POLYPHENOL OXIDASE AND PEROXIDASE IN FRUITS AND VEGETABLES

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I. INTRODUCTION

Polyphenol oxidase (PPO) and peroxidase (POD) are believed to be ubiquitous in the plant kingdom. They have been detected in most known fruits and vegetables. For the fruit and vegetable processor, the actions of PPO and POD are primarily connected to enzymatic browning of fresh and off-flavor generation in canned or frozen horticultural products, respectively. Both phenomena are of vital importance to the manufacturer as they impair not only the sensory properties and, hence, the marketability of a product, but often lower its nutritive value as well. It is no wonder that ever since their discovery in the past century these enzymes have been the subject of extensive research. As a result of this, considerable amount of data have been accumulated on their catalytic and molecular properties as well as on the role they play in the plants' life cycles and in some branches of food technology. However, the immense number of communications available on the topic at the present time still does not seem sufficient to provide a general understanding of the behavior of these enzymes. On the contrary, it can be said that, instead of completing the overall picture, new contributions often tend to upset it. This, as well as the unrelaxing interest of researchers in PPO and POD, might be due to the following:

1. Both enzymes catalyze more than one reaction and act on a great number of substrates;
2. They occur in multiple forms in the individual species;
3. The physiological role of either enzyme (but especially of peroxidase) in the plant is not yet clearly understood;
4. Their role in food processing is rather complex.

The object of the present review is to give a brief account on recent results achieved in research dealing with PPO and POD and to point out some contradictions found in the literature in order to draw the attention of researchers to some questions which need more thorough investigation.

II. POLYPHENOL OXIDASES (PPO)

A. Generalities

1. The Names of the Enzymes

Polyphenol oxidases belong to the group of oxidoreductases. They oxidize diphenols in the presence of molecular oxygen. The trivial name "polyphenol oxidase" has been applied in the early literature to denote two different enzymes. Both of them have been registered in the Enzyme Nomenclature under the number 1.14.18.1 and the systematic name of monophenol, dihydroxyphenylalanine: oxygen oxidoreductase.[1] They are, however, primarily different, with respect to substrate specificity. One of them oxidizes

phenolic compounds with ortho and vicinal (3,4,5-trihydroxy) OH-groups.[2] It is also able to act on monophenols by converting them into o-dihydroxy phenols. It was detected first in mushrooms over a century ago[3] and it occurs in practically all kinds of European fruits with the exception of some citrus species. However, its presence in berry fruit is of secondary importance.[4] Some trivial names of this enzyme, referring to the (principal) substrate(s) acted on, such as tyrosinase, cresolase, catechol oxidase, phenolase, o-diphenol oxidase, are still in use in the literature.

The other enzyme oxidizes o- and p-dihydroxy phenols and is most often referred to as laccase, after the Japanese lac tree *(Rhus vernicifera),* one of its main sources. It differs from o-diphenol oxidase in substrate specificity as it does not hydroxylate monophenols, and also in its sensitivity towards some inhibitors (CO, 2,3-naphthalenediol, PVP, and cationic detergents).[5] It is less frequently encountered in fruits and vegetables than o-diphenol oxidase, but its presence has been reported in some peach cultivars,[6] in mushrooms[7,8] and in tomatoes.[9]

This review will be concerned mainly with o-diphenol oxidase and, unless otherwise stated, the abbreviation PPO will denote *this* enzyme.

2. Occurrence of PPO in Nature

PPO is widely distributed in nature. In addition to its general occurrence in plants, it can also be found in microorganisms—especially in fungi and some animal organs.[7] The PPO content of different plants varies widely with the species or cultivar. From a great number of fruits and vegetables olives were found to have the highest PPO activity (especially on catechol substrate) along with a slight laccase activity.[10]

The localization of the enzyme in the plant cell depends on the species, age, and—in fruits or vegetables—on maturity. In green leaves, a considerable part of PPO activity is localized in the chloroplasts.[2,11] In potato tubers nearly all subcellular fractions were found to contain PPO, in amounts approximately proportional to protein content.[12] Substantial amounts of the enzyme were released from the tuber tissue by an endopectate-trans-eliminase from *Erwinia carotovora* specifically acting on α-1, 4-D-glycosiduronic linkages.[13] In the stem of spinach beet, most of the activity was sedimentable and associated with plastid membranes and mitochondria.[14] In freshly harvested apples, the enzyme was found to be localized almost exclusively in chloroplasts and mitochondria. The enzyme preparations obtained from these two particulate fractions differed slightly with respect to substrate specificity.[15]

In some species, e.g., spinach, alfalfa, wheat, oats, peas, and sugarcane leaves, the enzyme was present in a *latent form* in the chloroplast, and needed trypsin or red light to be activated.[11] In vine leaves a latent PPO tightly bound to cell particles could be activated by caffeine.[16] The particulate or chloroplast enzyme from other sources such as potatoes, mushrooms, beans, tomatoes, and corn leaves did not exhibit latency,[11,16] while PPO in the water extract of broad bean leaves required exposure to acidic or basic pH for activation.[17] Thus, latency does not seem to be related to the localization of the enzyme in the cell nor to its solubility, and activators differ according to its source.

The distribution of PPO in the different parts of fruits and vegetables may be considerably different and the ratio of particle-bound and soluble enzymes varies with maturity. Enzyme activity was found to be higher in the skin of grapes than in the flesh or sap. Its value decreased during ripening, the decrease being most pronounced in the skin.[18] Similar findings were reported for 16 peach, 3 cherry, and 3 plum cultivars: overall PPO activity was 77 to 96%, present as particulate-insoluble enzymes which decreased during ripening on the tree.[19] The enzyme could be detected in all the parts of apple fruit. Some found its activity to be highest around the core,[20] while others found it highest in the skin.[21] During ripening, the concentration of the particulate enzyme decreased under

simultaneous appearance of a soluble fraction.[15] The distribution of the enzyme in pears was found to be similar to that in apples. Clarified juices of both fruits were practically devoid of PPO activity; this remained almost entirely in the pulp.[21] In sweet cherries, enzyme activity values measured at pH 4.7 and 7.0 and abruptly rose when the fruit reached full maturity.[22] In olives, enzyme activity decreased while o-diphenol concentration increased during ripening. The enzyme was found to be tightly bound to chloroplasts in mature green fruits while it was present essentially in the soluble form in black olives.[23] Palmer found only particle bound, insoluble PPO in bananas, while Padron and co-workers were able to extract the enzyme from the pulp of this fruit with water.[24,25] In contrast to most fruits, the greatest part of PPO was localized in the pulp and only a small fraction in the peel of the banana fruit as well as in the leaves and bark of the plant.[25] In most fruits the insoluble part of the enzyme was found to be dominant. The activity of the soluble part amounted to 20 to 30% of the total value in peaches, 15 to 17% in sweet cherries, 13% in apricots, and 8 to 15% in apples. In several plum and pear cultivars only water-insoluble enzyme activity could be detected.[26]

The PPO content of plants might be affected also by agricultural techniques (e.g., activity levels in sugar beet leaves increased upon treating the soil with copper as a trace element.)[27] The addition of other trace elements (Zn, Cd, Hg, Cr, Mn, Co, W, and V) to the soil had a stimulating effect on the catalase, ascorbic acid oxidase, POD, and PPO activities of the vine plant during the later periods of vegetation.[28] There are, however no data as to the effect of these treatments on the enzyme levels in sugar beet or grapes. On the other hand, spraying peach trees with 50 ppm of ethrel (2-chloroethyl phosphonic acid) 4 weeks before harvest prevented browning of the pulped or sliced fruits for 24 hr even if blended after freezing.[29] Similarly, ethrel treatment of apple trees suppressed PPO activity and browning of cut surfaces of apple slices.[30]

3. The Role of PPO in Nature and in Food Processing

The role of PPO in nature is manifold even if considerations are restricted to its occurrence in plants.[31] It is said to participate in the respiration chain of higher plants as one of the terminal oxidases; however, the importance of this role has been questioned.[32,33]

Far more important is the part the enzyme plays in the resistance of plants to microbial or viral infections and, probably, to adverse climate. The vast literature dealing with the role of the PPO-poly-phenol system in plant pathology has been extensively reviewed by several authors.[31,34,35]

Tissues infected with Potato virus X were more susceptible to enzymatic browning and had a higher phenol content than healthy tubers. On the other hand, lipid content was lower in the infected tissues.[35] Inoculation of sweet potato tissue with a pathogenic or a nonpathogenic isolate of the fungus *Ceratocystis fimbriata* lead to a similar increase in both PPO and POD activities of the tissues. The inoculation of a susceptible variety with a nonpathogenic isolate induced in the layers surrounding the site of inoculation an immunity to subsequent infection with the pathogen.[37] Exposure of the roots of a susceptible sweet potato variety to low concentrations of ethylene equally induced resistance to ceratocystis infection, as well as an increase in PPO and POD activities. Ethylene treatment of potatoes or parsnip roots resulted in the stimulation of PPO only, while it did not bring about any alteration in the activity of either enzyme in root tissues of carrots, radishes, or turnips.[38] Cutting or infection of sweet potato roots produced upon incubation a new PPO component not present in intact tissues. The formation of this compound and the increase in activity were inhibited by various antibiotics. This leads to the assumption that the increase in activity was due to *de novo* protein synthesis.[39]

A rise in PPO and a decrease in POD activity were observed in post-harvest sugar beet stems infected with the storage rot pathogen *Phoma betae.* Oxidation products of catechol inhibited the growth of the organism in liquid culture.[40]

In apple tissues infected with the soft rot mould *Penicillium expansum,* enzymic browning did not occur owing to the inhibition of the PPO system. The rotted tissues contained increased amounts of some phenols such as caffeic acid, p-coumaric acid, and ferulic acid, the latter two of which are known to inhibit the oxidation of the natural phenolic constituents of the apple.[41,42] Phenolic compounds extracted from maturing apple tissues were found to be inhibitory to the growth of *Botrytis cinerea* spores as well as to mycelial growth of *B. cinerea, P. expansum,* and *Alternaria* sp. The decrease in phenolic content as observed during ripening coincided with an increase in susceptibility of the tissue to rot.[43]

A simplified, rough explanation of the role of PPO in the resistance of plants to infections is that the quinones formed upon the action of the enzyme undergo secondary polymerization reactions yielding dark, insoluble polymers. The tissues "impregnated" with these polymers act as barriers in the way of the spreading infection.[35] This is considered by some authors to be the main function of the enzyme.[44] The decrease in lipid content is important from this aspect as it might alter the permeability of the membranes and facilitate the access of the enzyme to its substrates, thus promoting the formation of the "barriers".[36] According to another theory, intermediates of the oxidative polymerization of polyphenols might inactivate or bind some labile plant enzymes and viruses, respectively (e.g., cucumber mosaic virus).[45]

Plants resistant to adverse climatic conditions have, in general, higher PPO activities than susceptible varieties, e.g., higher activity levels were found in the leaves of the frost-resistant plum tree *Prunus ussuriensis*[46] or in vine cultivars which displayed a higher resistance to unfavorable wintering conditions[47] than in the respective plants susceptible to frost damage.

The hydroxylation reaction of the enzyme plays a part in phenol biosynthesis by transforming tyrosine into DOPA, p-coumaric acid into caffeic acid, p-coumarylquinic acid into chlorogenic acid, or by inserting an o-dihydroxy group into the B-ring of flavonoids.[5]

PPO participates, although indirectly, in auxin biosynthesis. The primary products of its action on o-dihydroxyphenols, the o-quinones, react with tryptophan to form indole acetic acid via indolepyruvic acid.[48] Thus, it might play a role in plant growth regulation together with the auxin degrading enzyme, POD.

The quinones formed upon PPO action may also participate in reactions similar to those leading to nonenzymatic browning and humification and thus contribute to producing the organic matter of soil.[50,51] Laccase occurring in wood-rot fungi probably plays a role in lignin depolimerization.[49]

In connection with the PPO enzyme, food technologists are primarily concerned with the phenomenon of enzymatic browning. This topic has been reviewed recently.[5] Enzymatic browning is an indirect consequence of PPO action. The primary products of the oxidative reaction catalyzed by the enzyme, the o-quinones, (a) react with each other to form high molecular weight polymers, (b) form macromolecular complexes with amino acids or proteins, and (c) oxidize compounds of lower oxidation-reduction potentials.[52] The nonenzymic reactions (a) and (b) lead to the formation of brown pigments, the color of which is darker the higher their molecular mass; the products of reactions of type (c) are colorless. Enzymic browning is, in some branches of food technology, a desirable process, e.g., in the manufacture of black tea,[53] sultana grapes,[54] or prunes.[55] A positive correlation also was found between PPO activity of green coffee beans and the quality of the beverage.[56]

PPO action also may favorably affect the taste and flavor of fermented beverages. In the manufacture of cider and fermented pear juice the condensed and polymerized oxidation products of the naturally occurring phenols sediment out and can be removed by filtration or clarification, whereby the astringency of the beverage can be considerably reduced.[5] The quinones formed by the enzyme from grape phenols, mainly from catechins, may react with amino acids and yield volatile aldehydes as final products.[57]

Enzymic browning occurs in fruits and vegetables upon bruising during handling or transportation, or when such products are kept exposed to the air in cut, sliced, or pulped states, or when thawed after freezing. This is, of course, a highly undesirable process and has to be prevented.

High PPO activity as present in some types of flour may bring about the darkening of bread or pasta products.[58,59] The reactions of enzymatically formed quinones with proteins (e.g., casein) reduce the digestibility and available lysine content of the latter.[60,61] This may affect the nutritive value of foods and feeds. Enzymic discoloration also is unfavorable in the manufacturing of beets and cane sugar for it not only might affect the color of the final product, but the polymers formed render crystallization of the sugar difficult and lower its yield.[62]

Highly undesirable is the result of the reactions belonging to group (c) in which the quinones formed by PPO oxidize compounds of lower oxidation-reduction potentials, being again reduced to dihydroxyphenols at the same time. Thus, they continue to provide "fresh" substrate to the oxidative action of the enzyme until it gets inactivated by reaction products, or the compounds of lower oxidation-reduction potentials are depleted. This is how ascorbic acid is acted on by the quinones and one of the reasons why this compound is able to prevent the appearance of discoloration. Until its complete depletion, quinones are permanently reduced and not able to react with each other or with proteins or amino acids to form irreversible colored polymers. Thus, the occurrence of enzymic browning indicates total loss of vitamin C in the tissues affected.[63] Anthocyanins are degraded by PPO-generated quinones in a similar way, as has been shown, for eggplant as well as sour and sweet cherries.[64,65,66,67] Ascorbic acid prevents anthocyanin oxidation by virtue of its lower oxidation-reduction potential.[67]

B. Biochemistry Of PPO

1. The Reactions Catalyzed

PPO is a copper-containing enzyme which catalyzes two entirely different reactions: (a) the hydroxylation of monophenols to the corresponding o-dihydroxy compounds and (b) the oxidation of o-dihydroxy phenols to o-quinones. The reactions require molecular oxygen.

Enzyme preparations from different sources possess these two activities in different ratios. The ratios of oxidizing: hydroxylating activity were found to range from 1—10 to > 40.[68] The ratios may change during isolation and purification or treatment by physical methods; the hydroxylating activity might even get lost entirely.[2,5,69,70] Most of the PPO preparations, e.g., those from potatoes,[71,72,73] apples,[15] sugar beet leaves,[74] broad beans,[75] and mushrooms[76] possess both activities, while those from tea leaf,[77] tobacco,[76] mango,[78] banana,[24] pear,[79] and sweet cherrry[80] have been reported not to act on monohydroxy phenols. The lack of the ability of the latter preparations to hydroxylate monophenols has not been, so far, attributed to extraction or purification operations, nor is there evidence pointing to such a possibility.

The hydroxylation reaction starts on a pure monohydroxy phenol substrate after a lag period which can be reduced or entirely eliminated by small amounts of o-dihydroxy phenols, e.g., chlorogenic acid[81] or reducing agents such as ascorbic acid, NADPH or NADH, tetrahydrofolic acid, etc.[2] This induction phase cannot be observed in fruit or

vegetable homogenates where o-dihydroxy phenols are always present. With increasing amounts of chlorogenic acid the tyrosinase activity of potato PPO was found to go through a maximum. This was attributed to the different affinities of the enzyme towards the two substrates (K_M values for L-tyrosine and chlorogenic acid were $1.4 \cdot 10^{-3}$ mol l^{-1} and $1.4 \cdot 10^{-4}$ mol l^{-1}, respectively).[81]

PPO was reported by some authors to hydroxylate flavonoid compounds as well. Partially purified preparations from *Malus pumila* and *Pyrus communis* hydroxylated phloretin and phloridzin, introducing the hydroxy group in position C-3 of ring B; the enzyme from spinach beet leaves *(Beta vulgaris* L. ssp. *vulgaris)* hydroxylated at pH 5.3 and, in the presence of ascorbic acid, the flavonols kaempferol, dihydroxy kaempferol, and the flavanone naringenin to quercetin, dihydroxy quercetin and eriodictiol; preparations from cell cultures of parsley hydroxylated p-coumaric acid and, to a lesser extent, naringenin without having any effect on dihydroxy kaempferol and the flavone apigenin.[2,12]

For a long period of time there was a difference of opinions on whether hydroxylation of monophenols and oxidation of o-dihydroxy phenols were catalyzed by the same enzyme. The main views supported by the different parties were:

1. hydroxylation is not an enzymatic process, but a secondary reaction of the quinones formed by PPO from o-dihydroxy phenols.
2. both reactions are due to enzyme action performed by two separate protein molecules.
3. both reactions are enzymatic, the question whether catalysis is brought about by the same or by two separate enzymes being of secondary importance.
4. the reactions are catalyzed by one enzyme possessing two active sites.
5. both reactions are performed by the same active site of the enzyme whereby the valence of copper, the prosthetic group of PPO, varies permanently.[32,44,52,83]

The opinion generally agreed upon today is that both hydroxylation of monophenols and dehydrogenation of o-dihydroxy phenols are catalyzed by the enzyme PPO. However, one of the strongest arguments supporting this theory (that the two activities could not be separated by ultracentrifugal or electrophoretic techniques) seems to have been disproven recently. The protein moieties performing hydroxylation and dehydrogenation in a preparation from parsley cells were reported to be separable by ion-exchange chromatography on a DEAE-cellulose column.[83a]

An interesting feature of PPO is that it becomes inactivated during the reaction, which has been shown by a preparation from bananas.[25] The phenomenon is attributed to the action of the quinone produced which is assumed to form a covalent linkage with the enzyme in the vicinity of the active site.[3]

The amount of the prosthetic group of the enzyme was found to be 4 atoms of Cu per molecule in mushroom PPO, and 1 atom per molecule in *Vicia faba* PPO.[76,84] The concentrations of copper in preparations from mushroom, potato, sweet potato, and tobacco were 0.2 to 0.3%, 0.2%, 0.27% and 0.32% (w/w), respectively.[76,77,84,85] The hydroxylation requires Cu in the cuprous form which is provided by the dehydrogenation step. This might account for the elimination of the induction period by o-dihydroxy phenols or reducing agents. Total or partial removal of copper from the protein moiety, e.g., by ion exchange, leads to inactivation or reduction of activity which can be restored by the addition of Cu.[52,68]

The oxidation of o-dihydroxy phenols by PPO occurs, most probably, according to an ordered sequential mechanism.[3] Oxygen was reported to be the first substrate bound to the enzyme from potatoes[81] and others found the mechanism to vary with the substrate.[79]

Recently, the reaction has been shown to occur via free radical formation whereby the copper of the active site changes its valency.[85a]

A simple overall equation describing the reaction catalyzed by PPO can be given as follows:

catechol p-cresol o-benzoquinone 4-methyl catechol

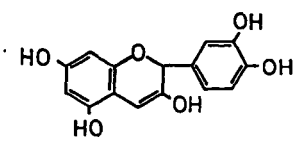
Laccase is also a copper-containing enzyme which is a deep blue color in the pure state. As already mentioned, it can be distinguished from PPO by its substrate specificity and its specificity to inhibitors. It readily oxidizes p-phenylene diamine and quinol (compounds not attacked by PPO) while it does not act on the PPO substrates tyrosine and p-cresol. However, according to some authors, laccase, too, is able to utilize p-cresol as a substrate.[86,87]

2. Substrates of Polyphenol Oxidase

Fruits and vegetables contain a wide variety of phenolic compounds. However, only a relatively small part of these serve as substrates to PPO. Apart from affecting the appearance of these products through the enzyme-catalyzed reaction leading to discoloration, polyphenols also have an effect on the taste of fruits. Higher contents, especially of catechins or proanthocyanidins, manifest themselves in astringency.[63] Polyphenols may lead to discolorations through nonenzymatic reactions as well. Some of them readily undergo autoxidation and subsequent polymerization yielding brown macromolecules.[88] The colorless proanthocyanidins may form red anthocyanidins, the pink or reddish-brown condensation products which cause the pink discoloration of canned pears, peaches, or apples.[63,89] Phenols with two or three adjacent hydroxyl groups easily react with iron salts. At pH values above four, the reaction products are blue-gray or black and impart a metallic taste to the food. After-cooking blackening of potatoes or black cloudiness in wine are attributed to such reactions.[90,91] Flavones and flavonols react with Al^{3+} ions to give colored pigments, anthocyanins form colored chelates with Sn^{2+}, Al^{3+}, and Fe^{3+} ions causing discoloration of tinned fruits or vegetables.

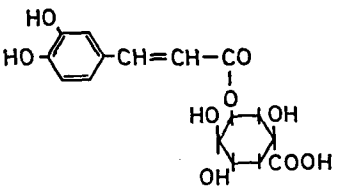
Polyphenols might give rise to the formation of sediments or turbidity in beverages, e.g., wine[63,92] and therefore have to be removed since they might inhibit the action of the pectolytic enzyme preparations used in fruit juice manufacture.[93,94] On the other hand, commercial pectolytic enzyme preparations possessing a hydroxycinnamic ester-splitting activity as an impurity impair the characteristic flavor of wine by transforming some of its natural phenolic ingredients.[95] Some plant phenols like quercetin or caffeic acid compounds are antioxidants and thus contribute to the stabilization of vitamin C (e.g., in black currants).[96]

The most important natural substrates of PPO in fruits and vegetables are catechins, cinnamic acid esters, 3,4-dihydroxy phenylalanine (DOPA), and tyrosine.[5,63,97] *Catechins* are 3-hydroxy flavanes of the general formula



The optical and stereoisomers corresponding to this formula and occuring in nature are (+)−catechin and (−)−epicatechin. (−)−Catechin and (+)−epicatechin as well as racemates of the diastereoisomers catechin and epicatechin could be obtained by heating (+)−catechin and (−)−epicatechin in a dilute solution of sodium carbonate. Detailed accounts on various aspects of this class of compounds have been given by several authors.[52,98,99]

From the cinnamic acid esters (or depsides) *chlorogenic acid* (3-*O*-caffeoyl-D-quinic acid) is the most widespread natural PPO substrate. Its formula is as follows:



The caffeic (3,4-dihydroxycinnamic) acid moiety of chlorogenic acid has been reported to be hydroxylated from p-coumaric acid by PPO.[100] The isomers of chlorogenic acid—iso-chlorogenic acid, neochlorogenic acid, pseudo-chlorogenic acid, and "Band 510" — are also encountered in nature as substrates of PPO. A comprehensive review on chlorogenic acids has been published by Sondheimer.[33]

L-DOPA is the product of tyrosine hydroxylation as catalyzed by PPO. *Tyrosine,* being a phenol and at the same time an amino acid constituent of proteins, is found practically in every plant tissue. However, PPO from some sources (e.g., apples) which is able to hydroxylate p-cresol, does not act on tyrosine although it oxidizes L-DOPA.[101,102,103] Sugar-beet PPO, while ineffective on tyrosine, was found to oxidize DOPA; the beet tissues contained, however, no detectable amounts of the latter but contained large amounts of tyrosine.[62] This, too, supports the findings of a number of researchers, according to which the best substrates of PPO are not always those occurring in the plant the enzyme has been obtained from.[15,104]

The sphere of the naturally occurring substrates of PPO is limited by the fact that the enzyme does not act on glycosides.[97] However, in the presence of "transfer substances" such as chlorogenic acids and catechins, flavonol glycosides are oxidized at a measurable rate, probably through the formation of dimers as a first step.[105]

The site of the substitution of mono- and dihydroxy phenols is also an important factor from the aspect of attackability by PPO. Monophenols are hydroxylated only if they have a para-substituted $> CH_2$-group, and p-substituted 3,4-dihydroxy phenols are oxidized at higher rates than 2,3-dihydroxy phenols.[2,5,97] Substituents in position 3 (3-methyl catechol, 2,3-dihydroxy benzoic acid, 2,3-dihydroxy-4-methoxibenzoic acid, 2,3-dihydroxy phenylsulphonic acid) cause a decrease in affinity of the enzyme for the substrate, probably owing to steric hindrance. The presence of an electron-donating group in position 4 (as in chlorogenic acid, 4-methyl catechol, etc.) increases, an electron abstracting group (as in 4-nitrocatechol, 3,4-dihydroxy benzoic acid, 3,4-dihydroxy benzaldehyde, etc.) reduces the reactivity of the substrate.[80] The nature of the p-substituent affects the rate of oxidation as well.[106] From these results, it was concluded that oxidation rate was governed by an electrophilic stage and that phenol was probably oxidized in the keto form.[80,106]

The extent to which naturally occurring phenolic substrates contribute to enzymatic browning of individual fruits or vegetables depends on the *localization* and *concentration* of the phenol as well as on the color intensity of the macromolecular pigments obtained from the different quinones. In most but not all fruits and vegetables, phenol

concentration is higher in the outer layers. In apples and pears, skin contained higher amounts of phenol than flesh, small fruits had a higher content of phenolics than large ones, and phenol concentration was higher in winter apples than in summer or autumn cultivars.[107] In carrot, radish, horseradish, and tomato tissue, phenol content was higher in the outer (surface) layers, whereas no differences between inner and outer layers were found in this respect in celery and red beet.[108,109]

A fundamental difference in the phenolic composition of fruits and vegetables is the fact that, while catechins, proanthocyanidins (dimers and oligomers of catechins), and leucoanthocyanidins (polyhydroxy-flavan-3,4-diols) are common constituents of most fruits,[63,110] they occur but exceptionally in vegetables.[111] In edible mushrooms, none of the groups of phenolic compounds present in higher plants could be detected:[112] the main substrates of PPO are tyrosine and L-DOPA.[113]

The contribution of a given endogenous substrate to enzymatic discoloration depends on its concentration and on the nature of the other substrates present in the tissue. According to Herrmann,[109] traces of a phenolic substrate are of no consequence, while concentrations of 100 mg kg^{-2} may lead to serious changes. In apples, (+) − catechin, (−) − epicatechin and chlorogenic acid were identified as substrates of PPO. The catechins were found to be oxidized more rapidly than chlorogenic acid, epicatechin contributing more to browning than the other two compounds. However, since the concentration of chlorogenic acid in apples is several times that of the catechins, its role in browning is decisive.[114] In potatoes, tyrosine, chlorogenic acid, and caffeic acid were found to form colored products upon enzyme action. However, the characteristic (red-brown) color shades obtained in vivo could be reproduced only with tyrosine. Thus, enzymatic browning of potatoes is considered to be essentially a tyrosine reaction.[115]

In some fruits and vegetables the main substrate of PPO is a compound not commonly occurring as a phenolic constituent of plant material. E.g., the principal substrate in bananas was identified as dopamine (3,4-dihydroxy phenylethylamine);[116] that in dates, as 3-*O*-caffeoylshikimic acid (dactylifric acid).[117] In yam-tuber tissues a catechin-like substrate of PPO was identified as catecholamine.[118] Phenolic substrates of the enzyme from various sources as reported in the literature are compiled in Table 1.

The data of the table show substrate specificity to depend not only on the genus but, to a certain extent, also on the cultivar and on the part of the fruit or vegetable the enzyme has been extracted from. The pH of the activity determination affects the utilizability of a substrate as well. The selectivity of the enzyme for monohydroxy phenols seems to be higher than for o-dihydroxy phenols. K_M values for the most common substrates of PPO preparations from different sources, as shown in Table 2, support these considerations.

The affinity of PPO towards a given substrate may vary within very wide limits, even if isoenzymes of the same origin are considered. This suggests that these differences might be due, at least partly, to steric factors connected to differences in protein structure. According to several authors,[121,129,142] no relationship could be found between K_M and V_{max} values obtained for different substrates with a given PPO preparation. Therefore it was recommended by some authors[129] to express the efficiency of a given substrate for a given enzyme preparation in terms of $V_{max}/2K_M$.

The substrate specificity of laccase depends on the enzyme source, too. p-Phenylenediamine, N,N-dimethyl-p-phenylenediamine, hydroquinone, N-phenyl-p-phenylenediamine, o-phenylenediamine, p-aminophenol, and catechol were reported as substrates (in the order of decreasing values of V_{max}) of the enzyme from *Rhus vernicifera*, while catechol and p-cresol were found to be oxidized by *Polyporus versicolor* laccase.[87] A certain overlapping of substrate specificity of PPO and laccase seems to exist, making distinction between the two enzymes difficult.

Table 1
SUBSTRATES OF POLYPHENOL OXIDASES (PPO) FROM VARIOUS SOURCES

Source	Compounds found to be substrates	Remarks	Ref.
Apple	Chlorogenic acid (100), catechol (60), (+)-catechin (60), caffeic acid (50), L-DOPA (18), 3,4-dihydroxy benzoic acid (18), p-cresol (13)[a]		119
Apple chloroplasts	Chlorogenic acid (100), catechol (40-45), p-cresol (7-9)[b]	Hydrocaffeic and p-coumaric acids not acted on	101
Apple chloroplasts	4-Methyl catechol, chlorogenic acid, L-DOPA, (−)-epicatechin, caffeic acid, 3,4-dihydroxybenzoic acid,[h] p-cresol[h]	Pyrogallol poor substrate	15, 102
Apple peel, (Red delicious)	4-Methyl catechol, chlorogenic acid, catechol, (+)-catechin[c]	Quercetin, tyrosine poor, quinol no substrate	103
Pear flesh	Chlorogenic acid (100), catechol (86), (+)-catechin (60), caffeic acid (43), L-DOPA (17), 3,4-dihydroxy benzoic acid (6), p-cresol (5)[a]		119
Pear, (Bartlett)	Catechol (100), chlorogenic acid (12), caffeic acid (10), (+)-catechin (7), protocatechuic acid (2)[b]	Phenol, m- and p-dihydroxy benzols not attacked	120
Pear, (Bartlett)	Catechol, chlorogenic acid, 4-methyl catechol, (+)-catechin[d]		79
Apricot	Isochlorogenic acid, caffeic acid, 4-methyl catechol, (−)-epicatechin, chlorogenic acid, (+)-catechin, pyrogallol, catechol[e]		121
Peach, (Redhaven)	Pyrogallol (116), 4-methyl catechol (105), catechol (100), caffeic acid (53), chlorogenic acid (50), gallic acid (5)[b]	Tyrosine and p-coumaric acid not acted on	104
Peach, (Halford)	(+)−Catechin, catechol, chlorogenic acid, caffeic acid, dopamine[f]		122
Peach, (Ford, Mary Gold, Elberta)	Chlorogenic acid, pyrogallol[f]		123
Plum	Chlorogenic acid, catechin, caffeic acid, catechol, DOPA[f]		55
Sweet cherry	Chlorogenic acid (142), 4-methyl catechol (133), 4-chlorocatechol (119), 3,4-dihydroxy-cinnamic acid (128), 3,4-dihydroxyphenylacetic acid (105), 3,4-dihydroxypropiophenone (105), catechol (100)[a]		80
Grape	(+)-Catechin, chlorogenic acid, catechol, caffeic acid, (±)-catechin, DOPA, protocatechuic acid, resorcinol, hydroquinone, phenol[g]		124
Grape, (Concord)	Catechol (100;100), pyrogallol (95;95), caffeic acid (90;90), DL-DOPA (90;80), D-catechin (75;70), dopamine (50;40), gallic acid (5:1), chlorogenic acid (3;2)[b,i]	*Monophenols not attacked*	125
Mandarin (Satsuma)	Pyrogallol		126

Table 1 (continued)
SUBSTRATES OF POLYPHENOL OXIDASES (PPO) FROM VARIOUS SOURCES

Source	Compounds found to be substrates	Remarks	Ref.
Mango	Dopamine-HCl, 4-methyl catechol, caffeic acid, catechol, (+)-catechin, chlorogenic acid, DL-arterenol-HCl, L-DOPA, DL-DOPA, p-cresol, tyramine-HCl, *L-tyrosine, DL-tyrosine*	Enzyme from irradiated, stored (14 days) fruit	127
Avocado, (Lerman and Fuerte)	4-Methyl catechol, dopamine, catechol, pyrogallol, chlorogenic acid, DL-DOPA, caffeic acid[f]		128
Potato peelings	Quercetin, rhamnetin, fisetin, chlorogenic acid, luteolin, 4-methyl catechol, 3,4-dihydroxy phenylpropionic acid, 3,4-dihydroxy cinnamic acid, catechol, rutin, taxifolin, catechin, 3,4-dihydroxy phenylacetic acid, maclurine, quercitrin, 3,4-dihydroxy benzoic acid[e]		129
Potato tubers	p-Coumaric acid, p-coumaryl glucose	Soluble enzyme	130
Potato	Chlorogenic acid, catechol, caffeic acid, L-DOPA, p-hydroxyphenyl propionic acid, p-cresol, p-hydroxyphenyl pyruvic acid, m-cresol[f]		43
Sweet potato	Chlorogenic acid, caffeic acid, caffeylamide		85
Broad bean leaf	4-Methyl catechol, catechol, pyrogallol, DOPA, adrenaline, caffeic acid, chlorogenic acid, gallic acid, protocatechuic acid, p-cresol, tyramine, p-coumaric acid	At pH 4.8—5.2 DOPA, chlorogenic, and gallic acids were oxidized at a higher rate; at pH 6.5—6.8 protocatechuic and caffeic acids, methyl caffeate and methyl gallate	75
Broad bean pod	L-DOPA and all diphenols tested	p-cresol not oxidized	131
Parsnip	Chlorogenic acid (100), catechol (11), tyrosine (12)[b]	No browning with cinnamic, ferulic, quinic acids, rutin, quercetin, DOPA	132
Parsnip	DOPA, catechol, chlorogenic acid	Tyrosine not oxidized	133
Eggplant	Chlorogenic acid		134
Mushroom	Catechol, L-DOPA, dopamine, D-DOPA, L-adrenaline, D-adrenaline, L-noradrenaline, D-noradrenaline		113

Note: DOPA = 3,4-dihydroxy phenylalanine.

[a] Figures in brackets: relative reaction rates.
[b] Figures in brackets: relative activities.
[c] In the order of decreasing reaction rates.
[d] In the order of decreasing V_{max}.
[e] In the order of decreasing $V_{max}/2K_M$.
[f] In the order of decreasing activities.
[g] In the order of decreasing affinities.
[h] Acted on only by part of the enzyme fractions.
[i] The first figure refers to values obtained by colorimetric, the second to values obtained by O_2 uptake method.

Table 2
K_M VALUES, FOR VARIOUS SUBSTRATES, OF POLYPHENOL OXIDASE FROM DIFFERENT SOURCES[a]

Substrate	K_M (mmol · l^{-1})	Enzyme source	Ref.
Chlorogenic acid	1.66	Apple chloroplast	101
	16.1	Pear (Bartlett)	79
	1.20	Apricot	121
	2.50	Grape	124
	0.01	Potato (peelings)	129
	1.40	Potato (tuber)	43
	0.95; 3.10	Potato[b]	85
	2.40; 1.10	Sugar cane[b]	62
	0.22	Mushroom	135
Isochlorogenic acid	0.36	Apricot	121
Caffeic acid	0.63	Apple chloroplast	101
	0.50	Apricot	121
	5.50	Grape	124
	2.10	Potato (tuber)	43
	2.40; 2.90	Potato[b]	85
	6.00; 8.70	Sugar cane[b]	62
(+)-Catechin	1.48	Apple chloroplast	101
	2.10	Pear (Bartlett)	79
	0.74	Apricot	121
	1.00	Grape	124
(−)-Epicatechin	0.55	Apricot	121
p-Cresol	0.67	Potato	43
Catechol	4.60	Apple (Red Delicious flesh)	136
	20.9	Pear (Bartlett)	79
	36.0; 6.60; 4.20; 7.00;	Peach (Cortez)[b]	137
	120	Peach (Fay Elberta)	138
	29.0	Peach (Redhaven)	104
	15.0	Peach (Halford)	122
	2.40	Apricot	121
	3.06	Grape	124
	67	Grape (Concord)	125
	13.0	Plum	55
	0.11	Potato (peelings)	129
	4.80	Potato (tuber)	43
	17.0	Parsnip	133
	1.68	Eggplant	139
	4.00	Eggplant	140
	2.50; 3.00; 4.00;	Mushroom[b]	141
	22.0	Mushroom	135
	8.00	Pear (Bartlett)	79
4-Methylcatechol	1.40	Apricot	121
L-DOPA	11.8	Potato (tuber)	43
	2.0	Sugar beet	62

[a] K_M = the Michaelis constant.
[b] Different enzyme fractions.

3. pH and Temperature Optima of Activity

The optimum pH of PPO activity varies with the source of the enzyme and with the substrate in a relatively wide range, in most cases between pH 4.0 and 7.0.[143] PPO preparations from several sources were reported to be inactive below pH 4.0[127,144] The enzyme in potato homogenates was found to be inactive, on both pyrogallol and chlorogenic acid, at pH 5.0,[145,146] while pyrogallol was not oxidized by PPO from unripe Satsuma mandarins at pH values below 6.0.[126] On the other hand, a preparation from grapes retained over 50% of its maximum activity at pH 3.4, the normal pH of grape juice.[125] The enzyme from plums was nearly fully active at its natural pH (3.8), while practically all its activity was lost in neutral medium.[55] The type of buffer and the purity of the enzyme affect the pH optimum as well. Particulate and soluble enzymes seem to be essentially different in this respect.[103] Isoenzymes may have distinctly different pH optima. Enzyme preparations obtained from the same fruit or vegetable at various stages of maturity have been reported to differ in optimum pH of activity. Some examples supporting these statements are given in Table 3.

Not only the optimum, but the relationship between activity and pH over a wide range of pH values was found to differ according to genera, cultivars, and substrates, as shown in Figure 1.[146]

The figure shows also that, for different lots of a given cultivar the pH-dependence of activity is essentially the same, although the activity values may differ (curves 2a and 2b). The V_{max} values obtained with a given substrate were found to be unaffected by pH.[15]

According to the data in Table 3, most of the enzyme preparations have a single pH optimum of activity. A second optimum was found in some cases to be due to insufficient purification.[80]

The pH optimum of laccase activity depends highly on the enzyme source and on the substrate. Optima between pH 4.5 and 7.5 have been reported for enzymes from various fungi, and the highest activity of a given preparation was obtained at the pH values of 5.0 and 4.0, on the substrates catechol and hydroquinone, respectively.[87] Laccase of peaches had a clear optimum at pH 6.0, both on 4-methyl catechol and on quinol substrates.[6]

The temperature optimum of activity has been much less investigated for PPO than the pH optimum. The data available indicate that the temperature optimum of the enzyme depends essentially on the same factors as the pH optimum.

The activity of peach PPO (cv. Redhaven) was found to increase from 3°C to 37°C and then decline up to 45°C. At 3°C activity was about 50% of the maximum value. In apricots the enzyme reached its maximum activity at 25°C;[146] in bananas at 37°C.[24] The activation energy of the reaction catalyzed by banana-PPO, as calculated from the temperature dependence of activity, was found to be 18.6 kJ $mole^{-1}$ on catechol substrate.[24] For two cultivars of grapes, temperature optima were between 25—30°C and 10—15°C, respectively.[124,125] In apples of the cvs. Jonathan and Starking, maximum PPO activity on chlorogenic acid was obtained at 30°C and 25°C, respectively. On pyrogallol substrate, activity rose steeply with temperature, but no maximum was reached up to 35°C.[146] The activity of parsnip PPO on chlorogenic acid substrate at pH 5.2 was found to increase mainly between 22°C and 27°C, whereafter only a slight further rise was noticed up to 32°C.[133] In potatoes, PPO had its maximum activity on catechol at 22°C;[150] on pyrogallol, a nearly linear increase in activity was noticed between 15°C and 35°C.[146]

Table 3
pH-OPTIMA OF ACTIVITY OF POLYPHENOL OXIDASES FROM DIFFERENT SOURCES

Source	pH-optimum	Remarks	Ref.
Apple	7.0	Tissue extract	147
	5.0	Chloroplasts	101
	4.8—5.0	Mitochondria; method: O_2-absorption	148
	5.1 and 7.3	Mitochondria and chloroplasts	15
	5.1 and 7.0	Particulate enzyme from peel	103
	4.2 and 7.0	Solubilized and highly purified enzyme from peel	103
Pear	6.2	cv. Bartlett	120
Peach	6.0—6.5	cv. Redhaven; substrate: catechol	104
	6.8; 6.5; 7.2; 7.0	Isozymes of cv. Cortez; substrate: catechol	137
	6.2	cv. Halford; substrate: catechol	122
	5.9—6.3	cv. Fay Elberta; catechol; citrate-phosphate buffer	138
	6.5—6.8	cv. Fay Elberta; catechol; oxalate-phosphate buffer	138
Sweet cherry	4.0	4-Methyl catechol substrate	107
	4.2	Catechol substrate	107
	4.5	Chlorogenic acid substrate	107
	3.9	Chlorogenic acid, (+)-catechin, catechol substrates; preparation eluted from DEAE-cellulose with 0.05 M phosphate buffer at pH 7.0	67
	7.0	Catechol substrate; preparation eluted from DEAE-cellulose with 0.2 M phosphate buffer at pH 7.0	67
	7.5	(+)-Catechin, chlorogenic acid substrates; preparation eluted from DEAE-cellulose with 0.2 M phosphate buffer at pH 7.0	67
Plum	4.25	Citrate-phosphate buffer; crude preparation	55
Grape	6.2	Chlorogenic acid substrate	124
	6.5	Catechol substrate	124
	7.0	(+)-Catechin, pyrogallol	124
	5.9—6.3	cv. Concord, catechol substrate, crude extract	125
Mandarin	7.2—7.6	Pyrogallol substrate	126
Banana	6.0—7.0	For different active forms	148
Avocado	5.3—6.7	4-Methyl catechol, DOPA substrates; crude enzyme	149

Table 3 (continued)
pH-OPTIMA OF ACTIVITY OF POLYPHENOL OXIDASES FROM DIFFERENT SOURCES

Source	pH-optimum	Remarks	Ref.
	5.5—6.5	4-Methyl catechol, DOPA substrates; partially purified preparations from cvs. Lerman and Fuerte	128
Mango	6.5—7.0	Preparations from fresh and irradiated fruits	78, 127
Potato	5.8	Catechol substrate; acetone extract of enzyme	150
Sweet potato root	6.0; 6.1—7.0	Different enzyme fractions eluted from DEAE-cellulose	85
Broad bean pod	4.0		131
Parsnip root	4.4 and 5.2	Chlorogenic acid, catechol substrates; acetone extract	132
	8.6	L-DOPA substrate; aqueous extract	133
Green chili	7.0		151
Eggplant	5.0—5.2	Catechol substrate	140
	6.8	Catechol substrate	139
Mushroom	5.5—7.0	Catechol substrate	152
	6.0—7.0	p-Cresol substrate	152

4. Temperature Stability of PPO

PPO does not belong to the extremely heat-stable enzymes. Short exposures, in the tissues or in solution, to temperatures of 70 to 90° C are, in most cases, sufficient for partial or total irreversible destruction of its catalytic function. Exposures to temperatures below zero may also affect activity. PPO in fruit or vegetable tissues is generally inactivated, by heat or by chemicals, prior to exposure to low temperatures to prevent enzymatic browning, which otherwise would take place rapidly upon thawing as a result of cell rupture occurring during freezing and access of the enzyme to its endogenous substrates.[153]

Thermotolerance of PPO depends, similarly to substrate specificity, and pH- and temperature optima of activity, to a considerable extent, on the source of the enzyme. When considering the enzyme as present in tissues, heat penetration connected to particle size might considerably influence the efficiency of thermal inactivation.

It is often difficult to compare data of heat stability obtained for purified enzyme preparations and for the enzyme in tissues or juices, as biochemists dealing with the former generally express thermotolerance in terms of half-lives or conditions of complete irreversible inactivation, and food technologists prefer to use D and z values. (D is the duration in minutes of heat treatment at a given temperature required to reduce activity to 1/10 its original value, assuming an exponential relationship between activity and time of exposure; z is the rise in temperature that brings about a decrease in D by one logarithmic cycle.)[154,155]

Apple PPO was found to be markedly inactivated (at pH 5.0) at temperatures above 70° C. Its half-life at this temperature was 12 min, and activity was completely destroyed at 80° C. Preparations obtained from apple fruitlets and from mature fruit did not differ in this respect.[101] The D value for apple puree at 75° C was found to be 0.24 min and the z value 10° C.[156,157]

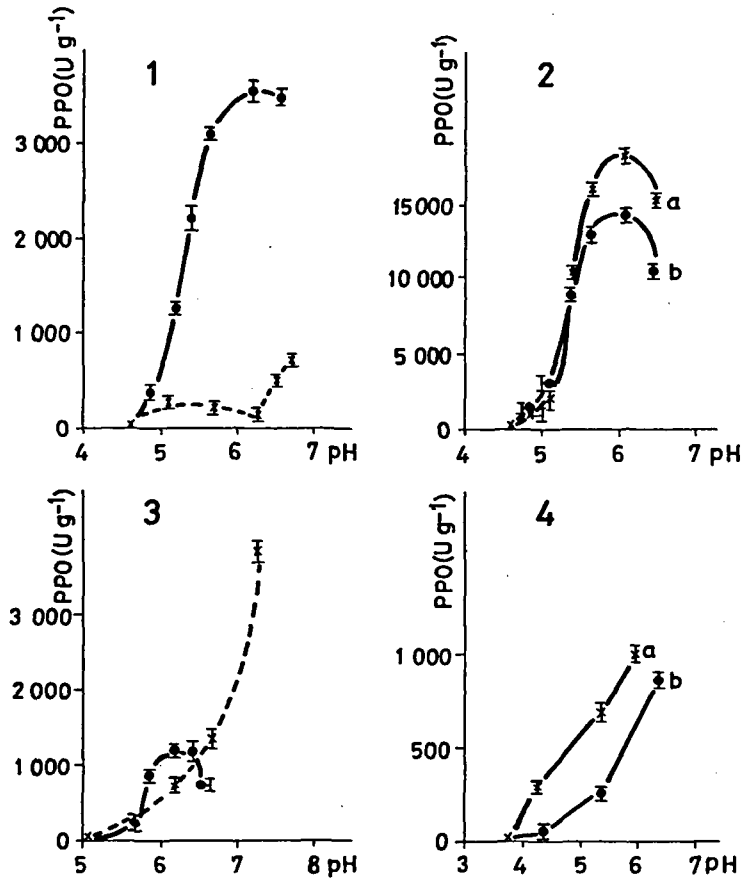


FIGURE 1. pH-dependence of polyphenol oxidase (PPO) activity in homogenates of Jonathan (1) and Starking (2a and b) apples, Gül baba potatoes (3) as well as of Ford (4a) and Elberta (4b) peaches. 2a and 2b represent two lots of the same cultivar. Substrates: chlorogenic acid (full line); pyrogallol (broken line) (From *Acta Alimentaria*, 7, 57, 1978. With permission.)

The inactivation of PPO in quince was found to take, for most cultivars investigated, 16 to 30 sec at 95° C and 25 to 80 min at 75° C. In one cultivar, activity could be destroyed at 75° C in 3 min.[158]

Out of 22 cultivars of different stone fruits, peaches were found to possess the least, and plums the most heat stable PPO. The higher heat stability of the enzyme in plums and cherries was accompanied by higher activity levels as compared to peaches.[159] PPO was found, by some authors, to be more heat stable in the juice than in the pulp of a given fruit,[159] while others observed the contrary.[20] No relationship could be established between pH and heat tolerance for PPO in stone fruits.[160] However, the enzyme proved more heat stable in unripe than in ripe fruit. In the stone fruits investigated, *z* values of PPO varied from 8.5 to 15° C, and instantaneous inactivation (within 6 sec) was achieved at temperatures from 89.5 to 110° C.[159] For sweet cherries of different cultivars with pH values between 3.44 and 3.76, *z* values ranged from 6.8 to 10.6° C, and temperatures of instantaneous inactivation from 77.2 to 91.7° C. PPO in sweet cherries was not inactivated by frozen storage: on thawing, fruits turned brown.[20]

The enzyme in apricots was reported to be relatively heat stable and the efficiency of thermal treatment was found to be dependent on pH. The hydroxylating activity (as

Table 4
HEAT INACTIVATION OF PPO IN HOMOGENATES OF DIFFERENT PEACH CULTIVARS FROM TWO LOCATIONS[163]

Cultivar	Degree of inactivation (%)	
	Location 1	Location 2
Mamie Ross	57.8	33.2
Rochester	88.0	79.8
Halehaven	74.8	22.3
Michigold	0	36.7

Note: Heat treatment was carried out at 50° C for 2 min; heating up and cooling were performed within 2 min each.

determined on p-cresol substrate) was more stable, in 5-min heat inactivation experiments, at pH 5 than at pH 6. At both pH values, complete inactivation of both activities occurred at 100° C. At 90° C, under similar conditions, 15, 4, and 5% of the original oxidizing activity (catechol substrate) were retained at pH 4, 5, and 6, respectively. (The hydroxylating activity was unstable at pH 3, and the oxidizing activity was most stable at pH 5, during 24 hr in the temperature range from 0° C up to 40° C, or during 17 hr from 0° C up to 60° C.) No essential difference was found between the thermal resistance of apricot PPO in solution or in the fruit tissue. Even very low residual activities were found to cause discoloration in canned fruit on storage.[161] A 10-min heat treatment at 90° C ensured complete inactivation of the enzyme in apricot puree, and prevented browning and off-flavor formation in the product on thawing after frozen storage.[162]

As already mentioned, peach PPO is, in general, of low thermostability. However, the cultivar and the location might influence inactivation to a considerable extent, as shown in Table 4.

Different molecular forms of PPO from the same source may have rather different thermostabilities. Three isoenzymes from peaches of the cv. Cortez had, at 55° C, half-lives of 5.5, 14.1, and 14.6 min, respectively; the fourth was stable over a period of 50 min and required treatment at 76° C for rapid inactivation.[137]

Grape juice PPO was found to lose activity relatively slowly up to 60° C. 65° C may be regarded as the critical temperature above which rapid inactivation starts. A heat treatment of 5 min at 65° C destroyed 74.5% of the original activity at pH 3.3, the natural pH of the juice. At pH 3.0, 81%, at pH 4.0, only 59% inactivation occurred under the same conditions.[164] Total inactivation could be achieved in 5 min only above 70° C.[165] Inactivation within 6 sec was reached, depending on the cultivar, at temperatures between 80° C and 89.5° C. In tissue homogenates of seedless grape cultivars, PPO was more heat stable and of higher activity than in the respective juices.[166] Crude grape PPO rapidly lost its activity at relatively low temperatures (between 20° C and 30° C) at pH values below 4.0; at pH 3.5, inactivation took 60 min; at pH 2.5, only 5 min.[167] At the pH of wine, PPO was found to be of poor stability even without heating. After long storage, activity could be detected in but 2 wine samples out of 20, while invertase was present in all of them.[168]

PPO in strawberries was reported to lose its activity upon prolonged storage of the fruits in the cold; when kept at −18° C, enzyme activity decreased first slower, then quicker to be reduced, after 7 1/2 months to 16% of the initial value. After 11 months of cold storage the samples occasionally contained traces of PPO activity.[169]

As even traces of activity may bring about discoloration, it is recommended, whenever feasible (e.g., with purees of products to be processed by pasteurization or appertisation) to heat the frozen good very quickly to a temperature above 65°C, where the enzyme gets more or less inactivated.[170]

Studies into the heat inactivation of banana PPO revealed, in the range of 42 to 80°C, first order kinetics of denaturation. Below 42°C the enzyme was very stable at pH 6.0: a 1-h exposure to this temperature brought about but 8% inactivation. In the range of 42 to 72°C, the activation energy of the inactivation reaction was 77 kJ/mol^{-1}, and in the range of 72 to 80°C, 355 kJ/mol^{-1}. The Q_{10} values and the entropy changes were also much more elevated in the latter range, showing that marked changes in the structure of the enzyme protein occurred only above 70°C. Thermodynamic data were similar in the pH-range from 5.0 to 8.0.[24] Complete inactivation of banana PPO could be achieved by exposure to 80°C for 15 min. Slow cooling brought about the regeneration of some molecular forms of the enzyme.[148]

Satsuma mandarins were reported to have an unusually heat stable PPO: after 30 min exposure to 80°C, the enzyme still retained more than 50% of its original activity.[126]

Heat stability of PPO has been much less studied in relation to vegetables than in relation to fruits, perhaps because the thermotolerance of PPO in vegetables is much inferior to that of POD. In fruits PPO was found to be more heat stable than POD.[171]

Inactivation of PPO in green peas before freezing was achieved in 29 min at 80°C, or 1 min at 95°C, while 2.5 min were required at 90°C.[172]

PPO extracts of green beans and green peas were completely inactive at −20°C. Inactivation was, however, reversible as activity reappeared after thawing. At −15°C some activity could be detected. The resistance to cold of the enzyme of these two vegetables was found to be remarkable: after thawing, activity remained essentially unaltered up to 395 and 310 days respectively, of storage at −20°C, whereafter a slow diminution or, with some string bean cultivars, even a slight increase was observed. The time-course of inactivation was found to approximate linearity for enzyme extracts of either vegetable. This, however, did not apply to the enzyme in the tissues.[173,174] The changes were attributed to gradual inactivation of the soluble and slow solubilization of the particle bound enzyme during storage.

PPO in green chilies was found to be remarkably heat resistant: it could be inactivated to 90% only by heat treatments of 5 and 2 min, at 90°C and 100°C, respectively.[151]

5. Effectors of PPO

a. Activators

While the literature on the inactivation of PPO and the prevention of enzymatic browning is very vast, relatively little has been published on the activation of the enzyme. This is undoubtedly connected to the fact that, in the majority of cases, PPO action is undesirable.

Anionic detergents (e.g., sodium dodecyl sulphate, or SDS) were found to reactivate the mitochondrial enzyme preparation from apple peels after inactivation by polyvinylpyrrolidone (PVP).[148] SDS was also reported to activate latent PPO in the crude and partially purified preparations obtained from different avocado cultivars.[175]

Short exposures to acid pH or exposure to urea resulted in a 4- to 10-fold reversible activation of grape PPO. Longer exposures to acid pH brought about irreversible activation. Activation by acid was higher at higher ionic strength. It occurred in intact chloroplasts as well as in partially purified enzyme preparations. Irreversible activation was accompanied by a decrease in relative electrophoretic mobility of the enzyme.[176] This supports the assumption that conformational changes might be the cause of activation. Urea was found to activate also the latent forms of broad bean PPO, enhancing especially its action on monophenols.[177]

The addition of Cu^{2+} ions to the media was found to increase enzyme activity in radish, while a similar treatment had no effect on potato PPO.[178,179]

L-DOPA activated the PPO-catalyzed oxidative cyclization of adrenaline and the formation of fluorescent intermediates.[113] Substrate induced activation was reported also in the case of sweet potato tissues.[180]

Although the activation of PPO from different sources has not yet been systematically studied, and is, perhaps, not quite understood, the majority of the few examples cited indicate protein association or dissociation to be involved in this process.[52] The active form of PPO seems to be an oligomer of a copper-containing enzyme monomer,[181] as will be shown later, and the role of the activator is to bring the enzyme to the degree of polymerization required for biological activity. The effect of substrates, or of the addition of the prosthetic ion, can be well understood, too.

However, enzyme activity has been reported to be induced by other factors as well, such as trace elements in fertilizers,[182] agents promoting ripening,[183] or physical treatments like ionizing radiation.[184,185,186] In these cases actual activation has not been proven. The effect might be connected to enhanced synthesis of enzyme protein in the plant; elimination of a natural inhibitor (e.g., by complexing with the metal ions); changes in the structure of the substrate or in cell wall permeability.[187] The different stimulating factors are not always known and are sometimes not easy to distinguish.

b. Inhibitors of PPO and Prevention of Enzymatic Browning

Inhibition of PPO and prevention of enzymatic browning are often treated as one and the same subject. However, browning may be prevented not only by inactivating the enzyme, but also by eliminating one of the two substrates necessary for the reaction (O_2 and the polyphenol), or by reacting with the products of enzyme action to inhibit the formation of the colored compounds produced in secondary, non-enzymatic reaction steps. In many cases, it is not easy to distinguish the different mechanisms underlying the action of a browning inhibitor; moreover, some inhibitors act simultaneously on the enzyme and the substrate or product.

The use of browning inhibitors in food processing is restricted by special requirements such as non-toxicity, wholesomeness, effect on taste, flavor, texture, etc. Good results are often achieved with mixtures of chemicals, the mechanism of action of which is not well understood. Recently Walker[5] has reviewed the different classes of inhibitors of enzymatic browning.

i. Inhibitors Acting Primarily on the Enzyme

As PPO is a metalloprotein with copper as the prosthetic group, it can be inhibited by metal chelating agents such as cyanide, carbon monoxide, sodium-diethyl-dithiocarbamate (DIECA), mercaptobenzthiazol, dimercaptopropanol, azide, or potassium methyl xanthate.[5,45,75,101,107,187] Some of these react also with the quinones formed.[45] Diethyl dithiocarbamate was found to be ineffective on banana PPO. This is thought to be related to the fact that no copper could be detected in the purified enzyme.[148] Ascorbic acid may also act directly on the enzyme by chelating with its prosthetic group.[45]

Distinct differences were found in the action of this group of inhibitors upon PPO from chloroplasts, mitochondria or the soluble fraction. While, e.g., the mitochondrial and soluble fractions of the dehydrogenating activity showed similar responses to potassium ethyl xanthate, thiourea and DIECA, the corresponding activity in chloroplasts was not inhibited by xanthate and only partially by DIECA. The hydroxylating activity of the mitochondrial and chloroplast fractions exhibited similar responses to these inhibitors; however, these responses were different from those observed with the dehydrogenating activity.[73] The inhibitory action of DIECA on the

hydroxylation reaction was completely reversed by the addition of o-dihydroxy phenols such as caffeic acid, protocatechuic acid, or catechol.[188]

Inorganic ions such as fluoride and azide inhibit the enzyme mainly in acidic medium when dissociation is uncomplete. Fluoride is a non-competitive, and azide a mixed-type inhibitor of broad bean PPO.[20,75] Some inorganic ions, e.g., borate, act by complexing with the substrate. With L-DOPA or L-adrenaline as substrates, the inhibition by borate was found to be of the competitive type.[189,190]

Benzoic acid and some substituted cinnamic acids were found to be mostly competitive inhibitors of the enzyme from sweet cherries, apples, pears, apricots, and potatoes.[191,192,193,194] K_i values increased in the order: cinnamic acid < p-coumaric acid < ferulic acid < m-coumaric acid < o-coumaric acid < benzoic acid. The results obtained with these compounds indicated PPO to have separate substrate and inhibitor binding sites, situated close together in the enzyme molecule.[5,191] Aromatic acid inhibitors need a free carboxylic group directly on the benzene ring or in conjugated position. Open chain acids must have at least two conjugated ethylene groups to be active.[194] Methylation was found to lower both the inhibition and the value of K_i. It was concluded that methylation or hydroxylation of the benzene nucleus of the inhibitor affected competition with the catalytic site.[192] p-Coumaric acid and ferulic acid formed by the soft rot organism *Penicillium expansum* might account for the light color of the infected apple tissue.[41]

Investigations with several inhibitors of the cinnamic acid type showed the type of inhibition and K_i to be dependent on the structures both of the substrate and the inhibitor. With chlorogenic acid as substrate, the action of cinnamic, p-courmaric, ferulic, and iso-ferulic acids was found to be non-competitive on a particulate, and competitive on a solubilized enzyme preparation from apples, whereas m-coumaric acid acted reversed. It is assumed that the removal of lipids or lipoproteins in the solubilization process modifies the structural organization of the enzyme and its ability for conformational changes.[192] The inhibition of mushroom PPO by benzoic acid and by cyanide, respectively, showed the former to be competitive with catechol and non-competitive with O_2, while the latter inhibitor showed a reversed behavior. This was interpreted as the result of the existence of two distinct substrate binding sites on the enzyme molecule, one of which had a high affinity for aromatic compounds, including phenolic substrates, while the other, which probably contained the enzyme copper, served for metal-binding agents and oxygen.[195]

K_i values of a given inhibitor can be different for isoenzymes of the same origin. This was shown for p-coumaric acid, a simple, linear, non-competitive inhibitor of pear PPO on chlorogenic acid substrate at pH 6.2, and for benzoic acid, a linear, competitive inhibitor of these enzyme fractions at pH 4.0[79] Also with this group of inhibitors, K_i was found to depend on whether an o-dihydroxy phenol or a monophenol was used as substrate.[43]

Other inhibitors acting on the enzyme have been reported to be small peptides, probably interacting with the copper of PPO, e.g., a galactose oxidase inhibitor from *Dactylium dendroides,*[196] sodium or potassium chloride,[115,197] substrates of denaturing and precipitating action.[198] A natural phenolic inhibitor of both PPO and POD was extracted from tea leaves; its effect could be reversed by albumin or some amino acids.[199]

Soluble polymers such as polyvinylpyrrolidone (PVP, MM 10,000—60,000)[5,45,148,200] act as competitive inhibitors of PPO. This action can be reversed by anionic detergents.

Thioglycollate, a powerful inhibitor of sugar cane PPO, reacts both with the enzyme and its substrates.[48,62]

2,3-Naphthalenediol is a specific competitive inhibitor of PPO which does not act on laccase and makes the distinction between the two enzymes possible.[201]

There is a group of PPO inhibitors in plant tissues acting by inhibition of protein biosynthesis as, e.g., antibiotics.[66,202] An interesting way of inactivating PPO consists in a treatment with an activator of proteolytic enzymes which then destroy PPO. Succinic acid-2,2-dimethylhydrazide retarded, by this mechanism, the enzymatic discoloration of cultivated mushrooms. Browning was reduced also by binding of the substrates to the inhibitor.[203] Inhibition of PPO by the contact freezing agent dichlorodifluoromethane is based on the protein denaturing action of this compound.[204]

ii. Compounds Reacting with Reaction Products or Substrates

The inhibitors of enzymatic browning, reacting with the reaction products or substrates, can be divided into the following groups: (1) reducing agents acting on the quinones formed by restituting the o-dihydroxy phenols. These compounds are consumed in the process of inhibition, and thus provide only temporary protection against discoloration, unless used in high concentrations, in which case reaction inactivation of the enzyme might occur prior to the depletion of the reducing agent. Some frequently used representatives of this group are ascorbic acid, SO_2, potassium metabisulphite, 2-mercaptoethanol, 2-mercaptobenzthiazole, thioglycollate; (2) quinone couplers forming stable colorless compounds with the quinones, thus providing permanent protection as long as they are not entirely consumed. Cysteine, glutathione, benzenesulphinic acid, DIECA, Na-ethyl xanthate are, among others, able to perform such reactions. High molecular, insoluble PVP strongly binds phenolic compounds. Some enzymes tranform the substrates of PPO by methylation or oxidative cleavage of the benzene nucleus. O-methyltransferase methylates the 3-position of 3,4-dihydroxy aromatic compounds, converting PPO substrates into inhibitors (e.g., caffeic acid into ferulic acid); it requires S-adenosylmethionine as cofactor. Protocatechuate-3,4-dioxygenase cleaves the benzene nucleus; it acts, however, only slowly on chlorogenic acid, one of the main substrates of plant PPO. Both enzymes are active at alkaline pH, while most fruits and vegetables are neutral or slightly acidic. These inhibitor enzymes are, for the time being, of little practical interest.[5,200,205,206,207] For the elimination of oxygen from fruit tissues, dipping in sugar syrup or vacuum infiltration of a concentrated sugar solution can be used.[208]

iii. Application of Individual Inhibitors

Potassium cyanide, in a concentration of 0.01 mol ℓ^{-1} strongly inhibited PPO from spinach chloroplasts, while azide had practically no effect.[209] The enzyme from potatoes was inhibited to 60% by both KCN and NaN_3 in concentrations of 0.1 mmol ℓ^{-1}.[73] 5 mmol ℓ^{-1} of KCN inhibited Concord grape PPO to 98%; NaN_3 of the same concentration, only to 60%.[125] Sugar cane PPO was completely inhibited by 1 mmol ℓ^{-1} KCN. Dialysis against water and subsequent addition of 0.2 μmol Cu $m\ell^{-1}$ brought about the regeneration of the enzyme in the plant tissue.[210] The inhibiting activity of cyanide is quickly lost in the presence of aldehydes, owing to the formation of cyanohydrins.[20]

By varying the levels of O_2, CO_2, and CO in the surrounding atmosphere, enzyme action can be inhibited to a considerable degree. In CO_2/CO (80:20), catechol oxidase activity of mushroom PPO was reduced by 68%.[211] Crude extracts and purified preparations from Red Delicious apples were competitively inhibited by CO_2 (pH 6.8, catechol substrate). 30% CO_2 brought about 70% inhibition at 20° C.[212] An atmosphere containing 5 to 10% O_2 and 1% CO_2, with or without 1% CO, was effective in preserving the color of lettuce during storage.[213] CO was also reported to be an efficient inhibitor of PPO in broad bean pods.[131] On the other hand, PPO in intact grapes or grape homogenates was reported to lose much less activity when kept in CO_2 (up to 30 days) than when kept in air.[214]

Diethyldithiocarbamate in concentrations ranging from 0.01 to 1 mmol ℓ^{-1} was found to inhibit apple PPO (as measured by O_2 absorption) to 28—87%.[101] Grape PPO could be inhibited by 0.1 mmol ℓ^{-1} of this compound.[102] A combined treatment with DIECA, bentonite, and PVP performed after pressing of the grapes was found most efficient to stabilize the color of wine.[214] The enzyme of green chilies was inhibited to 36% by 0.1 mmol ℓ^{-1} of this compound.[151]

2-Mercaptobenzthiazole was reported to be a potent inhibitor of PPO from different sources. Treatment of 1% or 2% solutions completely inhibited friction discoloration of d'Anjou pears.[215] 0.02 mmol ℓ^{-1} concentrations brought about prolonged inhibition of banana PPO, and the compound acted also on the enzyme from mushroom, potato, and tobacco leaf.[187]

2,3-Naphthalenediol in 1 mmol ℓ^{-1} concentration was sufficient to prevent browning of apple slices.[216] However, the effect of this compound on various isoenzymes was very different. With some isoenzymes it acted even as stimulator.[102] This inhibitor was found to be effective with soluble and particulate PPO from several plants or their fruits, e.g., peaches, potato tubers, lettuce seeds, and sugar beet leaves.[201]

One of the most widely used inhibitors of enzymatic browning is *ascorbic acid;* because it might increase the biological value of the food product, it has been added to and protects anthocyanins from oxidative degradation, thus contributing to color preservation of red fruits. Its action on the PPO — polyphenol system is complex, as it is a reducer of quinones, on the one hand, and a copper chelating agent on the other.[217,218] Moreover, it can be oxidized directly by the enzyme, thus acting as competitive inhibitor. Kinetic analysis showed its overall inhibitory action to be of the mixed type.[219] Competitive inhibition of PPO, as observed with some phenols (e.g., resorcinol), was enhanced by ascorbic acid.[217] It might also act as a pro-oxidant (in the presence of compounds of lower oxidation-reduction potential): the rate of O_2-uptake in the oxidation, by PPO, of L-tyrosine, DOPA, and protocatechuic acid was found to be increased by ascorbic acid.[220] The quinone-reducing capacity of ascorbic acid makes it evident that the efficiency of this inhibitor depends largely on its concentration: when applied at low concentrations, it may be quickly consumed in the reducing process and prevent the formation of colored polymers only for a limited time, which sometimes might even pass unnoticed. This is a possible reason of why several authors failed to notice inhibition of PPO by ascorbic acid.[24,221-223] If, however, ascorbic acid is added in high concentration, it can reduce the quinones formed to furnish fresh substrate to the enzyme till the latter undergoes reaction inactivation, which is an irreversible process.[101,223] Thus, high ascorbic acid concentrations might provide permanent protection against browning.

Apple-PPO was measurably inhibited by 0.4 mmol ℓ^{-1} ascorbic acid.[217] High concentration dips (5, 10, or 15%) for 20 sec protected peeled and cored apples against browning during storage of 3 to 4 days in polyethylene bags at 4 to 6°C and 85% relative humidity. The efficiency of the ascorbic acid solutions was cultivar-dependent.[224] Prolongation of the dipping time reduced the inhibitor-concentration required: 0.05 to 1% ascorbic acid preserved the color for 6 hr after thawing of apple slices soaked, prior to freezing, for 16 to 17 hr in the inhibitor solution.[225] A combination of 0.2% ascorbic acid and 0.3 to 0.4% calcium (as chloride) was found to provide even better protection. The pH of this dipping solution ought to be adjusted to a value between 7 and 9.[226] The prolongation of dipping time was found to increase the efficiency of the inhibitor to a greater extent than the increase in concentration.[227] Grape PPO could be completely inhibited by 5 mmol ℓ^{-1} ascorbic acid.[125]

Beside ascorbic acid, SO_2 or sulphite are most often used to prevent enzymatic browning of fruits and vegetables. These compounds have also a complex action on the PPO — polyphenol system. It was shown that sulphite inhibited browning by combining

irreversibly with the quinones to form colorless addition products, at the same time reducing the activity of the enzyme towards both mono- and dihydroxy phenols.[228,229] The inhibitor is gradually consumed in the quinone-coupling process; thus its action depends on its concentration, as well as on the nature and concentration of the phenols present. If only monophenols are present, a lower concentration of sulphite is efficient, since quinones are formed relatively slowly and a greater part of the inhibitor is available for direct action on the enzyme.[230] In the presence of o-dihydroxy phenols, it may happen that sulphite gets entirely consumed before complete enzyme inactivation has occurred. In such cases color formation is delayed and attenuated, but not entirely eliminated.[205] In the presence of both mono- and dihydroxy phenols, ascorbic acid interferes, by its reducing action, with the quinone coupling to sulphite, and thus promotes sulphite inhibition of the enzyme. The proper selection of the relative concentrations of the two inhibitors may, by virtue of direct sulphite inhibition of the enzyme, contribute to minimizing the loss of ascorbic acid.[229] The inhibition of tyrosine oxidation by bisulphite in potatoes was found to be pH-dependent. Below pH 5.0, the pH contributed considerably to inhibition. Inactivation was more rapid and residual activities lower, reactivation was not observed over a 7-day period.[231] It must, however, be noted, that potato PPO in the tissues is practically inactive at pH values below 5.0.[146]

The effectiveness of SO_2 on apple slices could be greatly enhanced by 0.5% malic acid. In such conditions, a 17-hr dip in 0.02% SO_2 was sufficient for complete penetration. Residual SO_2 was only 30 ppm, and not detectable after cooking.[225] Grape PPO could be completely inactivated by sodium metabisulphite.[124]

Some examples of the application, to foods, of selected browning inhibitors (partly dealt with in detail previously), are given in Table 5.

6. Extraction and Purification of PPO, its Homogeneity and Molecular Mass

a. Extraction

Three problems have to be dealt with in extracting PPO or laccase from plant material: (1) latency, (2) solubilization of cell-bound activity, and (3) prevention of enzyme-induced oxidation and subsequent polymerization and precipitation, on enzyme protein, of endogenous phenols.

Latency is a minor problem with plant PPO, as in most species the enzyme is present in the active form. Latent particulate enzyme could be activated, and, at the same time, solubilized by caffeine—sodium benzoate.[17] Latent PPO in crude and partially purified preparations of avocado could be activated with sodium dodecyl sulphate:[175] here, evidently dissociation to the active form occurred.

Solubilization is generally performed with detergents such as Tween-80® or Triton X-100®.[102,103,241a] The latter was found to be efficient for chloroplasts, while digitonin could be used with mitochondria of apples.[102] The incorporation of sodium deoxycholate in the extraction medium increased the activity extractable from sugar beet leaf chloroplasts 4 to 4.5-fold.[242] Butanol extraction, to remove lipoproteins to which the enzyme protein might be attached, was recommened for preparing PPO from apple peel.[103]

The greatest difficulty to be overcome in obtaining a soluble PPO preparation consists in preventing enzymatic phenol oxidation and pigment formation during comminution and extraction of the plant material. Pigments might precipitate on the enzyme protein and render the latter insoluble; moreover, irreversible reaction inactivation of the enzyme might take place. To minimize these dangers, all extraction steps should be carried out in the cold, possibly at −20 to −30° C; at any rate at temperatures below 0° C. Comminution and homogenization are often performed in liquid nitrogen or in nitrogen atmosphere.[62,127,243] In some cases flash-freezing and freeze-drying of the material are

Table 5
APPLICATION OF SELECTED INHIBITORS OF ENZYMATIC BROWNING TO FRUITS AND VEGETABLES

Name of fruit or vegetable	Name and concentration of inhibitor(s)	Remark	Ref.
Apple	SO_2, 50 ppm + benzoic acid	Inhibition is cultivar-dependent	205
Apple (Golden Delicious)	SO_2, 100 ppm + 1% calcium (as chloride)	pH 7—9; color stabilization for 9 weeks	234
Apple	30% sugar + 0.32—0.4% calcium (chloride)	Soaking for 1 hr at 35°C before freezing prevents browning after thawing	225
Apple juice	Cinnamic acid, 0.5 mmol ℓ^{-1}	Prevention of browning over 7 hr	193
Apple juice	Malic acid, 0.5—1%	pH 2.7—2.8	232
Apple or grape juice	SO_2, 10—50 mg ℓ^{-1} + bentonite, 0.4—1 g ℓ^{-1}	Bentonite adsorbs the enzyme protein	233
Apple, carrot, celery, cabbage	HCl + acid + alcohol + anionic surfactant	pH 1.5; vacuum infiltration before dehydration	235
Pear	NaCl, 1% or citric acid, 2%	Dipping	120
Grape juice	SO_2, 20 mg mℓ^{-1}		236
Grape juice and sweet wine	SO_2 50 mg mℓ^{-1} ÷ sorbic acid, 100 mg ℓ^{-1}	Color protection and prevention of fermentation	237
Fresh fruits (not specified)	Phosphoric acid—sodium bisulphite (4:1—1:2) or pyrophosphoric acid—sodium bisulphite (2:1)	Synergistic mixture	238
Wine	Insoluble PVP, 2 g ℓ^{-1}	Contact for 30 sec removes polyphenols; the polymer can be regenerated	239
Potato	Cysteine, 0.5 mmol ℓ^{-1}	Total inhibition	150
Potato	Sulphite, 600-2500 ppm.	Dip	205
Potato	Cysteine, 10^{-3}—10^{-2} mol ℓ^{-1}	Inhibition for 100 min	240
Green chilli	Cysteine, 1 mmol ℓ^{-1} or sodium metabisulphite, 0.1 mmol ℓ^{-1}	Inhibition: 60% and 45%, resp.	151
Olive (green)	0.4% NaOH	Dip, prevents formation of brown spots after mechanical injury	241

recommended as the first steps of enzyme extraction.[62,103,243] Acetone precipitation followed by buffer extraction, is one of the methods most often applied.[62,107,127,129,132,191,242] When comparing the efficiency of extraction and acetone precipitation, the latter method was found to give 20-fold activity with peaches. This extremely high increase in activity was attributed to the elimination of an inhibitor by acetone, or to degradation of the enzyme protein to the active form.[244] More enzyme activity could be extracted from an avocado acetone-powder at 24° C than at 4° C. Acetone precipitation did not produce artifacts.[149] However, e.g., with tea leaves, about 40% of the activity could not be extracted from the acetone-powder.[77]

Sometimes extraction with an appropriate buffer precedes the acetone precipitation step.[6,24,138] A great variety of buffers are used for extraction, and pH values vary with the enzyme source, but are, in general, adjusted to assure a slightly alkaline medium. The pH of the buffer might affect the enzyme form obtained.[245]

In order to protect the extraction system from enzymatic polyphenol oxidation reducing agents or quinone couplers, as well as reversible enzyme inhibitors, are incorporated in the media. Ascorbic acid, cysteine, sodium metabisulphite, DIECA, or

sucrose, sometimes mixtures of some of these, have been used.[5,6,136,246-248] A very effective means of preventing phenol oxidation and polymerization during enzyme extraction is the removal of the substrates from the media by binding them to an insoluble polymer. The most widely used phenol scavenger is *polyvinylpyrrolidone* (PVP), a very strong proton acceptor at neutral or acidic pH where phenols are not ionized.[84,248,249,250] At the same time PVP is a partially competitive inhibitor of PPO; however, its inhibitory action can be eliminated by washing it off from the enzyme preparation.[148] Polyethylene glycol (PEG) is sometimes preferred,[137,243,251] perhaps because its solubility in acetone presents certain advantages in obtaining a crude enzyme preparation.[249] Polyamide (nylon) and polycaprolaktam have also been successfully applied for phenol binding.[246,249] Other proteins than the enzyme, e.g., casein[127] might also be used to form hydrogen bonds with the phenols. Organic solvents acting as proton acceptors (ketones, esters, dimethylformamide, dimethylsulfoxide, and N-methylpyrrolidone) are also effective in phenol complexing.[249] Thus, acetone drying, often applied as a first step in enzyme extraction, is, at the same time, a powerful means in removing phenols, especially when it contains about 20% of water.

The removal of pectic substances of the fruit tissue can be achieved either by acetone or Ca-acetate precipitation;[137,146] eventually, as used in laccase extraction from peaches, by slight alkaline hydrolysis and precipitation by (cetyl trymethyl) ammonium bromide or application of a pectate lyase preparation.[6,252]

b. Purification

For the purification of the extracted crude PPO preparations, several methods have been described which vary according to the enzyme source and the degree of purity to be attained. Most often precipitation with ammonium sulphate of different saturation, gel chromatography on Sephadex G 100 or G 200, and ion exchange chromatography on the anion exchangers DEAE-cellulose or DEAE-Sephadex are applied, or combinations of some of these methods, with dialysis or Sephadex® G 25 chromatography steps in between to remove low molecular impurities.[6,24,65,85,119,129,136,148,191,241a,243,253] The order of the operations is interchangeable. In some cases, one or the other purification step is repeatedly applied; e.g., a purification procedure for mushroom PPO has been reported with two $(NH_4)_2SO_4$ precipitation steps, preceding and following, respectively, DEAE-cellulose chromatography.[100] The purification of peach and sweet cherry PPO involved repeated chromatography on DEAE-cellulose and on DEAE-cellulose and DEAE-Sephadex, respectively.[107,137] Adsorption on calcium phosphate gel, followed by adsorption chromatography on hydroxylapatite, has been applied to mushroom PPO,[141] and hydroxylapatite chromatography following elution from a DEAE-cellulose column for the purification of the enzyme of pears.[79] Recently, peach PPO has been reported to be purified by hydrophobic chromatography on Phenyl-Sepharose CL-4B.[244]

c. Homogeneity and Molecular Mass of the Enzyme

PPO from most sources has been reported to be present in different molecular forms. The number of these forms depends on the enzyme source and on the methods applied to extract and separate them. Some examples in Table 6 may give an idea on the complexity of the problem of PPO polymorphism.

The examples cited show that multiplicity of enzyme fractions arises from different sources. Part of the molecular forms are due to association-dissociation phenomena, as can be seen from their molecular masses. These phenomena have been attributed to (1) association of various degrees of polymerization of similar subunits; (2) various combinations of different subunits; (3) conformational changes of a single protein; or (4) combinations of the three possibilities mentioned.[26] The monomer is assumed to have a molecular mass of about 30,000, and to contain one atom of copper,[148,264] although

Table 6
MULTIPLE FORMS OF POLYPHENOL OXIDASE FROM DIFFERENT SOURCES

Source of the enzyme	Number of multiple forms[a]	Method of separation	Remarks	Ref.
Apple peel (Cox's Orange Pippin)	2	PEG, PVP, Triton X-100, DEAE-cellulose[b], SGE	Fractions homogenous in SGE; different anodic migration; similar substrate specificity	84
Apple (Golden Delicious)	3	PGE, 8%, pH 8.6	PGE-patterns are characteristic of the cultivar	254
Apple chloroplasts	3	Triton-X-100, SGE or DEAE-cellulose[b]	Different substrate specificity and response to inhibitors	102
Apple chloroplasts	3 (134,000; 67,000; 24,000)	Triton X-100, DEAE-cellulose[b]; agar gel electrophoresis; Sephadex G-100[b] (MM estimation)	Different susceptibility to inhibitors	255
Pear (Bartlett)	2	Sephadex G 25[b], DEAE-cellulose[b], hydroxylapatlite[b]; PGE (pH 9.3)	Different behavior in hydroxylapatite[b]	79
Cherry (Royal Ann)	3 groups	PEG + acetone, DEAE-cellulose[b], PGE	DEAE-cellulose[b] gave two, partly overlapping fractions; differences in substrate specificity, response to inhibitors, heat stability and molecular size	243
Cherry (Morbiane var. clingstone)	2	$(NH_4)_2SO_4$ saturation, DEAE-cellulose[b], PGE	Differences in MM and optimum pH (catechol)	67
Peach (Cortez)	4	Buffer extract, acetone precipitation, DEAE-cellulose[b]	Differences in substrate specificity, heat stability, optimum pH and response to inhibitors	157
Peach	2 + 3 "latent"	PGE	The "latent" forms appear on fixation of the gel in acetic acid after stain	244
Apricot	2	Buffer extraction (pH 5 and pH 7, respectively)	pH-dependent interconvertibility (association-dissociation)	245
Grape chloroplasts	3	PGE, IF	pI-s: 4.7, 4.9, 5.1; on storage, urea or low pH: appearance of slower moving bands of higher pI-s; no differences in catalytic properties	256
Grape skin and juice (six cvs.)	2—5	PGE	Number of bands only occasionally identical in skin and juice of a given cv.	257
Banana	2 (60,000; 12,000)	Buffer extraction, aceton precipitation, $(NH_4)_2SO_4$, Sephadex G100[b]; PGE	Urea treatment partly converts high MM into low MM form	24
Potato (Russet Burbanks)	Two (inhomogeneous	Preparative electrophoresis	Different specificity towards chlorogenic acid, catechol and p-cresol	258

Table 6 (continued)
MULTIPLE FORMS OF POLYPHENOL OXIDASE FROM DIFFERENT SOURCES

Source of the enzyme	Number of multiple forms[a]	Method of separation	Remarks	Ref.
Potato (Maritta)	17 (PGE); 9 (IF); 5 (GF); (36,000; 72,000; 145,000; 300,000; >540,000)	Crude $(NH_4)_2SO_4$ precipitate; PGE, IF, Sephadex G-100[b]	Only 5 forms active on monophenols; pI-s: 4—4.7 and 5.1—5.4; only the highest MM fraction obtained by GF was homogeneous in PGE	253
Potato (Rural Russet)	11	PGE (8%)	Different cultivars have different patterns of multiple enzyme forms	254
Potato (Mansa, Irmgaard)	11—15	Liquid N_2, buffer + PVP, ascorbic acid, Triton X-100, acetone: thin-layer IF on Sephadex G75	pI-s in the range of 4.8—6.8; 6 and 10 zones, respectively, showed hydroxylating activity	259
Potato	10 (132,000)	Buffer extraction, pH 5.3 with benzoic and ascorbic acids, sodium sulphate fractionation; Sephadex G200[b]; PGE	Fractions associate on standing, are not affected by urea and dissociate in SDS	260
Sweet potato roots	3	$(NH_4)_2SO_4$ fractionation, DEAE-cellulose[b]; SGE, agar gel electrophoresis	Identical immunochemical reactions, different substrate specificities and pH optima	85
Broad bean	4	Acetone precipitation, $(NH_4)_2SO_4$ saturation, SGE	Further purification did not yield homogeneous fractions; MM similar for all fractions	177
Eggplant	2	$(NH_4)_2SO_4$ fractionation, DEAE-cellulose[b]	One of the fractions preferentially degrades anthocyanins	65
Tomato (seven cultivars)	3—5	PGE	Three bands are common for all cultivars	261
Cucumber (two cultivars)	2—3	PGE	Catechol + proline stain	261
Onion (two cultivars)	5—7	PGE	Catechol + proline stain and DOPA; the two substrates stained proteins of different migrations, only two bands were identical	261
Sugar cane	2 (130,000; 32,000)	Sephadex G-200[b]	Different electrophoretic mobilities and substrate specificities	62

Table 6 (continued)
MULTIPLE FORMS OF POLYPHENOL OXIDASE FROM DIFFERENT SOURCES

Source of the enzyme	Number of multiple forms[a]	Method of separation	Remarks	Ref.
Mushroom	4 (α, β, γ, δ) (119,000, with SDS: 34,500)	Freezing, acetone fractionation, $(NH_4)_2SO_4$ saturation, continuous flow electrophoresis, hydroxylapatite[b]	From the four fractions three are homogeneous by sedimentation; these have similar amino acid compositions	262
	5	Prepared according to[263]; SGE	Obtained from fraction β[263]; band 3 gave several bands when incubated with buffers of various pH values, treated with SDS or when concentrated	263
	9	PGE, pH 8.6, 7—8%	Three bands showed hydroxylating activity; differences in heat stability and response to inhibitors were noted; urea had no effect; genera and species, soluble and particulate enzyme could be distinguished	254

Note: PEG = polyethylene gylcol; PVP = polyvinylpyrrolidone; SGE = starch gel electrophoresis; PGE = polyacrylamide gel electrophoresis; IF = isoelectric focussing; GF = gel filtration; SDS = sodium dodecyl sulphate; MM = molecular mass.

[a] Molecular masses in brackets.
[b] Chromatographic step.

considerably lower molecular masses of 12,000 and 10,000 daltons have been reported for the enzyme from banana and sugar beet chloroplasts, respectively.[24,265] These forms are, to a certain extent, interconvertible.[263,265] Interconversion can be induced by standing in solution, changes in pH, ionic strength, or concentration, as well as by protein dissociating agents.[24,245,256,260,263] These forms may differ in substrate specificity, pH optima, temperature stability, and response to inhibitors. Many studies deal with the ratios of the dehydrogenating and hydroxylating (catecholase:cresolase) activities. In general, only part of the multiple forms act on monophenols.[62,253,254,258] It has been established that the hydroxylating activity is connected to a higher degree of association and requires at least four subunits. However, at alcaline pH the predominating high molecular mass forms lose much of their hydroxylating activity, which is less stable under these conditions than the o-diphenol oxidizing activity.[268]

Beside these enzyme forms, there are others of identical molecular mass, differing only or mainly in electrophoretic properties.[84,259,267,267a] Several monomeric forms with different isoelectric points were detected by PAGE (polyacrylamide gel electrophoresis) in soluble PPO from potatoes.[267]

In some cases the patterns of the multiple forms were reported to be characteristic of the cultivar;[254] in others, all the cultivars tested showed the same pattern.[256] Zymograms obtained for PPO from various vegetables were found to vary with the year.[261] Moreover,

characteristic changes in the distribution of the different molecular forms under different storage conditions of fruits (apples) were reported to occur.[255]

Most of these findings seem, for the time being, rather contradictory and need further completion of knowledge on the molecular properties of the enzyme in order to permit of a deeper insight into the mechanism underlying their changes.

It shall be mentioned that the molecular mass of peach laccase was established as 70,000—90,000[6], and that laccase from various sources was found to have different molecular forms as well.[269,270]

7. Determination of Enzyme Activity

PPO activity can be determined by measuring (1) the rate of substrate disappearance, or (2) the rate of product formation. With either method, care must be taken to restrict measurement to the initial phase of the reaction, as reaction inactivation of the enzyme soon slows down o-dihydroxyl phenol oxidation.

When determining the rate of substrate disappearance, generally O_2 absorption is measured, either manometrically in a Warburg respirometer, or polarographically with an oxygen electrode. The two methods do not give identical results. The linear section of the time course of the reaction is considerably longer, and the O_2 adsorption values obtained under identical conditions are higher with the polarographic method.[271] The latter is considered by several authors the method of choice for determining PPO activity.[3,5,271]

The rate of product formation can be determined spectrophotometrically by measuring the optical density of the colored compounds formed from the quinones. These methods are very simple and lend themselves to routine analysis. Linearity is maintained for a relatively long period similarly to the polarographic method.[271] However, some authors are definitely against the use of spectrophotometric activity determinations, as these measure the secondary reaction products of PPO, and the secondary reactions are influenced by many factors difficult to control: the presence of ascorbic acid lowers the values obtained; while amino acid, protein degradation products, heavy metal ions, endogenous substrates of fruits, and autooxidation products of polyphenols may increase the levels of enzyme activity.[272] Spectrophotometric methods may be recommended whenever relationships between enzyme activity and enzymatic browning are to be established.

A wide variety of substrates can be used with spectrophotometric methods (e.g., catechol,[104,128,151] pyrogallol,[273] or natural substrates such as chlorogenic acid.[274] It has to be taken into account that the colored compounds formed from the oxidation products of the various phenols have their absorption maxima at different wavelengths, that the substrates may undergo auto-oxidation, especially at alkaline pH values, and that excess of some substrates (e.g., chlorogenic acid) causes strong inhibition of the enzyme.[274]

The hydroxylating activity has to be determined by the O_2 absorption method. When pure enzymes are tested using a monophenol substrate (most often p-cresol or tyrosine) in the absence of o-dihydroxy phenols, the lag phase of the reaction is not taken into account in calculating the activity value: the end of the induction period is considered as the beginning of the reaction, and the slope of the rising section of the O_2 absorption vs. time plot is taken as enzyme activity.[275]

In order to delay reaction inactivation of PPO during activity measurements, coupled reactions are made use of. The reaction mixture contains, beside the enzyme and the substrate, one or more compounds of more negative oxidation-reduction potential (e.g., hydroquinone and ascorbic acid), which immediately reduce the quinones formed. Thus catalytic amounts of the substrate are sufficient, as the concentration of this reactant remains practically constant. The oxidation-reduction potential of the system can be

measured polarographically.[276] Other versions based on this principle apply only ascorbic acid as easily oxidizable compound in the reaction mixture, and measure the decrease of its concentration at 265 nm, its absorption maximum.[135,277] The rate of disappearance of ascorbic acid is directly proportional to enzyme activity. The rate limiting step in the reaction system is the substrate ⇌ quinone transformation. Ascorbic acid oxidase interferes with the results. According to another version of this method, the time required for the complete consumption of a certain amount of ascorbic acid added to the reaction mixture, i.e., the appearance of the colored quinone products, is measured. This is called the chronometric method. The use of such a method is not recommendable, as there is no means of checking the linearity of the process. In fact, the time course of this reaction has been found to be non-linear.[3,271]

Ascorbic acid can be replaced by $K_4[FE(CN)_6].3H_2O$ and absorbance read at 420 nm. In this case the presence of ascorbic acid oxidase or plant material absorbing in the region of 265 nm does not interfere. Other advantages of the method are that ferrocyanide is less sensitive to changes in pH than ascorbic acid, especially in the acid range, and is also stable in solution. The drawback is that its sensitivity is less than half that of the ascorbate procedure when using the same substrate concentration.[278]

The gas chromatographic investigation of the phenolic substrates and reaction products showed that without added easily oxidizable material, higher K_M values were obtained with catechol substrate.[279] These investigations showed the reaction to follow Michaelis-Menten kinetics, and confirmed the inactivation of the enzyme during the reaction.

Several procedures have been devised to overcome reaction inactivation. These are based on the elimination of the quinones from the reaction mixture. Besthorn's hydrazone (3-methyl-2-benzothiazolone hydrazone hydrochloride) forms condensation products with the quinones, which are subsequently extracted from the reaction mixture with chloroform, and then the absorption of the organic phase is read at 500 nm.[150,280] An improvement of sensitivity consists in dissolving the condensation product in acetone instead of extracting it.[281] Another method makes use of the yellow compound 2-nitro-5-thiobenzoic acid anion, which consumes 1 mol of quinone per mol of thiol, and yields almost colorless adducts. Enzyme activity is assessed by following spectrophotometrically the decrease in absorbance of the yellow compound at 412 nm.[282] With 1,4-dihydroxybenzene as substrate instead of catechol, the method is suitable for determining *laccase* activity as well.[282]

C. Polyphenol Oxidase and its Substrates in Some Fruits and Vegetables

This section deals with the levels of PPO activity, and of its endogenous substrates, as well as with the enzymatic browning tendency in different cultivars of selected fruits and vegetables; the changes in the values of these characteristics during development, maturation and storage, as well as with their values in, and bearing on, the quality of processed products (e.g., juices) of plant origin.

1. Apples

Polyphenol oxidase activity, o-dihydroxy phenol content, and browning tendency of apples have been reported by several authors to be strongly cultivar-dependent.[283-285] The high or low browning tendency of a given cultivar is stable for several years,[286] and seems to be similar in fruits from different geographical regions with different climatic conditions, etc.[283-285] The browning rate of apple tissue from different cultivars is but slightly different in its response to various PPO substrates.[142] Some authors found enzymatic browning to be correlated with polyphenol content,[115,287] especially with chlorogenic acid and catechin levels, respectively;[288,289] others established relationships

between browning rate and PPO activity.[285,290] According to the reviewer's experience, the ratio of enzyme activity and substrate content determines whether browning rates of the cultivars of a given fruit correlate with PPO activity or with polyphenol content.[286]

The rate of discoloration following mechanical injury, comminution, etc., is first higher, then decreases, and finally drops to zero.[287,291,292] This is in conformity with reaction inactivation[3], and with the decrease in substrate content observed upon bruising.[287]

Total phenol content was reported to decrease during fruit growth, and to reach a steady level around maturity.[115,144] Browning, both actual and potential (measured in the presence of endogenous substrates only and of endogenous plus added substrates, respectively, as well as enzyme activity, were found to vary in a similar way.[144] This is in agreement with the findings according to which immature fruit goes brown more readily than mature fruit.[205] Some authors observed similar variations in chlorogenic acid and p-coumarylquinic acid contents,[111,293] while others found chlorogenic acid content to decrease throughout growth, maturation, and storage.[294] Catechin content was found to accumulate during fruit growth and then to drop abruptly before picking.[295] The decrease in the content of phenolic compounds during fruit growth was accompanied by a rise in susceptibility to *B. cinerea* infection.[43] In the climacteric phase, PPO activity was observed to have one or two local maxima, depending on the cultivar, which coincided with local minima of POD activity.[296] Isoenzyme patterns of PPO in both peel and pulp of different cultivars were reported to be different in the preclimacteric and post-climacteric phases.[297] Fertilizers did not affect isoenzyme composition of PPO in experiments carried out in two consecutive seasons with three apple cultivars.[298]

During storage, polyphenol content, PPO activity, and browning were reported by different authors to vary in different ways, obviously depending on cultivar, picking maturity, storage conditions, etc.[205] Total phenolics were reported to decrease,[284,299] chlorogenic acid to vary according to a maximum curve,[299] catechins to vary irregularly and to decrease, respectively.[111,295]

Soluble PPO content increased, mainly at the expense of the particulate fraction.[255] Browning was, in most cases, reported to increase during storage,[284,299,300] and PPO to decrease or to vary irregularly.[299,301] Storage in controlled atmosphere, or in polyethylene bags in the cold, seems to slow down these processes during storage.[295,300,301]

The differences in browning tendency of different apple cultivars are reflected in the browning of the juices prepared from them.[302] Proanthocyanidins play an important role as precursors of browning substrates in apple juice.[303] Pectolytic enzyme treatment was found to alter browning substrate composition, and, when carried out in oxidizing conditions without previous heat inactivation of the endogenous enzymes of the apples, might lead to a marked decrease in phenol content. This impairs the flavor of apple juice stored for several months.[304,305]

2. Pears

The enzymatic browning tendency of pears could be related to phenol content, especially to the levels of chlorogenic acid and catechin. The tissue around scleroid cells was found to contain less phenols.[306] Tissues of fruits from the sunlight-exposed side of the tree contained higher amounts of chlorogenic acid. Chlorogenic acid content was higher also in pears affected with the "cork spot", a physiological disorder.[307] In fruits harvested and kept at 0°C for 4 days, then after-ripened at 20°C, PPO activity first decreased and then steadily increased in parallel with the rate of browning observed upon comminution.[120] These findings seem to be in contrast to what has been described for apples.

3. Peaches

The literature on PPO of peaches has been reviewed separately.[308] Peach cultivars could be divided into two groups: those of strong browning tendency, i.e., high substrate content in which chlorogenic acid constituted less than 50% of total phenols, and those of slight browning tendency, i.e., low substrate content in which chlorogenic acid was practically the sole substrate. A direct proportionality was found between discoloration and chlorogenic acid content for both groups.[309] However, the intensity of discoloration was found to be related also to total polyphenol content and PPO activity. Peach cultivars of browning tendencies not differing significantly from each other were found to contain PPO of very different heat stability.[163,311]

During the early stages of fruit ripening (at stalk splitting), PPO activity was found to be very high; then it decreased and reached a stable value (about 1/5 of the initial) by the 5th or 6th week. The decrease in PPO activity was found to be accompanied by a decrease in o-dihydroxy phenols and a marked increase in laccase activity.[312] The shift, during maturation, of a single pH optimum of PPO at 6.2, to dual optima at pH 6.0 and 6.5, was interpreted in terms of synthesis of new isoenzymes.[104]

Lye-peeling of peaches was found to promote discoloration in the canned product. If heat processing was not performed immediately after lye-peeling, color stabilization was necessary after rinsing away the lye. This could be achieved with a 0.1% ascorbic acid or a 1% citric acid dip.[313]

4. Apricots

Browning rate, PPO activity, and o-dihydroxy phenol content of apricots were found to vary to a great extent with cultivar, location, and year, variations being more marked in enzyme and substrate concentrations than in browning rate. Browning correlated with o-dihydroxy phenol content.[314]

Total polyphenol, leucoanthocyanidin, and flavanol contents were reported to increase, during ripening, in most cultivars, and, correspondingly, were also higher in the juices produced from mature fruits.[315] Heat inactivation of apricot PPO during processing seems to be a very delicate operation: even traces of residual activity were found to have an adverse effect on canned or frozen apricots, while too strong heat treatment was equally disadvantageous from the aspect of sensory properties. Heat inactivation of PPO in halved apricots was achieved at 90°C in 2 min or 85°C in 5 min, whereas for frozen apricot puree 10 min at 90°C were recommended.[161,162]

5. Plums and Sour Cherries

PPO activity and polyphenol content were found to decrease in plums during ripening.[310] The isoenzyme pattern of sour cherry PPO was not influenced by fertilizers or pruning.[316]

6. Grapes

PPO of red, as well as of white grapes, was found to be localized mainly in the skin and stem. Activity decreased more or less in parallel to the polyphenol content. The decrease continued after harvest.[317] In Concord grapes *(Vitis labrusca),* PPO activity steeply increased, especially from the half-ripe state on till full maturity, then dropped abruptly. Correspondingly, beside two enzyme fractions present at all stages in the zymogram, additional new enzyme forms were developed during ripening.[125] Although the enzyme is more stable in CO_2 than in air,[317] activity was reduced during fermentation, owing to the inhibitory effect of alcohol: 11% ethyl alcohol completely inactivated the enzyme in the juice in 13 min. In homogenates the enzyme proved somewhat more resistant to alcohol: a concentration of 11.4% and 30 min were required to lower activity near to zero.[214]

The izoenzyme patterns of grape PPO of different cultivars were found, with certain restrictions, to be characteristic of the cultivar. One isoenzyme fraction was present in the PPO zymograms of all the cultivars tested; this band appeared with both DOPA and catechol — proline. The former substrate seems more suitable to characterize the cultivars.[318]

7. Miscellaneous Fruits

A disc eletrophoretic study of 16 *strawberry* species and three cultivars revealed one to seven molecular forms of PPO with DOPA as substrate. The patterns differed in the number and/or mobilities of active bands and were characteristic of the individual species and cultivars.[319]

PPO activity and browning tendency in banana fruits has been reported to be greatly influenced by the genotypes. Enzyme concentration was higher in the inner part of the pulp.[148] An increase in brownness with ripening was observed in the intact tissues of the fruits. At the same time, mass-related PPO activity did not change significantly, while endogenous substrate (dopamine) content decreased. Susceptibility to discoloration, as well as initial brownness, correlated with the dopamine content and not with PPO activity. The decrease in ascorbic acid content and increase in tissue permeability observed during ripening were assumed to be the causes of substrate oxidation by PPO in the intact tissues.[320] The phenols in the peels of banana fruits increased as the fruits stored at 21°C or 27°C approached the climacteric maximum. With fruits stored at 15°C no such change was observed.[321] Total phenolics of banana fruits harvested at the mature-green stage decreased, during storage, the quicker the higher the storage temperature. The peels lost total phenols proportionally to the duration of storage at 0°C. An inverse relationship was found between total phenol content and degree of blackening during storage at injurious chilling temperatures. It was assumed that not total phenols, only one or several specific phenols (e.g., dopamine), participated in the blackening reaction.[322] Gamma irradiation increased skin browning of bananas, probably by activating PPO. A good correlation was found between PPO and skin discoloration of the irradiated fruits.[323]

PPO activity in both mango peel and pulp was highest right after fertilization and decreased during ripening.[78] The skin of mangoes contained considerably more total phenols than the pulp, and this content decreased during storage.[322] PPO activity was minimum in ripe fruits, but increased to high levels upon gamma-irradiation, probably owing to *de novo* synthesis of the enzyme.[127] This is the more probable, as mango PPO from non-irradiated fruits was reported to be specific for dihydroxy phenols, while the enzyme from irradiated fruits (200 krad. 14-day storage) acted on p-cresol.[78,127] The intensity of discoloration, which increased with higher doses and advancing storage time, correlated well with PPO activity.[127]

Browning in halved avocado fruits could be detected, dependent on the cultivar, after 1, 4—6 and 12—18 hr, respectively. Discoloration was especially marked in frozen fruit after thawing. The differences in the rates of browning of different cultivars were directly related to the PPO activities of the crude enzyme preparations extracted from them.[149]

8. Potatoes

PPO activity and polyphenol content are not uniformly distributed in the potato tuber. PPO activity was found to be highest in the outer parts of the tuber (eye and peel), and phenolic compounds were present in the highest concentration in hilum, eyes, and peel.[324] Chlorogenic acid could be detected only in the eyes and peel and in the adjacent cortex. The distribution patterns were found to be cultivar-dependent.[324,325] During storage, PPO activity and polyphenol content increased in the outer parts, chlorogenic acid

content increased at low storage temperatures. PPO activity decreased in the inner parts of the tubers.[325]

The rate of discoloration was reported to be cultivar-dependent as well. Storage conditions had different effects, in this respect, on different cultivars: some showed minimum discoloration on storage at 7°C, others at 4°C, and a third group at temperatures varying from 4°C to 12°C. However, no correlation was found between chips color and pulp discoloration.[326]

PPO is assumed to play a role not only in brown discoloration of potatoes, but also in the formation of blackspot following mechanical injury.[327,328] Beside the cultivar, location and storage temperature affect pre-cooking discoloration.[328,329] It is interesting and shows the complexity of the phenomenon that no influence of PPO on blackspot formation could be demonstrated by factorial analysis and multiple regression analysis.[330] On the other hand, brown discoloration was found — by similar mathematical methods—to be dependent to 45% on PPO activity and total phenol content, other factors of influence being ascorbic acid, solids content, chlorogenic acid, and flavonols, as well as basic amino acids.[331]

Brown discoloration of potato pulp increased with increasing PPO content and decreased with increasing concentration of reducing substances (e.g., ascorbic acid). However, no distinct relationship could be established between these factors.[332] According to several authors, tyrosine content appears to be the major factor determining the rate of enzymatic browning of potatoes,[116,332-335] while no correlation was found with chlorogenic acid content.[333,334] Tyrosine content was shown to depend on genetic, climatic (humidity), and nutritional factors. It increases during storage, together with the rate of browning, and both characteristics reach higher values in potatoes stored at 20°C than in those stored at 5°C.[333] Since in comminuted potatoes kept in air, browning could be correlated to the loss of free tyrosine, but not to "chemically available" lysine content, it was concluded that quinones formed upon PPO action might polymerize to melanins directly, without coupling to proteins.[334]

During the growth period of potatoes, PPO activity increased and phenolic content decreased in one of two years investigated. In general, later harvested potatoes showed stronger discoloration.[336] Agrotechnics equally influenced discoloration of raw potato flesh: a strong negative correlation between potassium content of the tubers sampled at harvest and enzymatic discoloration could be established.[337] The fungicide pentachloronitrobenzene lowered free tyrosine content, leaving PPO activity practically unaffected. It is assumed that the fungicide might reduce discoloration.[338] The sprouting inhibitor maleic hydrazide was found to increase the susceptibility of the tubers to bruising and discoloration, probably by modifying lipid composition.[339]

Gamma-irradiation as used to prevent sprouting was reported to increase browning, chlorogenic acid content, and chlorogenic acid oxidation, as well as the hydroxylating activity of PPO, while it decreased ascorbic acid content.[70,340-343] The increased tendency to discoloration was interpreted as a result of cell damage, solubilization of cell-bound enzyme, and easier access to its substrates, as well as lack of quinone reduction by ascorbic acid. The increase in browning and chlorogenic acid content was dependent on the radiation dose, the harvest time, and the period of postharvest storage preceding irradiation.[340,344] The changes were less marked in late harvests and could be completely suppressed if irradiation took place 3 months after harvest.[342,344] PPO activity was affected but transiently, and mainly by radiation doses below that applied for sprout inhibition; the treatment resulted in the appearance of new molecular forms of lower electrophoretic mobilities.[341] Sprout-inhibiting doses caused variations in the catalytic properties of the various molecular forms by altering the catecholase:cresolase ratio. This was thought to suggest that transient activation was due to conformational changes, rather than to synthesis of new protein.[70,341]

9. Miscellaneous Vegetables

In the filtered extracts of *eggplants,* PPO activity was found to be cultivar-dependent: the purple eggplant showed the highest, the green one medium, and the white one the lowest levels of enzyme activity. PPO activity could be correlated with the copper content in the tissues.[345] It was higher in unripe than in ripe or overripe fruits. Chlorogenic acid was the main o-dihydroxy phenol component. During low temperature storage, o-diphenol content rapidly decreased as browning increased, except in overripe fruit, which did not show pulp browning.[134]

During tomato development, PPO was maximum at the preclimacteric stage and then decreased during ripening. This was expressed also in the electrophoretic patterns of the enzyme: preclimacteric fruit showed four, and nearly ripe fruit only two fractions. Potassium deficiency and blotchy ripening increased enzyme activity, while the latter reduced the number of multiple forms.[346] Seed germination could be accelerated by gamma-irradiation (2,000 R) which was accompanied by a marked rise in PPO activity.[347]

Parsnips turn brown soon after harvest. PPO activity resides mainly in the peel, while total phenol content is lowest in the tissue of the root surface and highest in the tissue of the vascular cylinder region. Chlorogenic acid concentration was found to be low and relatively highest near the surface and the vascular cylinder region. The intensity of browning was highest near the surface where enzyme activity was highest.[132,133]

Enzymatic browning of *carrots* is restricted to the surface, where phenol content is highest. Chlorogenic acid proved to be the main browning substrate, while L-tyrosine was only of secondary importance. Potential browning was found to correlate with phenol content.[348] Early seeded carrots showed a higher browning tendency than late seeded ones. Immature roots were more susceptible to browning than mature roots. The susceptibility to browning was not found to be influenced by the soil. It increased during the first month of storage in dependence on temperature.[349]

Chicory turned brown in 1 or 2 days when irradiated previously (100 krad) and stored in perforated polyethylene bags. The phenomenon was not accompanied by an increase in PPO activity or chlorogenic acid content, and could not be prevented by browning inhibitors such as cysteine or sodium chloride. Tissue damage might account for discoloration.[350]

In irradiated (100-300 krad) *endives* PPO activity increased right after irradiation, and was accompanied by quick browning of the cut white leaves. However, during 7-10 days of storage at 2°C and 10°C, respectively, enzyme activity increased slower in irradiated samples than in non-irradiated ones. Correspondingly, the irradiated samples preserved freshness and natural color of the leaves better.[351]

10. Mushrooms

The mushroom *Agaricus bisporus* (Lange) Sing was found to contain PPO in the active and latent, i.e., sodium dodecyl sulphate soluble forms. The activities of both forms increased during storage at three different temperatures (0°C, 10°C, and 20°C, respectively); however, the changes were but slight at the two lower temperatures. At 0°C, total phenol content steadily decreased, first faster, then slower, while at the two higher temperatures a rapid drop was followed by an increase. Discoloration was directly related to enzyme activity. The effect of low temperature (0°C) storage on PPO activity was interpreted in terms of suppressed maturation.[352] However, when maturation was slowed down by post-harvest irradiation, the increase in PPO activity did not vary in parallel.[353] During drying of mushrooms in the dark or at elevated temperature (50°C), PPO showed biphasic inactivation. By accelerating the initial phase of drying and of PPO inactivation by raising the temperature, discoloration could be greatly reduced. At higher temperatures (75°C) the biphasic inactivation curve became monophasic,

obviously because the heat sensitive fraction of the enzyme was instantaneously inactivated. When dried in the dark at room temperature, the first quick phase of activity drop coincided with considerable losses of water and reduction of respiration. This suggests that water activity might also play a role in the reduction of enzyme activity. Polyphenol content as related to solids decreased with progressing drying as well,[354] being probably consumed in the enzyme reaction.

III. PEROXIDASE (POD)

A. Generalities

1. Name of the Enzyme

Peroxidases (E.C.1.11.1.7, donor:hydrogen-peroxide oxidoreductase) are, similarly to PPO, members of the group of oxidoreductases.[1] They decompose hydrogen peroxide in the presence of a hydrogen donor. Peroxidases are very widespread in nature. They can be divided into two main classes: (1) iron-containing peroxidases, and (2) flavoprotein peroxidases. The enzymes belonging to class 1 can be subdivided into two groups: ferriprotoporphyrin peroxidases and verdoperoxidases. The former contain ferriprotoporphyrin III (hematin) as prosthetic group, and are brown when purified. They occur in higher plants (horseradish being one of the main enzyme sources), animals, and microorganisms. The prosthetic group of verdoperoxidases contains also an iron-protoporphyrin group—different, however, from ferriprotoporphyrin III. These enzymes are green in the purified state. They occur in animal organs and in milk (lactoperoxidase). The flavoprotein peroxidases contain flavine-adenine-dinucleotide as prosthetic group. They occur in microorganisms, e.g., *Streptococcus faecalis,* and animal tissues.[3,355,356]

The two groups of iron-containing peroxidases can be distinguished by acidic acetone treatment, which removes hematin from the protein moiety of ferriprotoporphyrin peroxidases, while it has no similar effect on verdoperoxidases.[3]

This review deals exclusively with ferriprotoporphyrin peroxidases as present in fruits and vegetables.

2. Occurrence of POD in Nature

As referred to in the preceding chapter, POD occurs in animals, plants, and microorganisms. In plants it is located in the cell partly in soluble form, in the cytoplasm, and partly in insoluble, cell-wall bound form.[357,358,359,360,361] Soluble POD can be extracted from tissue homogenates with a low ionic strength (0.05—0.18 mol ℓ^{-1}) buffer. The particulate enzyme can be present in two forms: ionically bound and covalently bound. The ionically bound form can be extracted with buffers of higher ionic strength (i.e., containing 1 mol ℓ^{-1} NaCl or 0.1—1.4 mol ℓ^{-1} $CaCl_2$); the covalently bound enzyme requires digestion with a pectolytic or cellulolytic enzyme preparation to be liberated.[361-364] The cell-bound POD-fractions of banana fruit were found to be localized primarily in mitochondria and other subcellular membranes. About 20% of the POD activity of horseradish roots were localized in the cell walls. 93% of this activity could be removed by washing with 2 mol ℓ^{-1} NaCl. About 1.4% of the total activity proved tightly bound to the cell wall; 75% of this portion could be solubilized by cellulase.[364] Green beans showed about identical levels of soluble and ionically bound POD, and about 1/5 these levels of covalently bound enzyme.[365] The covalently bound POD of etiolated green peas was found to be extremely stable and to contain molecular forms not present in the cytoplasm or in the ionically bound fraction.[361] In the membrane fraction of thylakoids from pea chloroplasts, peroxidase amounted to 2% of total membrane protein.[366] In pears, POD activity was concentrated in the parenchyma cells surrounding grit cells and

attached to cell walls throughout the pulp. Activity was highest in the core and cortical areas. Extractability of the enzyme increased with $CaCl_2$ concentration up to 0.2 mol ℓ^{-1} and declined at higher levels up to 0.3 mol ℓ^{-1}.[367]

POD can be induced by various chemicals: gibberellic acid increased its activity 1.75-fold in dwarf corn; phenylborate doubled it in mature tomato leaves; naphthalene-acetic acid increased it 36-fold in sugar cane; ethylene and water increased it 10- and 25-fold, respectively, in sweet potato.[9] Ethylene increased POD activity also in both the cytoplasmic and ionically bound cell-wall fractions of green peas. POD activity could be induced in sweet potato tissue by inoculation with *Ceratocystis fimbriata* or cut injury.[37,363] The increase in activity was connected to changes in the isoenzyme pattern and to the appearance of new isoperoxidases.[363] Cucumber mosaic virus infection of both cucumber and tobacco cultivars increased without inducing virus specific forms.[368] Virus infection of tobacco leaves was reported to reduce the heat stability of POD isoenzymes.[369]

3. The Role of POD in Nature and in Food Processing

The role POD plays in the life cycle of plants is one of the least elucidated topics concerning the enzyme. Taking into account some of the factors inducing its biosynthesis (cf. the preceding paragraphs), it has been concluded that physiological stress, wounding, fungal or viral infection bring about changes in POD isoenzyme patterns. However, these conclusions apply not solely to POD, but to other tissue enzymes as well;[370] moreover, they lack causality. In a recent review, Haard points out the role of POD in lignin biosynthesis, including the "Yuzuhada disorder" of pears, a physiological disorder which manifests itself in excessive development of sclereid cells.[371] Fiber formation in postharvest asparagus was accompanied by changes in isoperoxidase distribution. Both processes could be accelerated by exogenous ethylene. It was suggested that wound ethylene initiated a shift to peroxidase isoenzymes functional in lignification.[372] However, the ethylene-stimulated development of a lignin-like substance in swede roots *(Brassica Napo-Brassica)* was not accompanied by changes in POD activity.[373] POD-mediated ethylene formation was shown to be possible from α-keto γ-methylthiobutyric acid or methional in vitro; however, there is no evidence that ethylene biosynthesis from methionine occurs in plant tissue (apple) via these compounds.[374] It was also suggested that POD might be involved in the *hydroxylation of proline in cell walls,* as ethylene was found to increase the activity of the covalently bound enzyme fraction simultaneously with the hydroxyproline levels in the cell walls of etiolated green peas.[361]

Another important function of the enzyme is related to its indole acetic acid (IAA) oxidizing action, by which it might participate in plant growth regulation.[371] In fruits of climacteric ripening, e.g., pears and tomatoes, POD and IAA oxidase isoenzymes were found to be reinforced with progressing maturity; in non-climacteric fruits, e.g., blueberries, where ethylene levels did not noticeably change during ripening, only IAA oxidase isoenzyme concentration increased, while POD isoenzyme concentration decreased. Both ethylene and IAA were found to increase POD activity in tea shoots. However, the two compounds acted on different isoenzymes.[376] Aging of stem sections of dwarf peas was observed to give rise to the appearance of a new POD isoenzyme which could be repressed by IAA.[377] It was concluded that IAA might induce some and repress other POD isoenzymes. Some isoperoxidases were found to be more active than others in IAA destruction. It is assumed that IAA may regulate its own destruction by altering the levels of POD isoenzymes. Gibberellic acid was found to act in a similar way.[370]

Peroxidases are considered to be indices of ripening and senescence.[362] POD activity in Golden Delicious apples stored in controlled atmosphere showed two peaks, one corresponding to climacterium and the other to the start of senescence. The activity peaks

were, however, not accompanied by the upsurge of new isoenzymes. One isoenzyme fraction characteristic only of the phase of ripening was found.[378,379] POD activity was found to be higher in sugar beet tubers of slight medium fresh weight and high sugar content. This correlation was detectable in a few day-old plants, and might serve to predict sugar yield. It might also be of use in variety selection.[380] POD is assumed to play a part in chlorophyll degradation, a process accompanying ripening of most fruits and vegetables.[381] A carotene-bleaching enzyme exhibiting the characteristics of POD was obtained in the protein fraction of aqueous red pepper extracts.[382]

As can be seen, great steps forward have been made in establishing the physiological role of POD in plants. POD has been found to play a role in ethylene biosynthesis, hormone balance, membrane integrity, and respiration control, in ripening and senescence and changes connected with these processes.[362] However, the material accumulated so far is descriptive rather than interpretative, and more data are required to elucidate the interrelations between the assumed functions of the enzyme.

For the fruit and vegetable processor, POD is primarily connected to the formation of off-flavors during storage of canned products, especially in non-acidic vegetables which contain high levels of activity. Ranging among the most heat stable enzymes, it is used as an index of adequacy of pasteurization processes.[383] Some of the early and more recent literature concerning the topic has been lately reviewed by Burnette.[384] However, some of the early researchers failed to find POD action to cause flavor deterioration.[384a] A direct correlation between quality and POD activity of canned products could not be established till today. Moreover, the fact that POD requires, for its action, H_2O_2, which undoubtedly is destroyed in the tissues during heat treatment, makes the existence of such a correlation improbable.[385] This point will be discussed in detail in the following parts of this review dealing with heat inactivation, residual activity, and regeneration of POD.

B. Biochemistry of POD

1. The Reactions Catalyzed

Peroxidase catalyzes four types of reactions: (1) peroxidatic, (2) oxidatic, (3) catalatic, and (4) hydroxylation.[3] The overall equation of the peroxidatic reaction can be given as follows:[356]

$$ROOH + AH_2 \xrightarrow{POD} H_2O + ROH + A$$

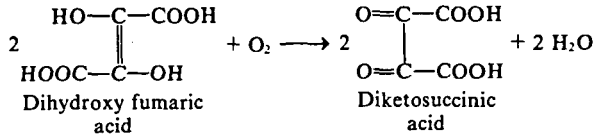
where R = H^+, CH_3 or C_2H_5, AH_2 = hydrogen donor in the reduced form, and A = hydrogen donor in the oxidized form.

In the course of the reaction, intermediate compounds are formed:

$$POD + H_2O_2 \longrightarrow \text{Compound I}$$
$$\text{Compound I} + AH_2 \longrightarrow \text{Compound II} + AH$$
$$\text{Compound II} + AH \longrightarrow POD + A$$

whereby the last reaction is rate-limiting.[356] As apparent from spectral changes, two further intermediates are formed in the reaction at high H_2O_2 concentrations; these are, however, not thought to be directly involved in the mechanism of action.[3] A great variety of compounds may act as hydrogen donor, including phenols (p-cresol, guaiacol, resorcinol), aromatic amines (aniline, benzidine, o-phenylene diamine, o-dianisidine), reduced nicotinamide-adenine dinucleotide, and reduced nicotinamide-adenine dinucleotide phosphate.[3,386]

The oxidatic reaction of POD may take place in the absence of hydrogen peroxide. It requires 0_2 and cofactors: Mn^{2+} and a phenol (mostly 2,4-dichlorophenol). A wide variety of substrates can be transformed in this reaction, such as oxalate, oxaloacetate, ketomalonate, dihyroxyfumarate or indoleacetic acid (IAA).[387-388] According to some authors, the oxidation of NADH and NADPH is cofactor-dependent, too.[389] The stoichiometry of the reaction is as follows:[3]



The reaction starts after a 2 to 3 min induction period, which can be reduced by increasing the enzyme concentration.[388] The induction period can be eliminated by catalytic amounts of H_2O_2.[390] The reaction shows anomalous kinetics: the time dependence of the oxidation of dihydroxy fumaric acid by O_2 in the presence of horseradish peroxidase (HRP) and Mn^{2+} ions was found to be sigmoidal. This shows the autocatalytic character of the process. The order of the reaction was found to be dependent on the initial concentration of the substrate (dihydroxy fumaric acid).[391] The IAA oxidase activity of the soluble POD from bananas varied with enzyme concentration according to a maximum curve, in contrast to HRP which varied linearly up to high enzyme concentrations.[362,391] IAA was found to be oxidized by the apoenzyme of HRP, from which the heme prosthetic group had been removed, and which was, therefore, devoid of peroxidase activity. It was concluded that Mn^{2+} and dichlorophenol were able to substitute for the heme group in this reaction.[392]

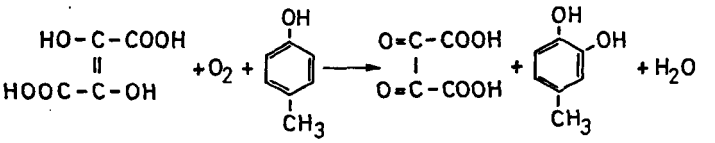
Different isoenzymes from various sources showed different ratios of peroxidatic/oxidatic activity.[388,393] The two activities gave different responses to inhibitors: the oxidation of phloroglucinol (pH 7.8) was barely affected, in the presence of Mn^{2+}, by cyanide, while the peroxidatic reaction with three different donor substrates (pyrogallol, p-anisidine, benzidine) was strongly inhibited; azide proved to be a specific inhibitor of phloroglucinol, while POD activity was more susceptible to boiling. These findings lead to the conclusion that separate active sites were responsible for the two catalytic actions.[394] The analysis of an "IAA-oxidase type" and a "HRP-type" isozyme from turnips showed, in the former, a carboxylate residue to be replaced by a basic group. It was postulated that the decrease in the number of the acidic groups in the hem cleft shifted the activity pattern from peroxide activation to oxygen activation.[395] In the experiments with purified isoenzymes from spinach leaves, the two activities were not always and absolutely found to coincide, and it was assumed that, in this case, the destruction of IAA was not strictly related to POD.[396]

The catalatic decomposition of H_2O_2 occurs in the absence of a hydrogen donor, according to the following equation:[3]

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

The rate of this reaction is negligible in comparison to the rates of the peroxidatic and the oxidatic reactions.[3]

The hydroxylating reaction produces o-dihydroxy phenols from monophenols and O_2, similarly to PPO. However, with POD, the reaction requires a hydrogen donor, e.g., dihydroxyfumaric acid, which provides for free radicals necessary for the action of the enzyme:[3]



According to Whitaker,[3] the oxidatic and hydroxylating reactions of POD are not a result of direct enzyme activity, but occur in secondary reactions brought about by the formation of $HO_2^{\cdot}$ free radicals. This seems to explain the oxidatic "activity" of the separated apoenzyme.

2. Substrates of POD

a. The Peroxide

As already mentioned, POD is highly specific to the peroxide substrate. Its main peroxide substrate is H_2O_2. The enzyme is inactivated by high concentrations of H_2O_2. Removal of excess H_2O_2, e.g., by catalase, restores activity.[384] The rate of inactivation is influenced by the concentrations of the enzyme and of H_2O_2. It was concluded that H_2O_2 attacked a non-heme, as well as a heme site of the enzyme.[397] Activity of grape POD was found to be maximum at a concentration of $6.37 \cdot 10^{-2}$ mol ℓ^{-1} H_2O_2 with a plateau in the curve between $0.57 \cdot 10^{-2}$ and $1.91 \cdot 10^{-2}$ mol ℓ^{-1} H_2O_2, giving less than half of maximum activity.[398] For POD in potato and kohlrabi homogenates, the concentrations yielding maximum activity were $0.74 \cdot 10^{-2}$ mol ℓ^{-1} and $1.1 \cdot 10^{-2}$ mol ℓ^{-1}, respectively, (pH 5.0).[399,400] HRP activity was maximum at $0.3 \cdot 10^{-2}$ mol ℓ^{-1} H_2O_2.[401] Hydroxymethylhydroperoxide was found to inhibit HRP, being at the same time a substrate of the enzyme. It has been concluded that POD requires a free HOO^- group in order to react with peroxides.[402]

b. The Donor Substrate

POD has a low specificity for the hydrogen donor substrate. This is interpreted as resulting from the different substrate specificities of the individual isozymes present in plant POD.[403] The number of the isoenzymes detected,[404] as well as heat stability and regeneration of a given POD, varies with the donor substrate applied.[384] This is of importance if the enzyme is used as a genetic marker,[404] or as indicator of the efficiency of heat treatment of fruits and vegetables.[384] Bound and soluble enzyme fractions were also found to differ in substrate specificity.[362]

Some substrates are preferentially used for a given purpose, e.g., guaiacol is traditionally applied to check thermal treatments, most often in a qualitative test. The concentration of guaiacol giving maximum reaction rate was found to be $1.4 \cdot 10^{-2}$ mol ℓ^{-1}. The K_M value of HRP for guaiacol was $0.7 \cdot 10^{-2}$ mol ℓ^{-1}. Benzidine is applied in histochemical stains,[399] as well as to detect isoenzymes after gel electrophoresis or isoelectric focusing. o-Dianisidine, o-phenylene diamine, o-tolidine, 3-amino-9-ethylcarbazole, 3,3'-diaminobenzidine tetrachloride (DAB), p-phenylene diamine, N,N'-dimethyl-p-phenylene diamine, and o-toluidine are also widely used substrates,[401,405,406,407,408] of which DAB was found to give a more sensitive assay for HRP than guaiacol or o-dianisidine,[401] and o-phenylene diamine to be most suited to detect isoperoxidases of green beans.[405] When using DAB as substrate, low concentrations of gelatin (0.1%) should be incorporated into the reaction mixture to prevent precipitation of the oxidized donor. The highest reaction rates can be obtained at a DAB concentration of $5 \cdot 10^{-4}$ mol ℓ^{-1}.[401] Mesidine (2,4,6-trimethylaniline) was also used as substrate of HRP.[397] Eugenol (2-methoxy-4-alkylphenol) was recommended for activity measurements as a non-carcinogenic donor substrate. This compound is the principal component of clove

oil, a precursor in lignin biosynthesis, and, therefore, a substrate of physiological significance.[409] Pyridoxal, pyridoxal phosphate, pyridoxine, and pyridoxamine in legume seedlings undergo oxidative destruction in the presence of POD and H_2O_2, and may, therefore, be considered as substrates.[410]

Since different isoenzymes give different responses to the various substrates,[404,405,408] it is advisable to test the suitability and sensitivity of several donors with a given POD.

The affinity of POD for a given donor substrate was found to depend on the source of the enzyme and its degree of purity.[398] Using o-phenylene diamine as donor, the K_M values (10^{-2} mol ℓ^{-1}) of HRP, crystalline HRP and grape POD were 0.13, 0.3, and 1.7 to 1.9, respectively. The concentration giving maximum reaction rate was $3 \cdot 10^{-2}$ mol ℓ^{-1}.[398]

3. pH and Temperature Optima of Enzyme Activity

The pH optimum of POD activity varies with the enzyme source, the isoenzyme composition, the donor substrate, and the buffer applied.[356,398,406] The broad pH optima observed with POD from some sources are due to the presence of isoenzymes of different pH optima.[406] Bound and soluble POD fractions from the same source may differ in pH optima as well.[362]

Activity decreases at low, as well as at high pH values. The loss of activity observed on acidification is attributed to the change in the protein from the native state to the reversible denatured state, brought about by detachment of the heme from the protein. At pH 2.4 and 25°C, low concentrations of chloride cause total detachment of the heme. The disturbance of the heme-protein interaction causes also a loss of protein stability. The transfer of the protein from the reversible denatured state to the irreversible denatured state is, therefore, influenced by pH.[411] That is why the heat stability of POD, e.g., in tomato stem tissue could be greatly reduced in a medium of low pH (2.5 to 4.5).[357] The activity changes occurring with changes in pH are related to structural changes in the enzyme molecule, as proven by circular dichroic spectra of Japanese radish peroxidase. These spectra indicated an α-helical structure for the native enzyme and some of its derivatives at neutral and alkaline pH. Acidification of the enzyme solution destroyed the helical structure, and a spectrum characteristic of the β-structure was obtained. The apoenzyme showed only the presence of the β-structure, even at neutral pH.[412] This supports the theory of the separation of apoenzyme and prosthetic group on acidification, as mentioned before.

The regeneration of the thermolabile POD fractions was found to depend only on the pH, irrespective of the duration and temperature (in the range of 60—90°C) of the inactivation process. The regeneration of HRP and spinach POD, after heat treatment, was maximum in the range of pH 5.5—8.0, while at values below pH 5.0 further losses of activity occurred on standing at room temperature.[413]

Storage at cooling and freezing temperatures (5, −4, and −40°C), as well as the method of freezing, did not affect the pH-optimum of green bean POD.[365] The non-enzymatic lipid-oxidizing function of POD was reported to be pH-dependent and practically not noticeable at values below pH 5.0.[414] The pH-optima of the peroxidatic and oxidatic activities of POD do, in general, not coincide.[406,415] The pH-optima of peroxidases from different sources are given in Table 7.

Temperature optima for potatoes, kohlrabi, and cauliflower POD (in homogenates were found to be 55°C and 35—40°C, respectively. Activity varied with temperature, up to these values, according to the Arrhenius equation. The apparent activation energies of the reaction, as calculated from plots of mass-related activity vs. reciprocal temperature (K) were 40.6 kJ mol^{-1} and 21.9 kJ mol^{-1} for kohlrabi and cauliflower POD, respectively.[399,400,417]

Table 7
pH OPTIMA OF SOME PEROXIDASES (PEROXIDATIC AND OXIDATIC ACTIVITIES)

Enzyme source	pH Optimum	Remarks	Ref.
Grape	5.4	Citrate-phosphate buffer, sharp maximum	398
	4.0—5.0	Borate buffer, 0.02 mol ℓ^{-1}, broad maximum	398
	5.0—6.0	Acetate buffer, 0.1 mol ℓ^{-1}, broad maximum	398
Banana	5.0—6.0	Extract purified by gel filtration	406
	4.5—5.0	Anionic fraction	406
	4.5	Cationic fraction	406
	6.5	IAA oxidase activity of extract purified by gel filtration	406
	4.5—5.0	IAA oxidase activity of anionic fraction	406
	5.0	IAA oxidase activity of cationic fraction	406
Pineapple	4.2	Activity independent from buffer concentration in the range of 0.1—0.2 mol ℓ^{-1}	415
	4.4	IAA oxidase activity	415
HRP	3.0	IAA oxidase activity	391
Green bean	5.0	Soluble, ionically and covalently bound enzyme	365
Cabbage	7.0	For enzymes from both healthy and *B. cinerea* infected tissues	415
Potato	5.0—5.4	In homogenate	399
Kohlrabi	5.1—6.3	In homogenate	400
Cauliflower	5.0—5.7	In homogenate	416

Note: IAA = indole-3-acetic acid.

4. Temperature Stability of POD

The behavior of POD during heating and cooling or freezing is the topic most investigated in relation to this enzyme. Heat inactivation of POD from many sources is, in certain conditions, a biphasic and partly reversible process. The enzyme is composed of units or fractions of different heat resistance, and part of its activity is restored during shorter or longer periods of storage at room or lower temperatures following thermal treatment.

The outstanding heat stability and the trend to regeneration of the enzyme, especially in non-acidic vegetables, induce the food processor to apply too severe blanching or pasteurization, conditions which might impair the quality of the canned or frozen product, causing deterioration of color, consistency, and flavor, as well as losses of valuable components, e.g., vitamins, proteins, amino acids, etc.[418,419] Thus, heat treatment of fruits and vegetables might affect both the nutritive value and the acceptability of the processed goods. Hence, overblanching ought to be avoided. This is a serious problem of the canning and freezing industries. No general rule has yet been established as to what extent the presence of residual or regenerated enzyme activity is permissible from the aspect of quality preservation of processed fruits and vegetables during storage. The presence of active enzymes has been shown, in many cases, to be connected to the formation of off-flavors and discoloration,[412] while several vegetables, blanched for very short times and containing slight levels of residual POD activity, were found to be superior, after frozen storage, to the batches devoid of enzyme traces.[420] Prevention of regeneration requires a heat load several times that necessary for inactivation.[421,422] Moreover, regenerated and residual activities are difficult to distinguish, and it is not known whether there exists any difference in their actions on the sensory properties of a product.

a. The Processes Occurring During Heat Treatment

The heat inactivation of an enzyme may be considered, theoretically, as a first-order decay process. In the ideal case, deactivation ought to be proportional to residual activity. However, such a model does not fit thermal destruction of enzyme activity, in general, and POD deactivation, in particular. The deviation from the ideal was interpreted as the result of differences in heat resistance within the enzyme molecule.[423] Heat inactivation of POD at temperatures not exceeding 80—90°C has long been observed to be biphasic, the two phases following first order kinetics each, with different rate constants.[413,424,425] This suggested the presence of heat stable and heat labile isoenzymes.[385,426,427] While the peroxidases of several vegetables, e.g., green peas, green beans, and spinach[427,428] were, in fact, proven to be a mixture of isozymes of different heat resistance, the biphasic inactivation curves were attributed by some authors to the formation, during heat treatment, of a new compound of higher thermostability from heat denatured enzyme protein and groups of POD that remained active.[429]

The following processes were found to be involved in the thermal denaturation of POD: (1) the dissociation of the prosthetic group from the holoenzyme, (2) a conformation change in the apoenzyme and (3) the modification or degradation of the prosthetic group.[430] With Japanese radish the process started above 60°C; step (1) coincided with a decrease in activity and was biphasic, the activation energies of the two phases of the reaction being 84 and 209.5 kJ $mole^{-1}$ (pH 7). The reversibility of this process was found to be pH-dependent: at pH 5 the reaction proved reversible, at pH values below 5 and at pH 9 it was irreversible. The irreversibility at acidic pH values was attributed to irreversible changes in the apoenzyme (process 2) and at alkaline pH to the modification of protohemin (process 3).[430] The holoenzyme was found to exhibit higher stability and resistance to thermal denaturation than the apoenzyme.[430] The opposite was found in heat treatment experiments with the separated apoprotein and prosthetic group of HRP. The recombination of non-heat-treated apoprotein and prosthetic group yielded nearly 100% of the original activity. The inactivation curves showed the apoenzyme to be more heat stable than the holoenzyme, the difference being more pronounced at 70°C than at 90°C. Neither partner was inactivated according to first order kinetics. Activity losses during heat treatment, as well as regeneration upon standing, were paralleled by changes in Soret-absorption (400—403 nm, characteristic of the protohematin group.) These changes indicated the occurrence of both reversible and irreversible processes.[431] The heme moiety of the molecule was found to remain unchanged.[432]

Inactivation was, further, established to involve the aggregation into oligomers with an average molecular mass of about double the value (87,000) of the native enzyme, as well as the unfolding of the molecules with subsequent stacking of the unfolded molecules.[432] As a result of unfolding, the exposure of the heme group increased and this led to an increase in the non-enzymatic lipid oxidizing capacity of the hemoprotein, the heme being still attached to the protein moiety. This phenomenon was found to occur in time and temperature conditions prevailing in food processing, and not only with peroxidase, but also with catalase (another hemoprotein).[413]

In some cases, e.g., with green beans, heat inactivation of POD was preceded, especially at lower temperatures, by a latency period,[427] or even an increase in activity, as shown in Figure 2 for the enzyme in kohlrabi *(Brassica oleracea* var. *Gongyloides)* extracts.[433] These phenomena were attributed to the transformation of the native enzyme, prior to inactivation, into a transient active form with an activity different from the initial which then got, in turn, inactivated.

At higher temperatures, e.g., in the range of 80 to 100°C, or between 100 and 120°C, thermal inactivation curves show generally first order kinetics.[385,413,424,433] This might, at

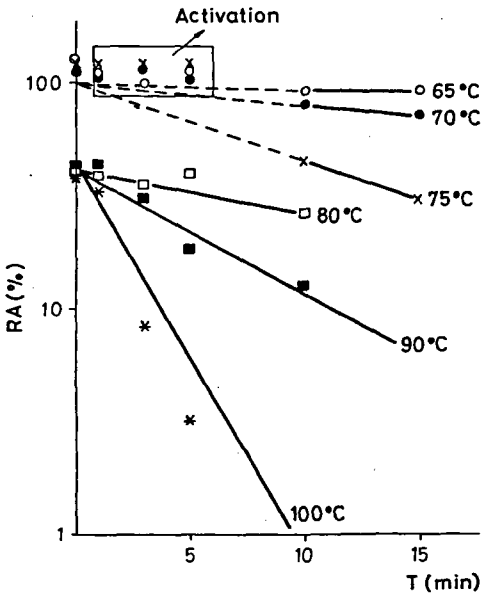


FIGURE 2. Heat inactivation of peroxidase from kohlrabi *(Brassica oleracea* var. *Gongyloides).*[433] Enzyme source: water extract of acetone powder prepared from freeze-dried kohlrabi. Activity determination: 7.4 mmol ℓ^{-1} H_2O_2, 0.08% o-phenylene diamine, pH 5.0, 25°C.[399] RA = residual activity.

least partly, be due to the fact that at higher temperatures the heat labile fraction is instantaneously inactivated, as shown in Figure 2. The experimentally established activation energy of HRP inactivation was found to be in the range of 85 to 100°C, 105 kJ·mole^{-1}, the free energy change 113—115 kJ·mole^{-1}, and the change in entropy was negative (31—33 J·mole^{-1} per degree). This was interpreted as indicating extremely slow energy transfer or higher rigidity of the activated than the native molecule, the latter being more likely than the former.[424]

b. Role and Behavior of Isoenzymes

The difference in heat resistance of the isoenzymes of POD from different sources has been proven by various authors using isoelectric focusing techniques.[428,434,435,436] It seems, however, that the differences in heat resistance of the isoenzymes vary considerably with the source. E.g., in experiments carried out with winter spinach, 1 min at 70°C inactivated all the isoenzymes in the pI-range of pH 5—6, while the acidic group (pI 3.5—5) proved much more stable, showing traces of activity even after 0.5 min at 100°C. No changes in molecular size were found to occur, thus heat resistance could not be explained in terms of aggregate formation and consequent blocking of active sites.[428] In green beans kept at 90°C for 20 min, the pIs of the two isoenzyme fractions showing heat resistance were found to be at pH 4 and 8, respectively.[434] Out of seven POD-isoenzymes separated from a crude kohlrabi extract four with pIs below pH 4.5, and one with a pI around pH 5 were found to resist a 10-min treatment at 90°C.[435] From the HRP isoenzymes the alkaline ones proved the least heat stable. When subjected to isoelectric focusing in the pH-range of 3 to 10, heat treatment of HRP at 90°C for 0—40 min brought about the formation of very large aggregates showing only traces of activity. Separated isoenzymes behaved in a similar way than when treated in a mixture.[436,437]

The heat inactivation of isolated isoenzymes was found to be biphasic as well.[438] During heat inactivation the formation of new isoenzymes was observed.[435,437,438] From these and some additional observations a model of POD inactivation was proposed involving the formation, above 90°C, of a macromolecular hemoprotein of increased heme content as related to the native enzyme. The higher the temperature, the higher the amount of the POD-inactive, lipid-oxidizing macromolecule formed.[428,438]

The theories concerning the mechanism of heat inactivation of POD, as advanced by different authors, fit in well with their own findings. However, when compared with each other, the observations, and, hence, the theoretical considerations deduced from them, are, in several respects, contradictory. The possibility cannot be precluded that the contradictions arise from structural differences in the enzyme molecules as present in different plant tissues. It seems probable that off-flavor formation in blanched canned or frozen vegetables during storage is not a consequence of residual POD activity. It can rather be assumed to be the result of non-enzymatic lipid oxidation performed by the aggregates formed from the isoenzymes of lower heat resistance which have been deactivated during thermal treatment. The role of residual POD activity might rather consist in indicating the insufficiency of the heat treatment applied in destroying the heme accumulated in the aggregates.[414] This assumption can be supported by the fact that the constituents of the off-flavor formed were identified as lipid oxidation products.[422,439] This being a result of lipoxygenase action is out of question, as this enzyme is not heat resistant nor does it show a tendency to regeneration.[439a] On the other hand, carrots frozen unblanched and stored for a year at −18°C maintained their taste and flavor superior to that of the blanched control lot in spite of the presence of POD activity which rose in the non heat-treated sample to a considerable value during storage.[440] (In the blanched sample no activity was restored.)

c. Factors Affecting Heat Inactivation

The factors affecting heat inactivation of POD can be subdivided into two groups: (1) those connected with the source of the enzyme, and (2) those resulting from external parameters of heat treatment.

The influence of the genus, species, or cultivar has already been mentioned. As an example illustrating this influence, it shall be mentioned that POD in potato and cauliflower homogenates could be completely and irreversibly inactivated by a 10-min heat treatment at 95°C, while the enzyme of kohlrabi still maintained about 0.3% of its original activity after 10-min exposures to 120°C.[399,400,416,421] Out of three kohlrabi cultivars, one showed increased POD activity after 10 min at 55°C, while in the other two cultivars activity slightly decreased in the same conditions.[400] However, according to others, residual activities of different cultivars did not differ, if the degree of comminution (particle size) and blanching conditions were identical.[441] Higher activity levels were found to be related to increased heat resistance.[442] The natural environment, i.e., the cell substances, affect heat stability of POD as well. They might have a protective effect on the enzyme, but might also reduce its thermal stability. The heat resistant fraction of pure HRP was smaller in green pea or green bean puree than in buffer solution. In green bean puree, the heat resistant fraction of HRP was found to decrease with increasing temperature of thermal treatment, while it fluctuated without any trend in green pea puree.[413] This might be important when determining residual activities by calculation.[438]

Fungal infection of vegetable tissues considerably lowered the heat resistance of POD from a susceptible, and increased that of POD from a resistant, cabbage cultivar as compared to the enzyme from healthy tissues.[415]

Although the observation was made with cereals, the marked increase in heat resistance of POD at low moisture contents might be important in the manufacture of

dried fruits or vegetables. In ground cereals of moisture contents below 4%, heat stability was found to be inversely proportional to moisture content. From extrapolation to 0% moisture it was obtained that thermal treatments of 20 hr at 108°C were necessary to inactivate POD in rice and wheat germs.[443]

Some external factors affecting the rate of inactivation of HRP were established by Lu and Whitaker:[411] added hematin (pH 7.0, 76°C) lowered, a rise in temperature increased, the rate of inactivation. The rate of inactivation was lowest at pH 7, eightfold this value at pH 4.0, and about twofold at pH 10. Initial rates of inactivation (pH 7.0) increased proportionally to sodium chloride concentration, up to 0.6 mol ℓ^{-1}. However, 2% NaCl had no effect on the heat stability of POD in pickles, which stability could be reduced by the addition of vinegar.[442] The heat resistance of the enzyme in apples and pears could be enhanced by sugars.[442]

The main external factors governing thermal inactivation of the enzyme (at a given pH) are time and temperature of the heat treatment. At a given temperature, longer heat exposures result in a more complete destruction of POD.[422] High temperature—short time (HTST) procedures are milder, and, although causing more rapid inactivation, often give rise to regeneration.[438]

Finally, the apparent heat resistance of POD was reported to depend also on the hydrogen donor substrate applied in activity determination.[383]

Thermal inactivation data of POD from different sources have been compiled in Tables 8 and 9.

The relative amount of the heat labile POD fraction was found to be different according to the source, as can be seen also from the data in Table 8. In a great number of vegetables it was reported to amount to 90 to 99% of total activity. In mushrooms, asparagus, and some potato cultivars, no heat stable fraction could be detected (inactivation was monophasic).[447]

d. Combined Treatments of Peroxidase Inactivation

Microwaves or ionizing radiation may reduce the heat load required for the inactivation of POD in blanching processes.

In white potatoes the enzyme could be inactivated by 1.5 min microwave and 3 min boiling water treatment or 2 min each of microwave and boiling water treatment. Reducing the time of microwave treatment to 1 min and raising the duration of heating in boiling water to 5 min did not completely destroy POD activity, but softened the consistency of the potatoes.[448]

Radiation doses of 1 to 2 Mrad were found to cause considerable changes in the isoenzyme patterns of HRP. New enzyme zones appeared in the pI-range of pH 4 to 6, while the most basic and most acidic isoenzymes were inactivated. This indicates different sensitivity of the various molecular forms to radiation. Ionizing radiation brought about the formation of aggregates, probably through a dimer (molecular mass = 100,000). In contrast to heat treatment, irradiation produced enzymatically active aggregates. The dimer and higher polymers had pI-s in the pH-range of 4 to 6. The pIs of the neutral and alkaline isoenzymes were shifted to the more acidic pH-range and vice versa; consequently all the isoenzymes were concentrated in the pI-range of pH 4 to 6. Results obtained with isolated isoenzymes were similar to those obtained with unfractionated HRP. From the aforesaid it is evident that aggregate formation upon irradiation occurs according to a mechanism different from that observed on heating.[437]

With doses resulting in similar inactivation, aggregation was less marked in solutions of lower concentrations. The synergistic effect of combined inactivation was more pronounced if irradiation preceded heat treatment, as the aggregates and modified monomers formed during irradiation were of a considerably lower heat stability than the native enzyme.[437]

Table 8
HEAT INACTIVATION CHARACTERISTICS OF PEROXIDASES FROM DIFFERENT SOURCES

Enzyme source	z (°C)	E_a (kJ $mole^{-1}$)	Remarks	Ref.
Horseradish	17	88.0	Heat labile fraction, 7.6% of total, by calculation	444
	27	142.5	Heat stable fraction, 92.4% of total, by calculation	444
	27.9	100.0	In acetate buffer, pH 5.6, RZ = 0.6	438
	31.4	88.9	In acetate buffer, pH 5.6, RZ = 3.2	438
	—	134	pH 7.0; heat labile fraction; about 50% of total	411
Green peas	9.8; 9.9	—	z Values of the two phases of inactivation	445
	9	—	Six min at 121°C are required for total inactivation	446
Spinach	13	—	pH 6, isolated enzyme	413
	17.5—18.0	—	pH 4—8; vegetable extract	413
	33	—	Homogenate, spring spinach	385
	45	—	Homogenate, autumn spinach	385
Kohlrabi	9.6	—	Water extract of acetone powder; heat labile fraction, 58—60% of total activity	433
	14.3	—	Water extract of acetone powder; heat stable fraction, 40—42% of total activity	433
Green beans	7.8—15.3	—	Different cultivars; pH 5.8—6.3; temperature required for total inactivation in 6 sec = 105.8—133.6°C	160
Eggplant	11.8	—	pH 5.03; temperature required for total inactivation in six sec = 117.2°C	160
Cherry	6.8	—	pH 3.46; homogenized solution; temperature required for total inactivation in six sec = 77.2°C	160
	10.6	—	pH 3.76; clarified juice; temperature required for total inactivation in six sec = 91.6°C	160

Note: z = Increase in temperature required to lower decimal destruction time by one order of magnitude; E_a = activation energy; RZ = purity of the preparation in terms of A_{403}/A_{275} (A = absorbance).

e. Regeneration

The regeneration of heat-inactivated POD has been detected in 1901 and some of its fundamental features extensively treated in 1944.[449,450] However, many questions related to this long-known phenomenon have not yet found a satisfactory answer. Opinions are divergent, among others, on whether regeneration can occur only in partially, or also in totally inactivated POD.[424,438,451] This controversy arises most probably from differences in sensitivity of the assay methods applied. In the light of present knowledge (confer the preceding chapters on inactivation), it seems probable that POD activity can be restored to a certain extent whenever parts of the protein moiety *and* the prosthetic group have not

Table 9
THERMAL DESTRUCTION DATA OF PEROXIDASE IN VARIOUS FRUITS AND VEGETABLES

	Thermal destruction			
Product	Time (min)	Temperature (°C)	Residual Activity (%)	Ref.
Peach (plain)	0.41	87	0	442
Pear (50% syrup)	4.5	87	0	442
Green bean	3.0	121	0	442
	2.0	95	0.7—3.2[a]	441
		100	0.2[b]	447
Green pea	1.0	100	0.3[b]	447
	1.5	95	0	172
	2.0	80	2.1—6.3[a]	441
Marrowfat pea	2.0	90—95	0.17—1.4[a]	441
Spinach	1.5	100	0.16[b]	447
Cabbage	2.0	95—100	2.9—8.2[a]	441
Red cabbage	2.0	95—100	7.5—11.5[a]	441
Cauliflower	2.0	95	13.4[a]	441
		100	4.8	441
Potato	1.5—3.0	100	0.4[b]	447
Carrot	2.0	100	0.02[b]	447
Asparagus	2.0	100	0.02[b]	447
Mushroom	1.0	100	0	447

[a] Gives best sensory properties after frozen storage (−18°C) for 9 months.
[b] RA in U g^{-1} (1 U = 1/μmole H_2O_2 min^{-1}). RA = residual activity.

been entirely destroyed, even if the concentrations of these parts are too low to be detected by the methods used.

As almost all the characteristics of POD, the trend to regeneration after heat inactivation depends on the species and cultivar. When heated to 99 to 177°C, the enzyme in green beans regained 6% of its original activity in 3 days, while no regeneration was observed in spinach POD after exposures to 121 to 143°C.[425] Regeneration of POD was observed, after 10-min heat treatments at 80 to 110°C, within 24 hr in horseradish and kohlrabi homogenates, whereas no activity was recovered in potato, cauliflower, and turnip suspensions. In the latter product, 24 hr of storage at room temperature following thermal treatment even led to a decrease in residual activity.[452]

Other factors affecting regeneration are the time and temperature of heat treatment, the conditions (pH and temperature) during storage of the inactivated enzyme, and the method of activity determination.[356] From these factors the duration of heat treatment is considered to play the primary role: the less reactivation can be observed, after exposure to a given temperature, the longer the thermal treatment. Sufficiently long heat treatments can prevent regeneration.[438,449,450] Regeneration of HRP was found to be much more marked after exposures to 100 to 120°C (pH 6) than after exposures to 60 to 90°C, the increase in activity being 17 and 5.4%, respectively.[413] Both rate and extent of regeneration were found to depend on the temperature of heat treatment.[400]

Regeneration seems to occur, up to a certain point, in parallel to storage temperature.[438,450] After 6 to 9 months at −18°C, regenerated activity in products properly blanched did not exceed 3.6% of the initial.[441] In sufficiently blanched green peas (6 min, 121°C) and green beans (6 sec, 177°C), activity increased, at room temperature, in 1 to 3 days to 6% of the original.[425,446] After exposure of HRP for 1 hr to

70°C, the average rate constant of regeneration was, at 40°C, 1.7-fold that observed at 30°C. At 50°C no regeneration occurred, but when storage temperature was lowered to 40°C, activity started to increase. No increase took place if the temperature was decreased to 40°C after 24 hr of storage at 50°C, i.e., the reversibly inactivated part of the enzyme became permanently inactivated.[438] The extent of regeneration of isoenzymes of different thermostability was reported to be different, too.[432]

Observations concerning the kinetics of regeneration are contradictory. According to some authors, the phenomenon cannot be described by usual kinetic equations[424]; according to others it follows a saturation curve[450]; a third group considers it a biphasic first order process.[438] It has been found to occur at the highest rate during the first 2 and 4 hr. in green peas and green beans, respectively.[445,450] Others reported regeneration to start after a lag of about 20 hr. Maximum regeneration was found to occur in 2 to 10 days, depending on the temperature of heat treatment, and was followed by a decrease in activity. This indicates that the regenerated molecule is less stable than the native one. Both regeneration and subsequent decrease in activity were the more rapid the higher the storage temperature.[424]

f. Effect of Low Temperatures

Freezing and subsequent storage of the frozen goods is generally carried out after blanching, as enzyme activities at −18°C or −20°C are not destroyed, only slowed down or reversibly inactivated (and regenerated upon thawing), and thus might lead to quality deterioration, e.g., in string beans, although freezing alone caused a loss of more than half of the POD activity. Blanching, even if some residual activity giving rise to regeneration is left, was found to ensure satisfactory sensory properties of the products during long periods (e.g., 1 year) of frozen storage.[420,453] This might be the reason why the effect of low temperatures on POD activity has been relatively not much studied.

Deviations from linearity of the Arrhenius-plot of (turnip) POD in the temperature range of +20°C to −30°C were interpreted as indicating increasing activation energies with decreasing temperatures; the phenomenon was attributed to the simultaneous presence of active and inactive enzyme being in reversible equilibrium. Inactivation at low temperatures was assumed to be due to the formation of intramolecular hydrogen bonds preventing sufficient unfolding of the molecule.[454] However, in cellular systems freeze damage and decompartmentalization might lead to apparent increases in activity.[454] Young cells with small vacuoles resist freezing better, even after blanching.[455]

In experiments carried out with green peas and string beans stored at various low temperatures (+5°C, −4°C, −20°C and −40°C, for periods ranging from 10 days to 18 months, the soluble, ionically bound and covalently bound POD fractions were found to vary in different ways, while total activity remained practically unchanged. This indicated that the enzyme was not denatured during cold or frozen storage, only transitions from one state of binding to the other took place.[173,174,365] The stability of POD at low temperatures was observed also with HRP preparations.[365] The possible causes of these phenomena, as considered by Gkinis and Fennema[365] are (1) the increase, brought about by freezing, in the ionic strength of the soluble phase which might affect binding, (2) disruption of the cell structure by freezing and consequent liberation of new ionic binding sites for POD, and (3) de-esterification of pectins as a result of which the cell wall binding capacity of POD might be altered. The isoenzyme composition showed but slight alterations during frozen storage. The covalently bound fraction was practically independent from storage temperature. The soluble and ionically bound fractions were affected more strongly by high negative than by low negative temperatures or by temperatures above 0°C. No formation of new isoenzymes or disappearance of isoenzymes originally present was found to occur.[365]

5. Inactivation of Peroxidase by Chemicals

Similarly to that of PPO, inactivation of POD can be achieved by chemicals acting on the enzyme itself, and by chemicals reacting with one of the substrates or the reaction products. The former group comprises metal chelators or reducing agents such as cyanide, sulphide, azide, nitric oxide, hydroxylamine, DIECA, sodium metabisulphite, or sodium dithionite.[205,382,383,415] The inactivating effects of hydroxymethyl hydroperoxide and acids have been treated in the chapters dealing with the substrates of the enzyme and the effect of pH on activity, respectively.

The inhibitors most often used in industrial practice are SO_2 and sulphites. The use of 0.1 to 0.15% sodium metabisulphite was found to prevent off-flavor formation in processed peas, but only partially reduced enzyme regeneration.[205] This, too, seems to indicate that factors other than residual or regenerated POD activity are responsible for off-flavor formation during storage of processed vegetables. The action of SO_2 on grape POD was found to depend on its ratio to the substrate H_2O_2. If the concentration of SO_2 was superior to that of H_2O_2, the reaction was stopped immediately. If H_2O_2 was added in excess to the inactivated enzyme, the reaction continued at the initial rate. If the concentration of added SO_2 was lower than that of H_2O_2, the reaction proceeded at the initial or at a lower rate, depending on SO_2 concentration.[398] (It shall be noted that the colored reaction product formed from the donor substrate o-phenylene diamine is irreversible and cannot be reduced by SO_2 to the colorless initial compound.) It has been concluded that the action of SO_2 consists merely in destroying H_2O_2. As the rate of the reaction $SO_2 + H_2O_2 \rightarrow SO_3 + H_20$ is higher than that of the formation of the enzyme-acceptor-donor complex, SO_2, by eliminating H_2O_2, blocks enzyme activity and maintains the donor substrate in its reduced form.[456]

Bisulphite was found to retard the inactivation of HRP by weak acids through stabilization of the linkage between the iron-containing prosthetic group and the protein. Cyanide, azide, and fluoride, which form reversible complexes with the heme iron, had a similar effect. Bisulphite was also assumed to form a complex with heme iron.[457]

The peroxidatic and the oxidatic reactions of POD showed different behavior with cyanide, azide, and dimethylformamide. The peroxidatic reaction with various donor substrates was strongly inhibited by cyanide, while the oxidatic reaction (phloroglucinol substrate) was, in the presence of $MnCl_2$, practically not affected. The reason is that heme Fe^{3+} is a stronger complexing agent than Mn^{2+}, the cofactor of the oxidatic reaction. Azide proved a specific inhibitor of the oxidatic reaction, which was also more strongly inhibited by dimethylformamide than the peroxidatic reaction.[394]

Natural competitive inhibitors of POD were extracted from unripe mango and banana fruits. They proved heat-labile and of proteinic nature.[458] Such inhibitors may cause apparent activation of POD during heat treatment (confer the chapter on heat inactivation.) Natural and synthetic naphthoquinones were found to inhibit POD as well. Some of them showed a stimulating effect in a certain concentration range. This range varied with the compound.[459]

Detergents were found to inactivate POD, and to affect its heat inactivation. Surface active compounds swelling in water, such as lecithin and monoglycerides, showed the strongest effect. Lecithin caused marked inactivation at 0°C and pH 4.0. The action of linoleic acid was stronger in air than in nitrogen atmosphere. This indicates that lipid peroxides as formed from linoleic acid upon heating might have a special effect. It is of utmost practical importance that detergents prevent regeneration of heat inactivated POD.[460]

Ionic polymers as applied in foods, e.g., carrageenans or pectins of different methyl pectate content, were reported to interact with POD: at pH 5.5, the pectins caused significant inactivation and the carrageenans a slight rise in activity. Furthermore, the

pectins shifted the activity maximum of the enzyme from pH 5.5 to pH 8.0. Thus they caused almost complete inactivation at low pH.[461] This might also be of importance in food processing, as the pH of most fruits and vegetables is in the acidic pH-range.

Metal ions (Ca, Cu, Fe^{2+}, Fe^{3+}, and Mn) in concentrations of 10^{-3} mol ℓ^{-1} proved to be specific metabolic effectors of the POD system, displaying a differentiated effect on the different molecular forms of the enzyme.[462] This shows the different response of isoenzymes to effectors, which is, most probably, not restricted to metal ions.

6. Extraction and Purification of Peroxidase, its Homogeneity, Molecular Mass, and Other Characteristics

a. Extraction and Purification

The extraction and purification of POD presents, in general, similar problems as that of PPO. In most fruits and vegetables the enzyme is present in soluble as well as in ionically and covalently cell-bound form. The ratios of these forms might vary during ripening.[362] The soluble fraction can be extracted with water or with a buffer of low ionic strength, the ionically bound fraction with a buffer of suitably elevated ionic strength, and the covalently bound fraction after digestion with a cell-wall macerating enzyme preparation (confer also the chapter on the occurrence of POD in nature.) Before extracting the enzyme, the plant material is often frozen in air or in nitrogen atmosphere. Freeze-drying, subsequent extraction with water, acetone precipitation, and repeated extraction were found to give a high yield of the soluble enzyme fraction of kohlrabi.[463] Direct extraction from peaches gave higher POD-activities than acetone powder extracts, probably owing to solvent denaturation or poor solubility of the powder.[244] Salting out, gel filtration, chromatography on both cation and anion exchangers, as well as affinity chromatography, were used to obtain purified POD preparations and separated isoenzymes from various sources, as will be shown on some examples below.

Polyphenols which might decrease activity and solubility of the preparations and cause discoloration could be eliminated by storage of the preparations at 4°C (up to 2 months) in 0.1 mol ℓ^{-1} phosphate buffer and subsequent preparative electrophoresis.[464]

Horseradish: seven isoenzymes were isolated by $(NH_4)_2SO_4$ precipitation (first 35%, then 90% saturation), followed by column chromatography on CM- and DEAE-cellulose. Equilibration buffer for the CMC-column: 0.005 mol ℓ^{-1} acetate, pH 4.4, elution buffers: (1) 0.005 mol ℓ^{-1} acetate, pH 4.4 and 0.1 mol ℓ^{-1} acetate, pH 4.4 (1:1 v/v, linear gradient), (2) 0.1 mol ℓ^{-1} acetate, pH 4.4 and 0.25 mol ℓ^{-1} acetate, pH 4.9 (1:1, v/v, linear gradient). Equilibration buffer for the DEAE-cellulose column: 0.005 mol ℓ^{-1} Tris, pH 8.4, elution buffers: 0.005 mol ℓ^{-1} Tris, pH 8.4 and 0.005 mol ℓ^{-1} Tris, pH 8.4, containing 0.1 mol ℓ^{-1} NaCl (1:1, v/v, linear gradient). 86% of the activity present in the crude homogenate were recovered in the seven isoenzymes. These could be divided into two groups: four were cationic and three were anionic. The cationic isoenzymes B and C accounted for 56%, and the anionic isoenzyme A—1 for 13% of the activity of the crude homogenate. The individual isoenzymes proved to be homogeneous in the ultracentrifuge and polyacrylamide gel discelectrophoresis. This purification procedure devised by Shannon and co-workers is considered a classical method and has been used by many others ever since.[465] A more recent procedure applies affinity chromatography on Sepharose®-bound Concanavalin A. The method gives 73% recovery of enzyme activity and considerable purification: the absorbance ratio (A_{403}:A_{280} = heme:protein) was increased from 0.62—2.45 to 2.8—3.1.[466]

Spinach: spring spinach was cooled to −50°C, and subsequently freeze-dried at a tray temperature of 30°C. The freeze-dried material was finely ground, resuspended in distilled water, and filtered after 30 min through cheese-cloth. The filtrate was saturated with $(NH_4)_2SO_4$ to 40 to 85%, the precipitate redissolved in 0.1 mol ℓ^{-1} phosphate buffer,

pH 7.0 and dialyzed against distilled water. The enzyme solution was cooled to −30°C and freeze-dried as above.[413] Autumn spinach was frozen, ground, and the enzyme extracted, after thawing, with 0.02 mol ℓ^{-1} phosphate buffer, pH 7.2, containing 1% ascorbic acid. Solubilization with $CaCl_2$ or EDTA did not yield differences in the isoelectric patterns; thus is was assumed that the extract contained also the particle-bound enzyme fraction.[428]

Green bean: the freshly harvested material was chilled to −20°C. The enzyme was extracted (1) from the pressed juice of partially thawed beans, (2) from acetone powder with 0.02 mol ℓ^{-1}, pH 7.2 phosphate buffer, and (3) by homogenization (5 times 30 sec, 0—5°C) in various buffers:

1. 0.02 mol ℓ^{-1} phosphate, pH 7.2
2. 0.3 mol ℓ^{-1} phosphate, pH 7.2
3. 0.05 mol ℓ^{-1} Na-maleate, pH 6.0
4. 0.05 mol ℓ^{-1} Na-maleate, pH 6.0, containing 0.2 mol ℓ^{-1} $CaCl_2$
5. 0.05 mol ℓ^{-1} Na-maleate, containing 0.08 mol ℓ^{-1} $CaCl_2$
6. 0.1 mol ℓ^{-1} HEPES (N-2-hydroxy-ethyl-piperazine-N'-2-ethanesulfonic acid) buffer, pH 7.4 + 0.01 mol/ℓ^{-1} dithioerithrite + 15% w/v insoluble PVP.

Before isoenzyme separation the extracts were centrifuged, if necessary, concentrated by ultrafiltration and centrifuged with Sephadex® G 25. All the variants gave identical isoelectric patterns.[405]

Banana: the soluble fraction was extracted from banana pulp with sodium maleate buffer (0.02 mol ℓ^{-1}, pH 6.0), casein dispersion and insoluble PVP (2:1), the ionically and covalently bound fractions with the same solution containing 0.2 mol ℓ^{-1} and 0.8 mol ℓ^{-1} $CaCl_2$, respectively. The pulp was mixed with the solutions in a ratio of 1:2 (w/v).[358] According to another method, slices of ripe bananas were frozen with liquid nitrogen, then ground for 3—5 min. Extraction media were added (1:1, w/v). This contained 0.1 mol ℓ^{-1} Tris-buffer, 17% sucrose, 0.1% cysteine-HCl, 1% ascorbic acid, and 0.8 mol ℓ^{-1} $CaCl_2$. The extract was centrifuged at 4°C and 75,000 X g for 30 min, then treated with 1% macerating enzyme during 4 hr at 20°C. This treatment lowered the viscosity of the extract. A preliminary fractionation was carried out on a Sephadex® G 25 column equilibrated with 0.005 mol ℓ^{-1} Tris buffer, pH 8.5. The POD-active fraction was pooled, kept at 4°C for 1 hr, whereafter the slimy substances were sedimented out by centrifugation. The supernatant was then chromatographed on a DEAE-cellulose column equilibrated with 0.005 mol ℓ^{-1} Tris buffer, pH 8.5. The cationic fraction which ran through the column was pooled. The anionic fraction which remained adsorbed to the column was eluted, preferably by batchwise elution, with 0.025 mol ℓ^{-1} Tris-HCl buffer, pH 8.5, containing 0.25 mol ℓ^{-1} NaCl in the final step.[406]

b. Homogeneity, Molecular Mass, and Other Characteristics

The heterogeneity of plant peroxidases has been known for about 40 years now. Theorell crystallized two peroxidases from horseradish in 1940, and Schwimmer separated two peroxidases from turnips in 1944.[355,450] Up-to-date separation techniques have made it possible to reveal a much higher number of molecular forms in POD of fruit and vegetable origin. These, although generally denoted as isoenzymes, were found to differ not only in electrophoretic mobilities or isoelectric points, but often in substrate specificity, resistance to heat and inhibitors, etc., sometimes even in molecular mass. Their number was found to depend in a given product, among others, on the methods used for their separation and detection; e.g., thin-layer chromatography on different gels revealed but one, disc electrophoresis three to four, and thin-layer isoelectric focusing, in

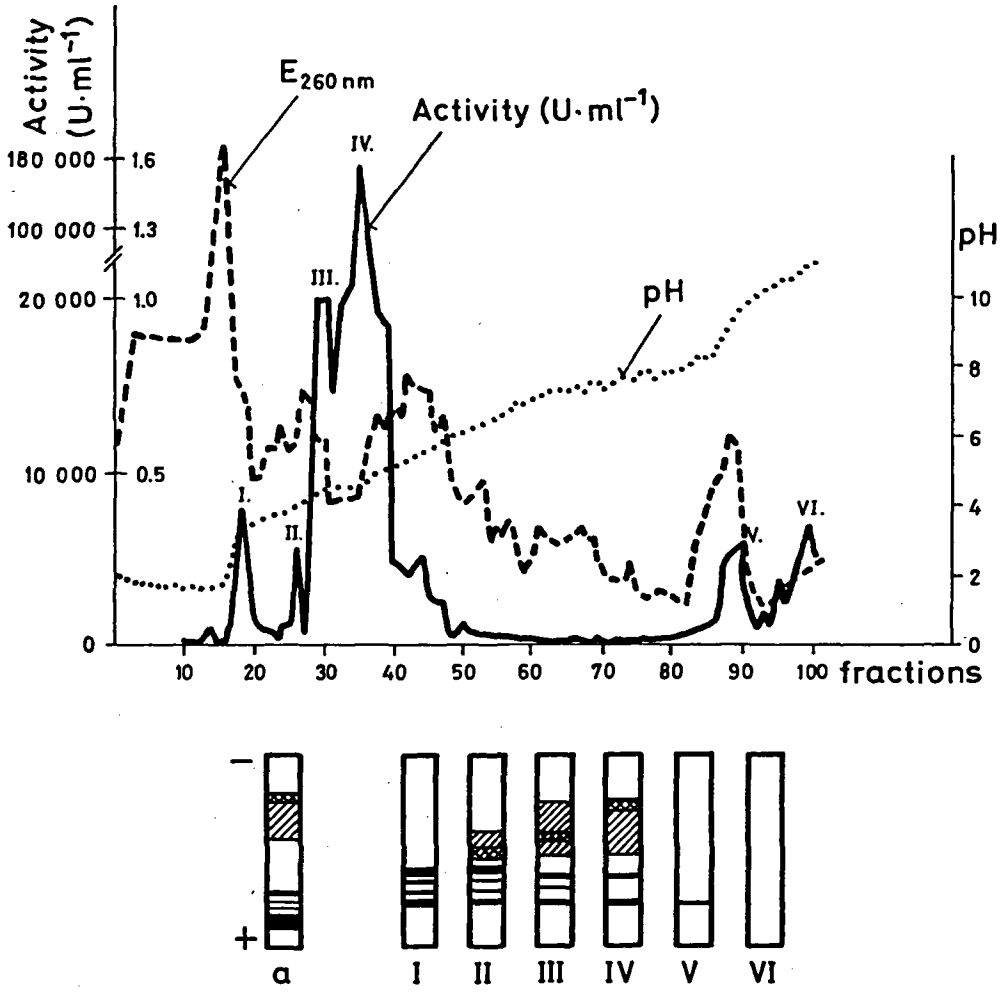


FIGURE 3. Isoenzymes of kohlrabi *(Brassica oleracea* var. *Gongyloides)* peroxidase.[463] Kohlrabi extract as in Figure 2, purified by gel filtration on Sephadex G 50. Upper drawing: fractionation by preparative density gradient isoelectric focusing (IF), pH-range 3—10. Isoelectric points and activities as related to total (%, in brackets), of the fractions: I. 3.3 (2.4); II. 4.1 (1.1); III. 4.4 (9.8); IV. 4.6 (72); V. 9.8 (3.7). Lower drawing: polyacrylamide gel (PAG) IF of the isoenzymes separated by preparative IF. Roman numbers as above. a: PAG IF pattern of enzyme extract passed through the Sephadex column. Activity determination: as in Figure 2. 1 U = $10^{-3}\Delta E\ min^{-1}$ (420 nm). E = extinction.

the pH-range 3 to 10, twenty isoenzymes in green bean POD.[405] Even with isoelectric focusing, the results depend on the media the pH-gradient has been established in. The majority of the POD fractions obtained from a kohlrabi extract by density gradient isoelectrofocusing (pH 3.5—10) proved heterogeneous on re-focusing in polyacrylamide gel, as illustrated in Figure 3.[463] The separation by electrofocusing in a thin layer of granulated gel was reported to be of considerably higher resolution than the density gradient method; moreover, there was a discrepancy in the isoelectric points obtained by the two methods for a given fraction. It was, therefore, suggested that the isoelectric points obtained by focusing methods be considered as *apparent* values.[467] The nature of the donor substrate and the concentrations of both substrates might affect the results as well.[405,406,468,469] All this makes comparison of the data as published by different authors difficult. The fact that a purified HRP preparation gave only 5 to 8 fractions in thin-layer

isoelectric focusing, while a less purified preparation gave more than 20,[470] indicates that utmost caution is advisable in evaluating such results.

Some data published on the number of POD isoenzymes, their molecular masses, and some other properties as obtained from different sources and by different methods, have been collected in Table 10.

The values of the molecular mass of peroxidases from various fruits and vegetables were found to range from 30,000 (Japanese radish) to 54,000 (fig).[405] It shall be recalled here that heat inactivation and regeneration as well as irradiation might alter the molecular mass of POD.

HRP in the purified form and in root extracts as well as POD isoenzymes released from root cell walls were reported not to contain hydroxyproline.[364] However, this amino acid was found to be present in traces in the anionic group of purified HRP isoenzymes.[465] Tyrosine content was also low; this accounts for the low absorbance at 280 nm and for the fact that in the zymograms not all the enzymatically active fractions give protein reactions.

POD is a carbohydrate-containing enzyme. The following carbohydrates have been identified in HRP: glucose, galactose, mannose, arabinose, xylose, fucose, and hexosamine.

7. Determination of Enzyme Activity

a. Peroxidatic Activity

Spectrophotometric methods based on the formation of a colored compound from the hydrogen donor substrate during the reaction are generally applied to determine POD activity, and to estimate, by visual method or densitometry, residual activities after heat treatment, as well as isoenzyme composition. Most of the assay methods have been developed by the early explorers of POD and have been summarized by Maehly and Chance.[480] A great number of variants of these methods as modified by present-day researchers can be found in the literature cited.

Care must be taken in selecting the concentrations of H_2O_2 and of the donor substrate, as well as the pH, so as to give maximum reaction rates, and, thus, high sensitivity (confer the related chapters). The selection of the appropriate wavelength is also of importance, as the absorption maxima of the various oxidized donors are different. The nature of the donor substrate might affect activity values as specificites of peroxidases from different sources are different.

Guaiacol is often used as donor substrate. It is transformed by the enzyme into tetraguaiacol. It has been claimed to be the most sensitive donor for determining residual activity after heat treatment.[147,244,383,413,442] It finds also wide application as a qualitative test.[383] However, *o-phenylene diamine* was found to be a more sensitive donor for assays of POD activity and detection of isoenzymes in vegetable extracts.[399,405,413,447,481] *Pyrogallol* ranges among the classical substrates. It is oxidized by POD to purpurogallin, hence the activity unit encountered in the early literature under the name of "Purpurogallinzahl".[383,480]

o-Dianisidine is preferentially used with purified enzyme preparations, as well as to detect isoenzymes after gel electrophoresis or isoelectric focusing.[388,406,435] *2,3',6-Trichloro-indophenol* in its leuco form can be used below pH 6.0 as hydrogen donor.[482] *3,3'-Dimethoxy-benzidine hydrochloride* at pH 4.0 and 6.0 (banana POD), and *3,3'-diaminobenzidine tetrahydrochloride* (pH 4.3, HRP) were found to be highly sensitive hydrogen donors.[358,401] *Benzidine* lends itself to detect POD in histochemical preparations.[399] A *fluorimetric* assay of POD is based on the catalytic formation, from *homovanillic acid* (pH 8.5), of the fluorescent compound *2,2'-dihydroxy-3,3'-dimethoxy* biphenyl-5,5'-diacetic acid. An advantage of the method consists in the fact that particulate material does not interfere.[483]

Table 10
MULTIPLE FORMS OF PEROXIDASE FROM DIFFERENT SOURCES

Source of the enzyme	Number of isoenzymes[a]	Method of separation	Remarks	Ref.
Horseradish	20 (in four groups); I: 4—5 fractions (3.5—4.5); II: 5—8 fractions (4.5—5.5); III: (5.5—7.6); IV: (7.6—9.0)	TLIF, pH 3—10	A_{403}/A_{275} = 0.6; IE in group II of low activity; group III contains main activity; groups I and II = anionic, groups III and IV cationic in IEC; all IE have similar MM (about 40,000)	470
	5—8 (in 2 groups); *a:* (5.5—7.6), *b:* (7.6—9.0)	TLIF, pH 3—10	A_{403}/A_{275} = 2.6—3.0; groups *a* and *b* correspond to groups III and IV of the less purified preparation; number and quantitative distribution of fractions differ in preparations of similar absorbance, but different origin	470
	5	Filter paper electrophoresis	The major component corresponds to POD II of Theorell; two are negatively charged at pH 5.0; all differ in substrate specificity	471
	7	$(NH_4)_2SO_4$ precipitation, CM- and DEAE-cellulose chromatography	Three fractions *(A)* migrate to (+) in basic and four to (−) *(C)* in acidic disc electrophoresis. *A* is more active in the oxidatic, *C* in the peroxidatic reaction. pH optima, specific activity, substrate specificity and susceptibility to azide differ	388
	4	CM-cellulose chromatography, acetate buffer pH 4.6	Main fractions of pI 4.0 and 8.8 contain 1/2 and 1/4 of total POD; they differ in substrate specificity. The acid POD contains less arginine	475 472
Horseradish, commercial preparations	4	CM-cellulose chromatography, acetate buffer, pH 4.4, SGE	Acidic isoenzymes present only in traces; basic isoenzymes have similar MM, absorption spectra, activities and amino acid compositions	472 473
Japanese radish	18	$(NH_4)_2SO_4$ precipitation, CM- and DEAE-cellulose chromatography, PAGE, pH 4.8 and 4.9	Pattern similar to that of HRP, but acidic IE prevail; acidic, basic and neutral isoenzymes differ in MM (in the range of 30,000—45,000) and in catalytic properties	475 476
Spinach	2		IE are different in CM—cellulose chromatography; spectral properties, dissociation constants of POD-cyanide complex and rate constants of POD reactions are similar	477

Table 10 (continued)
MULTIPLE FORMS OF PEROXIDASE FROM DIFFERENT SOURCES

Source of the enzyme	Number of isoenzymes[a]	Method of separation	Remarks	Ref.
Green beans	5—6	Thin-layer electrophoresis (Bio-Gel P-60), pH 9.0	Color intensities (OPDA) and mobilities differ; the most active 2 IE migrate to cathode	405
	20, in 3 groups: I. 7—8 fractions (3.5—5), II. 8—10 fractions (5—8), III. 6—7 fractions (8—9.5)	TLIF, pH 3—10	MM identical	405
	10 (soluble fraction), 3 (ionically bound), 7 (covalently bound)		Number of IE same in beans cooled at 5°C, chilled at −4°C or frozen at −40°C; changes in coloration occur during storage	365
Bean leaf	2 major cationic, 2 major anionic + three subpeaks	Disc electrophoresis		478
Tomato plant	3	Continuous-flow carrier-free electrophoresis, 10% sucrose in the buffer	Extreme dwarf tomato plant	479
Tomato stem	4 (soluble), 2 (ionically cell-wall bound)	SGE, pH 3.0	Different heat stabilities	357
Tomatoes (fleshy), seven cultivars	8	Acetone powder extracts; disc electrophoresis, basic gels	Identical patterns for all cultivars	260
Cucumbers, two cultivars	5	Acetone powder extracts; disc electrophoresis, basic system	Identical patterns for both cultivars	260
Onions, two cultivars	5 and 4	Acetone powder extracts; disc electrophoresis	Identical migrations	260
Grapes, six cultivars	5—9 in skin 3—7 in juice	Disc electrophoresis		258
Pineapple stem	2: one acidic, one basic (9.2)	Purification of basic fraction (164-fold): $(NH_4)_2SO_4$, CM-cellulose, hydroxylapatite + CM, gradient elution	MM: 35,200—42,000, by different methods	414

Table 10 (continued)
MULTIPLE FORMS OF PEROXIDASE FROM DIFFERENT SOURCES

Source of the enzyme	Number of isoenzymes[a]	Method of separation	Remarks	Ref.
Banana	9	Gel filtration, PAGE, CM- and DEAE-cellulose chromatography	Six bands of similar mobility, a fast moving doublet + 1 zone near the origin; the separated anionic fraction shows the same pattern without the fast moving bands; the cationic fraction enters only acidic gels and gives one fraction	406

Note: CM = carboxy methyl; DEAE = diethylamino ethyl; IE = isoenzyme; IEC = ion exchange chromatography; MM = molecular mass; OPDA = o-phenylene diamine; PAGE = polyacrylamide gel electrophoresis; SGE = starch gel electrophoresis; TLIF = thin-layer isoelectric focusing.

[a] Isoelectric points in brackets.

A great number of activity assays lately developed are referred to in the monography of Guilbault.[484]

b. Oxidatic Activity

2-Methyl-1,4-naphthoquinone dissolved in 0.01 N HCl was reported to be used as substrate; the determination was carried out at pH 6.0, measuring the increase in absorbance at 262 nm.[473] With *oxaloacetate* as substrate, oxygen consumption was followed polarographically with the use of a platinum oxygen electrode coupled to a millivolt recorder.[388] The cofactors $MnCl_2$ and 2,4-dichlorophenol have to be included into the reaction mixture. Activity is calculated from the linear section of oxygen consumption which follows the 2- to 3-min induction period.

Indole-3-acetic acid is most often used to assess the oxidatic (IAA oxidase) activity of POD. The reaction mixture may contain, e.g., 0.2 mmol ℓ^{-1} IAA, the necessary cofactors (e.g., 0.1 mmol ℓ^{-1} $MnCl_2$ and 0.1 mmol ℓ^{-1} 2,4-dichlorophenol) and the enzyme solution in 1 mmol ℓ^{-1} phosphate buffer (pH 6.1). Either the increase in absorbance at 261 nm is measured or a reagent (1% p-dimethylamino-cinnamaldehyde in 2 mol ℓ^{-1} HCl) is added to the mixture (1:1 v/v) and the absorbance of the purple reaction products is read, after 70 min in the dark, at 562 nm.[406,485] According to another method, the reaction mixture containing the substrate, the cofactors, and the enzyme is complemented with the Salkowski reagent ($FeCl_3$+ $HClO_4$). In this case, color development takes 2 hr.[486] Although with the use of color reagents and absorbance readings after 1 to 2 hr the induction period this reaction starts with does not appear, it seems more advisable to use kinetic measurements and eliminate the lag phase, if desired, by the addition of traces of H_2O_2.

C. Peroxidase in Some Fruits and Vegetables

Peroxidase activity varies in fruits and vegetables in wide limits as can be seen in Table 11. The data of the table show that, from the horticultural products tested in the study cited, cultivated mushrooms had the lowest, and horseradish the highest POD activity. Fruits had lower activities than most of the vegetables investigated.

Table 11
PEROXIDASE ACTIVITY IN SOME FRUITS AND VEGETABLES[399]

Name of Fruit or Vegetable	Activity[a](U g^{-1})
Cultivated mushroom	240
Apple	780—1610[b]
Pear	1,700—4,250[b]
Apricot	1,310—4,900[b]
Onion	1,764
Head of celery	3,450
Turnip	5,250
Early radish	5,360
Carrot	5,650
Green pepper	8,250
Potato	10,900
Asparagus	20,500
Early kohlrabi	30,400
Black radish	103,000
Winter kohlrabi	125,000
Horseradish	569,000

[a] Arbitrary units wet-weight basis. 1 U = 1×10^{-3} ΔOD min^{-1}.(OD = optical density, 420 nm). Activity determination: 7.4 mmol ℓ^{-1} H_2O_2, 0.08% o-phenylene diamine, pH 5.0.
[b] Different cultivars.

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Apples — POD activity was found to increase in apples during ripening.[362] It was reported to have a maximum in the cv. *Golden Delicious* at the climacterium, as already mentioned, and to increase during controlled atmosphere storage.[378] The increase in POD activity was observed also in cold storage in normal atmosphere, with different cultivars.[487] However, some authors found enzyme activity in apples stored at 0 to 2°C to increase or decrease, according to cultivar. Storage in polyethylene bags at 0 to −2°C slowed down the rate of these changes.[301] Mineral fertilizers (K^+, N^3, SO_4^{2-}, PO_4^{3-}, Cl^-) did not influence the POD isoenzyme patterns of apples. The patterns were found to be different in different cultivars.[298]

Pears — In pears, POD levels were found to be higher in the cv. *Bartlett* than in fruits with over-abundant grit cells. However, the relative amount of cell-bound POD was higher in the latter. This could be demonstrated also histochemically. The cell-bound part of the activity seemed to be related to grit-cell formation which, in turn, was related to Ca^{2+}-content. The phenomenon could be controlled by mineral nutrition.[367]

Peaches — POD was found to have two maxima during the ripening of peaches: one on the 2nd, and one on the 6th to 7th week.[244]

Apricots — The mass-related POD activity of apricots seems to be, to some extent, characteristic of the cultivar: in four cultivars from two locations and 2 years, the order of enzyme levels was the same.[488]

Sour cherries — The isoenzyme composition of POD in sour cherries was not affected by fertilizers.[316]

Grapes — In grapes, POD is present in soluble form, and, on crushing the berries, passes into the juice. Activity increases till full maturity, but then declines both in the juice and the particles. The enzyme content is considerable: it corresponds, on the

average, to an oxygen liberation, from H_2O_2, of 50 mℓ/ℓ of juice per min, and varies with the vineplant. In neutralized and PVP-treated grape juice, the enzyme was found to be resistant to heat, SO_2, and bentonite. Heat resistance was found to depend on pH. However, at any pH value, heat treatment was effective in lowering POD activity.[398,489]

Berry fruits — Zymograms of *strawberry* POD revealed up to four isoenzymes in 16 species and three varieties. Most species showed two isoenzyme bands.[319]

In ripening *blueberries,* POD and IAA oxidase activities were found to vary in opposite directions: coloration of IAA oxidase isoenzymes became more intense, while that of POD isoenzymes became fainter.[375]

Bananas — POD solubilized with buffers of low ionic strength from the pulp of ripening banana fruit remained more or less at a constant level during the process taking place under normal conditions (20°C, 85% relative humidity).[359] During the first 5—10 days of storage at 5°C, the activity of the soluble fraction started to increase in parallel to skin and pulp discoloration, as well as to the loss of ripening capacity when brought to normal conditions again. The rate of increase in activity of this fraction was similar in determinations carried out at pH 6.0 and 4.0. The cell-wall-bound fraction of the enzyme showed a marked decrease in activity, as determined at pH 6.0, during the first days of storage at 5°C. Afterwards, activity increased in an erratic way to culminate after 25 days and decrease again till the end of storage (35 days). Activity values determined at pH 4.0 showed practically no variations during ripening. The activity of the intercellular POD fraction increased, however, during normal storage (20°C, 85% relative humidity), to the 12-fold and 4-fold, as assessed at pH 4.0 and pH 6.0, respectively. This indicates that changes in the pH-dependence of activity might occur during storage. The strength of the bonds which fix banana POD to the cell wall are different in normally stored ripe fruits, normally stored green fruits, and those stored in the cold: the solubilization of the insoluble enzyme fraction can be achieved by 0.8 mol ℓ^{-1}, 0.3 mol ℓ^{-1} and 0.15-0.2 mol ℓ^{-1} $CaCl_2$ solutions, respectively, from the fruits stored under these conditions. These differences are expressed also in the isoenzyme pattern.[358]

Oranges — Some natural constituents of orange juice, such as ascorbic acid, caffeic, gentisic, and coumaric acids, were found to react with POD. In the juice their oxidation was slow, owing to low H_2O_2 content. When determined using the hydrogen donor p-phenylene diamine, H_2O_2 decomposition started with a lag phase caused by ascorbic acid interaction with an oxidation intermediate of the donor. An increase in activity of POD and pulp content in the juice due to processing conditions was accompanied by lower quality of the juice.[490]

Potatoes — The results obtained with six potato cultivars sampled at harvest, after storage for 2 and 4 months at 7°C, and after 3 weeks at 20°C, showed the significance of differences in POD activities of the bud-end, stem-end, and core to be cultivar-dependent. The significance of these differences was not affected by storage conditions. These influenced, however, the significance of differences in POD activities as found between cultivars.[325] The "potato enzyme" — phenol system and blackspot formation was found to be dependent, under certain conditions, on H_2O_2, POD, and catalase.[491]

Sweet Potatoes — Ethylene content in air caused a 100-fold increase in the POD activity of sweet potato slices. The increase was shown, by the incorporation of ^{14}C leucine and addition of a protein synthesis inhibitor, to be due to *de novo* synthesis of the enzyme protein.[492]

Asparagus — Fiber formation in postharvest asparagus was found to be related to changes in the POD isoenzyme pattern: the apperance of new isoenzymes was noticed, first at the butt end, and later at the tip end of the spear. Fiber development was more expressed in spears cut above ground level than in those cut below ground level. Both fiber formation and changes in the POD isoenzyme pattern were related to ethylene

formation.[372] Fiber formation was found to be related to cell-bound POD only, which could, however, not be controlled by $CaCl_2$ nutrition without adversely affecting asparagus quality.[371]

Green Beans — In green beans the resistance of POD to thermal treatment was increased by higher concentrations of Na_2CO_3 and NaCl (5% and 1% respectively.)[493]

Cabbage — The POD isoenzyme patterns of various cabbage cultivars showed different behavior after infection with *B. cinerea*. In patterns of a susceptible cultivar, no changes were noticed either in the number or in the mobility of the isoenzymes, while in those of a resistant cultivar, a new active band was revealed. Kinetic properties, susceptibility to inhibitors, pH optima, and heat resistance (confer the related chapter) were equally altered owing either to synthesis, in the infected tissues, of a POD of altered properties, or enzyme solubilization, perhaps changes in the properties of the enzyme present in the tissues prior to the infection.[415]

Spinach — The photoperiodic induction in spinach was found to be associated with a drop in IAA oxidase and POD activities, as well as with changes in the isoenzyme pattern. Female plants had a higher number of isoenzymes, while male plants showed higher enzyme activities. The two activities did not always coincide or vary in parallel.[396]

Chicory and Endives — The discoloration of chicory packed in polyethylene bags was related to POD activity. This relationship was not obvious in irradiated samples (100 krad). The activity did not change for 2 days after irradiation, and shelf life could be extended correspondingly, while activity increased strongly in the non-irradiated sample.[350] Also, in endives irradiated with 100-300 krad, POD activity did not change immediately after the treatment.[351]

Onions — POD activity in young onion (*Allium cepa* L.) bulbs (1.5—2 cm Ø) of three genotypes was found to be similar, while it differed in mature bulbs: those of short dormancy had higher activities and a higher number of isoenzymes than those of long dormancy. A preharvest spray of the sprouting inhibitor maleic hydrazide did not affect POD in bulbs of either type of dormancy.[494]

Tomatoes — In tomato fruits, activity increased during normal development and ripening. The isoenzyme pattern showed four bands. Neither enzyme activity levels, nor the isoenzyme pattern were affected by low levels of potassium, while blotchy ripening decreased activity and acted in a similar way on the isoenzymes.[346]

IV. CONCLUSION

From the literature reviewed it might be concluded that the investigations into the multiple forms of PPO and POD have largely contributed to a better understanding of the behavior of these enzymes in fruits and vegetables. The systematic study of the occurrence and formation, during the life cycle of the plants, as well as of the kinetic and molecular properties of the isoenzymes, might help to establish more exactly and profoundly their physiological role, the reasons of polymorphism in general, and the reasons why a given set of isoenzymes is present in one, and another set in another, cultivar, in particular. This could mightily promote the work of growers and processors aimed at producing high quality fresh, canned, frozen, dried, or freeze-dried fruits and vegetables. In the opinion of the reviewer this is the topic future work related to these enzymes should be focused on.

ABBREVIATIONS USED IN THE TEXT

DAB = 3,3'-diaminobenzidine tetrachloride
DIECA = sodium diethyl-dithiocarbamate

DOPA = 3,4-dihydroxy phenylalanine
HRP = horseradish peroxidase
HTST = high temperature — short time (thermal treatment)
IAA = indole-3-acetic acid
K_i = inhibition constant
K_M = Michaelis constant
MM = molecular mass (M_r)
NADH = reduced form of nicotine adenine dinucleotide
NADPH = reduced form of nicotine adenine dinucleotide phosphate
PAGE = polyacrylamide gel electrophoresis
PEG = polyethylene glycol
pI = isoelectric point
POD = peroxidase
PPO = polyphenol oxidase
PVP = polyvinylpyrrolidone
SDS = sodium dodecyl sulphate (electrophoresis)
V_{max} = maximum reaction rate
z = increase in temperature (°C) required to lower decimal destruction time (D) by one order of magnitude or one logarithmic cycle

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