PHYSICOCHEMICAL PROPERTIES AND FUNCTION OF PLANT POLYPHENOL OXIDASE: A REVIEW[1]

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ABSTRACT

Polyphenol oxidase (PPO)-catalyzed browning reactions are of significant importance in the fruit and vegetable industry. These reactions proceeding in many foods of plant origin cause deterioration and loss of food quality. A better knowledge of the factors that influence the action of PPO is imperative in order to control and manipulate its detrimental activity in plant products. This paper presents an overview of the current understanding of the reaction properties, biochemical characteristics and potential physiological roles of PPO in plants. Reaction properties will include general PPO reactions, specificities and molecular mechanisms of these reactions, and methods available to assess PPO activity. Physicochemical properties will evaluate substrate specificity, environmental influences such as pH and temperature, multiplicity, latency, and activators and inhibitors of PPO. The discussion will conclude with potential physiological roles of PPO in plants.

INTRODUCTION

A plethora of studies show that polyphenol oxidase (PPO; 1,2-benzenediol: oxygen oxidoreductase; EC 1.10.3.1), also known as tyrosinase, polyphenolase, phenolase, catechol oxidase, cresolase, or catecholase is widely found in nature (Whitaker 1994, 1996). PPO is typically present in the majority of plant tissues (Vamos-Vigyazo 1981; Zawistowski *et al.* 1991; Sherman *et al.* 1991, 1995;

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Fraignier *et al.* 1995; Haruta *et al.* 1999). Because of its involvement in adverse browning of plant products, PPO has received much attention from researchers in the field of plant physiology and food science. Enzymatic browning occurs as a result of the oxidation by PPO, of phenolic compounds to quinones and their eventual (nonenzyme-catalyzed) polymerization to melanin pigments (Mathew and Parpia 1971; Macheix *et al.* 1990; Sapers 1993; Nicolas *et al.* 1994; Sanchez-Ferrer *et al.* 1995; Whitaker 1995). It appears that oxidative browning reactions, proceeding in many foods of plant origin, generally cause deterioration in food quality by changing nutritional and organoleptic properties (Vamos-Vigyazo 1981; Matheis and Whitaker 1984; Zawistowski *et al.* 1991; Martinez and Whitaker 1995). These reactions significantly diminish consumer acceptance, storage life and value of the plant products.

In addition to its general occurrence in plants, PPO is also found in seafood (crustacean) products, such as shrimp (Simpson *et al.* 1987, 1988; Rolle *et al.* 1991; Chen *et al.* 1997) and lobster (Ferrer *et al.* 1989a, b; Rolle *et al.* 1990; Chen *et al.* 1991a; Ali *et al.* 1994). These highly prized and economically valuable products are extremely vulnerable to deteriorative enzymatic browning, also referred to as melanosis. Owing to its tremendous economic impact to the food industry, inhibition of PPO in seafood products has been widely studied (Ferrer *et al.* 1989c; Chen *et al.* 1991b, 1992, 1993; Kim *et al.* 2000). The millions of pounds of seafood, specifically shrimp, caught or imported into processing facilities must be treated with chemical preservatives to control and/or eliminate this discoloration process. For the interested reader, many of these facts are detailed by Kim *et al.* (2000). This paper will focus mainly on plant PPO. However, the general properties of PPO action are similar for both plant and seafood PPOs. Melanin biosynthesized in animals mainly by PPO acts as a skin and hair pigment-protecting agent against harmful solar radiation (Hill 1992). Additionally, PPO is responsible for various physiological roles in the development of crustaceans (Ferrer *et al.* 1989b) and insects (Gillespie *et al.* 1997; Sugumaran 1998), including sclerotization (cuticular hardening), wound healing and defense reactions. Even though oxidation of phenols and formation of melanins are normal physiological processes of PPO in plants, the significance of the enzyme activity in living intact plant tissues is not fully understood. One of the objectives of this overview is to elaborate on potential physiological roles proposed for plant PPO.

Further objectives are to comprehend the reaction properties and biochemical characteristics of PPO in plants because an understanding of the essential factors controlling the action of PPO is necessary in an attempt to inhibit or control its activity in fruit and vegetables during processing. Reaction properties will include general PPO reactions, specificities and molecular mechanisms of these reactions, and methods to assay PPO activity. Physicochemical properties

will evaluate substrate specificity, environmental influences such as pH and temperature, multiplicity, latency, and activators and inhibitors of PPO.

IMPORTANCE OF PPO IN THE FOOD INDUSTRY

Problem

The catalytic action of PPO has an enormous impact on the quality of several fruit and vegetable crops and results in alteration of color, flavor, texture, and nutritional value (Vamos-Vigyazo 1981). It is a limiting factor in the handling and technological processing of crops as peeled, sliced, bruised or diseased tissues rapidly undergo browning. Some commercially important edible plant products susceptible to adverse browning reactions include fruits such as apple (Harel *et al.* 1964; Barrett *et al.* 1991; Yemenicioglu *et al.* 1997), avocado (Kahn and Pomerantz 1980; Espin *et al.* 1997a), banana (Gooding *et al.* 2001), cucumber (Miller *et al.* 1990), grape (Cash *et al.* 1976; Rathjen and Robinson 1992), pineapple (Das *et al.* 1997), mango (Robinson *et al.* 1993), peach and apricot (Fraignier *et al.* 1995), and vegetables such as eggplant (Perez-Gilabert and Carmona 2000), cabbage (Fujita *et al.* 1995, 1997), lettuce (Heimdal *et al.* 1994), potato (Sanchez-Ferrer *et al.* 1993b; Cho and Ahn 1999) and table beet (Escribano *et al.* 1997). However, browning in some other instances such as in the processing of black tea (Eskin 1990; Ullah 1991), coffee (Amorim and Silva 1968; Amorim and Melo 1991) and cocoa (Lee *et al.* 1991; Lopez and Dimick 1991) is beneficial to some extent as it enhances the quality of the beverages through its forming flavorful products.

Biochemical Foundation of the Problem

The simplified scheme of PPO reactions and subsequent nonenzymatic condensation process involved in food deterioration is shown in Fig. 1. Enzymatic browning occurs in two steps: the first one is the enzymatic oxidation of monophenols or *o*-diphenols to yield *o*-quinones, which is followed by condensation or polymerization reactions (Lerner 1953; Mathew and Parpia 1971; Sapers 1993; Whitaker 1994, 1995; Sanchez-Ferrer *et al.* 1995). Severe browning of plant products arises in stress conditions due to subcellular decompartmentation and oxygen penetration leading to PPO-substrate contact. These reactions are complex given that a large number of monophenolic and/or diphenolic compounds catalyzed by PPO may in turn form a variety of products (quinones and condensation products) (Harel *et al.* 1964; Mathew and Parpia 1971; Vamos-Vigyazo 1981; Goodenough *et al.* 1983; Lee and Jaworski 1988; Eskin 1990; Whitaker 1995; Sugumaran 1998; Jimenez and Garcia-Carmona 1999a, b). The *o*-quinones generated by the reaction of PPO with phenolic

substrates are themselves colored. However, typical reddish brown color characteristic of enzymatic browning is due primarily to nonenzymatic secondary reactions of *o*-quinones resulting in formation of complex polymers known as melanins (Lerner 1953; Pierpoint 1966; Mathew and Parpia 1971; Vamos-Vigyazo 1981; Mayer and Harel 1991; Sapers 1993; Sanchez-Ferrer *et al.* 1995; Whitaker 1995). The sequence of biochemical reactions leading to formation of melanin from the oxidation of phenolic amino acid tyrosine is given by Lerner (1953) and illustrated in Fig. 2.

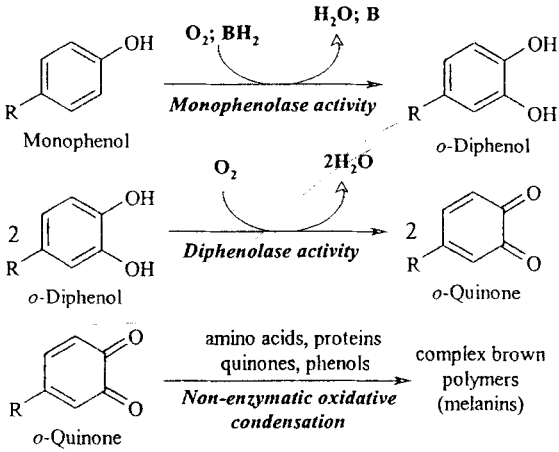

FIG. 1. SCHEMATIC REPRESENTATION OF ENZYMATIC AND SUBSEQUENT NONENZYMATIC CONDENSATION PROCESSES FOR BROWNING

Oxidation products of phenolic compounds have potential to interact with food proteins leading to covalent condensations (Loomis and Battaile 1966; Mason and Peterson 1965; Pierpoint 1966; Mathew and Parpia 1971; Nicolas *et al.* 1994). Cross-linking reactions could result in changes in structural, functional and nutritional characteristics of food proteins (Matheis and Whitaker 1984). Reduction in the nutritive value of food proteins is due to the interactions of quinones with side chains of essential amino acids in plant proteins (Matheis and Whitaker 1984; Felton *et al.* 1989, 1992b). The -SH and $-NH_2$ groups of amino acids are mostly susceptible to binding or alkylation by quinones. A decrease in the available lysine content of casein in the presence of phenolic compounds and PPO was reported (Matheis and Whitaker 1984; Felton *et al.* 1992b). Felton *et al.* (1989) also observed an 8% reduction in measurable lysine in tomato protein treated with chlorogenic acid and PPO. Additionally, food

proteins containing phenolic tyrosine groups or attached to a phenolic acid through a pseudopeptide bond are also subject to modification by serving as substrates for PPO (Matheis and Whitaker 1984). For example, oxidation of tyrosine residues on alcohol dehydrogenase and aldolase by mushroom PPO was shown to result in about 50% and 80% loss in their catalytic activities, respectively (Cory and Frieden 1967). Subsequent studies by Ito *et al.* (1984) identified the reaction products of PPO action on tyrosinase residues in proteins: 5-S-cysteinyl-3,4-dihydroxyphenylalanine (5-S-cysteinyl-dopa) was found to be the major product in yeast alcohol dehydrogenase and bovine serum albumin, which contain cysteine residue(s), whereas dopa is formed in bovine insulin, which lacks cysteine residues. Moreover, quinones formed during PPO-oxidation reactions may undergo redox recycling, which generate free radicals, and can damage DNA, proteins, amino acids or lipids (Felton *et al.* 1992b; Hill 1992).

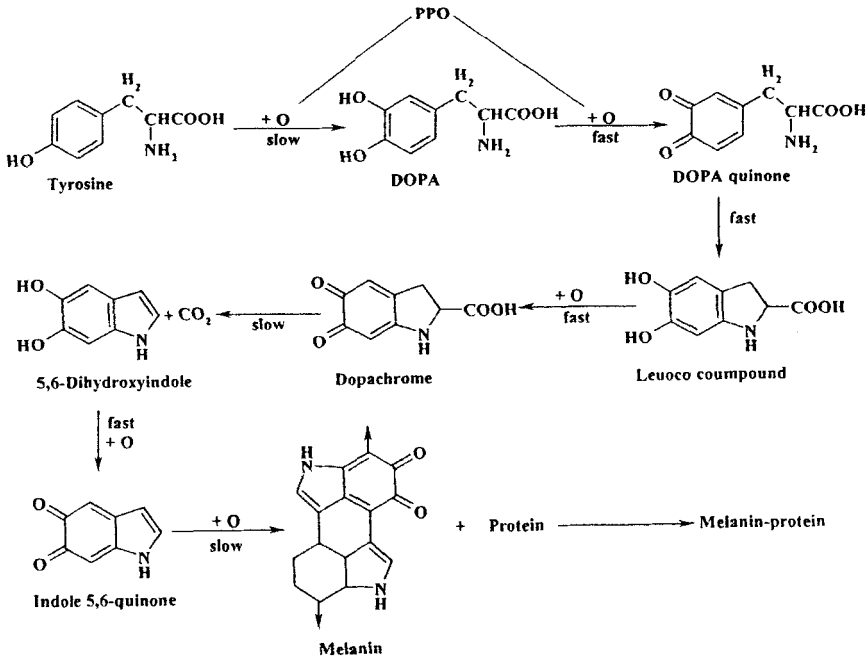

FIG. 2. ENZYMATIC OXIDATION OF THE PHENOLIC AMINO ACID TYROSINE TO MELANIN

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REACTION PROPERTIES OF PPO

Reactions Catalyzed

PPO is a generic name for a group of enzymes capable of catalyzing reactions for several phenols to produce *o*-quinones (Mathew and Parpia 1971; Whitaker 1994). PPO accomplishes *o*-hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols into *o*-quinones (diphenolase activity) with oxygen as the primary oxidant (Mason 1957; Mayer and Harel 1979; Kahn and Pomerantz 1980; Vamos-Vigyazo 1981; Janovitz-Klapp *et al.* 1990; Zawistowski *et al.* 1991). The involvement of copper as a prosthetic group of PPO is essential for its activity (Solomon and Lowery 1993; Van Gelder *et al.* 1997). Monophenolase activity, also called hydroxylase or cresolase, is always coupled to diphenolase activity, also termed catecholase or oxidase. However, diphenolase activity is not always preceded by hydroxylase activity (Rodriguez-Lopez *et al.* 1992; Whitaker 1994).

The two general reactions, where phenols and oxygen are the substrates and BH_2 stands for an *o*-diphenolic compound acting as the electron donor, are shown in Fig. 1. In the absence of an *o*-diphenol (BH_2), there is a characteristic lag or induction period in the monophenolase reaction prior to attaining a steady state rate (Kahn and Pomerantz 1980; Sanchez-Ferrer *et al.* 1993c). When the existing active PPO generates some BH_2, the reaction continues as normal (Rodriguez-Lopez *et al.* 1992; Sanchez-Ferrer *et al.* 1993c; Whitaker 1994; Lerch 1995). Duration of the pronounced lag and the steady-state rate are experimentally influenced by a number of factors including temperature and pH, and BH_2, oxygen, enzyme and monophenol concentrations (Martinez-Cayuela *et al.* 1988; Valero *et al.* 1988; Sanchez-Ferrer *et al.* 1988, 1989b, 1990, 1993c; Espin *et al.* 1997b; Perez-Gilabert and Carmona 2000; Laveda *et al.* 2001).

Monophenolase activity is usually primed by addition of a small amount of ascorbic acid or an *o*-diphenol to the reaction medium (Kahn and Pomerantz 1980; Martinez-Cayuela *et al.* 1988; Valero *et al.* 1988; Espin *et al.* 1997b; Perez-Gilabert and Carmona 2000; Laveda *et al.* 2001). A catalytic amount of *o*-diphenol helps to shorten or eliminate the lag period by acting as a co-substrate and neither acts as a substrate for diphenolase activity nor modifies the steady-state rate of the monophenolase activity under the defined experimental conditions (Martinez-Cayuela *et al.* 1988; Valero *et al.* 1988; Sanchez-Ferrer *et al.* 1993c; Sojo *et al.* 1998b; Perez-Gilabert and Carmona 2000; Laveda *et al.* 2001). In certain cases, where the activity is completely in the latent state, addition of SDS to the reaction medium may also shorten the lag period (Jimenez and Garcia-Carmona 1996; Sojo *et al.* 1998b). Overall hydroxylation reaction involves introduction of one atom of oxygen onto the monophenol and

reduction of the other to water (Mason 1957). Isotope studies with $^{18}O_2/H_2{}^{16}O$ and $^{16}O_2/H_2{}^{18}O$ demonstrated the origin of oxygen appeared in monophenol as molecular oxygen, not water (Mason 1956).

In the case of diphenolase activity (Fig. 1), BH_2 is not required because the existing *o*-diphenols are sufficient for the reaction (Whitaker 1995). Two molecules of *o*-diphenol are oxidized to two molecules of *o*-quinone. Since both atoms of the oxygen molecule are reduced to water, diphenolase activity is referred to as a four-electron transfer oxidase (Mason 1957). Evidence concerning the appearance of a lag period dependent on pH in the expression of catecholase activity from grape PPO put forward the hysteretic nature of the enzyme (Valero and Garcia-Carmona 1992, 1998). The lag period was shown to be independent of enzyme concentration but dependent on substrate concentration, suggesting the ionization of a group located outside the active site in a domain of the protein that undergoes a slow pH-induced conformational transition during assay and/or perhaps *in vivo* (Valero and Garcia-Carmona 1992, 1998).

Laccase (*p*-diphenol:oxygen oxidoreductase, E.C. 1.10.3.2) is another phenol-oxidizing enzyme present in some higher plants. It was found in grape, secreted by *Botrytis cinerea* often present on grapes (Interesse *et al.* 1984), in mushroom (Dawley and Flurkey 1993; Flurkey *et al.* 1995), peaches (Mayer and Harel 1968) and in the sap exuded from mango fruit (Robinson *et al.* 1993). Laccase-type PPO is active on both *o*- and *p*-diphenol substrates, and is differentiated from PPO by its unique ability to catalyze the oxidation of the latter (Fig. 3), some molecular properties and response to inhibitors (Mayer and Harel 1979, 1991; Dawley and Flurkey 1993; Robinson *et al.* 1993; Flurkey *et al.* 1995). Differentiation of laccase and PPO using selective inhibitors, in conjunction with selective substrates, was shown by Flurkey *et al.* (1995) in mushroom species.

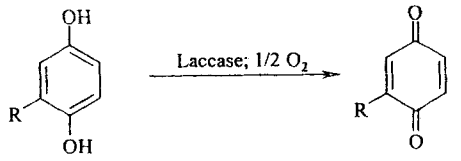

FIG. 3. LACCASE CATALYZED REACTION

Reaction Specificity

PPO preparations from the fungus Neurospora (Lerch 1983) and a number of plants such as apple (Harel *et al.* 1964; Goodenough *et al.* 1983), avocado

(Kahn and Pomerantz 1980), broad bean (Robb *et al.* 1964; Jimenez and Garcia-Carmona 1996), cherimoya (Martinez-Cayuela *et al.* 1988), dog-rose (Sakiroglu *et al.* 1996), eggplant (Perez-Gilabert and Carmona 2000), grape (Valero *et al.* 1988; Sanchez-Ferrer *et al.* 1989a), kiwi (Park and Luh 1985), mushroom (Bouchilloux *et al.* 1963) and potato (Sanchez-Ferrer *et al.* 1993c) have both types of activities, while those from cocoa bean (Lee *et al.* 1991), lettuce (Heimdal *et al.* 1994), longan (Jiang 1999), tea (Gregory and Bendall 1966), peach (Flurkey and Jen 1980), pear (Rivas and Whitaker 1973), pineapple (Das *et al.* 1997), wild potato trichome (Kowalski *et al.* 1992), field bean (Paul and Gowda 2000), mung bean (Shin *et al.* 1997) and sunflower (Raymond *et al.* 1993) lack the hydroxylation properties and act only on *o*-diphenols. Diphenolase activity is generally the most prevalent form of PPO in higher plants. When both monophenolase and diphenolase activities are present, the ratio of monophenolase to diphenolase activity varies from 1:10 to 1:40 depending on plant sources (Vamos-Vigyazo 1981; Perez-Gilabert and Carmona 2000). Diphenol oxidation predominates over monophenol oxidation at near physiological pH in eggplant (Perez-Gilabert and Carmona 2000), potato (Sanchez-Ferrer *et al.* 1993c) and banana (Sojo *et al.* 1998b).

The issue of whether all PPOs can perform monophenolase function continues to be a challenge. Early works on spinach, for example, showed PPO to possess only diphenolase activity (Goldbeck and Cammarata 1981; Meyer and Biehl 1980), whereas a subsequent study by Sanchez-Ferrer *et al.* (1989b) reported that spinach PPO has both activities. Similarly, strawberry PPO, reported to act only on *o*-diphenols by Wesche-Ebeling and Montgomery (1990b), was later shown to possess monophenolase activity (Espin *et al.* 1997b). In both cases, discrepancy in reaction properties of PPO was accounted for by differences in extraction and purification procedures and/or sensitivity of assay method. Thomas and Janave (1986) believed that failure to detect monophenolase activity in some banana isoforms is due to inactivation during the extraction and purification processes.

Ability to act on monophenols might be an intrinsic property of PPO (Mayer and Harel 1991) but reaction properties of native multifunctional enzymes may be subject to *in vitro* alterations due to its labile monophenol-oxidase nature (Mason 1956; Constantinides and Bedford 1967; Nakamura *et al.* 1983; Interesse *et al.* 1984; Park and Luh 1985; Sanchez-Ferrer *et al.* 1989b, 1993c; Mayer and Harel 1991; Espin *et al.* 1997b; Shin *et al.* 1997; Sojo *et al.* 1998b; Perez-Gilabert and Carmona 2000). Physical and chemical changes during the preparative procedures may provoke alterations in the relative positions of the two copper atoms (Mason 1956). Additionally, the copper-free portions in the active site of the enzyme may be altered with ensuing loss of monophenolase activity (Mason 1956).

In some cases, the absence of monophenolase activity can be due to latency. Latency is well defined for broad bean PPO (Moore and Flurkey 1990; Robinson and Dry 1992; Jimenez and Garcia-Carmona 1996). It was shown that the latent monophenolase activity of broad bean PPO could be detected after activation by SDS as its catecholase activity (Sanchez-Ferrer *et al.* 1990; Jimenez and Garcia-Carmona 1996). Monophenolase activity of latent banana pulp PPO is also affected by SDS where increasing SDS concentration results in increasing activity with maximum activity in the presence of 2 mM detergent (Sojo *et al.* 1998b). Recently, Laveda *et al.* (2001) reported the monophenolase activity of peach PPO after activating with trypsin. Alternatively, monophenolase activity may require different optimal assay conditions than diphenolase activity (Sanchez-Ferrer *et al.* 1988, 1989a; Perez-Gilabert and Carmona 2000). Failure to eliminate a lag period under the experimental conditions (Kahn and Pomerantz 1980; Sanchez-Ferrer *et al.* 1993c) or poor sensitivity of the activity assay methods (Espin *et al.* 1998a) could explain the lack of monophenolase activity reported for several PPOs in the literature. It is also believed that removal of natural diphenols, which shorten or eliminate the lag period, during purification could mask monophenolase activity (Sanchez-Ferrer *et al.* 1988, 1993c).

Determination of PPO Activity

Sensitive determination of PPO activity is essential in order to identify its biochemical properties and function and, in turn, to understand how to prevent its deteriorative action in agricultural crops. While studying PPO, it is important to recognize secondary reactions, for instance the oxidation of phenolic substrates by peroxidase (EC 1.11.1.7). Peroxidase catalyzes oxidation of *o*-diphenols to their corresponding quinones in the presence of hydrogen peroxide as an oxidizing agent (Vamos-Vigyazo 1981; Miller *et al.* 1990; Nicolas *et al.* 1994; Richard-Forget and Guillard 1997). Even though the contribution of peroxidase activity to browning of fruits and vegetables is believed to be minor due to limited or insufficient availability of endogenous hydrogen peroxide (Felton *et al.* 1989; Nicolas *et al.* 1994), the evidence suggests that peroxidase enhances the degradation of phenols by using PPO-generated hydrogen peroxide and quinones (Richard-Forget and Guillard 1997). Addition of catalase and alcohol to the reaction solution reduces possible interferences from peroxidase activity (Mayer and Harel 1979; Fraignier *et al.* 1995; Richard-Forget and Guillard 1997). Tropolone could also be used to detect peroxidase activity in the reaction medium, as this compound is a very effective inhibitor of PPO (Nicolas *et al.* 1994). In the presence of hydrogen peroxide, tropolone is also reported to serve as a substrate for peroxidase (Nicolas *et al.* 1994).

A number of quantitative methods available to assess PPO activity are outlined in Table 1. PPO activity toward monophenolic substrates can be

determined spectrophotometrically at 400-500 nm by measuring the rate of formation of the corresponding *o*-quinone (Interesse *et al.* 1980; Saluja and Sachar 1982; Sanchez-Ferrer *et al.* 1988, 1989b, 1993c; Valero *et al.* 1988; Sojo *et al.* 1998b; Perez-Gilabert and Carmona 2000; Laveda *et al.* 2001). The use of absorbance near 280 nm, where *o*-diphenols show strong absorbance, is another way of assessing *o*-diphenol formation from monophenolic substrates (Fig. 1). Lately, a continuous spectrophotometric method, utilizing 3-methyl-2-benzothiazolinone hydrazone (MBTH) as the nucleophilic agent with *o*-quionones, was shown to assay monophenolase and diphenolase activity of PPO (Espin *et al.* 1995, 1997a, b, 1998a, b, 2000). However, some MBTH-quinone adducts showed solubility problems under certain assay conditions. The radioassay technique measuring the rate of formation of ^{3}HOH from [3,5-^{3}H]-tyrosine is an alternative and highly sensitive method for assaying the rate of the hydroxylation reaction (Pomerantz 1966; Kahn and Pomerantz 1980). It is believed that assessing the rate of tritium removal from the *o*-position of a monophenol during the reaction is a better approach for monitoring monophenol hydroxylation only (Whitaker 1995).

TABLE 1.
METHODS FOR ASSAYING RATE OF PPO REACTIONS[1]

Method	Principle of the Procedure
To assay diphenolase activity	
Manometric	Measurement of oxygen consumption with a Warburg apparatus (Harel *et al.* 1964; Mayer *et al.* 1966)
Polarographic	Measurement of oxygen consumption with an oxygen sensitive electrode (Harel *et al.* 1964; Mayer *et al.* 1966)
Chronometric	Determination of the time when the first color is observed in the presence of ascorbic acid (Mayer *et al.* 1966)
Spectrophotometric	Measurement of product formation at a wavelength near 400-500 nm (Mayer *et al.* 1966)
To assay monophenolase activity	
Spectrophotometric	Measurement of o-diphenol formation at a wavelength near 280 nm or quinone formation at a wavelength near 400-500 nm (Sanchez-Ferrer *et al.* 1988)
Radioassay	Measurement of the rate of tritium incorporation into product, using triated tyrosine (Pomerantz 1966; Kahn and Pomerantz 1980)

[1]Some references are given in the text.

The most commonly utilized routine assays in the literature for monitoring diphenolase activity rely on the spectrophotometric measurement of quinone

formation from *o*-diphenolic compounds at 400 to 500 nm (Interesse *et al.* 1980; Sanchez-Ferrer *et al.* 1989b; Moore and Flurkey 1990; Raymond *et al.* 1993; Fraignier *et al.* 1995; Marques *et al.* 1995; Laveda *et al.* 2000; Serradell *et al.* 2000) or oxygen consumption polarographically with an oxygen-sensitive electrode (Harel *et al.* 1964; Gregory and Bendall 1966; Goldbeck and Cammarata 1981; Interesse *et al.* 1984; Janovitz-Klapp *et al.* 1990; Robinson and Dry 1992; Heimdal *et al.* 1994; Sheptovitsky and Brudvig 1996; Richard-Forget and Guillard 1997). These methods are rapid, practical and perhaps more accurate relative to the other two-diphenolase assays (Table 1) (Harel *et al.* 1964; Mayer *et al.* 1966).

These techniques for determining PPO activity are central to determining initial rates because PPO undergoes inactivation during its catalysis (Whitaker 1994). The initial rate of the enzyme-catalyzed reaction with *o*-diphenols is linear for only a short period. This may be due not only to irreversible binding of the oxidized products to the active site of PPO through a Michael reaction (Wood and Ingraham 1965) but also to quick reaction inactivation, so called suicide inactivation (Whitaker 1994, 1995). Suicide inactivation is believed to occur as a consequence of the reaction of *o*-semibenzoquinone free radicals (Fig. 4), intermediate products in the oxidation reaction (Korytowski *et al.* 1987), with the active site's histidyl groups bound to binuclear copper (Osuga *et al.* 1994; Whitaker 1994, 1995). Free-radical-catalyzed fragmentation of the active site's histidine residue(s) results in release of the enzyme-bound copper and consequently PPO becomes inactive. Inactivation of *Neurospora crassa* PPO accompanying catechol oxidation is characterized by loss of copper and histidine residue (His306) in the active site (Dietler and Lerch 1982).

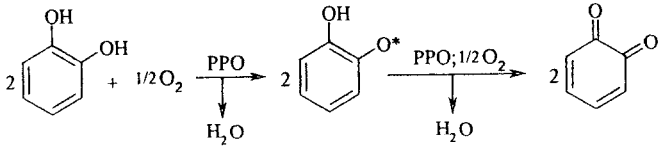

FIG. 4. FORMATION OF FREE-RADICAL INTERMEDIATE IN PPO-CATALYZED REACTION WITH *o*-DIPHENOLS

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However, enzyme activity assays cannot be used to differentiate PPO isoforms or to identify latent enzyme forms without priming with activators

(Lanker *et al.* 1987). Electrophoresis can be used for detection and comparison of active PPO isoforms from a source or among several sources (Angleton and Flurkey 1984; Lax *et al.* 1984; Watson and Flurkey 1986; Barrett *et al.* 1991; Fraignier *et al.* 1995; Marques *et al.* 1995; Shin *et al.* 1997). Immunological approaches that distinctively identify PPO protein give an additional insight into the detection of active as well as inactive and latent forms of the enzymes (Lieberei *et al.* 1981; King and Flurkey 1987; Lanker *et al.* 1987; Fraignier *et al.* 1995; Marques *et al.* 1995; Murata *et al.* 1995; Shin *et al.* 1997).

Molecular Mechanism of Action of PPO

The overall mechanism of monophenol and diphenol oxidations proposed for *Neurospora crassa* PPO is shown in Fig. 5 (Lerch 1995). The mechanism of the catalytic action for the enzyme was deduced based on the geometric and electronic structure of the copper active site (Himmelwright *et al.* 1980; Lerch 1983, 1995; Wilcox *et al.* 1985; Solomon *et al.* 1992), which structurally resembles that found in hemocyanin, an oxygen transport protein (Himmelwright *et al.* 1980; Gaykema *et al.* 1984).

The active site of the enzyme consists of two copper atoms each in the ligand field with three conserved histidine residues (Lerch 1983; Huber *et al.* 1985) and exists in three forms, met-PPO (Cu^{+2}), deoxy-PPO (Cu^{+1}), and oxy-PPO (Cu^{+2}). The resting form of the enzyme is believed to be met-PPO (Himmelwright *et al.* 1980; Solomon *et al.* 1992). It is reduced to deoxy-PPO via oxidation of one molecule of catechol to *o*-benzoquinone. The active site of the enzyme for the monophenol oxidation pathway (Fig. 5) then combines with oxygen to give an oxy-PPO intermediate. Monophenolic substrates can only react with oxy-PPO forming the ternary PPO-oxygen-monophenol complex (Lerch 1995). Formation of *o*-benzoquinone from monophenol is followed by its release from the enzyme. PPO is converted into deoxy PPO and ready for another round without cycling through the resting form. In the diphenol oxidation pathway (Fig. 5), diphenolic substrates react not only with oxy-PPO but met-PPO as well (Lerch 1995). Met-PPO is first reduced to its deoxy form with the oxidation of one molecule of *o*-diphenol and then combines with oxygen leading to the formation of oxy-PPO, which in turn forms *o*-diphenol-PPO complex. After the oxidation of catechol to *o*-benzoquinone, the enzyme is reduced to its met form.

The kinetic data indicated that the enzyme first binds oxygen and later monophenol in an ordered sequential mechanism (Wilcox *et al.* 1985). Although a random sequential mechanism was suggested for binding *o*-diphenol and oxygen to the enzyme (deoxy-PPO) (Wilcox *et al.* 1985), it is thought that when the chemical recycling steps of the proximal pathway are taken into account the mechanism appears to be an orderly process (Sanchez-Ferrer *et al.* 1995). The

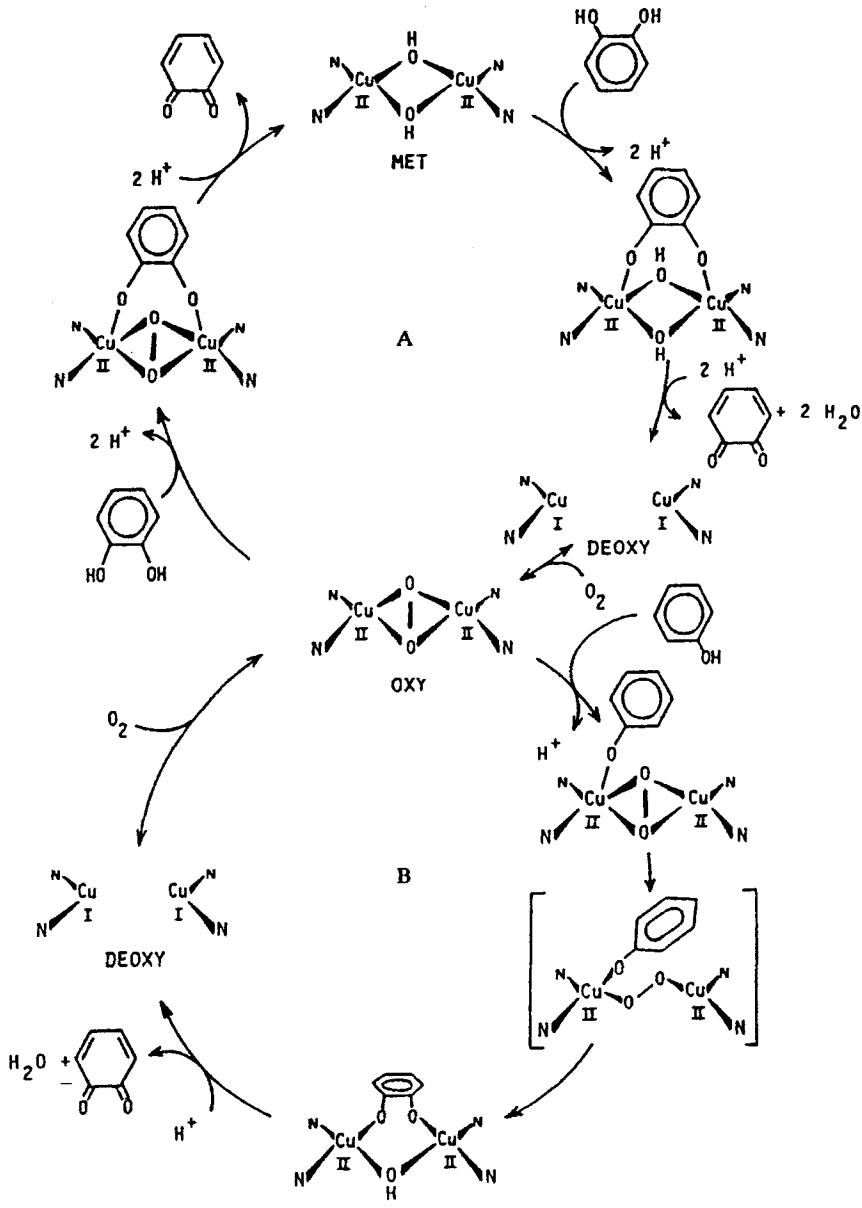

FIG. 5. THE PROPOSED DIPHENOL (A) AND MONOPHENOL (B) OXIDATION PATHWAYS OF *NEUROSPORA CRASSA* PPO

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ordered sequential Bi Bi mechanism in which oxygen is the first substrate to be bound by PPO during diphenolase activity is generally assumed (Janovitz-Klapp *et al.* 1990; Whitaker 1994). However, the order of the removal of the products is not fully clear.

Studies by Wilcox *et al.* (1985) comparing the kinetic constants for oxidation of a variety of monophenolic and diphenolic substrates indicated that the bulky substituents of monophenols significantly decrease the hydroxylation rate but those of diphenols show little effect on their oxidation rates. It is suggested that monophenols undergo axial-to-equatorial arrangement in the protein pocket surrounding the copper site for catalysis; signifying the possibility of the steric hindrance of ring substituents (Wilcox *et al.* 1985). However, oxidation of diphenols to *o*-quinones by PPO is believed to have less geometric and electronic requirements. Afterward, quantitative studies (NMR assays and kinetic data analysis) were conducted to evaluate the possible effect of different side substituents of the benzene ring on the catalytic rate of PPO from apple, pear (Espin *et al.* 1998b), and mushroom (Espin *et al.* 2000). It appeared from these experimental results that a ring substituent with a high electron donor capacity in the monophenolic substrate significantly increases the nucleophilic power of the oxygen atom of the hydroxyl group on the monophenol toward the active site copper (electronic effect). Hence, the monophenols with a high electron-donor side-chain are oxidized more quickly. However, the size of the side-chain substituent (steric effect) on the catalytic velocity was not significant due to the low nucleophilic power of the monophenols. For *o*-diphenols, electron donor capacities of the substituents were shown to have little impact on the oxidation rates, but the size of the side-chain (steric effect) becomes very important (Espin *et al.* 1998b, 2000).

By considering the kinetic aspects as well as the occurrence of three different states of the enzyme (met-PPO, deoxy-PPO and oxy-PPO) based on the reaction pathway for *Neurospora crassa* PPO (Fig. 5), models for the reaction mechanism explaining monophenolase and diphenolase activities of PPO from plant sources have been proposed (Fig. 6; Rodriguez-Lopez *et al.* 1992, 1993). The reaction mechanisms are applicable to monophenolase and/or diphenolase activities of PPOs from apple, pear (Espin *et al.* 1998b), avocado (Espin *et al.* 1997a), banana (Sojo *et al.* 1998b), eggplant (Perez-Gilabert and Carmona 2000), mushroom (Espin *et al.* 2000), peach (Laveda *et al.* 2001) and strawberry (Espin *et al.* 1997b). These models additionally confer the chemical redox recycling of two *o*-quinones generated by the action of the enzyme to one *o*-diphenol and one aminechrome. The reader is also referred to Sanchez-Ferrer *et al.* (1995) for an overview on both kinetic and structural aspects of the reaction mechanism for PPO.

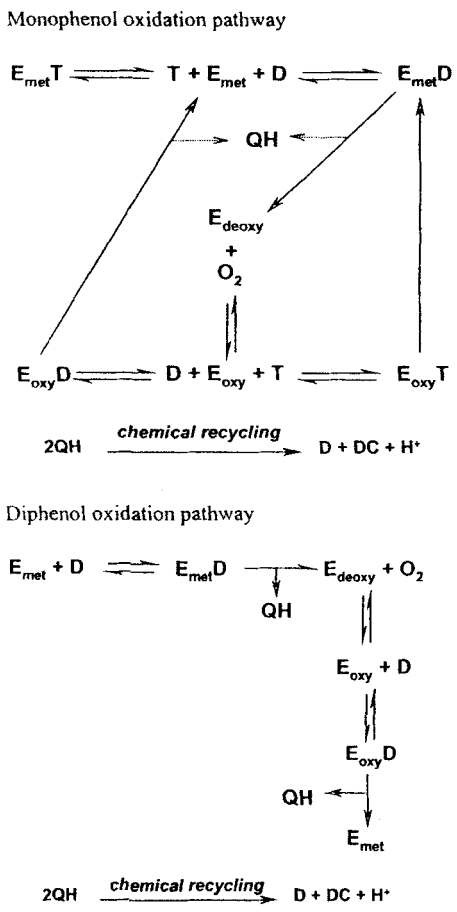

FIG. 6. REACTION MECHANISM OF PPO ON MONOPHENOLS AND *o*-DIPHENOLS COUPLED TO NONENZYMATIC REACTIONS FROM *o*-QUINONE
E_{met}, met-PPO; E_{deoxy}, deoxy-PPO; E_{oxy}, oxy-PPO; T, monophenol; QH, *o*-quinone-H^+; D, *o*-diphenol; DC, aminechrome. Reproduced with permission from Rodriguez-Lopez *et al.* (1993), Biochem. J. *293*, 861. Copyright 1993 the Biochemical Society.

The pathway for monophenols starts with monophenolase activity of PPO (Fig. 6). The characteristic lag period observed in the activity is described as the time required to generate the amount of *o*-diphenol necessary to form oxy-PPO from met-PPO (Rodriguez-Lopez *et al.* 1992; Sanchez-Ferrer *et al.* 1995). The lag period increases with increasing monophenol concentration since binding of monophenol to met-PPO (with no catalytic activity on monophenols) renders the formation of more met-PPO-monophenol (dead-end complex, Fig. 6), requiring

more time for the steady state. The *o*-diphenols generated from the chemical recycling of the quinones drive the dead-end complex to the catalytic cycle, producing the level of diphenol necessary to maintain the steady state (Rodriguez-Lopez *et al.* 1992; Sanchez-Ferrer *et al.* 1995). The lag period, however, decreases with increasing enzyme concentration as more oxy-PPO will be present in the medium and the steady state amount of *o*-diphenol necessary to eliminate the lag phase is reached more quickly.

Even though it is not yet fully clear whether the mechanisms stated are applicable to all PPOs, it is evident that whatever the mechanism of the enzyme action, copper is essential for PPO activity. Removal of copper from the enzyme by copper-chelating reagents results in a relatively inactive enzyme whose activity can be restored by the addition of excess copper (Lerner *et al.* 1950; Park and Luh 1985; Kowalski *et al.* 1992; Yoruk and Marshall 2003). Based upon amino acid sequencing of PPOs from various plant, animal, fungal and bacterial sources, all have two conserved copper-binding sites, called CuA and CuB (Cary *et al.* 1992; Shahar *et al.* 1992; Hunt *et al.* 1993; Newman *et al.* 1993; Dry and Robinson 1994; Boss *et al.* 1995; Joy *et al.* 1995; Lerch 1995; Haruta *et al.* 1998; Chevalier *et al.* 1999; Gooding *et al.* 2001). Sequence and structural features of plant PPOs were the objective of reviews by Van Gelder *et al.* (1997) and Steffens *et al.* (1994). This binuclear copper is the site of interaction between the enzyme and its substrates (Dietler and Lerch 1982; Wilcox *et al.* 1985; Rodriguez-Lopez *et al.* 1993; Solomon and Lowery 1993; Steffens *et al.* 1994). In addition, sequence comparison studies have revealed high homology around the copper-binding ligands of arthropodan and molluscan hemocyanins and several PPOs (Cary *et al.* 1992; Shahar *et al.* 1992; Hunt *et al.* 1993; Dry and Robinson 1994; Steffens *et al.* 1994; Boss *et al.* 1995; Joy *et al.* 1995; Thygesen *et al.* 1995; Van Gelder *et al.* 1997; Haruta *et al.* 1998; Chevalier *et al.* 1999). Comparative studies on several plant PPOs show that they also have structural similarity in terms of immunological criteria (Flurkey 1986; Fraignier *et al.* 1995; Haruta *et al.* 1999). It is believed that the knowledge of hemocyanin whose structure is confirmed with X-ray analysis (Gaykema *et al.* 1984) will be helpful in predicting a structural model for the active site of PPOs in the absence of X-ray crystallographic information (Van Gelder *et al.* 1997).

PHYSIOCHEMICAL PROPERTIES OF PPO

Substrate Specificity

Phenolic compounds are the primary substrates of PPO. Several phenolic compounds serve as substrates (Table 2). Types and relative concentrations of

natural phenols vary widely for different plant sources. For example, catechin is a major phenolic compound found in grapes (Jaworski and Lee 1987), and tea (Ullah 1991), whereas chlorogenic acid is in apple (Murata *et al.* 1995), potato (Sanchez-Ferrer *et al.* 1993b), sunflower (Raymond *et al.* 1993), and sweet potato (Lourenco *et al.* 1992; Nozue *et al.* 1998). Yang *et al.* (2000) and Sojo *et al.* (1998a) reported that enzymatic browning in banana pulp is caused by the oxidation of the phenolic compound, dopamine, by endogenous PPO. Catechin, epicatechin and caffeic acid derivatives are believed to be common natural substrates of several other fruit PPOs (Macheix *et al.* 1990). Some studies demonstrated that different phenolic compounds show different degrees of browning. In grape for instance, monomeric catechins and dimeric procyanidins brown more deeply than do other phenolics (Lee and Jaworski 1988). Catechin was also reported to be the major contributor of browning in apples (Murata *et al.* 1995).

TABLE 2.
RELATIVE SUBSTRATE SPECIFICITY OF PPO

	Percent Activity					
Substrates	Apple[1]	Peach[2]	Sunflower seeds[3]	Strawberry[4]	Field bean[5]	Grape[6]
Di- or triphenols						
Catechol	100	100		9	100	5.9
4-Methylcatechol	181	103		80	140	74
Chlorogenic acid	102		32.3	11	0	51
L-DOPA		23			22.6	5.4
D,L-DOPA	12		8			4.1
Catechin	54	539		100	0	21
Protocatechuic acid		15		4		
Caffeic acid		7	87.3	13	0	100
Gallic acid		5	100		0	0
Pyrogallol	38	182	100	62	24	0
Monophenols						
Tyrosine	3	0	0	0	0	0
p-Cresol		0	0		0	0
p-Coumaric acid		0		0		

[1]Zhou *et al.* (1993); [2]Flurkey and Jen (1980); [3]Raymond *et al.* (1993); [4]Wesche-Ebeling and Montgomery (1990b); [5]Paul and Gowda (2000); [6]Lee *et al.* (1983)

The level of PPO activity toward DOPA as a substrate varies widely in the plant kingdom (Sherman *et al.* 1991). However, these differences could be due to the differing substrate specificity of PPOs from different plants. Distinct variations in the activities of PPOs from different plant sources for different

types of substrates are shown in Table 2. PPO is active to those substrates with a high preference to the enzyme. Nature of the side chain, number of hydroxyl groups and their position in the benzene ring of the substrate have a major effect on the catalytic activity of the enzyme (Harel *et al.* 1964; Macheix *et al.* 1990; Park and Luh 1985). Activity toward *o*-diphenols only was found for PPOs from grape (Lee *et al.* 1983), field bean seed (Paul and Gowda 2000), strawberry (Wesche-Ebeling and Montgomery 1990b) and sunflower seed (Raymond *et al.* 1993). The activity of apple PPO on tyrosine is much lower than on *o*-diphenols (Zhou *et al.* 1993), which is typical for PPOs.

It appears that substrate specificity of PPO is also dependent on species and cultivars. For example, in the case of DeChaunac grape PPO, caffeic acid is oxidized at much faster rates than other structurally related substances (Table 2; Lee *et al.* 1983). The substrates 4-methylcatechol and chlorogenic acid also show relatively high levels of activity. However, the activity of PPO from Koshu grapes is greatest with chlorogenic acid followed by caffeic acid and *d*-catechin (Nakamura *et al.* 1983), whereas Concord grape PPO oxidizes catechol more rapidly than caffeic acid (Cash *et al.* 1976). In addition, PPO isoforms in a tissue of interest may also exhibit differential substrate specificities and variations in their relative activities toward monophenols and *o*-diphenols (Bouchilloux *et al.* 1963; Harel *et al.* 1964, 1965; Constantinides and Bedford 1967; Wong *et al.* 1971; Park and Luh 1985; Thomas and Janave 1986; Oba *et al.* 1992). For instance, two isoforms isolated by Oba *et al.* (1992) in banana bud extracts showed maximum relative activity toward dopamine. However, these enzymes exhibited variations in their activities toward chlorogenic acid, L-dopa, and (+)-catechin.

pH Effects in PPO Activity

The changes in ionization of prototropic groups in the active site of an enzyme at lower acid and higher alkali pH values may prevent proper conformation of the active site, binding of substrates, and/or catalysis of the reaction (Segel 1976; Tipton and Dixon 1983; Whitaker 1994). In addition, irreversible denaturation of the protein and/or reduction in stability of the substrate as a function of pH could also influence the catalytic activity of enzymes. Kinetic behavior of PPO was reported to alter depending on the pH of the assay due to pH-induced conformational changes in the enzyme (Janovitz-Klapp *et al.* 1989; Valero and Garcia-Carmona 1992, 1998).

The pH optimum of PPO varies widely with plant source but is generally in the range of 4.0-8.0 (Table 3). Cherry and strawberry PPOs show a narrow pH optimum with estimated maximum at about pH 4.5 with 4-methylcatechol as substrate (Wesche-Ebeling and Montgomery 1990b; Fraignier *et al.* 1995). Several fruit PPOs including almond, apricot, peach and plum generally have

maximal activities around pH 5.0 (Fraignier *et al.* 1995). However, PPO from apricot and plum exhibit maximum activity in a wider acidic pH range. Apple and grape PPO have broad acidic pH optima with estimated values between pH 3.5 to 4.5 (Valero *et al.* 1988; Marques *et al.* 1995). The optimum at pH 3.5 is well below the normal optima compared to other fruits (Table 3). However,

TABLE 3.
pH OPTIMUM OF PPO FROM SEVERAL PLANT SOURCES

Enzyme Source	Substrate	pH Optimum	Reference
Almond	4-Methylcatechol	5.0	Fraignier *et al.* (1995)
Apple	4-Methylcatechol	3.5-4.5	Marques *et al.* (1995)
	Chlorogenic acid		
Apricot	4-Methylcatechol	5.0-5.5	Fraignier *et al.* (1995)
Avocado	4-Hydroxyanisole	5.0	Espin *et al.* (1997)
Cherry	4-Methylcatechol	4.5	Fraignier *et al.* (1995)
Cocoa	Catechol	6.8	Lee *et al.* (1991)
Cucumber	Catechol	7.0	Miller *et al.* (1990)
Dog-rose	Catechol	8.5	Sakiroglu *et al.* (1996)
	Pyrogallol	7.0	
	L-Tyrosine	7.0	
	p-Cresol	5.0	
Eggplant	4-Methylcatechol	~5.0-6.5	Perez-Gilabert and Carmona
	tert-Butylcatechol	~5.0-6.5	(2000)
	p-Cresol	7.5	
Field bean	Catechol	4.0	Paul and Gowda (2000)
seeds	4-Methylcatechol	4.0	
	L-DOPA[1]	5.0	
Grape	4-Methylcatechol	3.5-4.5	Valero *et al.* (1988)
Kiwi	Catechol	7.3	Park and Luh (1985)
	(+) Catechin	8.0	
Lettuce	Chlorogenic acid	5.0-8.0	Heimdal *et al.* (1994)
Longan	4-Methylcatechol	6.5	Jiang (1999)
Mango	4-Methylcatechol	5.8	Robinson *et al.* (1993)
Olive	4-Methylcatechol	5.5-7.5	Ben-Shalom *et al.* (1977)
Peach	4-Methylcatechol	5.0	Fraignier *et al.* (1995)
Pineapple	Catechol	6.0-7.0	Das *et al.* (1997)
Plum	4-Methylcatechol	4.0-5.5	Fraignier *et al.* (1995)
Potato	Chlorogenic acid	4.5-5 and 6-6.5	Sanchez-Ferrer *et al.* (1993a)
	tert-Butylcatechol	4.5-5 and 6-6.5	
Spinach	Dopamine	8.0	Sheptovitsky and Brudvig (1996)
Strawberry	Catechol	5.5	Wesche-Ebeling and
	4-Methlycatechol	4.5	Montgomery (1990b)
Sunflower	Gallic acid	7.9	Raymond *et al.* (1993)
Tea	4-Methylcatechol	5.0	Gregory and Bendall (1966)
	Pyrogallol	5.7	
Wheat	4-Methylcatechol	5.3 and 6.9	Interesse *et al.* (1980)

[1]L-3,4-dihydroxyphenylalanine

the pH optimum of grape PPO among different varieties was shown to be quite variable ranging from 3.5 to 7.3 (Cash *et al.* 1976; Wissemann and Lee 1981; Lee *et al.* 1983; Nakamura *et al.* 1983; Interesse *et al.* 1984; Valero *et al.* 1988; Lamikanra *et al.* 1992). The acidic pH optimum for apple PPO was also determined by other researchers, but some found a single pH optimum at pH 4.5 to 5.0 (Goodenough *et al.* 1983; Janovitz-Klapp *et al.* 1989; Zhou *et al.* 1993) whereas others note the existence of two pH optima, one at around pH 7.0 and one at around pH 5.0 (Harel *et al.* 1964; Stelzig *et al.* 1972).

PPO from subtropical fruits such as longan and pineapple fruit is most active near neutral pH (Das *et al.* 1997; Jiang 1999), whereas PPO activity in kiwifruit is maximal at pH 8.0 with (+) catechin as substrate (Park and Luh 1985) (Table 3). The pH optimum of PPO from vegetable plants also varies depending on the plant source. Lettuce PPO shows a broad pH optimum of 5.0 to 8.0 (Heimdal *et al.* 1994). A single pH optimum around 8.0 was observed for spinach PPO and there was practically no activity below pH 6.0 (Sheptovitsky and Brudvig 1996). The effect of pH on the expression of diphenolase and monophenolase activities of eggplant is markedly different (Perez-Gilabert and Carmona 2000) (Table 3). In the case of the former activity, a broad optimum pH between about 5.0 and 6.5 for both 4-methylcatechol and *tert*-butylcatechol substrates was observed. However, in the later case, a single slightly higher pH optimum of 7.5 was detected.

As shown in Table 3, some PPOs show two pH optima such as those from potato (Sanchez-Ferrer *et al.* 1993b) and wheat (Interesse *et al.* 1980). The maximal PPO activities of potato are at pH 4.5-5.0 and 6.0-6.5 for both chlorogenic acid and *tert*-butylcatechol as substrates. This was attributed to the existence of enzyme-substrate complex in two ionization states or to the existence of two different pKs in the system since only one activity band was observed under electrophoresis (Sanchez-Ferrer *et al.* 1993b). PPO isoforms present in a plant extract may also exhibit different pH optima (Harel *et al.* 1964, 1965; Wong *et al.* 1971; Saluja and Sachar 1982; Park and Luh 1985; Oba *et al.* 1992; Marques *et al.* 1995; Nozue *et al.* 1998). For example, two fractions in a kiwifruit extract from a DEAE-cellulose column exhibit optimum activity at pH 6.8 and 7.3 in the presence of catechol (Park and Luh 1985). The pH optima of two isoforms purified from sweet potato were found to be 5.4 and 6.7 for the oxidation of chlorogenic acid (Nozue *et al.* 1998). Oba *et al.* (1992) also reported two PPO isozymes in banana bud extracts with optimum pHs of 6.8 and 5.5 for the oxidation of dopamine.

The optimum pH is influenced by a number of experimental factors such as extraction methods, temperature, nature of the phenolic substrate, and buffer system used during determination (Whitaker 1994). It is therefore difficult to extrapolate data obtained by other methods and using other substrates. Hence, Table 3 represents the approximate pH optimum values. The marked effect of

extraction methods on the pH optimum of PPO from olive fruit was shown by Ben-Shalom *et al.* (1977). PPO extracted from olive without any purification process exhibited a broad pH optimum between pH 5.5 and 7.5, while PPO purified from an olive acetone powder had a single pH optimum of 4.5. Nature of the substrate utilized in the activity assay is another factor, which may significantly influence the pH optimum of the enzyme. For example, PPO from tea leaves shows maximum activity at pH 5.7 with pyrogallol but a pH of 5.0 with 4-methylcatechol (Table 3). The substrate dependent pH optimum also exists in PPO from dog-rose (Sakiroglu *et al.* 1996), bean seeds (Paul and Gowda 2000) and strawberry (Wesche-Ebeling and Montgomery 1990b). This variation could be accounted for by the differences in the binding ability of the substrates to the active site under acidic and alkaline conditions (Tipton and Dixon 1983; Whitaker 1994).

The optimum pH for maximum PPO activity is also subject to changes when assayed in the presence of the modulator, SDS (Fig. 7). The general behavior of apple PPO with changing pH alters in the presence of SDS (Marques *et al.* 1995). Regardless of the substrate, the activity of apple PPO is inhibited at acidic pH and activated at pH above 5.0 in the presence of 3.5 mM SDS (Marques *et al.* 1995). The pH optimum of the enzyme is also shifted from low to a higher pH value with different values for different substrates (Fig. 7). This also appears to be the case for *Prunus* fruits (Fraignier *et al.* 1995), peach (Laveda *et al.* 2000), strawberry (Serradell *et al.* 2000) and broad bean (Moore and Flurkey 1990; Jimenez and Garcia-Carmona 1996) PPOs, where SDS causes a shift in the pH optimum of the enzyme from low to higher pH values (Fig. 7). In the case of PPOs from apple, strawberry and *Prunus* fruits, modification of the behavior of the enzyme with regard to pH by SDS is not considered to be by activation of latent PPO form (Fraignier *et al.* 1995; Marques *et al.* 1995; Serradell *et al.* 2000). In contrast, broad bean and peach PPOs is believed to exist in a latent state and activated severalfold by SDS (Moore and Flurkey 1990; Laveda *et al.* 2000). The difference in pH profiles of the latent and SDS-activated PPO forms was attributed to the displacement of the sensitive pKs of the enzyme caused by the interaction with negatively charged SDS molecules (Jimenez and Garcia-Carmona 1996; Espin and Wichers 1999b).

Temperature Effects in PPO Activity

Temperature is another important factor that significantly influences the catalytic activity of PPO. It is well known that a decrease in the kinetic energy of the reactant molecules at low temperatures corresponds to a slower reaction (Laidler and Peterman 1983; Lehninger *et al.* 1993). In addition, integrity of the delicate three-dimensional structure of the enzyme molecule is subjected to disruption and denaturation at high temperatures (Segel 1976; Whitaker 1994).

Variations in temperature may also alter the solubility of oxygen, one of the substrates required for PPO to perform its catalytic activity (Whitaker 1994).

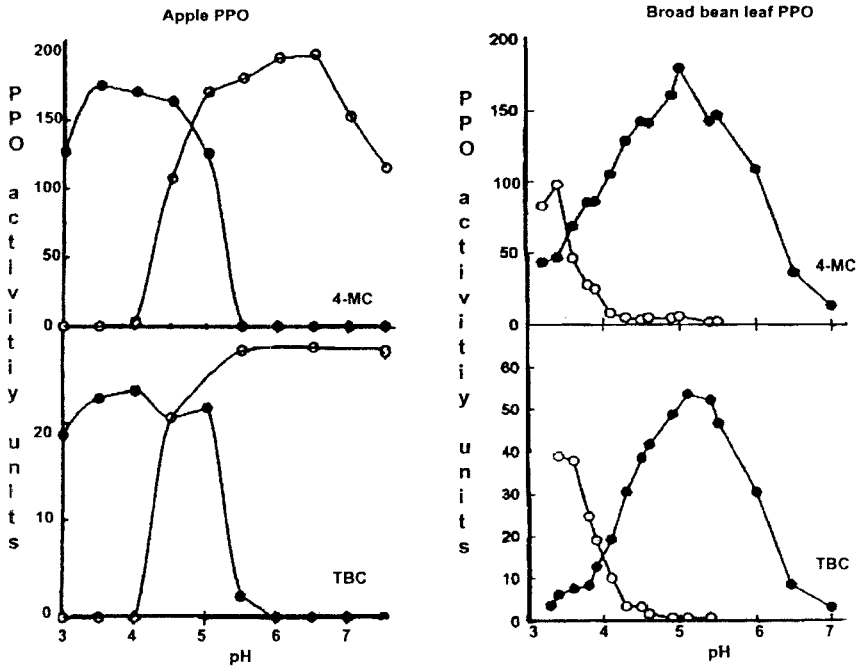

FIG. 7. EFFECT OF pH AND SDS ON PPO ACTIVITY
4-MC, 4-methylcatechol; TBC, *tert*-butylcatechol; without (•) and with (o) SDS for apple assays (from Marques *et al.* 1995); with (•) and without (o) SDS for broad bean assays (from Jimenez and Garcia-Carmona 1996).

Effect of temperature on PPO activity is shown in Table 4. The optimum temperature of PPO varies for different plant sources. PPOs from apple (Zhou *et al.* 1993), banana (Yang *et al.* 2000) and mango (Robinson *et al.* 1993) have temperature optima of 30C while PPOs from cocoa bean (Lee *et al.* 1991) and sunflower (Raymond *et al.* 1993) show 45C optima under the specified experimental conditions. The optimum temperatures for maximal PPO activities in lettuce (Heimdal *et al.* 1994) and grape (Valero *et al.* 1988) are in the range between 25-35C and 25-45C, respectively, and below and above the point the enzyme activity decreases gradually. PPOs from strawberry (Serradell *et al.* 2000) and cucumber (Miller *et al.* 1990) have relatively high optimum

temperature compared to those from other plant sources (Table 4). Optimum temperature of the activity is also affected by the substrate used in the assay. In a dog-rose extract, PPO activity toward monophenols exhibits higher temperature optima than toward di- and triphenols (Sakiroglu *et al.* 1996). The enzyme exhibits maximum activity at 25C with catechol, 15C with pyrogallol and 65 and 60C when tyrosine and *p*-cresol are the substrates, respectively.

TABLE 4.
EFFECT OF TEMPERATURE ON PPO ACTIVITY

Enzyme Source	Substrate	Temp opt (C)	Half-life (C)	Half-life min	Reference
Apple	Catechol	30	60	30	Zhou *et al.* (1993)
Banana	Dopamine	30			Yang *et al.* (2000)
Cocoa bean	Catechol	45	80	5	Lee *et al.* (1991)
Cucumber	Catechol	50			Miller *et al.* (1990)
Dog-rose	Catechol	25			Sakiroglu *et al.* (1996)
	Pyrogallol	15			
	Tyrosine	65			
	p-Cresol	60			
Grape	4-Methylcatechol	25-45	65	20	Valero *et al.* (1988)
Lettuce	Chlorogenic acid	25-35	80	5	Heimdal *et al.* (1994)
Longan	4-Methylcatechol	35	50	20	Jiang (1999)
Mango	4-Methylcatechol	30	80	35	Robinson *et al.* (1993)
Peach	Catechol				Wong *et al.* (1971)
PPO A			55	5.4	
PPO B			55	14.6	
PPO C			76	2.2	
PPO D			55	14.1	
Potato	Catechol	40	70	0.8	Cho and Ahn (1999)
Strawberry	Pyrocatechol	50			Serradell *et al.* (2000)
Sunflower	Gallic acid	45	80	15	Raymond *et al.* (1993)

Thermal inactivation of PPOs from several sources was shown to follow first-order kinetics (Wissemann and Lee 1981; Lee *et al.* 1983; Wesche-Ebeling and Montgomery 1990b; Robinson *et al.* 1993; Yemenicioglu *et al.* 1997). However, loss in PPO activity from sunflower seeds did not follow first-order kinetics at temperatures of 80 and 100C but at a lower temperature of 65C (Raymond *et al.* 1993). A complete study on heat inactivation kinetics for a number of apple cultivars at 68, 73, and 78C showed that PPO activity initially increased, attributed to the activation of a latent form, and then decreased with

heat, following a first order kinetic model (Yemenicioglu *et al.* 1997). They also highlighted the variability in the thermal stability of the apple cultivars; PPO in Amasya, for instance, is less heat-stable than PPO in Starking Delicious apples. In addition, multiple forms present in the tissue may also show differential heat stability (Table 4, Wong *et al.* 1971).

Exposure time and temperature required for inactivation of the enzyme are quite variable among different plant sources (Table 4). Studies on thermal stability of a grape PPO indicated that the enzyme shows about a 50% reduction in activity at 65C after 20 min and complete inactivation can be achieved at 75C after 15 min (Valero *et al.* 1988). At 60C, PPO from apple has a half-life of 30 min (Zhou *et al.* 1993). PPOs from lettuce (Heimdal *et al.* 1994), and cocoa bean (Lee *et al.* 1991) are relatively heat stable. Heat treatment up to 70C for 5 min did not affect lettuce PPO activity while at 90C no activity remained after 5 min (Heimdal *et al.* 1994). PPO in mango skin is also relatively thermostable, requiring more than 15 min at 80C for 50% loss of activity (Robinson *et al.* 1993). Thermal stability of PPO may also be influenced by nature of phenolic substrate used during determination (Park and Luh 1985; Wesche-Ebeling and Montgomery 1990b). For example, PPO activity in kiwifruit extracts toward (+) catechin was found to be more heat resistant than toward catechol (Park and Luh 1985). Additionally, Lourenco *et al.* (1992), while working on PPO from sweet potato, found that sucrose and salts in the reaction environment function as protective agents for the enzyme against thermal denaturation.

Multiplicity in Biochemical Properties

Existence of PPOs in multiple molecular forms (isoforms) was found in various plant sources. Multiple forms detected in such plants as apple, banana, dog-rose, grape, kiwifruit, lettuce, mushroom, peach, pineapple, potato, spinach, strawberry and sweet potato are shown in Table 5 and those found in several other plant sources are included in the discussion below. Such forms of PPO are recognized by their distinguishable differences in physical, chemical or enzymatic properties such as electrophoretic mobility, temperature and pH optimum, substrate specificity and pI (Table 5). Multiple forms differing in their enzymatic and biochemical properties are believed to be of enormous physiological significance *in vivo* (Harel *et al.* 1965; Constantinides and Bedford 1967; Harel and Mayer 1968; Shaw *et al.* 1991). Nonetheless, the issue of the innate existence of such forms in the tissues is somewhat contentious. As shown in Table 5, there is conflicting evidence regarding the number of molecular forms in a tissue of interest. In an apple extract, four active PPO molecular forms were found in an early work by Harel *et al.* (1965) and Harel and Mayer (1968) while subsequent studies by Marques *et al.* (1995) and Zhou *et al.* (1993)

reported two, and those by Goodenough *et al.* (1983) and Murata *et al.* (1995) found a single form.

TABLE 5.
NUMBER AND DISTINCTIVE PROPERTIES OF MULTIPLE FORMS OF PPO

Enzyme Source	No. Isoforms	Distinctive Properties
Apple	4	Tissue location, electrophoretic mobility, Km towards oxygen, substrate specificity, sensitivity to inhibitors (Harel *et al.* 1965)
	2	Chromatographic behavior (Zhou *et al.* 1993)
	2[1]	MW, pH optima, SDS activation (Marques *et al.* 1995)
	3	Electrophoretic mobility upon storage (Barrett *et al.* 1991)
	1	(Goodenough *et al.* 1983; Murata *et al.* 1995)
Banana	5	Electrophoretic mobility (Watson and Flurkey 1986)
	2	Electrophoretic mobility, pH optima, heat stability (Oba *et al.* 1992)
	1	(Yang *et al.* 2000; Sojo *et al.* 1998)
Dog-rose	2	Chromatographic behavior, elecrtophotic mobility (Sakiroglu *et al.* 1996)
Grape	8	Electrophoretic mobility, pH (Harel *et al.* 1973)
	3	Chromatographic behavior, pI (Interesse *et al.* 1984)
	2	Electrophoretic mobility (Sanchez-Ferrer *et al.* 1989)
	1	(Rathjen and Robinson 1992; Nakamura *et al.* 1983)
Kiwifruit	8	Electrophoretic mobility, substrate specificity (Park and Luh 1985)
Lettuce	2	Electrophoretic mobility, tissue location, ability to separate into subunits (Heimdal *et al.* 1994)
Mushroom	15	Tissue location, pI (Leeuwen and Wichers 1999)
	12	Electrophoretic mobility, substrate specificity, sensitivity to inhibitors, heat tolerance (Constantinides and Bedford 1967).
	4	Chromatographic behavior, substrate specificity, amino acid composition, copper content (Bouchilloux *et al.* 1963)
Peach	4	Electrophotic mobility, substrate specificity, heat stability, pH optima, sensitivity to inhibitors (Wong *et al.* 1971)
	3	Chromatographic behavior, carbohydrate content (Flurkey and Jen 1980)
Pineapple	3	Chromatographic behavior (Das *et al.* 1997)
Potato	11	Electrophoretic mobility (Constantinides and Bedford 1967)
	1	(Sanchez-Ferrer *et al.* 1993a)
Spinach	10	Electrophoretic mobility, substrate specificity, sensitivity to inhibitors (Lieberei *et al.* 1981)
	4	Electrophoretic mobility (Angleton and Flurkey 1984)
	2	MW estimation (Goldbeck and Cammarata 1981)
Strawberry	2	Chromatographic behavior, electrophoretic mobility, MW estimation (Wesche-Ebeling and Montgomery 1990a)
	1	(Serradell *et al.* 2000)
Sweet potato	2	MW, Km towards chlorogenic acid, pH optima, amino acid sequences (Nozue *et al.* 1998)
	1	(Lourenco *et al.* 1992)

[1]Multiplicity imitated by *in vitro* proteolysis

Likewise, Rathjen and Robinson (1992) and Nakamura *et al.* (1983) described only one PPO form from grapes, whereas up to eight bands staining for PPO activity were found by Harel *et al.* (1973), Interesse *et al.* (1984) and Sanchez-Ferrer *et al.* (1989a). This diversity also observed in several other PPO sources is widely questioned in the literature.

The uncertainty in number, size and biochemical characteristics of PPO forms could account for differences between developmental stages, subcellular location, storage conditions or for the formation of artifacts during isolation and purification processes due to modification of the native enzyme (Harel and Mayer 1968; Lerner *et al.* 1972; Cash *et al.* 1976; Lieberei and Biehl 1978; Meyer and Biehl 1980; Lieberei *et al.* 1981; Wissemann and Lee 1981; King and Flurkey 1987; Kowalski *et al.* 1992; Sanchez-Ferrer *et al.* 1993b; Fraignier *et al.* 1995; Marques *et al.* 1995; Shin *et al.* 1997; Marì *et al.* 1998; Leeuwen and Wichers 1999). These differing results may also reflect the differences between varieties. For example, electrophoretic analysis of PPO isolated from two-muscadine cultivars, Welder and Noble, display a single and two bands stained for PPO activity, respectively (Lamikanra *et al.* 1992). The different PPO isoforms were also reported not only in different papaya cultivars but in different tissues of the same papaya cultivar as well (Shaw *et al.* 1991).

Multiple forms are often regarded as artifacts with much different chemical or physiochemical characteristics than the native enzyme. Such molecular forms are suggested to arise from association-dissociation phenomena for PPOs from several sources (Harel and Mayer 1968; Jolley *et al.* 1969; Goldbeck and Cammarata 1981; Das *et al.* 1997). It is believed that interconversion between PPO forms can take place upon aging or further purification (Harel and Mayer 1968; Goldbeck and Cammarata 1981) and it can be enhanced by enrichment of the enzyme protein and by various treatments (Harel and Mayer 1968; Jolley *et al.* 1969; Das *et al.* 1997). In addition, stimulation of PPO in wheat seeds by the phytohormone, GA_3, was shown to induce the appearance of new multiple forms via association of the low molecular weight form following some structural modifications (Saluja and Sachar 1982). However, the hormone-induced multiple forms did not exhibit the phenomenon of association-dissociation by enrichment of the enzyme protein, which was contradictory to the behavior of mushroom PPO (Jolley *et al.* 1969). Meyer and Biehl (1980, 1981) proposed that spinach PPO forms cannot be converted artificially *in vitro* and the interconversion of PPO forms occurs only during senescence of spinach leaf.

The likely attachment of phenolic oxidation products (Gregory and Bendall 1966; Matheis and Whitaker 1984; Lanker *et al.* 1987; Macheix *et al.* 1990; Kowalski *et al.* 1992; Sanchez-Ferrer *et al.* 1993b, 1994; Sojo *et al.* 1998a) or carbohydrate materials (Flurkey and Jen 1980; Wesche-Ebeling and Montgomery 1990a; Raffert and Flurkey 1995) to the native enzyme *in vivo* or during extraction and purification may also result in formation of artifacts. The covalent

interactions between PPO protein and the catalytically produced quinones could generate oxidative artifacts with different isolelectric points and molecular weights from the native one (Interesse *et al.* 1984; Kowalski *et al.* 1992). Relative molecular weights of cross-linked forms could be as much as 20X that of the native one (Kowalski *et al.* 1992). Sanchez-Ferrer and his group (Sanchez-Ferrer *et al.* 1989a, 1993b, 1994; Sojo *et al.* 1998a) suggest that the temperature-induced phase separation method (a two-phase partitioning approach) eliminates the postpurification tanning of the enzyme and enables the characterization of native PPO forms.

In addition, PPO could also be modified during isolation procedures by covalent attachment of glycosides to PPO (Flurkey and Jen 1980; Raffert and Flurkey 1995). Alternatively, carbohydrate attachment to PPO could be a consequence of posttranslational events (Flurkey 1985, 1986; Raffert and Flurkey 1995). Glycosylation could influence physiochemical characteristics, such as solubility, thermal stability and resistance to proteases, of the enzyme (Flurkey and Jen 1980; Wesche-Ebeling and Montgomery 1990a). Furthermore, the modified native enzyme could be further subjected to nonspecific degradation. Partial degradation of nonspecific carbohydrates or glycoproteins attached to, or associated with, the enzyme by the action of hydrolytic enzymes may be responsible for different forms of the enzyme (Harel *et al.* 1973). PPO from several plant sources, including apple (Stelzig *et al.* 1972), broad bean (Raffert and Flurkey 1995), cabbage (Fujita *et al.* 1995, 1997), peach (Flurkey and Jen 1980), potato (Balasingam and Ferdinand 1970), strawberry (Wesche-Ebeling and Montgomery 1990a) and sunflower (Raymond *et al.* 1993) was reported to have a carbohydrate material. Recently, a mature apricot PPO clone was shown to possess one putative glycolysation site (Chevalier *et al.* 1999). The differences between molecular weights determined from PPO cDNA and observed under fully denaturing electrophoretic conditions are speculated to be indicative of glycolysation of the PPO protein in broad bean (Cary *et al.* 1992) and apricot (Chevalier *et al.* 1999). However, it is yet to be clarified if the enzyme is modified by association with carbohydrates during extraction or intrinsic glycoproteins are not taken into account while estimating molecular weight since the presumed one is lower.

Generation of artifacts from protease action has been widely reported (Harel *et al.* 1973; Flurkey and Jen 1980; King and Flurkey 1987; Marques *et al.* 1994; Fraignier *et al.* 1995; Shin *et al.* 1997). In the presence of trypsin protease inhibitors (PMFS, Trasylol), a decrease in number of peach PPO forms during purification process was observed by Flurkey and Jen (1980). In view of that, protease inhibitors are normally used during extraction and purification to prevent formation of multiple forms by limited proteolysis with endogenous proteases. However, multiple PPO forms even in the presence of protease inhibitors were noted in mung bean leaf (Shin *et al.* 1997), strawberry (Wesche-

Ebeling and Montgomery 1990a) and sweet potato extracts (Nozue *et al.* 1998). Studies on apple PPO have confirmed these findings by showing that the native enzyme is affected by proteolysis into a smaller active form resistant to further protease activity (Marques *et al.* 1994; Mari *et al.* 1998) and possesses different biochemical characteristics (Marques *et al.* 1995). In these studies, supposed native multiplicity is simulated by subjecting the native extract to SDS-proteinase K digestion. The native and the proteolysed forms have similar Km values for various substrates but different pH optima, 3.5-4.5 and 4.5-7.5, respectively. However, when the native form is assayed with SDS, it shows the same pH and inhibition patterns as the proteolysed form. Therefore, it is suggested that proteolysis has the same effect as SDS on the activity of native form (Marques *et al.* 1995). Altered kinetic properties of intact PPO versus the proteolysed form are also considered by Shin *et al.* (1997) in mung bean extracts. Additionally, studies on broad bean (King and Flurkey 1987; Robinson and Dry 1992; Jimenez and Garcia-Carmona 1996) and various *Prunus* fruits (Fraignier *et al.* 1995) showed that PPO can be cleaved without any significant loss of enzyme activity and thus lower molecular weight forms may arise from proteolysis. The possibility of involvement of *in vivo* proteolytic degradation in formation of multiple forms perhaps during senescence, injury (Fraignier *et al.* 1995; Mari *et al.* 1998) or in response to some unidentified trigger (Nozue *et al.* 1998) has long been questioned and is still not fully clear.

In some cases, multiplicity can be explained by activation of latent forms. The latent enzymes activated by endogenous proteases or by other means, discussed in the next section, may exhibit different physicochemical properties than the active enzymes given that both latent and active forms might exist at the same time (Interesse *et al.* 1980; Angleton and Flurkey 1984; Leeuwen and Wichers 1999). Alternatively, the different activating agents may render active PPO forms with different pH profiles and molecular weights (Jimenez and Garcia-Carmona 1996; Espin and Wichers 1999b) and with different catalytic and affinity properties toward their substrates (Espin and Wichers 1999a). Moreover, the changes in the tertiary structure of the enzyme by detergents or denaturing agents (Robb *et al.* 1964; Angleton and Flurkey 1984; Moore and Flurkey 1990) or the activation of latent enzymes during drastic isolation procedures or perhaps *in vivo* may also account for multiplicity (Lieberei and Biehl 1978; Meyer and Biehl 1980, 1981; Lieberei *et al.* 1981; Sanchez-Ferrer *et al.* 1989a, 1990; Guillard and Richard-Forget 1997; Leeuwen and Wichers 1999). The understanding of the activation process and characterization of the distinct forms in a given PPO source is crucial in order to develop efficient methods of controlling PPO activity.

Multiplicity in apparent size and number may also be induced via conformational changes in the enzyme during prolonged storage or upon exposure to urea and acid pH as the electrophoretic mobilities of several active

PPO bands from grape extract alter as a function of those experimental parameters (Lerner *et al.* 1972; Harel *et al.* 1973). More recent studies by Mari *et al.* (1998) on apple PPO showed that a change in tertiary structure of the protein alters the movement in electrophoresis. Therefore, multiplicity assigned based on PPO molecular weight estimates must be interpreted with caution. Interconversion of various forms of PPO determined under fully and partially denaturing electrophoretic conditions has largely been reported in the literature (Flurkey 1990; Cary *et al.* 1992; Robinson and Dry 1992; Fraignier *et al.* 1995; Marques *et al.* 1995). In apple, for example, the native (42 kDa) and proteolysed (27 kDa) forms detected under partially denaturing conditions were found to have molecular weights of 64 and 42 kDa, respectively (Marques *et al.* 1995). Studies on broad bean PPO also showed that the 45 kDa forms observed under partially denaturing conditions are converted to the 60 kDa PPOs under fully denaturing electrophoresis conditions (Cary *et al.* 1992; Robinson and Dry 1992). In general, molecular weights of PPOs vary significantly from source to source. The isoforms of PPOs from many plant sources were reported to range in molecular mass from 32 to over 200 kDa, mostly within the range of 35-70 kDa (Flurkey 1986; Sherman *et al.* 1991; Steffens *et al.* 1994; Fraignier *et al.* 1995; Van Gelder *et al.* 1997; Yang *et al.* 2000).

Investigations on genetic and molecular biology of PPO furnish additional insights into the multiplicity phenomenon. A study concerning the immunological relationship of multiple PPO forms differing in mono- and diphenolase activities and sensitivity to inhibitors in spinach suggests that these are genetic variants of a single protein based on their antigenic characteristics (Lieberei *et al.* 1981). Afterward, several nuclearly inherited multiple forms were identified in tobacco species and hybrids and the differences in isoforms were attributed to posttranslational modifications of the enzyme by other gene products (Lax *et al.* 1984). Although multiplicity is mostly reported as being induced by modification of an enzyme artificially during the isolation process or secondarily in the organism during processing of nuclearly coded protein (Lax *et al.* 1984), it may also be due to differential expression of different members of a gene family. This is based on the findings that PPOs from several sources including apple (Boss *et al.* 1995), banana (Gooding *et al.* 2001), broad bean (Cary *et al.* 1992), potato (Hunt *et al.* 1993; Thygesen *et al.* 1995), tomato (Shahar *et al.* 1992; Newman *et al.* 1993) and pokeweed (Joy *et al.* 1995) are encoded by multigene families. Differences in processing and translocation of the products of various genes across the chloroplast envelope may also account for multiplicity (Sommer *et al.* 1994).

Latency and Effect of Activators

In higher plants, PPO is believed to be membrane bound (Tolbert 1973;

Goldbeck and Cammarata 1981; Lieberei *et al.* 1981; Meyer and Biehl 1981; Vaughn and Duke 1984a; Sanchez-Ferrer *et al.* 1989b, 1990; Amorim and Melo 1991; Murata *et al.* 1997). The enzyme existing in a latent form on the thylakoid membranes is not involved in the synthesis of phenolic compounds, which are restrained to vacuoles, unless it is activated *in vivo* during senescence, injury or stress (Tolbert 1973; Lieberei and Biehl 1978; Meyer and Biehl 1980, 1981; Goldbeck and Cammarata 1981; Lieberei *et al.* 1981; Vaughn and Duke 1984a; Vaughn *et al.* 1988; Barrett *et al.* 1991; Murata *et al.* 1997). Limited activity of PPO in some plant tissues such as apple (Marques *et al.* 1995), apricot (Chevalier *et al.* 1999), cucumber (Miller *et al.* 1990), eggplant (Perez-Gilabert and Carmona 2000), potato tubers (Sanchez-Ferrer *et al.* 1993b), sunflower (Raymond *et al.* 1993) and various *Prunus* fruits (Fraignier *et al.* 1995) may be attributed to its tight binding to membranes for the reason that it is active upon its release from the membrane particularly by using nonionic detergents (Sanchez-Ferrer *et al.* 1994; Marques *et al.* 1994, 1995). However, latency may not be due just to membrane integrity because in some other plant PPOs such as broad bean (Kenten 1957; Robb *et al.* 1964; Moore and Flurkey 1990; Sanchez-Ferrer *et al.* 1990; Robinson and Dry 1992; Jimenez and Garcia-Carmona 1996), wheat (Interesse *et al.* 1980), spinach (Tolbert 1973; Lieberei and Biehl 1978; Goldbeck and Cammarata 1981; Sanchez-Ferrer *et al.* 1989b), grape (Sanchez-Ferrer *et al.* 1989a; Dry and Robinson 1994), potato leaf (Sanchez-Ferrer *et al.* 1993a), mango (Robinson *et al.* 1993), peach (Laveda *et al.* 2000, 2001), pear (Guillard and Richard-Forget 1997) and sago palm (Onsa *et al.* 2000) latency persists even after released from the plastid and requires activation.

The degree of latency varies widely with plant species and tissues. *In vitro* activation of such latent enzymes has been studied intensively for years and a variety of treatments or agents have been shown to release the enzyme from its latent state. Activation can be induced by frost and aging (Lieberei and Biehl 1978; Meyer and Biehl 1980), fatty acids (Goldbeck and Cammarata 1981; Guillard and Richard-Forget 1997; Onsa *et al.* 2000), alcohols (Guillard and Richard-Forget 1997; Espin and Wichers 1999a; Onsa *et al.* 2000), denaturants (Robb *et al.* 1964; Swain *et al.* 1966; Lerner *et al.* 1972; Saluja and Sachar 1982), detergents (Angleton and Flurkey 1984; Sanchez-Ferrer *et al.* 1989a, b, 1990, 1993a; Moore and Flurkey 1990; Robinson and Dry 1992; Jimenez and Garcia-Carmona 1996; Sojo *et al.* 1998a; Espin and Wichers 1999b; Laveda *et al.* 2000), acid and alkali (Kenten 1957), protease treatment (Tolbert 1973; Saluja and Sachar 1982; King and Flurkey 1987; Sanchez-Ferrer *et al.* 1989b, 1990; Robinson and Dry 1992; Laveda *et al.* 2001), sonication (Leeuwen and Wichers 1999) and mild heat treatment (Sheptovitsky and Brudvig 1996). Removal of PPO-bound inhibitors may also result in activation of latent enzymes (Kenten 1957; Lieberei and Biehl 1978; Interesse *et al.* 1980, 1984; Angleton

and Flurkey 1984; Lamikanra *et al.* 1992). Meyer and Biehl (1980, 1981), on the other hand, suggest that the latent forms can be activated by interconversion of multiple forms.

Activation of latent PPO, a consequence of partial enzymatic proteolysis of PPO protein, has long been questioned. Latent spinach PPO can be activated with both native and denaturated trypsin (Tolbert 1973). Since catalytically active trypsin is not essentially the main element for the activation process, nature of the mechanism of trypsin activation of latent spinach PPO is unknown (Tolbert 1973). The later studies by Sheptovitsky and Brudvig (1996) suggest that membrane associated active spinach PPO may have a proteolytic function, though nature of the cleavage mechanism was not studied. Trypsin-mediated activation of PPO in wheat seeds is believed not due to proteolytic action of trypsin but to a nonspecific protein-protein interaction (Saluja and Sachar 1982). It is mostly believed that endogenous proteases involved in activation of latent PPO is by cleaving a certain region, i.e., proteolytic digestion, of the latent protein (King and Flurkey 1987; Rathjen and Robinson 1992; Robinson and Dry 1992; Dry and Robinson 1994; Laveda *et al.* 2001). Therefore, protease inhibitors are usually used to avoid further PPO activation by endogenous proteases during isolation procedures.

Several studies have been conducted to reveal the mechanism of proteolytic activation. In broad bean, only one latent PPO form with a molecular mass of 60 kDa, which is active upon treatment with SDS, was observed in the presence of protease inhibitors (Robinson and Dry 1992). Additionally, *in vitro* proteolytic cleavage of the 60 kDa enzyme yields a 42 kDa active PPO form and inactive peptides of 15 to 18 kDa. Therefore, activation of the latent enzyme by partial proteolysis was attributed to loss of the protease-sensitive sites at the carboxy-terminal end (Robinson and Dry 1992). It is not yet clear whether this proteolytic cleavage can also occur *in vivo*. However, *in vivo* carboxy-terminal processing within the chloroplast appears to be a prerequisite for the activation of the latent grape PPO as the inactive 60 kDa precursors produces a 40 kDa PPO (Rathjen and Robinson 1992; Dry and Robinson 1994). *In vitro* proteolysis does not produce the 40 kDa PPO forms, i.e., these are not isolation artifacts. The smaller form bearing two copper-binding regions seems to form in the chloroplast upon the removal of carboxy-terminal extension following the cleavage of the transit peptide from the amino-terminus (Dry and Robinson 1994). There exists a positive correlation between the amount of the 40 kDa proteins and PPO activity. Therefore, the presence of the 60 kDa protein in young but not mature berries, and its accumulation in the white regions of the mutant grapevine having negligible PPO activity, were given as further evidences for the requirement of *in vivo* processing of the larger precursor (Rathjen and Robinson 1992).

The anionic detergent, SDS, has been widely used as an activator of latent PPO from several plant sources (Angleton and Flurkey 1984; Sanchez-Ferrer *et al.* 1989a, b, 1990, 1993a; Moore and Flurkey 1990; Jimenez and Garcia-Carmona 1996; Sojo *et al.* 1998a; Espin and Wichers 1999b; Laveda *et al.* 2000). Most enzymes lose their biological activity upon treatment with SDS because of the drastic alteration of the tertiary and quaternary structure of proteins. Resistance of PPO to SDS is perhaps due to presence of disulfide bonds strengthening PPO structure (Fraignier *et al.* 1995; Marques *et al.* 1995; Mari *et al.* 1998). Degree of SDS activation varies greatly with plant materials. For example, PPO activity increased about 65- to 119-fold in broad bean (Sanchez-Ferrer *et al.* 1990; Jimenez and Garcia-Carmona 1996), 19-fold in grape (Sanchez-Ferrer *et al.* 1989a), 25-fold in peach (Laveda *et al.* 2000), 10-fold in banana (Sojo *et al.* 1998a), 4-fold in potato leaf (Sanchez-Ferrer *et al.* 1993a) and 7-fold in mango (Robinson *et al.* 1993) by SDS under the defined experimental conditions. As discussed below, PPO activation by SDS is dependent on a number of parameters: detergent concentration and pH being the ones typically highlighted in the literature.

PPO activation is generally achieved at low SDS concentrations and higher detergent concentrations significantly inhibit the enzyme activity (Robb *et al.* 1964; Sanchez-Ferrer *et al.* 1989b, 1993a). Comprehensive studies on latent PPO from broad bean extracts showed that latent enzyme is activated in a sigmoidal manner with increasing SDS concentrations, and SDS concentrations greater than the critical micelle concentration for the detergent under certain assay conditions show inhibition on PPO activity (Moore and Flurkey 1990). The similar manner of activation was also observed in case of mushroom (Espin and Wichers 1999b), peach (Laveda *et al.* 2000), table beet (Escribano *et al.* 1997) and mango (Robinson *et al.* 1993) PPOs. In addition, degree of activation of latent potato leaf PPO by SDS was shown to be dependent on the substrate used in the assay, where highest activation is achieved with the most hydrophobic substrate at pH 4.0 (Sanchez-Ferrer *et al.* 1993a). Conversely, Jimenez and Garcia-Carmona (1996) detected no significant differences in the activation values of broad bean PPO for any substrates used in the assay.

Experiments on the joint effect of pH and SDS on PPO activity, aforementioned, revealed that the detergent causes a shift in pH optimum of the enzyme from low to higher pH values (Moore and Flurkey 1990; Jimenez and Garcia-Carmona 1996; Escribano *et al.* 1997; Laveda *et al.* 2000). However, this behavior does not seem ubiquitous as similar pH optimum profiles with and without SDS were obtained for latent potato leaf PPO (Sanchez-Ferrer *et al.* 1993a) and Sago palm PPO (Onsa *et al.* 2000). In broad bean extracts, however, assays in the presence of SDS abolish the optimum pH around 4.0 and a new maximal optimum appears around pH 5.0 (Fig. 7) (Jimenez and Garcia-Carmona 1996). Observed activity at acid pH values without SDS is attributed to being

induced by acid shocking (Kenten 1957). In general, maximal activation by SDS is attained above pH 4.0 and there is no detectable activation or complete inhibition below 4.0 (Moore and Flurkey 1990; Jimenez and Garcia-Carmona 1996; Escribano *et al.* 1997; Laveda *et al.* 2000). This was attributed to the existence of a specific pH-sensitive SDS binding center (Jimenez and Garcia-Carmona 1996). In this case, effect of SDS on PPO activity is suggested to be restricted only to the pH values above 4.0 (Jimenez and Garcia-Carmona 1996). Activation of SDS on fruit PPOs at higher pH values is considered not to be due to latency per se but rather the joint outcome of SDS and pH (Fraignier *et al.* 1995; Marques *et al.* 1995).

A detailed study examining the possible mechanism of SDS activation on latent broad bean PPO by Moore and Flurkey (1990) has given an additional insight into the activation phenomena. The mode of PPO activation by SDS was attributed to the limited conformational changes owing to binding of small amounts of SDS as the detergent alters both enzymatic and physical characteristics of the enzyme. This concurs with the conclusion of earlier workers that SDS initiates the activation of latent PPO by inducing limited conformational changes in the enzyme structure (Robb *et al.* 1964; Swain *et al.* 1966). An additional support to these findings comes from kinetic studies (Espin and Wichers 1999b) on mushroom PPO whose >95% total activity was previously found in the latent state (Leeuwen and Wichers 1999) and activated with SDS by a slow conformational change. It appears from these studies that access to the catalytic center is controlled by a regulatory PPO domain or region, shifted by SDS in response to pH or cut by proteolysis (Marques *et al.* 1995; Jimenez and Garcia-Carmona 1996).

Studies comparing the behavior of the SDS activated and protease activated PPO forms in response to pH showed that these two forms exhibit different pH profiles (Jimenez and Garcia-Carmona 1996). There is little or no detectable activity below pH 4.0 as stated previously when the enzyme is activated by SDS, whereas the protease-activated form is extremely active at acidic pH values. This shows that the action of the enzyme at different pH values depends on the modulator used to provoke activation. The SDS-mediated activation process can be fully reversible in the presence of cyclodextrins, which form a noncovalent complex with the detergent (Laveda *et al.* 2000). Activation of PPO by changes in pH (Lerner *et al.* 1972; Valero and Garcia-Carmona 1992) and by fatty acids (Goldbeck and Cammarata 1981), alcohols (Espin and Wichers 1999a; Onsa *et al.* 2000) and denaturants (Robb *et al.* 1964; Swain *et al.* 1966; Saluja and Sachar 1982) was also attributed to conformational changes in the enzyme structure.

Nevertheless, the ratio of latent and active PPO forms in the intact living tissues is still controversial since it is unclear if the enzyme is isolated in its natural form (Interesse *et al.* 1980; Sanchez-Ferrer *et al.* 1994). The new mild

temperature-induced phase partitioning method is believed to offer the possibility of extracting PPO in its natural latent state (Sanchez-Ferrer *et al.* 1989b, 1990, 1993a, 1994; Sojo *et al.* 1998a). However, it is not known specifically if the activation is a normal process occurring *in vivo*. In general, aging (Tolbert 1973; Lieberei and Biehl 1978; Goldbeck and Cammarata 1981), association/dissociation phenomena (Meyer and Biehl 1980), or endogenous compounds likely present in the plant tissues of interest including fatty acids (Goldbeck and Cammarata 1981), proteases (Tolbert 1973; King and Flurkey 1987; Rathjen and Robinson 1992; Dry and Robinson 1994; Jimenez and Garcia-Carmona 1996), terpenes (Robinson *et al.* 1993), benzyl alcohol (Espin and Wichers 1999a), or dissociation of low molecular weight inhibitors (Kenten 1957; Lieberei and Biehl 1978) may be the physiological activators of latent PPO. In addition, it is suggested that PPO activity may be regulated *in vivo* by oxygen concentration and pH as the level of both changes in the chloroplast of the intact tissue (Lerner *et al.* 1972; Valero and Garcia-Carmona 1992). Degree of latency also remains obscure and may differ depending on plant source, stage of development and type of activators (Tolbert 1973; Meyer and Biehl 1980; Lanker *et al.* 1987; Sanchez-Ferrer *et al.* 1989b, 1993a; Espin and Wichers 1999a, b; Leeuwen and Wichers 1999). Recently, Soler-Rivas *et al.* (1997) reported that latent PPO could be activated by pathogen attack, signifying the possible involvement of an activation process in a response mechanism to infection that may also occur *in vivo*.

Inhibitors

Millions of dollars in crop losses occur yearly due to enzymatic browning (Martinez and Whitaker 1995; Whitaker 1996; Kim *et al.* 2000). Enormous economic impact of PPO-induced deleterious browning reactions in fruit and vegetables, as well as seafood, such as shrimp and lobster, necessitates its control in order to maintain quality and extend product shelf-life. Several approaches can be shown experimentally to diminish or prevent browning of injured tissues. As reviewed by McEvily *et al.* (1992), inhibitors available to avoid PPO activity fall into six groups based on their mode of action:

(1) Reducing agents (ascorbic acid and analogues, sulfites)
(2) Chelating agents (ethylenediaminetetraacetate (EDTA), sodium diethyldithiocarbamate (DIECA), sodium azide)
(3) Complexing agents (cyclodextrins, chitosan)
(4) Acidulants (ascorbic acid, citric acid, malic acid, phosphoric acid)
(5) Enzyme inhibitors (substrate analogs, halides)
(6) Enzyme treatments (proteases, *o*-methyltransferase)

These compounds diminish or inhibit the browning reaction rate by means of eliminating from the reaction an active reaction element(s), that is, enzyme, substrate(s), copper or a reaction intermediate (o-quinones) (Vamos-Vigyazo 1981; Richardson and Hyslop 1985; Macheix *et al.* 1990; Nicolas *et al.* 1994; Ahvenainen 1996; Ferrar and Walker 1996). PPO from different sources may react similarly with inhibitor compounds. However, effectiveness of inhibitors against different PPOs could significantly vary and, therefore, specific control measures for individual systems would also be needed (Ferrar and Walker 1996).

Reducing agents are broadly used in the food industry. They inhibit melanin formation by preventing the accumulation of *o*-quinones, or they may form stable colorless products (Eskin *et al.* 1971; Nicolas *et al.* 1994; Osuga *et al.* 1994; Ashie *et al.* 1996; Kim *et al.* 2000). Of those agents, one of the most potent inhibitors of enzymatic browning is sulfur dioxide (SO_2) or sulfites, particularly sodium sulfite, sodium bisulfite, and sodium metabisulfite (Eskin *et al.* 1971; Sapers 1993; Kim *et al.* 2000). Sulfiting agents were utilized broadly in the fruit and vegetable industry as anti-browning agents because of their effectiveness and low price (Eskin *et al.* 1971; Eskin 1990). It is believed that sulfites not only simply act as a reducing agent but also have ability to directly inhibit PPO. They also interact with quinones preventing their further participation in forming brown pigments (Ashie *et al.* 1996). However, due to safety concerns, their use in fresh fruit and vegetables was banned by the Food and Drug Administration (Sapers 1993; Martinez and Whitaker 1995). They are still allowed for use on shrimp to delay the formation of blackspot and maintain the quality during storage or processing (Kim *et al.* 2000).

Ascorbic acid (vitamin C) and its isomer erythorbic acid, the best-known alternative reducing agent to sulfite, is commonly used as an antibrowning agent in the manufacturing of fruit juices, purees, frozen sliced fruits, and canned fruits and vegetables. However, browning typically proceeds after the exhaustion of ascorbic acid (Sapers 1993; Osuga *et al.* 1994; Ashie *et al.* 1996). There is substantial interest in using ascorbic acid along with other inhibitors for better control of PPO-induced browning. For example, a combination of ascorbic acid and citric acid was found more effective than the former alone (Eskin *et al.* 1971; Sapers 1993). This is probably due to stability of ascorbic acid in an acidic environment and inhibition of the acidic environment on the catalytic activity of the enzyme. Citric acid may also function as PPO inhibitor through its chelating action (Eskin *et al.* 1971) and ascorbic acid through its site-directed specificity toward histidine residues on the PPO protein (Golan-Goldhirsh *et al.* 1992; Osuga *et al.* 1994).

The use of acidulants is another approach widely used in food processing to diminish browning. Acids naturally present in some edible food products, such as ascorbic, citric, malic, or phosphoric acid shift the food pH to 3 or lower (Eskin *et al.* 1971; Park and Luh 1985; Eskin 1990; Osuga *et al.* 1994). Lemon juice is widely added to foods in substantial quantities to prevent enzymatic browning. PPO is generally catalytically more active in a pH range from 4.0-8.0 (Table 3) and the enzyme activity drastically drops at a higher acid environment. Mode(s) of pH-induced inhibition is attributable to protonation of catalytic groups essential for catalysis, conformational changes in the active site of the enzyme, irreversible denaturation of the protein, and/or reduction in the stability of the substrate as a function of pH (Segel 1976; Tipton and Dixon 1983; Whitaker 1994). Additionally, acidity may also diminish the strong binding of the enzyme to its active site copper, escalating ability of citric acid to react with copper through chelation (Osuga *et al.* 1994).

An alternative way of controlling adverse browning is the use of physical treatments. These include heating, freezing, refrigeration, dehydration, irradiation and high pressure (Ashie *et al.* 1996; Kim *et al.* 2000). However, these methods may also have some disadvantages, such as subcellular decompartmentation leading to enzyme-substrate contact and deterioration in texture (Macheix *et al.* 1990). Packaging under controlled atmospheres and the use of edible films and coatings as part of the food product, which prevents the penetration of oxygen, are other appealing methods for extending postharvest storage life of processed fruit and vegetables as oxygen is needed to trigger browning (Ahvenainen 1996).

Only a few browning inhibitors have the potential for use in the food industry because of off-flavors/odors, food safety, economic feasibility and perhaps effectiveness of inhibition as well (Eskin *et al.* 1971; McEvily *et al.* 1992; Sapers 1993). Therefore, there is interest to find additional effective, natural and safe anti-browning agent(s) for preventing adverse browning reactions in fruit and vegetables. Substantial investigations are performed on natural anti-browning substances, including amino acids (Kahn 1985), Maillard reaction products synthesized from various amino acids with glucose (Tan and Harris 1995), honey (Oszmianski and Lee 1990; Chen *et al.* 2000), pineapple juice (Lozano-de-Gonzalez *et al.* 1993), rhubarb juice (Son *et al.* 2000a) and commercial pectin preparations (Tong *et al.* 1995). Oxalic acid, a natural PPO inhibitor present in rhubarb juice and pectin, was recently shown to be a good chelator for the active site's copper (Son *et al.* 2000b; Yoruk and Marshall 2003). These natural inhibitors of PPO are not yet commercially utilized for food practice.

The more recent approach to reduce browning capacity of plants and, in turn, to improve crop quality is the control of PPO levels *in vivo* by means of antisense RNA strategy (Steffens *et al.* 1994; Martinez and Whitaker 1995). The

idea behind this approach is that the mRNA encoded by the antisense gene hybridizes with mRNA encoded by endogenous gene and consequently no translation occurs (Martinez and Whitaker 1995). Significant advances made on the molecular biology and genetics of PPO are discussed in this paper and elsewhere (Steffens *et al.* 1994; Van Gelder *et al.* 1997). In light of the molecular and genetic data available, it is believed that manipulation or regulation of the PPO gene expression in order to diminish adverse effects of PPO on quality of fruit and vegetable crops without application of food additives will be the future challenge. Anti-sense down-regulation of PPO, for instance, in potato has already been performed by Bachem *et al.* (1994) and Coetzer *et al.* (2001). Genetic transformation has also been performed by Murata *et al.* (2000) who showed that a transgenic apple (*Malus* × *domestica*) shoot in which PPO expression is reduced has lower browning potential than a control shoot.

LOCATION AND PHYSIOLOGICAL FUNCTION OF PPO IN PLANTS

Even though oxidation of phenols and formation of melanins are normal physiological processes of PPO, significance of the enzyme activity in living intact plant tissues is not fully understood. Since one must take into account the subcellular location of PPO to ascribe a physiological function to the enzyme (Mayer and Harel 1979), location of PPO has always been of special interest to researchers in the field. PPO is found in a variety of subcellular fractions such as peroxisomes, mitochondria and microsomes (Harel *et al.* 1964; Shomer *et al.* 1979; Mayer and Harel 1979; Martinez-Cayuela *et al.* 1989). In the mid 1900s, Arnon (1948, 1949), while studying spinach beet, presented evidence that PPO is exclusively localized in the chloroplast. Several studies concerning plastidic location of PPO have been accumulated in the literature since then. Considerable evidence that plant PPO is a nuclear-coded protein (Lax *et al.* 1984), located in the plastids has been provided (Vaughn and Duke 1984a; Vaughn *et al.* 1988; Sherman *et al.* 1991; Murata *et al.* 1997). Of the evidence, the compelling one on tentoxin-treated plants lacking PPO activity (Vaughn and Duke 1981, 1982, 1984b) makes the presence of a different form of the enzyme in aplastidic tissues implausible. The toxin was shown to inhibit the import of precursor PPO protein into plastids (Sommer *et al.* 1994).

It appears from the investigations that PPO is synthesized on cytoplasmic ribosomes and is practically inactive until integrated into the plastid. A partially unfolded protein stabilized by chaperones could be the translocation-competent form integrating into thylakoid membranes (Yalovsky *et al.* 1992; Marques *et al.* 1995). Nuclear-coded precursor proteins of higher molecular weight possessing a transit peptide sequence at the amino-terminal end are directed to the chloroplast. Such signal sequences are eventually removed by specific stromal peptidases (Koussevitzky *et al.* 1998) during the process of incorporation

and a lower molecular weight mature protein is produced (Sommer *et al.* 1994). This observation has been verified for a number of species, including apricot (Chevalier *et al.* 1999), apple (Boss *et al.* 1995; Haruta *et al.* 1998), broad bean (Cary *et al.* 1992), grape (Dry and Robinson 1994), potato (Hunt *et al.* 1993), spinach (Hind *et al.* 1995), tomato (Newman *et al.* 1993; Sommer *et al.* 1994) and pokeweed (Joy *et al.* 1995). Molecular weights of precursor and mature PPO proteins are approximately 68 kDa and 60 kDa, respectively.

Tomato PPO is routed to the thylokoid lumen in two steps (Sommer *et al.* 1994). In the first step, a 67 kDa precursor is imported into stroma and then processed to 62 kDa by stromal peptidases. In the final step, the resulting intermediate is translocated into the lumen and processed to a 59 kDa mature protein. The ratio between the intermediate and mature forms depends on the plant species, age and growth conditions (Sommer *et al.* 1994). However, there is variability in subplastidic location of the enzyme. PPO is believed to bind tightly to thylakoid membranes (Goldbeck and Cammarata 1981; Vaughn and Duke 1984a; Sanchez-Ferrer *et al.* 1990; Marques *et al.* 1994; Sheptovitsky and Brudvig 1996) but soluble forms in thylakoid lumen were also reported (Newman *et al.* 1993; Sommer *et al.* 1994). Detection of PPO reaction products within the lumen suggests that PPO is located on the lumenal face of thylakoid membrane (Shomer *et al.* 1979; Sherman *et al.* 1991). PPO may also be present in both soluble and membrane-bound forms (Harel *et al.* 1964; Lieberei *et al.* 1981; Escribano *et al.* 1997). The soluble form may be the result of membrane-bound forms released spontaneously as ripening and senescence progress (Ben-Shalom *et al.* 1977; Meyer and Biehl 1980, 1981; Lieberei *et al.* 1981; Barrett *et al.* 1991) or there may exist PPO forms not associated to the membrane at all. Within the tomato PPO gene family, only three out of seven of them were reported to possess sufficient hydrophobic character for being membrane-associated and the rest was devoid of membrane-spanning domains (Newman *et al.* 1993).

Involvement of PPO in oxidation of phenolic compounds in various plants is indisputably its most familiar role. Severe oxidative browning does not occur in healthy intact plant tissues perhaps because PPO activity *in vivo* is limited by lack of phenolic substrates physically separated from the enzyme within the vacuoles (Vaughn and Duke 1984a; Vaughn *et al.* 1988; Macheix *et al.* 1990; Amorim and Melo 1991; Lopez and Dimick 1991). It is usually believed that senescence or injury results in destruction of the biological barriers between PPO and polyphenols and the enzyme is active only when it unites with its phenolic substrates (Vaughn and Duke 1984a; Richardson and Hyslop 1985; Vaughn *et al.* 1988; Felton *et al.* 1989; Eskin 1990; Barrett *et al.* 1991; Lopez and Dimick 1991; Murata *et al.* 1997). However, the question is whether there are other factors, beside disruption of membrane integrity, that are involved in formation of such active enzymes capable of synthesizing or oxidizing

polyphenols or that affect the amount of active enzyme form and the level of enzyme activity during senescence, developmental stages or injury.

Developmental Regulations

There is a general agreement that during normal growth and development, PPO activity is much higher in unripe fruits and young leaves than in mature fruits and mature leaves (Ben-Shalom *et al.* 1977; Vamos-Vigyazo 1981; Lanker *et al.* 1987; Felton *et al.* 1989; Mayer and Harel 1991; Nicolas *et al.* 1994; Steffens *et al.* 1994; Murata *et al.* 1995; Serradell *et al.* 2000), suggesting its possible role in protection of growing plants against infection or injury. The general decrease in PPO activity with tissue age may be attributable to conformational changes, degradation by proteases, a decline in concentration of latent enzyme activators or of phenolic substrate biosynthesis, (Barrett *et al.* 1991; Murata *et al.* 1995; Laveda *et al.* 2000), or to inactivation of PPO due to tanning reactions (Lanker *et al.* 1987; Macheix *et al.* 1990). However, a large number of studies on plants proposed that level of enzyme activity is controlled at the transcriptional level, as PPO transcripts are highly expressed at early developmental stages and expression decreases as development continues (Rathjen and Robinson 1992; Shahar *et al.* 1992; Hunt *et al.* 1993; Dry and Robinson 1994; Boss *et al.* 1995; Thygesen *et al.* 1995). For potato tubers, PPO gene expression with maximal levels in developing tubers is prolonged moderately beyond early development and throughout tuber growth and differentiation (Thygesen *et al.* 1995). This is perhaps due not only to developmental but also to tissue-specific expressions of different PPO genes. About seven genes exhibiting tissue-specific expression patterns in various vegetative and floral organs during growth were also identified from tomato plant (Shahar *et al.* 1992; Newman *et al.* 1993).

In some other cases, there appears to be petite or no PPO gene expression at advanced development stage, i.e. PPO synthesized early in developmental stages is maintained relatively stable *in vivo* (Hunt *et al.* 1993; Dry and Robinson 1994; Chevalier *et al.* 1999; Gooding *et al.* 2001). Absence of translatable PPO mRNA at late ripening stages of apricot fruit in contrast to significant PPO activity suggests that translational and posttranslational controls may be involved in regulation and level of PPO activity (Chevalier *et al.* 1999). Likewise, significant levels of PPO activity with little PPO gene expression throughout ripening of banana fruit suggests activation of preexisting enzymes as a result of a loss in membrane integrity during natural ripening of the fruit (Gooding *et al.* 2001). Variation in PPO activity during spinach leaf development was also attributed to activation of a preexisting latent PPO, which is synthesized before but not after onset of senescence (Meyer and Biehl 1980, 1981; Lieberei *et al.* 1981). PPO activation in the leaves was shown to be

greater during senescence due to reduced binding of latent enzyme to thylakoid membranes than before or after onset of senescence (Meyer and Biehl 1980, 1981). In addition to physical and chemical disintegration of thylakoid membranes, activation of such preexisting latent enzymes, under certain conditions, may also be induced by limited conformational changes (Lerner *et al.* 1972; Goldbeck and Cammarata 1981; Valero and Garcia-Carmona 1992; Espin and Wichers 1999a), dissociation of PPO-bound inhibitor complex (Kenten 1957; Lieberei and Biehl 1978; Interesse *et al.* 1980, 1984; Angleton and Flurkey 1984; Lamikanra *et al.* 1992;) or partial proteolysis (King and Flurkey 1987; Sanchez-Ferrer *et al.* 1990; Robinson and Dry 1992; Dry and Robinson 1994; Jimenez and Garcia-Carmona 1996).

In the case of grape, it is suggested that the latent proenzyme of higher molecular weight, synthesized mainly at the early development stage, is proteolytically processed to the active catalytic unit during growth and differentiation (Rathjen and Robinson 1992; Dry and Robinson 1994). On the other hand, detection of strongly antigenic but enzymatically inactive PPO forms in an apple extract led Marques *et al.* (1995) to propose posttranslational regulation of partially unfolded chaperone-stabilized inactive PPOs via folding into the active form rather than activation by proteolytic degradation since the inactive form is not a larger precursor.

Regulations in Response to Injury or Disease

In general, the defense mechanisms of plants, which inherently offer protection against pathogens, pests and injury, are a multifaceted network (Bell 1981; Vaughn *et al.* 1988; Macheix *et al.* 1990; Johal *et al.* 1995). Evidence showed that constitutively abundant PPO associated with the glandular trichomes of *Solanum* and *Lycopersicon* species has a specific *in vivo* function in insect-resistance mechanism (Kowalski *et al.* 1992; Yu *et al.* 1992). Trichome-based resistance involves entrapment of small insect bodies in polymerized trichome exudates. Exadutes released from the trichomas and disintegrated by insect contact undergo PPO-mediated oxidative polymerization resulting in hardened exudates entrapping the insects and causing death (Kowalski *et al.* 1992). In addition, perpetuation of relatively high levels of PPO activity and PPO gene expression throughout tuber development of potato suggest that PPO could be important in protecting the growing parts of plants against disease or injury (Thygesen *et al.* 1995). Elevation of PPO activities for numerous plants in response to pathogen and insect assaults is considered an additional line of the plants' defense against pathogens and pests (Mayer and Harel 1979; Bashan *et al.* 1987; Felton *et al.* 1989; Thipyapong and Steffens 1997). PPO activities could be induced locally at infected or wounded sites of the tissues (Boss *et al.* 1995; Ray and Hammerschmidt 1998) or systemically perhaps to protect the

plant against further attack (Thipyapong *et al.* 1995;) or both (Bashan *et al.* 1987; Constabel *et al.* 1995; Thipyapong and Steffens 1997). Hence, either constitutive, induced or both types of PPO activity may be involved in the putative protective defense strategy. Thipyapong and Steffens (1997) indeed reported that antisense down-regulation of constitutive and induced PPO activity results from increased pathogen susceptibility.

Vigorous surveys have been conducted to determine the significance of PPO activity in induced defense mechanism of several plants. Wound-inducible PPO activity was verified in a number of plants, including apple, potato, tobacco, tomato, etc. (Boss *et al.* 1995; Constabel *et al.* 1995; Thipyapong *et al.* 1995; Thipyapong and Steffens 1997; Constabel and Ryan 1998). The increase in PPO activity in infected or wounded tissues of many plants supports the assumption that PPO-mediated oxidative browning could be an important defense response of plants against infection or wounding. However, induction of PPO activity in an injured plant is not widespread, as PPO activities of a number of plants do not increase after wounding (Constabel and Ryan 1998; Gooding *et al.* 2001).

An increase in the amount of PPOs and PPO mRNAs was observed in wounded tissues of apple, and potato (Boss *et al.* 1995; Thipyapong *et al.* 1995). Constabel *et al.* (1995) later proposed that the expression of PPO gene(s) is regulated by wound-inducible octadecanoid-signaling pathway. The products of the inducible signaling pathway, systemin and methyl jasmonate, promote defense mechanism of wounded tomato plants by activating the synthesis of 66 kDa PPO proteins. Thipyapong and Steffens (1997) also showed that only one of seven known tomato PPO genes is differentially regulated in response to methyl jasmonate, artificial injury, and bacterial and fungal infection in young and older leaves. Since methyl jasmonate was shown to induce the gene expression only in young leaf tissues, they contemplate the involvement of additional signaling mechanism(s). The wound signaling molecule, methyl jasmonate, was also shown to increase PPO activity in tobacco, poplar and potato (Constabel and Ryan 1998). On the other hand, several plant PPOs do not respond to methyl jasmonate induction at all (Constabel and Ryan 1998; Gooding *et al.* 2001). Since those PPOs are activated with neither wounding nor treatment with methyl jasmonate, it is suggested that wound-responsive induction is typically associated with methyl jasmonate-induction. Then again, possibility of differential inducibility of PPOs by other types of tissue damage or of evolution of the enzyme for another undiscovered role rather than defense against insects is considered (Constabel and Ryan 1998).

Some researchers call attention to an additional mechanism for elevation of PPO activities: preexisting PPOs presumed to be relatively stable in latent form *in vivo* could be activated or altered in response to wounding (Boss *et al.* 1995; Thipyapong *et al.* 1995) or pathogen attack (Soler-Rivas *et al.* 1997). The free fatty acids released from membrane lipids under stress may also involve wound-

induced PPO activation processes (Goldbeck and Cammarata 1981). Robinson *et al.* (1993) suggest that terpenes in mango sap activate skin PPO during sapburn injury of the fruit. A study on the effects of *Pseudomonas tolaasii* or its toxin, called tolaasin, on PPO activity suggests that latent forms can be activated upon pathogen attack by a nonproteolytic mechanism (Soler-Rivas *et al.* 1997). In some cases, increased enzyme activity may be attributable to appearance of new multiple forms (Mari *et al.* 1998; Saluja and Sachar 1982; also see section Multiplicity). Cleavage of an active PPO protein to a smaller and still active PPO form, resistant to further proteolysis and exhibiting a broad optimum pH, upon disruption of the infected cells is considered an alternative defense mechanism against pathogens (Mari *et al.* 1998). Furthermore, stimulation of PPO activity by phytohormones associated with formation of new multiple forms, a consequence of the association of preformed low molecular weight multiple forms, may be another type of defense response (Saluja and Sachar 1982).

Whatever the mechanism is to control the level of PPO activity, disruption of membrane integrity, loss of latency, and transcriptional or posttranscriptional control during normal growth and development, PPO is relatively present at all developmental stages of plants and could even be more abundant after wounding or infection. The studies discussed put forward the role of PPO in protection of plants against diseases and invading pathogens or insect pests. This implication is essentially based on the remarks that secondary reaction products of PPO prevent spread of the infection. The most extensively accepted potential effects of the reaction products on disease resistance could be classified in three groups:

(1) Oxidative polymerization of quinones form insoluble melanin over wounds, sealing off infected tissues (Vamos-Vigyazo 1981; Vaughn *et al.* 1988; Zawistowski *et al.* 1991).

(2) Bacteriocidal and fungicidal effect of hydroxyphenolics and quinones to assaulting microorganisms and toxic effects of the polymerized phenolics on invading viruses in injured tissues (Mayer and Harel 1979; Vaughn *et al.* 1988; Macheix *et al.* 1990; Scalbert 1991; Shaw *et al.* 1991; Zawistowski *et al.* 1991).

(3) Covalent modification of proteins by quinones as an antinutritive defense mechanism (Felton *et al.* 1989, 1992a, b). The quinones generated by reaction of PPO with a variety of phenolic substrates can modify dietary proteins by reacting with amino, sulfhydryl, phenolic and imidazole groups (Mason and Peterson 1965; Matheis and Whitaker 1984), reducing their nutritive value to insect herbivores (Felton *et al.* 1989, 1992a, b).

Nevertheless, PPO perhaps is not the only enzyme contributing to resistance of plants to diseases or insect herbivores. Food proteins can also suffer protein cross-linking in the presence of peroxidase and its products (Matheis and Whitaker 1984). A study by Ray and Hammerschmidt (1998) on potato tuber supports involvement of constitutive PPO activity in cooperation with wound-inducible peroxidase activity in a plant's defense resistance responses. Richard-Forget and Guillard (1997) reported involvement of peroxidase in enzymatic browning through its synergistic effect with PPO. It appears that the oxidation products of PPO, H_2O_2 and quinones, are used by peroxidase as oxidizing agents or substrates. High thermostability of both PPO and peroxidase also suggests that these enzymes together play a key role in darkening of processed cucumber products (Miller *et al.* 1990). A new unique type of PPO, called phloroglucinol oxidase, also having strong peroxidase activity and oxidizing only phloroglucinol and phloroglucinolcarboxylic acid from cabbage was characterized by Fujita *et al.* (1995, 1997). It was suggested that the enzyme has separate active sites for PPO and peroxidase activities. Additionally, synthesis and accumulation of natural phenolic compounds following mechanical or biological stress could also play a role in protection of plants either directly through their antibacterial properties or indirectly acting as natural substrates of PPO and peroxidase (Macheix *et al.* 1990). Therefore, control of phenylalanine ammonia lyase (PAL; EC 4. 3. 1. 5) activity, involved in the biosynthetic pathway of phenolic compounds, could also be important in preventing onset of adverse enzymatic browning in crops (Martinez and Whitaker 1995).

Aside from possible contribution of PPO as a part of a protective mechanism of plants against invading pathogens or insect pests, several other roles for PPO were also proposed. High induction of pokeweed PPO in ripening, betalain-containing fruit, was related to its involvement in the betalain pathway (Joy *et al.* 1995). Investigations by Steiner *et al.* (1999) provided additional evidence supporting the hypothesis that PPO is involved in betalain biosynthesis of higher plants. Its likely role in auxin biosynthesis was also considered by Shaw *et al.* (1991). Matheis and Whitaker (1984) reported that reaction of proteins with PPO- or laccase-generated *o*- and *p*-quinones, respectively, may be involved in formation of humus. Furthermore, thylakoid location of the enzyme led researchers to suggest that PPO may play a role in photosynthesis of functional chloroplast (Vaughn and Duke 1984a; Vaughn *et al.* 1988). Associations of the enzyme with photosystem complexes suggested a likely role in photosynthetic electron transport (Lax and Vaughn 1991; Sheptovitsky and Brudvig 1996). It may function by regulating pseudocyclic photophosphorylation (ATP production with oxygen as a terminal electron acceptor) (Tolbert 1973; Vaughn and Duke 1984a; Sherman *et al.* 1995). However, pH dependent activity of photosystem II membrane-associated PPO

suggested that PPO is important mostly for dark processes in thylakoid lumen (Sheptovitsky and Brudvig 1996).

Dietary PPO in fruits and vegetables may also offer some positive health effects to humans. A recent study by Cowan *et al.* (2000) pointed out possible role of dietary PPO in preventing dental caries by abolishing the attachment of *Streptococcus sobrinus* to glucans on the tooth surface. PPO activity was also considered valuable for riddance of bad breath and of other stinking odors in the environment by removing thiol compounds (Negishi and Ozawa 1997).

CONCLUSION

PPO has been studied intensively for more than a century. Significant advances have been made on biochemistry, molecular biology and genetics of the enzyme to date. The biochemical properties involved in the action of PPO are determined from numerous plant sources. Much attention is given to significant variations in physiochemical properties of the enzyme from different plant sources. Models explaining monophenolase and diphenolase activities of PPO have provided an additional insight to our understanding of a detailed picture of the molecular structure of PPO reactions. Genes encoding PPOs have been isolated and characterized from a variety of plants and all these studies verify the plastidic location of the nuclearly coded protein. However, revealing all the complexity of PPO gene regulation remains a prime challenge for researchers in the field. Additionally, the food industry still faces the problem of how to prevent enzymatic browning while considering food safety, regulations, marketability of the treated products, and the cost associated with the prevention process.

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