et al.*, 1998.)* © *2010 American Chemical Society.*




**SCHEME 10.1** Kinetic reaction mechanism of polyphenol oxidase (PPO) on monophenols and *o*-diphenols. Em: *met*PPO; Ed: *deoxy*PPO; Eo: *oxy*PPO; M: monophenol; D: diphenol; Q: *o*-quinone. *(Reprinted with permission, Espín* et al.*, 1998). © 1998 American Chemical Society.*

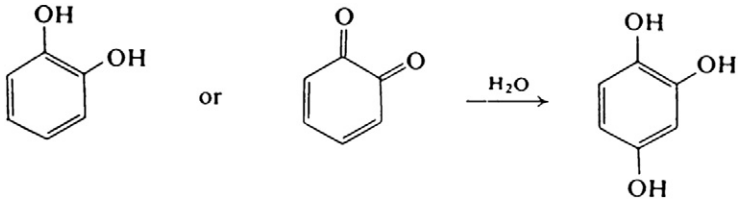
diphenols bind to both of them. As shown in the reaction mechanisms, monophenolase activity is intimately coupled to diphenolase activity and produces two electrons. These are required to incorporate one oxygen atom into the monophenol substrate.

The structural and kinetic mechanisms for the hydroxylation of monophenols (M) and the oxidation of *o*-diphenols (D) to *o*-quinones (Q) catalyzed by PPO have been established (Fenoll *et al.*, 2004; Espín *et al.*, 1998; Rodriguez-Lopes *et al.*, 1992). Monophenolic substrate initially coordinates to an axial position of one of the coppers of $E_o$ that leads to the hydroxylation of monophenol by the bound peroxide, loss of water and formation of the enzyme−diphenol ($E_mD$) complex. This complex can either render free diphenol or undergo oxidation of the diphenolate intermediate that bound to the active site, giving a free quinone and a reduced binuclear copper on the enzyme site ($E_d$). *Oxy*PPO is then regenerated after the binding of molecular oxygen to $E_d$ (Rodriguez-Lopez *et al.*, 1992). When a diphenol is present in the medium, this substrate binds to both $E_o$ and $E_m$ to give $E_oD$ and $E_mD$ intermediates, which, in turn, give rise to two quinones (Orenes-Piñero *et al.*, 2005) (Scheme 10.1).

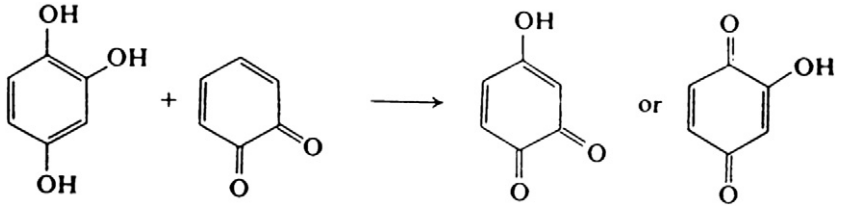
The presence of a dead-end complex, $E_mM$, is related to the occurrence of a lag period in the monophenolase activity of PPO (Rodriguez-Lopez *et al.*, 1992) that can be reversed by the addition of small quantities of reducing agents or *o*-diphenols as cosubstrates.

Quinone formation is both enzyme and oxygen dependent. Once this has taken place, the subsequent reactions occur spontaneously and no longer depend on the presence of PPO or oxygen. Joslyn and Ponting (1951) summarized those chemical reactions which may account for the formation of brown melanins.

The first reaction is thought to be a secondary hydroxylation of the *o*-quinone or of excess *o*-diphenol:



The resultant compound (triphenolic trihydroxybenzene) interacts with *o*-quinone to form hydroxyquinones:




Hydroxyquinones undergo polymerization and are progressively converted to red and red–brown polymers, and finally to the brown melanins which appear at the site of plant tissue injury (Matheis and Whitaker, 1984; Whitaker, 1972).

The ratio of diphenolase to monophenolase activity depends on the plant source and can vary from 1:10 to 1:40 (Vamos-Vigyazo, 1981). Sanchez-Ferrer *et al.* (1988) partially purified PPO from Monastrel grapes and identified both cresolase and catecholase activity. Rocha and Morais (2001) also found cresolase and catecholase activity in Jonagorad apple PPO. Cresolase activity was described for strawberry and pear by Espín *et al.* (1997a, b). Several plants, however, have been found to be devoid of monophenolase activity, including coffee (Mazzafera and Robinson, 2000), broccoli florets (Gawlik-Dziki *et al.*, 2007), butter lettuce (Gawlik-Dziki *et al.*, 2008), and cashew apple (Queiroz *et al.*, 2011).

## C. Biological Significance of Polyphenol Oxidase in Plants

The role of PPO in the living intact cell has until recently remained somewhat obscure. Early studies suggested its involvement as a terminal oxidase in respiration (James, 1953) or in the biosynthesis of lignin (Mason *et al.*, 1955). This was later discounted in studies by Nakamura (1967), who examined the role of three enzymes isolated from the latex of the Japanese lacquer tree (*Rhis vermicifera*): phenolase; peroxidase; and laccase. Of these enzymes only peroxidase was involved in lignification. Recent studies have demonstrated the role of laccase in lignification (Srebotnik and Hammel, 2000; Arora *et al.*, 2002; Shleev *et al.*, 2006). The mechanism is discussed in Section II, E.

Another possible role for PPO is in the biosynthesis of betalain. Red beet (*Beta vulgaris*) and common portulaca (*Portulaca portiflora*) present PPO that hydroxylates tyrosine, forming 3,4-dihydroxylphenylalanine (DOPA), and oxidizes DOPA to dopaquinone (Steiner *et al.*, 1996, 1999). Enzymatic activity is complemented by dioxygenase activity, leading to betalain formation. However, more evidence is needed to confirm this function.

PPO is restricted to the plastids. The enzyme appears to be in a latent form and bound to the thylakoid membrane, where the photochemical reactions of photosynthesis occur. The latent form can be activated in the presence of fatty acids (Siegenthaler and Vaucher-Boniour, 1971), detergents (Sellés-Marchart *et al.*, 2006), or trypsin (Tolbert, 1973). Pinto *et al.* (2008) isolated soluble and insoluble fractions of PPO from cowpea plants and reported activation by sodium dodecyl sulfate, a detergent, only in the soluble fraction, suggesting that insoluble PPO was purified in the active form.

In intact cells PPO seems to have little activity towards phenolics, which are located in the vacuole somewhat isolated from the plastid. Since the enzyme functions normally only when the cells are damaged or undergo senescence, it may also have a protected role, as proposed originally by Craft and Audia (1962). PPO is involved in phenolic metabolism when the plastid and vacuole contents are mixed. This occurs during senescence, when the integrity of the cell is disrupted, and the activation was reported by Goldbeck and Cammarata (1981). However, recent studies have demonstrated higher activity during development of the plant. Yu *et al.* (2010) evaluated PPO activity in longan fruit pericarp collected from 30 days after full bloom to mature fruit. They observed the highest enzymatic activity at first stage, followed by a sharp decrease, and then a slow increase in the final stage. These data are also in agreement with those described previously by Yang *et al.* (2000) and Sun *et al.* (2009). Ayaz *et al.* (2008) investigated PPO activity in medlar fruits during ripening and overripening and showed that, in mature fruit, the reaction velocity was higher than in overripe fruit. This could indicate that the enzyme was more active than in ripe and overripe fruits. A molecular study by J. Y. Kim *et al.* (2001) showed differential expression of two PPO genes in apple. The authors investigated the mRNA of PPO extracted from flower, fruit, and leaf in different stages and blotting analyses showed higher expression in the earlier stages (Figure 10.4).

Enzymatic browning also results following injury to the fruit or vegetable, causing disruption of the plastid, activation of latent PPO, and catalysis of phenolics released from the vacuole. Activation of PPO by biotic or abiotic stress had been demonstrated by several studies. Apple PPO showed maximum activity after 24 hours of

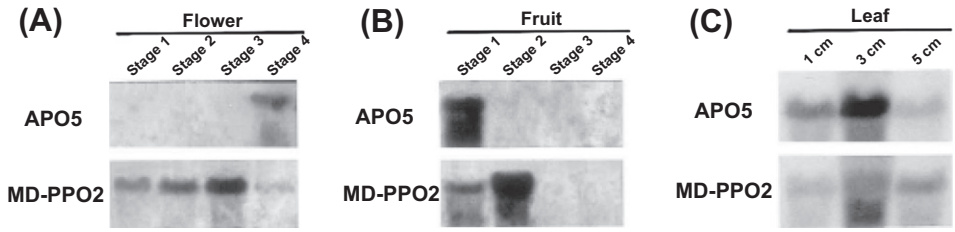



**FIGURE 10.4 Tissue-specific expression of two Fuji apple polyphenol oxidase (PPO) mRNAs.** Total RNAs were isolated from the different developmental stages of (A) flower, (B) fruit, and (C) leaf tissues as indicated *(Reprinted from J. Y. Kim* et al., *2001). © 2001, with permission from Elsevier.*

wounding, whereas in cowpea PPO, the activity reached the maximum after 48 hours of wounding (J. Y. Kim *et al.*, 2001; Pinto *et al.*, 2008). Temperature is an important factor in determining the level of activation since PPO is thermosensitive. Queiroz *et al.* (2011) studied the effect of mechanical injury and storage for 24 hours at different temperatures on cashew apple PPO (Table 10.1). Five-fold higher activation was observed in fruits kept at 2°C and 27°C, but low activity at the higher temperature studied (40°C). Environmental stress can also modulate PPO activity. Stress situations are related to enhanced activity of phenylalanine ammonia-lyase, which regulates the synthesis of phenolic compounds, increasing the content of PPO substrates in plant cell (Dixon and Paiva, 1995). Tegelberg *et al.* (2008) found higher enzymatic activity in leaves of *Betula pendula* after exposure to elevated carbon dioxide (700 ppm) associated with elevated temperature (2.5°C higher than ambient temperature) and elevated ultraviolet (UV)-B radiation (7.95 $kJ/m^2$/day). On the other hand, Thipyapong *et al.* (2004b) reported higher tolerance to water stress in transgenic tomato with suppressed PPO than in non-transformed plants and in overexpressing PPO. However, in these conditions the authors also observed upregulation of two PPO genes, probably related to stress resistance. Rivero *et al.* (2001) submitted watermelon and tomato to cold and heat stress and observed that PPO activity was decreased and phenylalanine ammonia-lyase activity increased, leading to an accumulation of polyphenols in stressed plants. These findings demonstrate the different ways that plants use to defend against an undesirable situation.

The enzyme appears to play an important role in the resistance of plants to infection by viruses, bacteria, and fungi (Tyagi *et al.*, 2000; Mohammadi and Kazemi, 2002; Wang and Constabel, 2004). Under these conditions the activity of the enzyme increases with the production of insoluble polymers which serve as a barrier to the spread of infection in the plant. Alternatively, some of the intermediates in the oxidative polymerization of polyphenols prevent or reduce infection by inactivating or binding some labile plant enzymes or viruses. Mahanil *et al.* (2008) observed increased resistance to the common cutworm [*Spodoptera litura* (F.)] in transgenic tomato plants overexpressing PPO (Figure 10.5). The study also showed that increased PPO activity led to higher larval mortality. According to the authors, the efficiency of conversion of both ingested food and digested food of third instars was found to be significantly different among tomato genotypes with differing PPO activity levels, suggesting that PPO activity rendered foliage less nutritious. Other studies in tomato have shown the role of PPO in disease resistance; antisense suppression of PPO increases susceptibility, and PPO overexpression increases resistance, to *Pseudomonas syringae* pv. *tomato* (Li and Steffens, 2002; Thipyapong *et al.*, 2004a).

**TABLE 10.1** Polyphenol Oxidase Activity by Wounded Cashew Apple

| Enzyme Extract | Specific Activity (U/min/mg Protein) | Activation |
|---|---|---|
| 0 hour (control) | 0.62 | – |
| 2°C/24 hours | 2.92 | 4.8 |
| 27°C/24 hours | 3.33 | 5.4 |
| 40°C/24 hours | 1.07 | 1.7 |

Reprinted from Queiroz *et al.* (2011). © 2011, with permission from Elsevier.

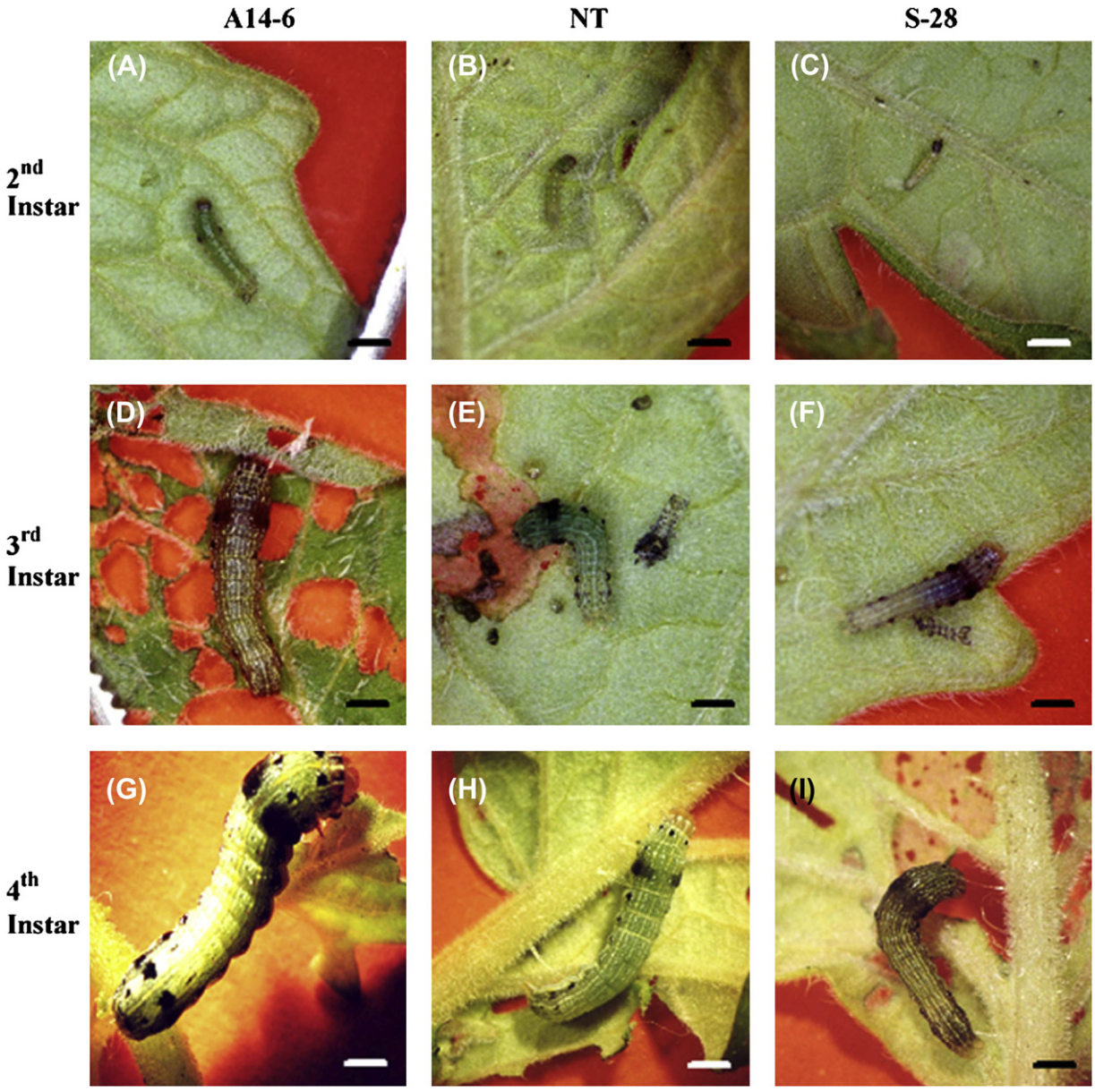


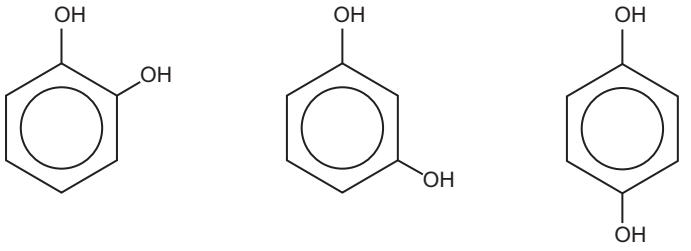

**FIGURE 10.5 (A–C) Second instar, (D–F) third instar and (G–I) fourth instar larvae of *Spodoptera litura* (F.) feeding on node 8 leaves of tomato plants with varied polyphenol oxidase (PPO) activity levels.** A14-6: transgenic line with suppressed PPO activity; NT: non-transformed control; S-28: transgenic line overexpressing PPO activity. Scale bars = 2.5 mm *(Reprinted from Mahanil* et al.*, 2008). © 2008, with permission from Elsevier.*

## D. Phenolic Compounds in Food Material

Phenolic compounds are natural substances that contribute to the sensorial properties (color, taste, aroma, and texture) associated with fruit quality (Marshall *et al.*, 2000). They form one of the main classes of secondary metabolites, with a large range of structures and functions, but generally possessing an aromatic ring bearing one or more hydroxy substituents. The phenolic composition of fruits is determined by genetic and environmental factors but may be modified by oxidative reactions. They are synthesized during plant development, but their synthesis is stimulated in stress conditions by activation of phenylalanine ammonia-lyase. Thus, these compounds play a role in defense and adaptive mechanisms of plants. The phenolic compounds which occur in food material and which participate in browning may be classified into four groups, namely, simple phenols, phenolic acids, cinnamic acid derivatives, and flavonoids.

### 1. Simple Phenols

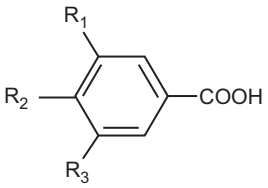
The simple phenols include monophenols such as L-tyrosine and *o*-diphenols such as catechol, resorcinol, and hydroquinone. However, among these compounds only catechol can be oxidized by PPO, because hydroxyl is at the *ortho* position. Catechol was identified in *Diospyro kaki* roots by Jeong *et al.* (2009) and PPO showed high affinity towards this phenolic compound (Özen *et al.*, 2004).




catechol resorcinol hydroquinone

### 2. Phenolic Acids

This class comprises the acids synthesized from precursor benzoic acid and they are widely distributed in plants. Gallic acid is present in an esterified form in tea flavonoids. Gallic and protocatechuic acids were found in cashew apple (Michodjehoun-Mestres *et al.*, 2009; Queiroz *et al.*, 2011) and protocatechuic was the main free phenolic acid identified in medlar fruits (Gruz *et al.*, 2011).

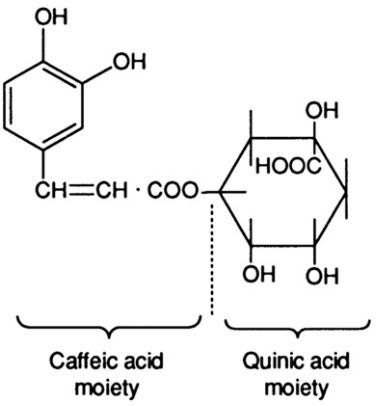



Benzoic Acids

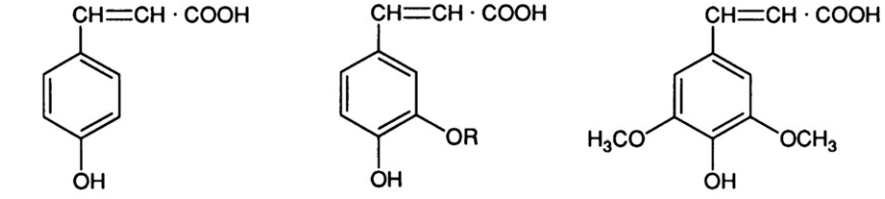
Gallic acid $R_1$=$R_2$=$R_3$=OH
Protocatechuic acid $R_1$=H, $R_2$=$R_3$=OH
Vanillic acid $R_1$=H, $R_2$=OH, $R_3$=$OCH_3$
Syringic acid $R_2$=OH, $R_1$=$R_3$=$OCH_3$

### 3. Cinnamic Acid Derivatives

The most important member of this group of compounds in food material is chlorogenic acid, which is the key substrate for enzymatic browning, particularly in apples and pears (Gauillard and Forget, 1997; Song *et al.*, 2007). Although potato is rich in chlorogenic acid, it is not a determinant factor for blackspot development. Two potato cultivars (cv. 'Bildtstar' and cv. 'Lady Rosetta') were bruised and the pigments identified. Quinic acid was detectable in hydrolysates of the pigments from 'Bildtstar', but not in those of 'Lady Rosetta', which indicated that chlorogenic acid may take part in blackspot formation, but is not essential for the discoloration (Stevens and Davelaar, 1996). These data were confirmed by Lærke *et al.* (2002), who found a correlation between black end products and free tyrosine, but none between black color and chlorogenic and caffeic acids in potato cultivars (cv. 'Dali' and cv. 'Oleva'). Discoloration derived from chlorogenic acid is attributed to the oxidation of complexes formed between iron and caffeic and chlorogenic acids.

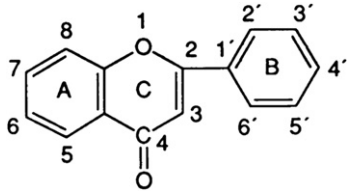



Other members of this group of compounds include *p*-coumaric, caffeic, ferulic, and sinapic acids. The universal distribution and high concentration of cinnamic acids in fruits may be due to their function as precursors in the biosynthesis pathway of more complex polyphenols.

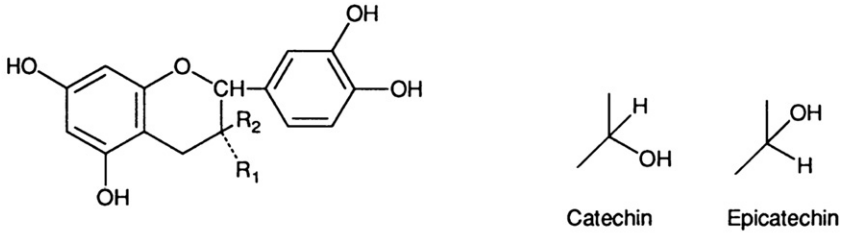



### *4. Flavonoids*

This group is the most widespread and structurally diverse among polyphenols. All members of this group of compounds are structurally related to flavone:



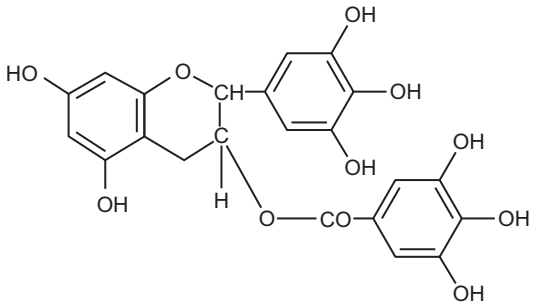

In food material, the important flavonoids are the catechins, anthocyanins, and flavonols. Catechin has the following structure:



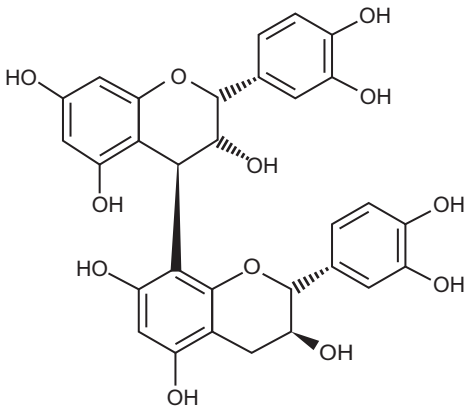

(+)–Catechin ($R_1$ = H; $R_2$ = OH)

(–)–Epicatechin ($R_1$ = OH; $R_2$ = H)

An extra hydroxyl group attached to the 5′ position on the B ring of catechin and epicatechin gives rise to gallocatechin and epigallocatechin, respectively. The catechin gallates are esters of catechins and gallic acid, the ester linkage being formed from the carboxyl group of gallic acid and the hydroxyl group attached to position 3 of the catechin C ring. An example is (−)-epigallocatechin gallate, which is the major polyphenol in dried tea leaves.

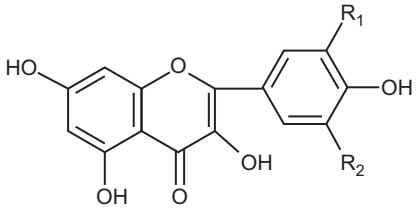



Such compounds are not the major phenols in fruits; however, they are important constituents of fruits in oligomeric or polymeric forms as proanthocyanidins or condensed tannins. Procyanidin is a dimer and is present in apple, grape, and cherry (Robards *et al.*, 1999).




Procyanidin B-1

The flavonols also participate in browning reactions and are widely distributed in plant tissues. The most commonly occurring flavonols are kaempferol, quercetin, and myricetin:




Kaempferol ($R_1 = R_2 = H$)

Quercetin ($R_1 = OH$; $R_2 = H$)

Myricetin ($R_1 = R_2 = OH$)

Flavonols possess a light yellow color and are particularly important in fruits and vegetables in terms of the astringency they impart to the particular food. They occur naturally as glycosides, examples of which are rutin and the glycosides of quercetin, the latter occurring in tea leaves and apple skins (Hulme, 1958).

All of the compounds discussed so far are substrates for PPO. Such oxidation reactions are important in tea fermentation, in the browning of cling peaches (Luh *et al.*, 1967), and in the drying stage of the curing of fresh cacao seeds (Roelofsen, 1958). It appears to be an important step in the development of the final color, flavor, and aroma of cacao and chocolate. PPO plays a beneficial role in the case of tea and cacao fermentation, in contrast to its role in the browning of fruits and vegetables.

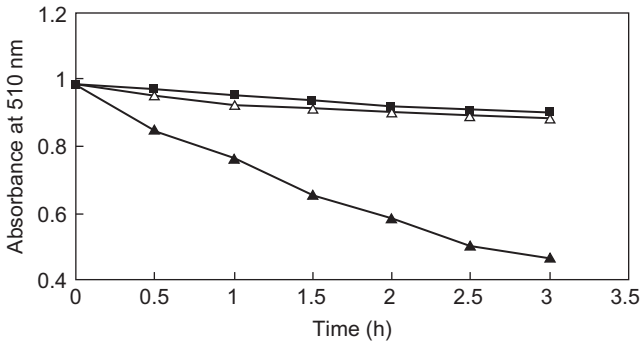
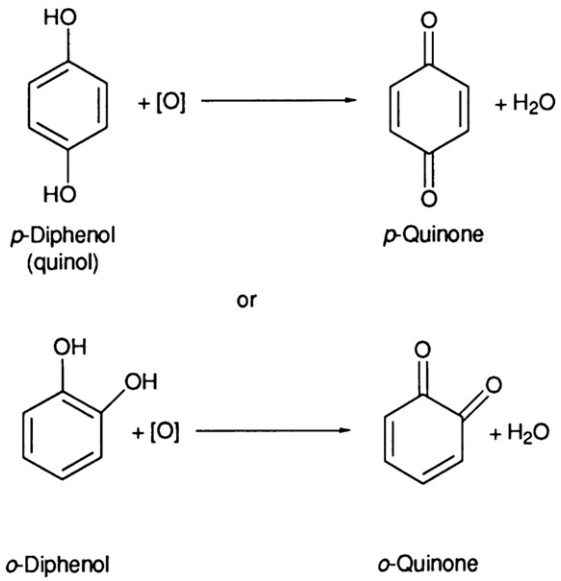



**FIGURE 10.6 Changes in absorbance of bayberry anthocyanin at 510 nm during its degradation by bayberry polyphenol oxidase (PPO) in the presence (▲) and absence (Δ) of gallic acid and after thermal denaturation of the PPO in the presence of gallic acid (■)** *(Reprinted from Fang* et al.*, 2007).© 2007, with permission from Elsevier.*

Several studies have been conducted to identify other endogenous substrates for PPO in fruits. Epicatechin is the endogenous substrate for litchi (lychee) and longan PPOs (Sun *et al.*, 2006; Shi *et al.*, 2008). Other flavonoids, such as eriodictyol, myricetin, and fisetin, can also be oxidized by PPO (Jiménez *et al.*, 1998; Jiménez and García-Carmona, 1999; Jiménez-Atiénzar *et al.*, 2005a, b). Anthocyanins are not directly oxidized by PPO or are poor substrates (Mathew and Parpia, 1971), but they could be degradated by a coupled oxidation mechanism. Ruenroengklin *et al.* (2009) suggested that litchi PPO directly oxidized epicatechin, then oxidative products of epicatechin, in turn, catalyzed litchi anthocyanin degradation, and finally resulted in the browning reaction, which can account for pericarp browning of postharvest litchi fruit. Similar results were found by Fang *et al.* (2007) which demonstrated that the rate of anthocyanin degradation in bayberry was stimulated by the addition of gallic acid. In the absence of gallic acid, the rate of cyaniding 3-glucoside discoloration was almost the same as when the enzymatic extract was inactivated by heating and gallic acid was present (Figure 10.6).

## E. Laccase

Early studies on enzymatic browning of phenolic compounds identified two types of activity which were initially termed tyrosinase and laccase. The respective systematic names formerly used were *o*-diphenol: oxygen oxidoreductase (EC 1.10.3.1) and *p*-diphenol: oxygen oxidoreductase (EC 1.10.3.2), which are now combined as monophenol monooxygenase (EC 1.14.18.1). The diagnostic feature of laccase is its ability to oxidize *p*-diphenols, a property not possessed by tyrosinase or PPO (Mayer and Harel, 1979). It is present in many plants, fungi, and microorganisms; however, most of the known laccases are of fungal origin, in particular from the white rot fungi



(Thurston, 1994; Revankar and Lele, 2006; Fonseca *et al.*, 2010). The basic laccase-catalyzed reaction responsible for the oxidation of *p*-diphenol is:

Laccase is responsible for the oxidation of flavonoids and browning during *Arabidopsis* seed development (Figure 10.7) and is one of the major enzymes responsible for oxidation of lignin, an amorphous polymer which functions as a cementing material in wood cells. The enzyme acts in cooperation with other ligninolytic (lignin-degrading) fungal enzymes such as lignin peroxidase, manganese peroxidase, and versatile peroxidase (Arora *et al.*, 2002). Laccase alone can only oxidize the phenolic lignin units. Therefore, laccase is often applied with an oxidation mediator, a small molecule able to extend the effect of laccase to non-phenolic lignin units and to overcome the accessibility problem (Srebotnik and Hammel, 2000; Shleev *et al.*, 2006). The mediator is first oxidized by laccase and then diffuses into the cell wall, oxidizing lignin inaccessible to laccase. The use of these mediators in laccase reactions enables the application of the enzyme in the forest industry, removing pitch, phenolic contaminants, and dyes from wood-based materials and water; laccase technology is applicable to virtually the entire production chain of paper products from pulping to recovery of secondary fibers and effluent treatment (Widsten and Kandelbauer, 2008).

## F. Specificity of Polyphenol Oxidase

As discussed previously, PPO catalyzes two different reactions, either the hydroxylation of monophenols to *o*-dihydroxyphenols or the oxidation of *o*-dihydoxyphenols to *o*-quinones. The most appropriate chemical structure with respect to PPO activity, when the reaction rate is at a maximum, appears to correspond to the *o*-dihydroxy structure as evident in such compounds as catechol, caffeic acid, and the catechins (Rocha and Morais, 2001; Gawlik-Dziki *et al.*, 2007). Oxidation of *o*-diphenols to the corresponding *o*-quinones is a general reaction of all known PPO, irrespective of whether the source material is potato, sweet potato (Hyodo and Uritani, 1965), lettuce (Gawlik-Dziki *et al.*, 2008), apple (Harel *et al.*, 1966), tomato (Hobson, 1967), banana (Ünal, 2007), artichoke (Aydemir, 2004), tobacco (Shi *et al.*, 2002), cashew apple (Queiroz *et al.*, 2011), litchi (Ruenroengklin *et al.*, 2009; Yue-Ming *et al.*, 1997), or green olives (Segovia-Bravo *et al.*, 2009). Monophenols

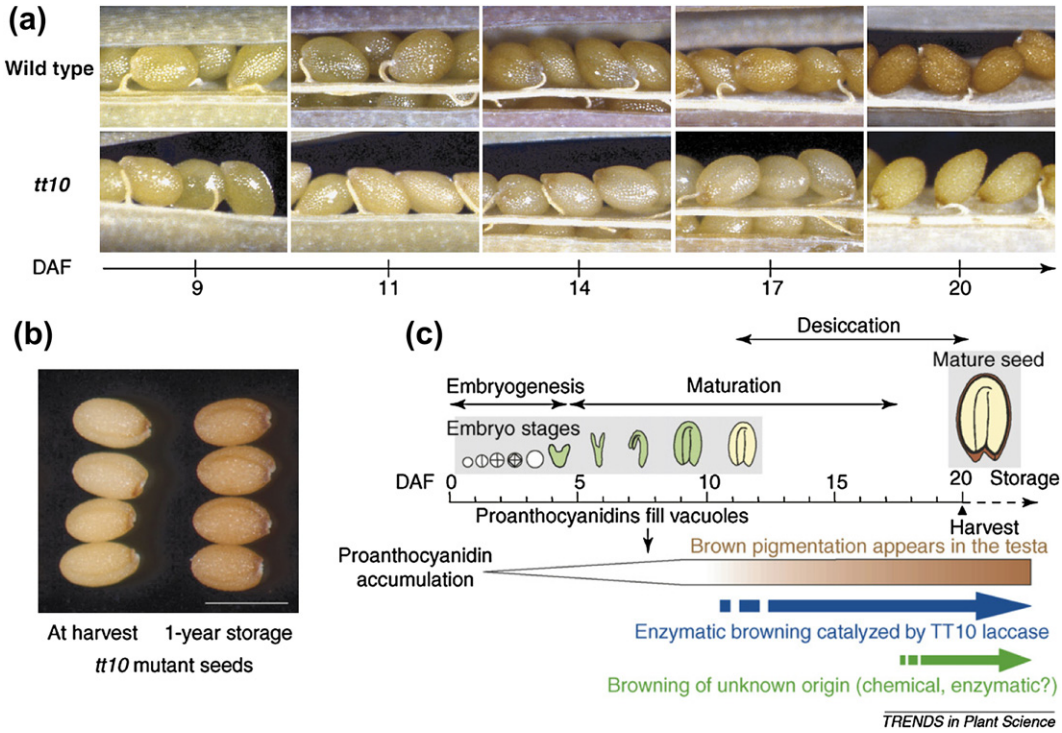



**FIGURE 10.7 Seed coat pigmentation in *Arabidopsis*: illustration of a browning process.** (a) Photographs showing the appearance of a brown pigmentation in the testa of the wild-type genotype during seed desiccation. The brown pigment is absent from the transparent testa 10 (tt10) mutant defective in a laccase enzyme. (b) Mutant tt10 seeds slowly become brown after harvest and eventually resemble wild-type seeds. Scale bar = 550 mm. (c) Schematic drawing indicating the occurrence of brown pigmentation during *Arabidopsis* seed development. DAF: days after flowering *(Reprint from Pourcel* et al., *2006).© 2006, with permission from Elsevier.*

are more slowly acting substrates as they have to be hydroxylated before their oxidation to the corresponding *o*-quinones. The oxidation of monophenols is less widespread than that of diphenols, being catalyzed, for example, by potato and mushroom enzyme preparations. The relationship between cresolase and catecholase activities is not yet fully understood. It appears that many PPOs are specific to a high degree in that they attack only *o*-diphenols. Aydemir (2004), in a study on artichoke PPO, found that it exhibited the highest specificity towards catechol, followed by 4-methycatechol and pyrogallol. According to Espín *et al.* (1998), the best substrates are those with low molecular size substituent side-chain and high electron donor capacity. Erat *et al.* (2006), studying PPO activity in *Ferula* sp., found the best results using catechol and (−)-epicatechin with enzyme extracted from leaf and stem, respectively. Sellés-Marchart *et al.* (2006) found chlorogenic acid to be the most efficient substrate, followed by 4-methylcatechol, 4-*tert*-butylcatechol, epicatechin, catechol, and isoproterenol, for loquat fruit (*Eriobotrya japonica* Lindl.). PPO from Henry chestnuts catalyzed the oxidation of catechol and pyrogallic acid, but had no effect on cresol or tyrosine (Xu *et al.*, 2004). The substrate with the highest activity, for Amasya apple (*Mallus sylvestris* Miller cv. Amasya) PPO, was catechol, followed by 4-methylcatechol, pyrogallol, and 3,4-dihydroxylphenylalanine (L-DOPA) (Oktay *et al.*, 1995). Other kinetic parameters found by researchers are described in Table 10.2.

**TABLE 10.2 Characteristics of Polyphenol Oxidases from Plant Sources**

| Plant | Substrate | $K_m$ (mM) | Optimum temperature (°C) | Optimum pH |
|---|---|---|---|---|
| Apple[a] | Catechol | 34 | 15 | 7.0 |
| | 4-Methylcatechol | 3.1 | | |
| Artichoke[b] | Catechol | 10.2 | 25 | 6.0 |
| | 4-Methylcatechol | 12.4 | | |
| Banana[c] | Catechol | 8.5 | 30 | 7.0 |
| Broccoli[d] | Catechol | 12.3 | – | 5.7 |
| | 4-Methylcatechol | 21.0 | | |
| Cashew apple[e] | Catechol | 18.8 | – | 6.5 |
| Grape[f] | Chlorogenic acid | 3.2 | 25 | 5.0 |
| | Catechin | 4.3 | | |
| Mango[g] | Catechol | 6.3 | 30 | 7.0 |
| Melon[h] | DOPAC [l] | 7.2 | 60 | 7.0 |
| Strawberry[i] | Catechol | 5.9 | 25 | 5.0 |
| Vanilla[j] | Catechol | 85.0 | 37 | 3.4 |
| | 4-Methylcatechol | 10.6 | 37 | 3.0 |
| Yacon root[k] | Caffeic acid | 0.2 | 30 | 6.6 |
| | Chlorogenic acid | 1.1 | | |

[a] *Oktay* et al. *(1995)*
[b] *Aydemir* et al. *(2004)*
[c] *Ünal (2007)*
[d] *Gawlik-Dziki* et al. *(2007)*
[e] *Queiroz* et al. *(2011)*
[f] *Rapeanu* et al. *(2006)*
[g] *Wang* et al. *(2007)*
[h] *Chisari* et al. *(2008)*
[i] *Dalmadi* et al. *(2006)*
[j] *Waliszewski* et al. *(2009)*
[k] *Neves and Silva (2007)*
[l] *3,4-dihydroxyphenylacetic acid.*
Adapted from Queiroz *et al.* (2008).

# III. POLYPHENOL OXIDASE IN FOODS AND FOOD PROCESSING

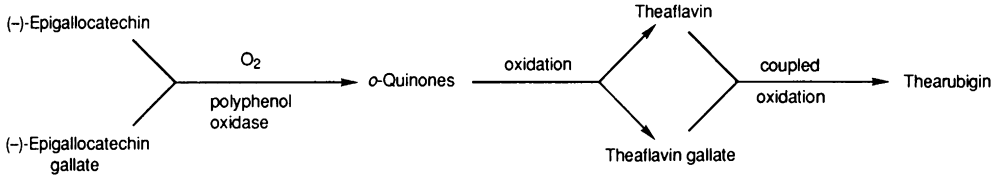
## A. Role in Tea Fermentation

The production of black tea is dependent on the oxidative changes that *Camellia sinensis* leaf polyphenols undergo during processing. Such changes are particularly important for the development of color as well as the reduction of the bitter taste associated with unoxidized tannin (polyphenol compound). Several types of tea, namely white, green, oolong, and black tea, come from the same plant, *C. sinensis*. It is the processing that the tea leaves go through which determines what type they will become and will also alter the phenolic composition of the tea.

The main tea leaf polyphenols, determined by partition chromatography, include (+)-catechin, (−)-epicatechin, (+)-gallocatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate. Of these compounds, (−)-epigallocatechin gallate is the major component in the tea shoot. During tea processing, Muthumani and Kumar (2007) observed that considerable quantities of epigallocatequin gallate, epigallocatechin, and epicatechin gallate were oxidized to form theaflavins and their gallates. Changes in catechin and epicatechin contents were not observed, probably owing to the formation of free gallic acid and catechin from the other catechin fractions such as epigallocatequin gallate, epigallocatechin, and epicatechin gallate, by oxidative degallation. Different results were published by Munoz-Munoz *et al.* (2008). These authors found that the best substrates for PPO in tea leaves are epicatechin followed by catechin, owing to better access of enzyme to hydroxyl radicals.

The production of black tea, the most popular form of the beverage, is carried out in four stages. The first stage is called withering, when the shoots from the tea plant are allowed to dry out. This is followed by rolling with a roller, which disrupts the tea leaf tissue and causes cell damage, providing the necessary conditions for the development of the oxidative processes. The next step is the fermentation of the fragmented tea leaves, which are held at room temperature in a humid atmosphere with a continuous supply of oxygen. These conditions are optimal for PPO action on the tea leaf catechins, which, in addition to reducing astringency, converts the green color of the rolled tea leaves to give coppery-red and brown pigments. Fermentation is terminated by firing, where the tea is dried at 90−95°C and the moisture reduced to 3−4%.

The critical biochemical reaction during tea fermentation is oxidation of catechins by PPO to the corresponding *o*-quinones. These quinones are intermediate compounds which are subjected to secondary oxidation leading to the production of theaflavin and theaflavin gallate, the yellow−orange pigments in black tea, and to a group of compounds referred to as thearubigins. These thearubigins are dark brown and the main contributors to the familiar color of black tea, and they are the oxidative products of theaflavins. A simplified scheme for the oxidative reactions occurring during tea fermentation is outlined in Scheme 10.2. The theaflavin content of tea was shown by Hilton and Ellis (1972) to correlate with the tea taster's evaluation. This was consistent with earlier studies by Roberts (1952) and Sanderson (1964), who noted a positive correlation between tea quality and PPO activity. The enzyme was later purified by Hilton (1972). The oxidative degradation of phloroglucinol rings of the theaflavins by peroxidase caused a loss of theaflavins and a decline in tea quality (Cloughley, 1980a, b). Consequently, the presence of both these enzymes affects the quality of tea. Van Lelyveld and de Rooster (1986) examined the browning potential of black tea clones and seedlings. They found a much higher level of PPO in a high-quality hybrid clone (MT12) compared to a low-quality seedling tea (Table 10.3). The reverse was true for peroxidase, in which lower quality tea had more than double the activity of peroxidase. This suggested that the combination of higher theaflavin levels and PPO activity was responsible for the better quality associated with the MT12 clone. Subramanian *et al.* (1999) studied the role of PPO and peroxidase in the formation of theaflavins. They demonstrated that PPO generates hydrogen peroxide ($H_2O_2$) during oxidation of catechins and peroxidase utilizes the formed $H_2O_2$ for subsequent oxidation of the products of PPO-catalyzed reactions, decreasing the content of theaflavins. The fermentation time is also important to the formation of theaflavins and thearubigins. Muthumani and Kumar (2007) reported an optimum time of 45 minutes, when theaflavins reached their maximum content; after this time their content declined to 20%, leading to a lower quality tea.




**SCHEME 10.2** Oxidative transformations of (−)-epigallocatechin and its gallate during tea fermentation.

**TABLE 10.3** Specific Activity (Δ OD/min/mg protein) of MT12 and Seedling Tea Leaves

| Clone | Peroxidase[a] | Polyphenol Oxidase[b] |
|---|---|---|
| M12 | 1.821 | 0.055 |
| Seedling | 0.735 | 0.020 |

[a]*Significant at* $p < 0.05$
[b]*Significant at* $p < 0.01$.
From Van Lelyveld and de Rooster (1986).

Green tea is particularly popular in Oriental countries, such as Japan. It is an unfermented tea with a light color and a characteristic degree of astringency owing to a high content of catechins. This is achieved by the application of heat during the early stages of tea manufacture, which inhibits or prevents oxidation. Red and yellow teas are intermediate between black and green teas and are semifermented products (partially fermented before firing). An example of the latter is the Chinese variety oolong. Tea leaf catechins generate theaflavins and thearubigins during the longer processing time. White and green teas have higher levels of catechin, whereas oolong and black teas are rich in theaflavins and thearubigins (Table 10.4).

## B. Shrimps and Crustaceans

Enzymatic browning, having been studied extensively in fruits and vegetables, has also been implicated in the discoloration, called melanosis or blackspot, of shrimps and other crustaceans (Zamorano *et al.*, 2009; Giménez *et al.*, 2010), which renders these products unattractive to the consumer and lowers their market value. In crustaceans, PPO is mainly located in the cuticle, specifically on the internal surface, inside chromatophores. During iced storage of crustaceans, the inactive PPO stored in homocytes and digestive glands can also be activated by the action of proteolytic enzymes leaching from the digestive tract. Moreover, protein hydrolysis by these proteases provides substrates for active PPO (Ali *et al.*, 1994).

A recent study by Zamorano *et al.* (2009) evaluated PPO activity *in situ* and in partially purified extracts from different organs of deepwater pink shrimp (*Parapenaeus longirostris*). They found higher enzyme activity in carapace extracts, but marked melanosis developed on the cephalothorax and head after 1 day at 4°C (Figure 10.8). Even after 7 days at 4°C, there was no melanosis in the carapace, confirming that the development of melanosis in the different tissues depends on another factor in addition to PPO levels. In native non-denaturating conditions, a band of 500 kDa was identified which was capable of oxidizing dihydroxyphenylalanine. Table 10.5 shows some biochemical characteristics of PPO extracted from marine sources.

**TABLE 10.4** Flavonoid Composition of Tea (Percent by Dry Weight)

| Component | Green Tea | Black Tea |
|---|---|---|
| Total flavonoids | 15–25 | 15–25 |
| Total catechins | 12–18 | 2–3 |
| (−)-Epicatechin | 1–3 | < 1 |
| (−)-Epicatechin gallate | 3–6 | < 1 |
| (−)-Epigallocatechin | 3–6 | < 1 |
| (−)-Epigallocatechin gallate | 9–13 | 1–2 |
| Flavonols | 2–3 | 1–2 |
| Theaflavins | < 1 | 4 |
| Other polyphenols | 2–4 | 7–15 |

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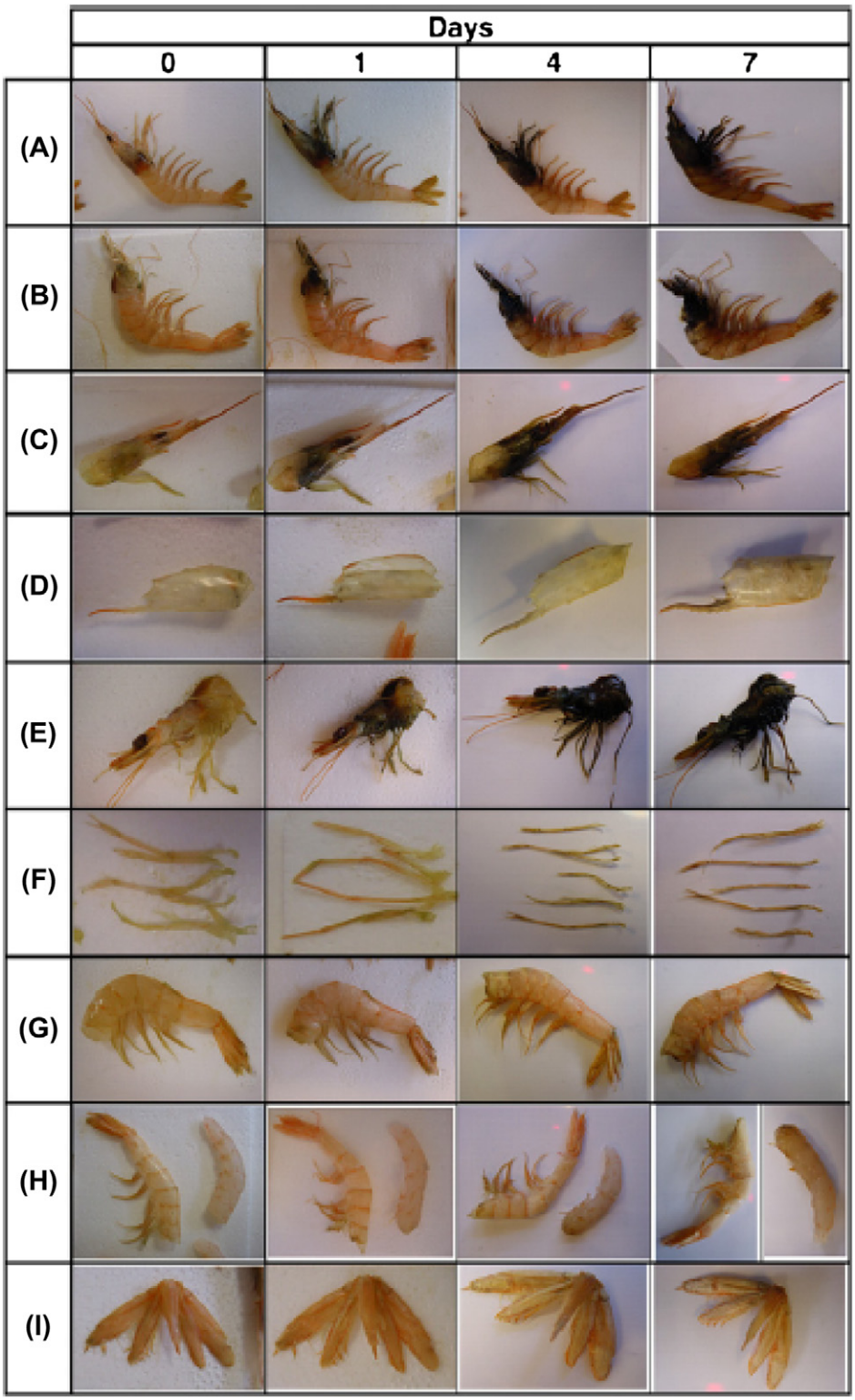



**FIGURE 10.8 Melanosis appearance in different anatomical parts of deepwater pink shrimp during 0, 1, 4, and 7 days of storage at 4°C.** (A) Whole shrimp; (B) Whole shrimp with the carapace removed; (C) Head (carapace + cephalothorax + pereopods and maxillipeds); (D) Carapace; (E) Head with the carapace removed; (F) Pereopods + maxillipeds; (G) Whole abdomen (including the exoskeleton, muscle, pleopods, and telson); (H) Abdomen with the muscle removed (left) and corresponding muscle (right); and (I) Telson *(Reprinted from Zamorano et al., 2009). © 2009, with permission from Elsevier.*

Melanosis inhibitors are used in marine food to prevent browning and extend the shelf-life of products. Traditional methods include the use of sulfite-based formulations, but sulfite causes adverse reactions. Therefore, other compounds, including those of natural origin, have been studied as alternatives to sulfur compounds. Martínez-Alvarez *et al.* (2007) sprayed 4-hexylresorcinol-based formulations on Norway lobsters. After 12 days of storage they

**TABLE 10.5** Characteristics of Polyphenol Oxidases from Marine Sources

| Crustacean | Substrate | $K_m$ | Optimum temperature (°C) | Optimum pH |
|---|---|---|---|---|
| Deepwater pink shrimp[a] | DOPA | 1.85 | 15–60 | 4.5 |
| Prawn[b] | DOPA | – | 40–60 | 5.0 and 8.0 |
| Norway lobster[c] (carapace) | Catechol | 19.40 | 60 | 7.0 |
| Norway lobster[c] (viscera) | Catechol | 5.90 | 60 | 8.0 |

DOPA: 3,4-dihydroxylphenylalanine.
[a]*Zamorano* et al. *(2009)*
[b]*Montero* et al. *(2001)*
[c]*Giménez* et al. *(2010)*.

observed lower PPO activity and melanosis and improvement in the appearance of the lobsters. Cystein and glutathione inhibited the oxidation of L-DOPA catalyzed by kuruma prawn PPO (Benjakul *et al.*, 2006). Among natural substances, recent studies have demonstrated the potential use of phenolic compounds inhibiting browning. Shrimps treated with 2% ferulic acid had, after 10 days of storage, a lower melanosis score and higher scores for color, flavor, and overall likability, compared with the control and sodium metabisulfite-treated shrimps (Nirmal and Benjakul, 2009). Treatment combining catechin and ferulic acid before freeze–thawing was efficient in retarding melanosis in Pacific white shrimp. Phenolics also acted to decrease the growth of psychrophilic bacteria and retard lipid oxidation during the subsequent refrigerated storage (Nirmal and Benjakul, 2010).

Novel technology such as high hydrostatic pressure was studied by Montero *et al.* (2001), achieving partial inactivation of prawn PPO after treatment at 300–400 MPa for 10 minutes. The mechanism of enzyme inactivation by high pressure is discussed in Section IV, D.

## IV. CONTROL OR INHIBITION OF ENZYMATIC BROWNING

The inactivation of PPO is required to minimize product losses caused by browning. Several methods and technologies have been studied. Heat treatment and addition of antibrowning agents are usually applied, but several researchers have proposed the application of other methods as alternatives to thermal processing for PPO inactivation (Queiroz *et al.*, 2008).

Inhibitors of PPO activity can be based on their mode of action, for example, the exclusion of reactants such as oxygen, denaturation of enzyme protein, interaction with the copper prosthetic group, and interaction with phenolic substrates or quinones.

### A. Exclusion of Oxygen

The simplest method of controlling enzymatic browning is by immersing the peeled product, such as potato, in water before cooking. This can be done very easily in the home to limit access of oxygen to the cut potato tissue. This procedure has been used for a long time on a large scale for the production of potato chips and French fries (Talburt and Smith, 1967). The method is limited as the fruit or vegetable will brown on re-exposure to air or via the oxygen occurring naturally in the plant tissues. The removal of oxygen from fruit or vegetable tissue could lead to anaerobiosis if it is stored for extended periods, which in turn could lead to abnormal metabolites and tissue breakdown. In the case of frozen sliced peaches, the surfaces are treated with excess ascorbic acid to use up the surface oxygen.

More recently, modified atmospheres have also been used to prevent enzymatic browning. Since browning is an oxidative reaction, it can be retarded by eliminating oxygen from the cut surface of the vegetables (Limbo and Piergiovanni, 2006). Modified atmosphere packaging has the potential to extend the shelf-life of fruits and vegetables, mainly by limiting the oxidation processes. Low-oxygen and elevated carbon dioxide atmospheres can reduce surface

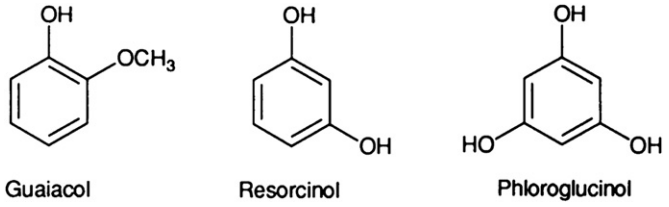
browning. This decrease in the browning phenomenon is accompanied by several physiological effects such as a decrease in respiration rate and a delay in the climacteric onset of the rise in ethylene (Solomos, 1997). Day (1996) hypothesized that high oxygen concentrations may cause substrate inhibition of PPO or, alternatively, high levels of colorless quinones formed may cause feedback inhibition of the enzyme. Gorny *et al.* (2002) found that low-oxygen (0.25 or 0.5 kPa), elevated carbon dioxide (air enriched with 5, 10, or 20 kPa $CO_2$), or high-oxygen (40, 60, or 80 kPa) active atmospheres alone did not effectively prevent surface browning of fresh-cut pear slices. Studies on the influence of modified atmosphere packaging and the use of antioxidant compounds on the shelf-life of fresh-cut pears have mainly focused on the sensory qualities of the commodity (Sapers and Miller, 1998; Gorny *et al.*, 2002; Soliva-Fortuny *et al.*, 2002, 2004). According to Oms-Oliu *et al.* (2008), knowledge about the impact of dipping treatments and packaging conditions on antioxidant properties of fresh-cut pears is still incomplete, especially in regard to high-oxygen active packaging. Y. Kim *et al.* (2007) evaluated the effects of hot water treatment in combination with controlled atmosphere (CA) storage and the resultant changes in quality and antioxidant polyphenolics in mango. They observed that the external quality of mango fruit during ripening was greatly improved by low-oxygen and/or high-carbon dioxide storage conditions and hot water immersion treatments. Carbon dioxide storage delayed fruit ripening, as evidenced by physicochemical changes, and the hot water treatment plus CA storage was an effective treatment combination to extend the postharvest life of mangoes, without adversely changing the nutritional profile of the fruit.

Limbo and Piergiovanni (2006) studied the effects of high oxygen partial pressures in combination with ascorbic and citric acid on the development of the enzymatic browning of peeled and cut potatoes ('Primura' variety), packaged in flexible pouches and stored at 5°C for 10 days. They observed that the treatments with high oxygen partial pressures had some positive effects on enzymatic browning only if the initial atmosphere was close to 100 kPa and the dipping acid concentrations were chosen with care. As Wszelaki and Mitcham (2000) have shown, near 100 kPa oxygen atmospheres could be difficult to maintain either in a package or on a larger scale, as well as being dangerous owing to flammability. A study conducted by Saxena *et al.* (2008) showed that the synergistic effect of antibrowning and antimicrobial compounds with reduced oxygen and elevated carbon dioxide atmosphere could enhance the keeping quality of fresh-cut jackfruit bulbs by minimizing deteriorative changes in physiological, sensorial, and microbial attributes. Martínez-Sánchez *et al.* (2011), studying the influence of oxygen partial pressures ($pO_2$) and light exposure during storage on the shelf-life of fresh-cut Romaine lettuce, noticed that the consumption of oxygen in samples exposed to 24-hour light differed significantly from that of those stored in a 12-hour light/12-hour dark photoperiod or in 24-hour darkness ($10.6 \pm 7.0$, $18.3 \pm 3.5$, and $25.8 \pm 8.6$ nmol $O_2$/kg/second, respectively). Packages exposed to light showed higher $pO_2$ than packages stored in darkness, while those exposed to the photoperiod had intermediate values. This study showed that under light conditions respiration activity was compensated by photosynthesis, resulting in a higher $pO_2$. Thus, browning of fresh-cut Romaine lettuce can be promoted by light exposure during storage as it increases headspace $pO_2$. In fact, uncontrolled light conditions during storage and commercial distribution can contribute to the induction of the browning process, which can cause the product to be unsaleable.

## B. Chemical Inhibitors of Polyphenol Oxidase

Different effectors can control enzymatic browning and these compounds are classified, based on the inhibition mechanism, as reducing agents, chelating agents, acidulants, enzyme inhibitors, enzyme treatments, and complexing agents (Özoğlu and Bayındırlı, 2002).

Several compounds possess chemical structures closely related to *o*-diphenols but do not function as substrates of PPO. These include methyl-substituted derivatives such as guaiacol and ferulic acid (Finkle and Nelson, 1963; Nirmal and Benjakul, 2009), and *m*-diphenols such as resorcinol and phloroglucinol, which have been shown to have an inhibitory effect on PPO activity, either in a competitive way (Montero *et al.*, 2001; Waliszewski *et al.*, 2009) or by a slow-binding inhibition mechanism (Jiménez and García-Carmona, 1997), depending on the substrate.




Guaiacol Resorcinol Phloroglucinol

Since PPO is a metalloprotein in which copper is the prosthetic group, it is inhibited by a variety of chelating agents, including sodium diethyldithiocarbamate (DIECA), sodium azide, potassium ethylxanthate, and ethylenediaminetetraacetate (EDTA). While sodium azide and EDTA inhibit PPO, they appear to be less specific chelators than DIECA or potassium ethylxanthate (Kavrayan and Aydemir, 2001; Aydemir, 2004; Gawlik-Dziki *et al.*, 2007; Pinto *et al.*, 2008; Gao *et al.*, 2009). The latter compounds were reported by Anderson (1968) to combine with the quinones produced by PPO in addition to chelating copper.

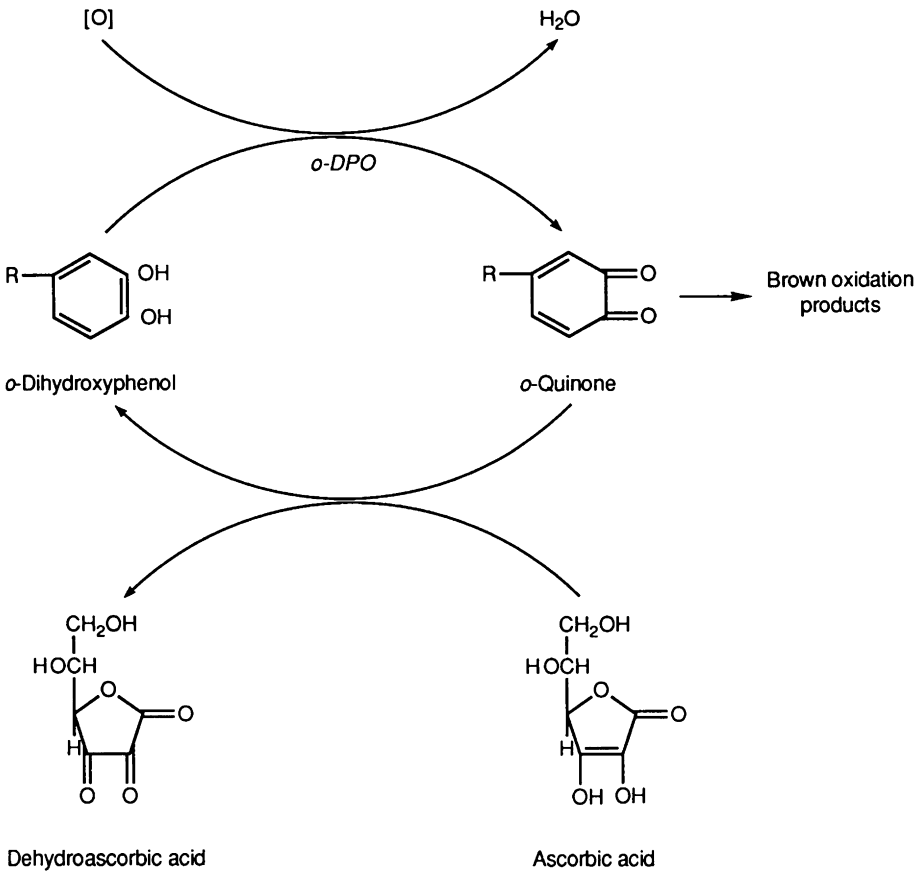
Sulfites are strong inhibitors and have been used for a long time in the food industry. However, an excess of sulfite-based formulations can cause adverse reactions, mainly in asthmatic people. Since it was reported by the 51st meeting of the Joint Expert Committee on Food Additives that the intake of sulfites could be above safe limits, their use as food additives is not recommended. Researchers have been carrying out studies to find potent antibrowning agents which cause no damage to the organism. L-Cysteine has been shown to be an effective inhibitor of PPO by acting as a quinone coupler as well as a reducing agent. It reacts with *o*-quinone intermediates to produce stable and colorless products (İyidoğan and Bayındırlı, 2004). Since Kahn (1985) reported L-cysteine to be the most effective inhibitor of avocado, banana, and mushroom PPO, many studies have shown its effect on the PPO activity of artichoke, apple juice, mango purée, grape, and lettuce (İyidoğan and Bayındırlı, 2004; Guerrero-Beltrán *et al.*, 2005; Rapeanu *et al.*, 2006; Altunkaya and Gökmen, 2009). However, effective concentrations of cysteine produce an undesirable odor, limiting its use in food processing. Altunkaya and Gökmen (2009), trying to select the most effective antibrowning compound on fresh lettuce, evaluated the effectiveness of ascorbic acid, cysteine, citric acid, and oxalic acid. They found that lettuce treated with oxalic acid and ascorbic acid maintained a higher level of phenolic compounds than citric acid and cysteine. Interestingly, cysteine had no positive effect on the prevention of oxidation of phenolic compounds even though it prevented browning in lettuce.

The application of acids to control enzymatic browning is used extensively. The acids employed are those found naturally in plant tissues, including citric, malic, phosphoric, and ascorbic acids. This method is based on the fact that lowering the tissue pH will reduce or retard the development of enzymatic browning. The optimum pH of most PPO lies between pH 4.0 and 7.0, with little activity below pH 3.0, as illustrated in Table 10.2. Muneta (1977) examined the effect of pH on the development of melanins during enzymatic browning. Although, as discussed earlier, the initial reaction involving the formation of quinone is enzyme catalyzed, polymerization of these quinones to the brown or brown−black melanins is essentially non-enzymatic. Since both are pH dependent, Muneta (1977) studied the effect of pH on the non-enzymatic reactions leading to the formation of melanins in potatoes. The formation of dopachrome from dopaquinone was monitored in phosphate buffers at pH 5.0, 6.0, and 7.0. Very rapid melanin development occurred at pH 7.0, compared to a rather slow process at pH 5.0. This observation could be important to the processor, particularly if the lye-peeled potatoes are inadequately washed, resulting in a high surface pH that facilitates enzymatic browning and melanin formation.

Citric acid has been used in conjunction with ascorbic acid or sodium sulfite as a chemical inhibitor of enzymatic browning (Limbo and Piergiovanni, 2006; Ducamp-Collin *et al.*, 2008; Hiranvarachat *et al.*, 2011; Queiroz *et al.*, 2011). Cut fruit or vegetables are often immersed in dilute solutions of these acids just before processing. This is particularly important in the case of lye-peeled cling fruits, where the acid dip counteracts the effect that any residual lye might have on enzymatic browning. Citric acid also inhibits PPO by chelating the copper moiety of the enzyme. Compared to citric acid, however, malic acid, the principal acid in apple juice, is a much more effective inhibitor of enzymatic browning.

A particularly effective inhibitor of PPO is ascorbic acid (Özoğlu and Bayındırlı, 2002). It does not have a detectable flavor at the level used to inhibit this enzyme, nor does it have a corrosive action on metals. This vitamin acts as an antioxidant because it reduces the quinone produced before it undergoes secondary reactions that lead to browning, and also decreases the pH for the enzyme activity (Guerrero-Beltrán *et al.*, 2005). The mode of action of ascorbic acid is outlined in Scheme 10.3 (Walker, 1976). Ascorbic acid can inhibit the enzyme by decreasing pH and acting as an antioxidant. Thus, it reduces quinones to diphenols, reversing the reaction and decreasing brown pigments. Degl'Innocenti *et al.* (2007) evaluated lettuce, escarole, and rocket salad and their sensitivity to enzymatic browning. They observed that the resistance of rocket salad to browning was associated with the highest ascorbic acid content compared to other species. A study by Hsu *et al.* (1988) compared the effectiveness of several ascorbic acid derivatives, including dehydroascorbic acid, isoascorbic acid, ascorbic acid-2-phosphate, and ascorbic acid-2-sulfate, with that of ascorbic acid. On the basis of kinetic studies they found ascorbic acid and isoascorbic acid to be the most effective inhibitors of mushroom PPO, followed by dehydroascorbic acid. According to Özoğlu and Bayındırlı (2002), ascorbic acid has been shown to

**SCHEME 10.3** Reduction by ascorbic acid of the primary quinone oxidation products of enzymatic browning *(Walker, 1976)*.




be more effective than its isomer isoascorbic acid. The effect of ascorbic acid can be considered temporary because it is oxidized irreversibly by reaction with intermediates, such as pigments, endogenous enzymes, and metals such as copper. In apple juice, an inhibitory effect of ascorbic acid at a concentration of 1.8 mM was observed for 4 hours.

An adequate amount of ascorbic acid must be added to food material to delay enzymatic browning. In fruit juices treated with ascorbic acid, autooxidation of ascorbic acid, or natural ascorbic acid oxidase activity, will use up any dissolved oxygen in the fruit juice. Thus, oxygen would become the limiting factor determining the rate of enzymatic browning. The addition of ascorbic acid at a concentration of 300 mg per pound (0.454 kg) of fruit was shown by Hope (1961) to control browning as well as reduce headspace oxygen in canned apple halves. This method was particularly effective in controlling enzymatic browning in spite of the thickness of the apple tissue and its comparatively high oxygen content.

Muneta (1977) suggested sodium acid pyrophosphate as an alternative inhibitor of enzymatic browning. It has several advantages over the organic acids, being much less sour than citric acid, as well as minimizing after-cooking blackening of potatoes by complexing iron. An additional benefit of chelating iron is to limit its role in the catalysis of rancidity in fried or dehydrated potatoes.

Kojic acid is found in many fermented Japanese foods. Although it is present in certain foods as a natural fermentation product, the use of kojic acid in the food industry may be restricted by the difficulties of large-scale production and its high cost. İyidoğan and Bayındırlı (2004) showed a significant antibrowning effect of kojic acid in concentrations ranging from 1 to 4 mM. This agent inhibited PPO and bleached melanin through chemical reduction of the browning pigment to colorless compounds.

Benzoic and cinnamic acids, aromatic carboxylic acids, are PPO inhibitors owing to their structural similarities with the phenolic substrates. Undissociated forms of these acids are able to inhibit PPO through complexation with copper at the active site of the enzyme (Marshall *et al.*, 2000). Rapeanu *et al.* (2006) reported a weak inhibition at 0.5 mM (23%) when benzoic acid was tested in grapefruit.

Cyclodextrins are a family of cyclic oligosaccharides composed of α-1,4-linked glucopyranose subunits. Cyclodextrins are produced from starch by enzymatic degradation. These macrocyclic carbohydrates with apolar internal cavities can form complexes with and solubilize many normally water-insoluble compounds (Singh *et al.*, 2002). β-Cyclodextrins bind substrate in its hydrophobic core. The most important functional property of cyclodextrins is their ability to form inclusion complexes with a wide range of organic guest molecules, including PPO substrates (Irwin *et al.*, 1994). Because they can form inclusion complexes the properties of the materials with which they complex can be modified significantly. As a result of molecular complexation phenomena cyclodextrins are widely used in many industrial products, technologies, and analytical methods (Martin Del Valle, 2004). Özoğlu and Bayındırlı (2002), using concentrations ranging from 0.3 to 1.8 mM, showed no effect of β-cyclodextrin, indicating that a higher amount of the compound was necessary to achieve inhibition. In addition, PPO activity in apple juice was affected by the type of cyclodextrin (López-Nicolás *et al.*, 2007). To achieve efficiency in PPO inhibition, food industries may pay attention to the major phenolic compounds present in the product and the type of β-cyclodextrins used.

Sodium chloride is a strong oxidizing agent which can generate chlorine dioxide under acidic conditions. Below pH values of about 5 a strongly pH-dependent inhibitory effect is observed and the degree of inhibition increases with the acidity of the reaction medium. In contrast, an activation effect is observed at higher pH values (Valero and García-Carmona, 1998; M. H. Fan *et al.*, 2005). Severini *et al.* (2003) evaluated the effect of sodium chloride on the prevention of enzymatic browning in sliced potatoes and observed that all the considered blanching treatments allowed PPO inactivation. With regard to color, the use of calcium chloride, at low concentrations, would seem better than the use of sodium chloride.

These differences in the mechanisms allow the use of combinations of antibrowning agents that may result in enhancement of inhibition. A combination of ascorbic acid, cysteine, and cinnamic acid showed a synergistic effect compared to the individual compounds in cloudy apple juice (Özoğlu and Bayındırlı, 2002). İyidoğan and Bayındırlı (2004) obtained 89% of enzymatic inhibition in Amasya apple juice treated with 3.96 mM L-cysteine, 2.78 mM, kojic acid, and 2.34 mM 4-hexylresorcinol (4-HR). Also in apple juice, a synergic effect was observed between β-cyclodextrin and 4-HR which was not observed for the combination of β-cyclodextrin and methyl jasmonate. In the first combination, β-cyclodextrin reduced the concentration of free substrate that could be oxidized, while 4-HR interacted directly with the enzyme by a competitive mechanism. In the second case, β-cyclodextrin complexed with the substrate and methyl jasmonate, increasing the amount of free substrate and decreasing the concentration of methyl jasmonate that could inhibit PPO activity (Alvarez-Parrilla *et al.*, 2007). The inhibition by methyl jasmonate observed in this study might be an exception, since some authors have described PPO activation by this compound (Koussevitzky *et al.*, 2004; Melo *et al.*, 2006).

Some natural agents have an antibrowning effect. Honey contains a number of components that act as preservatives, such as α-tocopherol, ascorbic acid, flavonoids, and other phenolic compounds. Honey from different floral sources reduced PPO activity by 2−45% in fruit and vegetable homogenates. When combined with ascorbic acid, this inhibitory effect is enhanced (Chen *et al.*, 2000). It was also shown that native procyanidins, flavanol polymers that occur naturally in plants, inhibited PPO activity in cider apple juices and the inhibition intensity increased with degree of polymerization of the procyanidins. The mechanism is probably due to the binding of the polyphenol to the protein, affecting the catalytic activity of the enzyme or by forming an inactive enzyme−polyphenol−substrate complex (Le Bourvellec *et al.*, 2004). Lee and Park (2005) tested Maillard reaction products, synthesized from various amino acids and sugar solutions, on potato PPO activity and observed an inhibitory effect which was dependent on the amino acid (arginine > cysteine > histidine > lysine) and the type of sugar used (monosaccharides > disaccharides).

Proteins, peptides, and amino acids can affect PPO activities by reacting with the *o*-quinones and by chelating the copper at the active site of PPO. Girelli *et al.* (2004) measured PPO activity in the presence of various glycyl-dipeptides and they found that these compounds can affect PPO activities by reacting with *o*-quinones and by chelating the copper at the active site of PPO. Glycylaspartic acid, glycylphenilalanine, glycylglycine, glycyllysine, glycyltyrosine, and glycylhistidine affected the formation of quinone at all concentrations used, varying from 1 to 50 mM. In a study conducted by Shi *et al.* (2005), different inhibition types between cinnamic acid and its derivatives were demonstrated. Strength and inhibition types were: cinnamic acid (non-competitive) > 4-hydroxycinnamic acid (competitive) > 4-methoxycinnamic acid (non-competitive). No inhibitory effect of 2-hydroxycinnamic acid on the diphenolase activity was found. According to the authors, these inhibitors were attached to a different region from the active site and hindered the binding of substrate to the enzyme through steric

hindrance or by changing the protein conformation. Chen *et al.* (2005) demonstrated that PPO was inhibited by several *p*-alkoxybenzoic acids and that *p*-methoxybenzoic acid was the most potent inhibitor. Song *et al.* (2006) studied the inhibitory effects of *cis*- and *trans*-isomers of 3,5-dihydroxystilbene. Although both compounds inhibited PPO activity, the *cis*-form had higher inhibitory ability because it can bind more tightly to the active site of the enzyme than its isomer.

Oms-Oliu *et al.* (2010) reviewed some recent advances in the maintenance of fresh-cut fruit quality with respect to the use of chemical compounds, including plant natural antimicrobials and antioxidants, as well as calcium salts for maintaining texture. They focused on the use of natural preservatives, which are of increasing interest because of the toxicity and allergenicity of some traditional food preservatives, and on the difficulties in the application of these substances to fresh-cut fruit without adversely affecting the sensory characteristics of the product. Edible coatings are presented as an excellent way to carry additives since they have been shown to maintain high concentrations of preservatives on the food surface, reducing the impact of such chemicals on overall consumer acceptability of fresh-cut fruit.

## C. Thermal Processing

Heat treatment is the most widely used method for stabilizing foods because of its capacity to destroy microorganisms and to inactivate enzymes. Blanching is the most common method used to inactivate vegetable enzymes (Marshall *et al.*, 2000). It causes denaturation and therefore inactivation of the enzymes but also causes destruction of thermosensitive nutrients, and it is rarely used for soft fruits (Lado and Yousef, 2002) because it results in losses in vitamins, flavor, color, texture, carbohydrates, and other water-soluble components.

The inactivation of PPO as well as other spoilage enzymes can be achieved by subjecting the food article to high temperatures for an adequate length of time to denature the protein. In general, exposure of PPO to temperatures of 70−90°C destroys their catalytic activity, but the time required for inactivation depends on the product (Chutintrasri and Noomhorm, 2006). Fortea *et al.* (2009), studying thermal inactivation of grape PPO and peroxidase, described that they showed similar thermostability, losing over 90% of relative activity after only 5 minutes of incubation at 78°C and 75°C, respectively. Khandelwal *et al.* (2010) estimated the concentrations of polyphenol in different cultivars of four pulses commonly consumed in India and examined the effects of domestic processing. They demonstrated that processing reduced the concentrations of phenolic compounds by 19−59%. Chutintrasri and Noomhorm (2006), studying thermal inactivation of pineapple PPO, described that the enzyme activity reduced by approximately 60% after exposure to 40−60°C for 30 minutes. Denaturation increased rapidly above 75°C. Thus, residual activity was about 7% after 5 minutes at 85°C and 1.2% after 5 minutes at 90°C. In another study, to determine the effect of heating conditions on enzymatic browning, Krapfenbauer *et al.* (2006) evaluated heated apple juice from eight different varieties of apples at high temperature (60−90°C) and short time (20−100 seconds) (HTST) combinations. The results showed that HTST treatment at 80°C already inactivated PPO, whereas pectinesterase activity was reduced to half and could not be inactivated completely, even at 90°C. The highest residual pectinesterase activity was found at 60°C. Heating at 70°C caused stable pectinesterase activity and even a slight increase for 50 and 100 second heating times. In Victoria grape PPO (Rapeanu *et al.*, 2006), complete inactivation was reported after 10 minutes at 70°C (Figure 10.9). The activity of PPO from *Castanea henryi* nuts after incubation at 70°C for 30 minutes was 8% (Xu *et al.*, 2004).

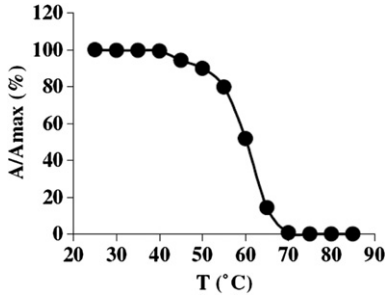



**FIGURE 10.9 Thermal stability of Victoria grape polyphenol oxidase extract. Residual activity was measured after a 10-minute treatment at different temperatures.** *(Reprinted from Rapeanu* et al.*, 2006). © 2006, with permission from Elsevier.*

The heat inactivation of enzymes in foods is not only dependent on time but is also affected by pH. The optimum temperature for PPO varies considerably for different plant sources as well as among cultivars, as shown in Table 10.2, and with the substrate used in the assay.

## D. High-Pressure Processing

High hydrostatic pressure (HHP) treatment of fruit and vegetable products offers a natural, environmentally friendly alternative to pasteurization and the chance to produce food of high quality, greater safety, and increased shelf-life (Welti-Chanes *et al.*, 2005). HHP reduces microbial counts and inactivates enzymes (Bayındırlı *et al.*, 2006). The HHP treatment is expected to be less detrimental than thermal processes to low-molecular-mass food compounds such as flavoring agents, pigments, and vitamins, as covalent bonds are not affected by pressure (Butz *et al.*, 2003). In cashew apple juice processed by HHP at 250 or 400 MPa for 3, 5, and 7 minutes, there was no change in pH, acidity, total soluble solids, ascorbic acid, or hydrolyzable polyphenol content. These data demonstrate that HHP can be used in the food industry, generating products with higher nutritional quality (Queiroz *et al.*, 2010). Perera *et al.* (2010), studied cubes of two different varieties of apple, which were vacuum packed in barrier bags with 0−50% (v/v) pineapple juice and subjected to HHP at 600 MPa for 1−5 minutes (22°C). They observed no visible color changes during 4 weeks of storage in these bags. However, they also noticed that the texture and the in-pack total color change after 5 hours' air exposure were significantly affected by the apple variety, HHP time, and percentage of pineapple juice used. The combined treatment significantly reduced residual PPO activity, whereas pectinmethylesterase activity was not affected in either variety.

HHP can affect protein conformation and lead to protein denaturation, aggregation, or gelation, depending on the protein system, the applied pressure, the temperature, and the duration of the pressure treatment. Protein denaturation is associated with conformational changes and it can change the functionality of the enzyme by increasing or losing biological activity or changing substrate specificity. The effectiveness of treatment depends on the type of enzyme, pH, medium composition, temperature, time, and pressure level applied (Hendrickx *et al.*, 1998).

For several PPOs, it has been reported that pressure-induced inactivation proceeds more rapidly at lower pH and that it is influenced by the addition of salts, sugars, or chemical antibrowning effectors (Rapeanu *et al.*, 2005). It is known that PPO is more resistant to pressure than thermal treatment, when compared to other enzymes (Y.-S. Kim *et al.*, 2001). In general, HHP is more effective at pressures above 600 MPa (Figure 10.10), but the combination of these methods increases the effectiveness of inactivation. The best results from studies aiming to decrease PPO activity in banana purée (Palou *et al.*, 1999), litchi (Phunchaisri and Apichartsrangkoon, 2005), and carrot juice (Y.-S. Kim *et al.*, 2001) were obtained at pressures higher than 400 MPa combined with mild heat (50°C). In contrast, low pressure (up to 400 MPa) induced PPO activation in pear (200−400 MPa, 25°C, 10 minutes) (Asaka and Hayashi, 1991) and apple juice (100 MPa, 1 minute) (Anese *et al.*, 1995).

HHP can be used to create new products (new texture or new taste) or to obtain analogue products with minimal effects on flavor, color, and nutritional value, and without any thermal degradation (Messens *et al.*, 1997). Pressure can also influence biochemical reactions by reducing molecular spacing and increasing interchain reactions (Marshall *et al.*, 2000).

## E. Gamma Irradiation

Irradiation is a physical treatment involving direct exposure to electrons or electromagnetic rays, for food preservation and improvement of safety and quality. For these reasons, it can be used to extend the shelf-life of fruits and vegetables. Radiation inactivates microorganisms, guaranteeing disinfection and delaying the ripening process and senescence (Lacroix and Ouattara, 2000). In addition, retention of ascorbic acid and synthesis of polyphenols during storage of irradiated food have been reported (Moussaid *et al.*, 2000; Zhang *et al.*, 2006).

Low-dose γ-irradiation is commonly applied to fruit and vegetable products to extend shelf-life. Prakash *et al.* (2000) noticed that treatment with a dose of 0.35 kGy decreased 1.5 and 1 log of total aerobic microorganisms and yeasts and molds in cut Romaine lettuce, respectively, and that this dose did not adversely affect sensory attributes, such as visual quality or off-flavor development. Latorre *et al.* (2010) observed the effect of low doses of γ-irradiation (1 or 2 kGy) on PPO activity of fresh-cut red beetroot and concluded that it produced biochemical changes in cellular contents as well as in the cell wall constitutive networks which could not necessarily be sensed by consumers. They also mentioned changes involving an increase in the antioxidant capacity of red beetroot tissue, showing that the studied doses could be used in the frame of a combined technique for red beet processing. D. Kim *et al.* (2007) related

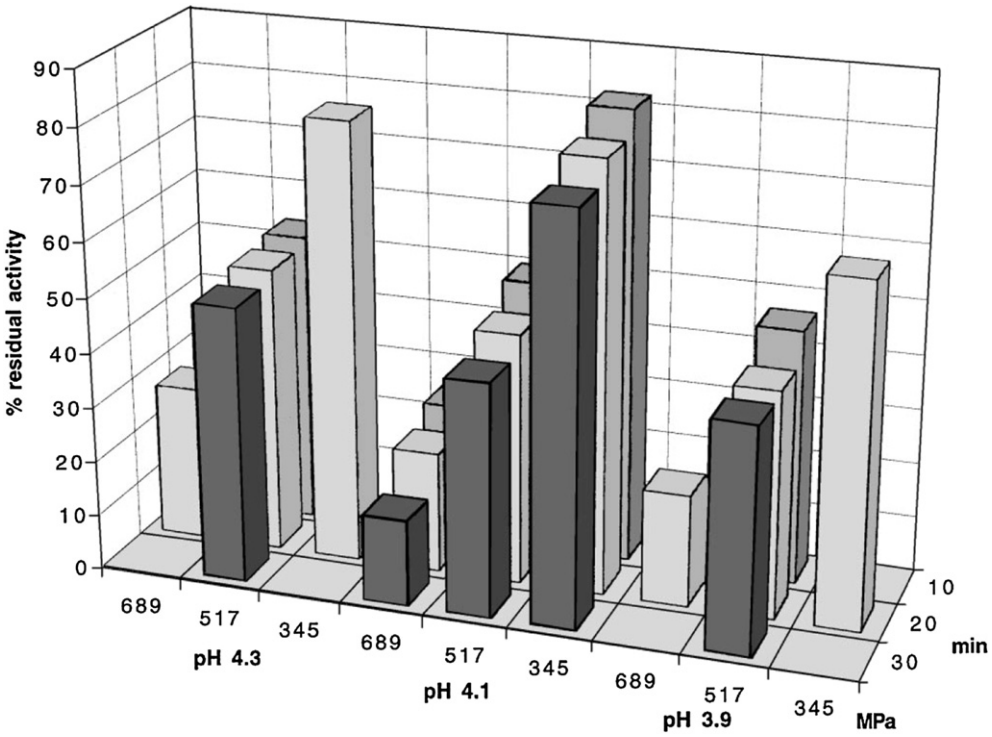



**FIGURE 10.10 Effect of high hydrostatic pressure treatments and initial pH on residual polyphenol oxidase activity of avocado purée.** *(Reprinted from Lopez-Malo* et al.*, 1998). © 1998, with permission from Elsevier.*

that total aerobic bacteria in fresh kale juice, prepared by a general kitchen process, were detected in the range of $10^6$ cfu/ml, and about $10^2$ cfu/ml of the bacteria survived in the juice in spite of γ-irradiation treatment with a dose of 5 kGy. In the inoculation test, the growth of the surviving *B. megaterium* and *E. acetylicum* in the 3−5 kGy-irradiated kale juice retarded and/or decreased significantly during a 3 day postirradiation storage period. However, there were no significant differences in the residual PPO activity and browning index between the non-irradiated control and the irradiated kale juice during the postirradiation period. Lu *et al.* (2005) showed a decrease in PPO activity of 73% in fresh-cut celery treated with 1.0 kGy (0.5 kGy/hour) after 3 days under refrigeration (4°C). In 9 days, the enzyme showed an activity around 25% lower than the control sample.

The use of irradiation in combination with other methods, or chemical antibrowning inhibitors, can result in products that are microbiologically safe and of high quality (X. Fan *et al.*, 2005).

## F. Pulsed Electric Field

Pulsed electric field (PEF) is a non-thermal food preservation technology, used as an alternative to other conventional techniques, which consists of introducing the food into a chamber containing two electrodes that apply high-voltage pulses (20−80 kV) for a short time (microseconds).

In the past few years, several studies have demonstrated the ability of intense treatments to obtain safe and shelf-stable liquid foods. Novel applications such as the improvement of mass transfer processes or the generation of bioactive compounds using moderate field strengths are currently under development (Soliva-Fortuny *et al.*, 2009).

This emerging food processing technology is being investigated owing to its ability to inactivate undesirable microorganisms and enzymes, with limited increase in food temperature. As a result, more stable foods with fewer changes in composition, physicochemical properties, and sensory attributes can be obtained (Martín-Belloso and Elez-Martínez, 2005). The PEF applied to the food causes electroporation, that is, the irreversible loss of cell membrane functionality, leading to inactivation of the microbial cell (Zhong *et al.*, 2005; Cserhalmi *et al.*, 2006), and it also induces changes in the secondary structure of some enzymes. After PEF treatment, the loss of the relative α-helix fractions of PPO and peroxidase were calculated and these results showed that PPO was more susceptible to the treatment than peroxidase (Zhong *et al.*, 2007).

There are many studies in the literature focusing on the effect of PEF in the inactivation of microorganisms (García *et al.*, 2003, 2005; Evrendilek *et al.*, 2004; Li and Zhang, 2004), but few about the effect of this technology on PPO activity (Giner *et al.*, 2001, 2002; Zhong *et al.*, 2007). The inactivation of commercial PPO depends on the electric field strength and treatment time. The greatest reduction in PPO activity was 76.2% at 25 kV/minute for 744 μs (Zhong *et al.*, 2007). Giner *et al.* (2001, 2002) reported a decrease of 97% on the PPO activity in apple extract at 24.6 kV/cm for 6000 μs, 72% in pear at 22.3 kV/cm for the same treatment time, and 70% in peach PPO at 24.3 kV/cm for 5000 μs. Riener *et al.* (2008) observed a decrease of 71% in PPO activity using a combination of pre-heating to 50°C and a PEF treatment time of 100 μs at 40 kV/cm in freshly prepared apple juice. This level of inactivation was significantly higher ($p < 0.05$) than that recorded in juice processed by conventional mild pasteurization, where the activity of PPO decreased by 46%.

According to Yang *et al.* (2004) the sensitivity to PEF treatment varies from enzyme to enzyme. The sequence of sensitivity to PEF of five enzymes tested was: pepsin > PPO > peroxidase > chymotrypsin and lysozyme. The inactivation effect of PEF on enzymes was affected by electric field strength, electrical conductivity, and pH.

## G. Other Technologies

Although most studies have focused on HHP, irradiation, and PEF treatments, other emerging techniques may hold promise for food technology.

Ohmic heating is defined as a process in which electric currents are passed through foods in order to heat them. The heating method affects the temperature distribution inside a food, and directly modifies the time−temperature relationship for enzyme deactivation. Ohmic heating is distinguished from other electrical heating methods by the presence of electrodes contacting the foods, the frequency, and the waveform of the electric field imposed between the electrodes. Icier *et al.* (2008) evaluated fresh grape juice ohmically heated at different voltage gradients (20, 30, and 40 V/cm) from 20°C to 60, 70, 80, or 90°C and the activity of PPO. The critical inactivation temperatures were found to be 60°C or lower for 40 V/cm, and 70°C for 20 and 30 V/cm. The activation energy of PPO inactivation for the temperature range 70−90°C was 83.5 kJ/mol. According to Castro *et al.* (2004), the inactivation kinetics of PPO were first order for conventional and ohmic heating treatments. These authors demonstrated that, using this technology, the time needed for enzyme inactivation was reduced. Icier *et al.* (2006) showed that ohmic heating can also be used for blanching food products. After treatment by ohmic blanching, pea purée peroxidase was inactivated in a shorter processing time than by conventional water blanching.

Supercritical carbon dioxide is a non-thermal technology in which a pressurization step ensures that the applied gas penetrates the microbial cells and subsequent explosive decompression results in rapid gas expansion within the cells, physically destroying them (Corwin and Shellhammer, 2002). Supercritical carbon dioxide has an effect on enzyme inactivation due to conformational changes caused by gas in the secondary and tertiary structure (Gui *et al.*, 2007). According to a study conducted by Gui *et al.* (2007), cloudy apple juice exposed to supercritical carbon dioxide at 30 MPa and 55°C for 60 minutes presented a reduction in PPO activity of over 60%, compared to a 27.9% reduction obtained under atmospheric conditions at 55°C, indicating that the combined effects of pressure, temperature, and time occurred after carbon dioxide treatment. Corwin and Shellhammer (2002) demonstrated the inhibition of PPO activity, with a residual activity of 57.6% (500 MPa, 3 minutes at 50°C), even when the carbon dioxide was added before HHP. In this study, addition of carbon dioxide decreased the PPO activity significantly at all pressures (0, 500, and 800 MPa) and temperatures (25 and 50°C) beyond the effects of pressure alone. Niu *et al.* (2010) evaluated the qualities of cloudy apple juices from apple slices treated with high pressure carbon dioxide and mild heat, and observed that PPO was completely inactivated and its minimal residual activity at 65°C was 38.6%.

Ultrasound is a non-thermal technology; it causes enzyme inactivation by cell lysis using vibration energy, which produces cavitation bubbles and temporarily generates spots of extremely high pressure and temperature when imploded (Morris *et al.*, 2007). Ultrasonication has been found to be more effective in inhibiting enzyme activity when combined with other processes, such as high pressure and/or heat, contrary to the minimal inhibitory effects of individual application. The effects of ultrasound and ascorbic acid on changes in activity of PPO and peroxidase of fresh-cut apple during storage were investigated by Jang and Moon (2011) and Jang *et al.* (2009). They found that the combined treatment inactivated monophenolase, diphenolase, and peroxidase, whereas individual treatment with ultrasound or ascorbic acid had an inverse and limited inhibitory effect on the enzymes. This investigation revealed that simultaneous treatment with ultrasound and ascorbic acid had synergistic inhibitory effects on several enzymes related to enzymatic browning.

Blanching in hot water or steam has been widely used for thermal inactivation of undesirable enzymes, including PPO. Because thermal treatments are also responsible for undesirable changes in tissue texture, several other methods have been tried to inhibit PPO activity and avoid color changes in fruits (Lamikanra, 2002). Microwave heating is an alternative method for liquid food pasteurization and, compared to the conventional method, microwaves can heat products internally, and have greater penetration depth and faster heating rates that could potentially improve the retention of thermolabile constituents in food (Heddleson and Doores, 1994; Deng *et al.*, 2003). According to Cañumir *et al.* (2002), microwave energy induces thermal effects in microorganisms and enzymes similar to those of conventional heating mechanisms. Matsui *et al.* (2007) submitted solutions simulating the chemical constituents of coconut water to a batch process in a microwave oven, and observed that PPO activity in water and in sugar solution reduced after treatment. In salt solution, the stability of PPO was significantly affected and the contact between salt and enzyme promoted a drastic reduction in the initial activity. The authors found that, at temperatures above 90°C, the combined effects of salts and microwave energy reduced enzymatic activity to undetectable levels. However, at 90°C, the inactivation effect could be due to temperature alone. In a further study, Matsui *et al.* (2008) showed that thermal inactivation of PPO during microwave processing of green coconut water was significantly faster than conventional processes reported in the literature.

Short-wave ultraviolet light (UV-C) radiation is an alternative and inexpensive method to reduce the number of microorganisms on the surface of fresh and cut fruits and vegetables (Yaun *et al.*, 2004; Fonseca and Rushing, 2006; Shama, 2006). The potential of UV-C light for commercial use in minimal processing of fruits depends on its ability to contribute to food safety without causing undesirable quality changes. Few studies have been conducted on the influence of UV-C on produce quality (color, texture, taste, and aroma) during storage. The reported effects are quite diverse depending on the type of produce and the dosages applied (Shama, 2006). Although it has been claimed that UV-C treatment does not produce undesirable by-products that could change taste, odor, and color, skin discoloration in tomatoes, browning of calyxes in strawberries, and increasing susceptibility to brown rot in peaches have been reported (Shama and Alderson, 2005). Erkan *et al.* (2001) found that high doses of UV-C caused a slight reddish-brown discoloration on the surface of zucchini squash. Gómez *et al.* (2010) examined the effect of UV-C irradiation at different doses, associated with some pretreatments (hot water blanching, dipping into a solution containing ascorbic acid and calcium chloride) in order to minimize browning on the surface color of fresh-cut apple disks. They found that the color and compression parameters were dependent on UV-C dose, storage time, and type of pretreatment. Samples exposed to only UV-C light turned darker and less green compared to fresh-cut apple slices or to samples on day 0, and this effect was more pronounced at the greatest UV-C dose. Light microscopic images showed the breakage of cellular membranes in UV-C-treated samples, which may explain the increase in browning of irradiated apples.

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