# The biochemistry and control of enzymatic browning

M. Victoria Martinez and John R. Whitaker

Half of the world's fruit and vegetable crops is lost due to postharvest deteriorative reactions. Polyphenol oxidase (PPO), found in most fruit and vegetables, is responsible for enzymatic browning of fresh horticultural products, following bruising, cutting or other damage to the cell. Chemical methods for controlling enzymatic browning include the use of sodium bisulfite, ascorbic acid and/or packaging under controlled atmospheres. Current approaches to understanding and controlling enzymatic browning are presented in this review article, with special focus on the use of antisense RNA as a control method.

Browning results from both enzymatic (PPO) and non-enzymatic oxidation of phenolic compounds. Browning usually impairs the sensory properties of products because of the associated changes in color, flavor and softening (due probably to the action of pectic enzymes). Once cell walls and cellular membranes lose their integrity, enzymatic oxidation proceeds much more rapidly. Browning is sometimes desirable, as it can improve the sensory properties of some products such as dark raisins and fermented tea leaves.

Browning in fruit and in some vegetables, such as lettuce and potato, is initiated by the enzymatic oxidation of phenolic compounds by PPOs. The formation of shrimp black spot is another example of browning due to PPO activity. The initial products of oxidation are quinones, which rapidly condense to produce relatively insoluble brown polymers (melanins). Some non-enzymatic causes of browning in foods include the Maillard reaction, autooxidation reactions involving phenolic compounds and the formation of iron–phenol complexes.

The most important factors that determine the rate of enzymatic browning of fruit and vegetables are the concentrations of both active PPO and phenolic compounds present, the pH, the temperature and the oxygen availability of the tissue. Understanding the details of the enzymatic browning process is necessary in order to control it and to obtain a final product that is acceptable to consumers.

## Polyphenol oxidase: An overview

Polyphenol oxidase (1,2-benzenediol:oxygen oxidoreductase; EC 1.10.3.1) is a Cu-containing enzyme, which is also known as catechol oxidase, catecholase, diphenol oxidase, *o*-diphenolase, phenolase and tyrosinase.

PPO is present in some bacteria and fungi, in most plants, some arthropods and all mammals. In all cases, the enzyme is associated with dark pigmentation in the organism, and seems to have a protective function[1]. The fact that PPO is not found in many bacteria, some plants and albinos suggests that it is unlikely to play a vital role in metabolism; thus, it is possible to study its function *in vivo* by working with different types of mutants. Recombinant PPOs have been expressed in organisms that are different from the one that they orginated from or in albino strains of the organisms[2].

In this article we will focus on plant PPOs. PPOs are found in almost all higher plants, including wheat[3], tea[4], potato[5], cucumber[6], artichoke[7], lettuce[8], pear[9], papaya[10], grape[11], peach[12], mango[13] and apple[14], as well as in seeds such as cocoa[15].

In plants, both soluble and membrane-bound PPOs have been described. Histochemical techniques reveal PPOs to be located in the chloroplasts. The PPO gene is encoded in the nucleus and translated in the cytoplasm; the proPPO formed is then transported to the chloroplast[16] where it is cleaved by a protease, producing the active form.

Molecular weights predicted for mature PPOs from cDNA sequences are 58 and ~63 kDa for the mouse and human, respectively, and 128 kDa for mushroom PPO. In plants, predicted molecular weights range from 57 to 62 kDa (Refs 5, 17). Fewer mature protein molecular weights have been directly determined. *Neurospora crassa* and *Streptomyces glaucescens* PPOs are single polypeptide enzymes of 46 and 30.9 kDa, respectively[18,19]. Mushroom PPO is generally thought to contain four subunits with a total molecular weight of 128 kDa, although under some conditions monomeric through to octameric forms are found[20].

So far, all of the PPOs discovered have the ability to convert *o*-dihydroxyphenols to *o*-benzoquinones, using $O_2$ as the second substrate (catecholase activity), but not all PPOs hydroxylate monophenols. The proposed mechanisms of oxidation of both monophenols and diphenols are shown in Fig. 1.

## The PPO substrates

A wide range of *o*-dihydroxyphenols are substrates for the PPOs in higher plants; therefore there is a great deal of potential for browning because of the presence of oxidizable OH groups (oxidizable OH groups are those phenolic OHs that are adjacent, *ortho*, to each other) (Fig. 2). The enzyme phenylalanine ammonia lyase (PAL; EC 4.3.1.5) is involved in the biosynthetic pathway

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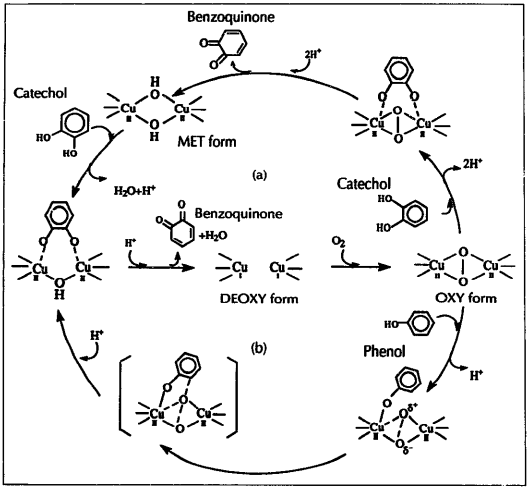


Fig. 1

Proposed kinetic mechanism for polyphenol oxidase in *Neurospora crassa*: (a), oxidation of *o*-dihydroxyphenols, for example catechol, to *o*-benzoquinones; (b), hydroxylation of monophenols, for example phenol, to *o*-benzoquinones. These *o*-benzoquinones will further autooxidize and polymerize via a non-enzymatic mechanism. Possible intermediates are shown. For catechol oxidation, start with the DEOXY form at the center of the figure and move counterclockwise through the upper half (a), then back to the DEOXY form. For monophenol oxidation, start with the DEOXY form and move clockwise through the lower half (b). (Reproduced with permission from Ref. 20.)

of phenolic compounds. When minimally processed lettuce was treated with ethylene, induced PPO and PAL activities increased 1.2–1.7-fold and 2.5–5.3-fold, respectively. Browning intensity correlated with the increased enzyme activity and with the final visual quality of the lettuce[8]. Similar results have been reported for other vegetables such as artichoke[7]. This suggests that the control of PAL activity, and thereby the biosynthesis of phenolic compounds at the site of injury to the fruit and vegetables, is also important in controlling enzymatic browning caused by postharvest treatments.

## Control of enzymatic browning in the food industry

Heat inactivation of PPO is feasible by applying temperatures of >50°C but may produce undesirable colors and/or flavors as well as undesirable changes in texture. Temperatures of >60°C for 3 min are sometimes used to heat treat red grapes before vinification[21].

Polyphenols can be removed by β-cyclodextrins and by insoluble poly(vinyl polypyrrolidone) or poly(ethylene glycol)[22].

Several inhibitors of PPO have been used, mainly benzoic acids and their derivatives. Diamine derivatives of coumarin and 4-hexylresorcinol are effective inhibitors of black-spot formation in shrimp; 4-hexylresorcinol also inhibits mushroom PPO[22] but is not a good inhibitor of grape PPO (M.V. Martinez and J.R. Whitaker, unpublished). 4-Hexylresorcinol only partially prevented browning in apple slices as compared with bisulfite or ascorbate[23].

Two factors already mentioned, pH and oxygen, influence PPO activity as well as subsequent non-enzymatic browning. The adjustment of the pH with citric (lemon juice is frequently used), malic or fumaric acids to pH 4 or below can be used to control browning in juices, fruit slices, avocado, guacamole, etc., as long as the acidity can be tolerated taste-wise[22]. There may be a further decrease in PPO activity below pH 4 due to less tight binding of copper in the active site of the enzyme, permitting chelators, for example citric acid, to remove the copper[22]. A high percentage of molecular $O_2$ can be replaced with either $N_2$ or $CO_2$ to slow down or prevent browning.

The use of reducing compounds, is to date, the most effective control method for PPO browning. Studies with mushroom PPO have revealed that ascorbate, bisulfites and thiol compounds have a direct inactivating effect on PPO[22], in addition to their ability to reduce benzoquinones to *o*-dihydroxyphenols – the reducing compounds are oxidized in the process. The reducing compound sulfite is used by the industry by placing fruit slices in controlled-atmosphere chambers with burning sulfur, which reacts with oxygen to produce bisulfite. There is increasing concern regarding allergic reactions to sulfites in certain individuals, and therefore the residual concentrations of sulfites have been regulated for different commodities. As a result of Food and Drug Administration (FDA) regulations in 1995, sulfites are no longer used in salad bars[24].

As oxygen is required by PPO at the site of wounding to initiate the browning reaction, the use of $O_2$-impermeable packaging or edible films may be useful in preventing the onset of browning. The exclusion of $O_2$ is also used in juices and wines by bottling them under nitrogen. Prevention of mechanical bruising during the shipping of fresh fruit is important to prevent $O_2$ accessibility; compression and vibration can be prevented by the use of pulp board to cushion individual fruit pieces.

## New approaches for the control of enzymatic browning

Despite the fact that the involvement of PPO in browning has been studied for more than a century, many questions still remain about the enzyme itself as well as the browning mechanism. Any new approach for controlling PPO activity needs to be based on basic research. X-ray crystallography and site-directed mutagenesis may help decipher the complex interactions essential at the active site[25]. Site-directed mutagenesis of histidine residues 62 and 189 has shown these residues to be important in Cu binding[26]. Research on the biochemical processes that occur on wounding is important to establish the function of PPO *in vivo*[27]. If we wish to decrease the production of an enzyme *in vivo*, we need to know the possible effects of that manipulation. Current research on genetic engineering methods such as antisense RNA and gene silencing (see below) will help increase our understanding of the functions of PPO and how to control them to improve crop quality.

Molecular biology techniques have helped explain the confusion regarding the multiple forms of PPO isolated from many fruit and vegetables. In tomato, a gene family comprising at least seven nuclear genes has been described[17]; there are differences in their 5′ promoter regions that may regulate their differential expression. Five different PPO cDNAs were found in a potato tuber cDNA library[28], suggesting that there are at least five different PPO genes or allelic variants of the PPO gene. Three cDNA clones were found for *Vicia faba* (broad bean) PPO[29]. In grape, only one gene has been postulated based on Southern analysis[11].

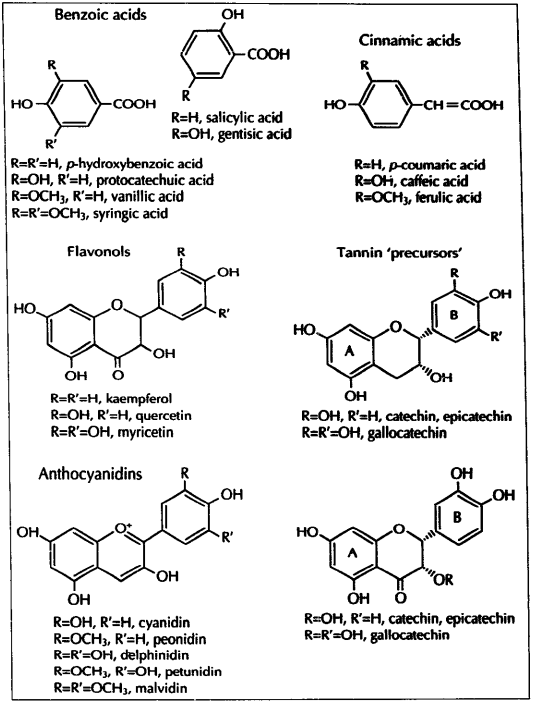


**Fig. 2**
Families of phenolic compounds commonly found in both fruit and vegetables.

There are two conserved amino acid sequence regions in all published PPO sequences (see Fig. 3). Most of the histidines are present in these regions (with five conserved histidines in the two regions of all PPO sequences determined). The two regions seem to correspond to the active site of the enzyme and show good correlation with the accepted enzymatic mechanism and previous physicochemical data[20].

## Antisense RNA approach for the control of PPO

A novel approach for the control of PPO *in vivo* is the use of antisense techniques[30]. Recently, antisense RNAs have been found to selectively block the gene expression of other plant enzymes, such as polygalacturonase and peroxidase in tomato[31]. A gene, or a significant part of it, is introduced into the plant cells in a reverse orientation. The simplest explanation of how such an approach controls the expression of a particular protein is that the mRNA encoded by the antisense gene hybridizes with that encoded by the endogenous gene and thus the protein product is not made (Fig. 4).

The expression of PPO in potatoes has been decreased by using vectors carrying antisense PPO cDNAs[28]. Either full-length PPO cDNAs or a 5′ 800 base-pair section of two classes of genes found in an expression library from potato tubers were used to make the constructs. About 70% of the transformed plants had lower PPO activity than the controls. On visual scoring, a significantly lower level of discoloration was noted. When PPO was inserted in the sense orientation, very high

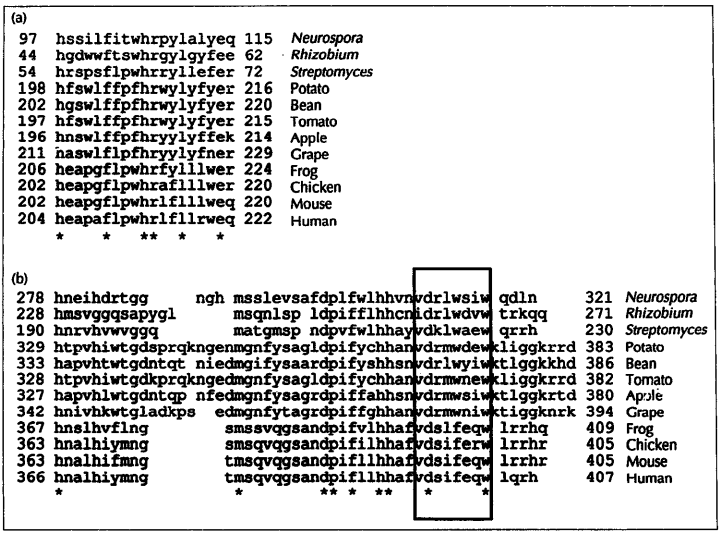


**Fig. 3**

Alignment of two significantly conserved regions, (a) and (b), in the amino acid sequences of some polyphenol oxidases (PPOs). Deduced amino acid sequences show five histidines thought to be associated with the PPO active site. The asterisks (*) indicate 14 amino acid residues that are conserved in all 12 PPO sequences. The boxed sequence has been used to design specific rapid amplification of cDNA ends – polymerase chain reaction (RACE–PCR) primers for cloning PPO from *Vitis vinifera* cv. Grenache (M.V. Martinez and J.R. Whitaker, unpublished).

PPO activity was found in the lines expressing the construct. In this case, sense suppression did not occur. Some of the transgenic lines chosen for field trials did not grow; however, the authors suggested that this might be due to somaclonal variation (genetic changes that occur in somatic cells, that is derived from the leaf, during growth in culture) rather than to decreased expression of PPO. However, the transgenic lines that grew did so as vigorously as the normal plants, produced chlorophyll to the same extent and produced tubers that were normal except that they did not brown when bruised. More field experiments, as well as sufficient testing to meet FDA regulations, will be required before these potatoes can be commercialized, but the absence of aberrant phenotypes suggests that this approach may be applied to a variety of crops.

Antisense RNA techniques have several uses in plant research. They can be used to find answers to questions such as the *in vivo* function of a particular gene(s) and its biochemical mode of action. They can also be put to more practical use for crop improvement. Gene silencing in transgenic plants uses antisense techniques, and has received much attention in recent years. The expression of a transgene (i.e. a gene that has been introduced into plant cells through molecular biology techniques) or an endogenous gene seems to be affected by the presence of a homologous transgene, resulting in gene silencing – the disappearance of expected phenotypic results. *Cis*-inactivation, paramutation and co-suppression are the three postulated modes of homology-dependent gene silencing[32]; these types of gene silencing may be due to transcriptional or posttranscriptional processes.

Antisense experiments have led to, and are associated in some cases with, attempts to control the expression of particular RNAs by the expression of a synthetic ribozyme that is specific for them. In a cell-free system, ribozymes specific for acetyl-CoA carboxylase mRNA (ACC mRNA) cleaved ACC mRNA at the expected sites[33]. Preadipocyte cells showed a substantial reduction in the amount of ACC mRNA as compared with non-ribozyme-expressing cells when they were transfected with the ribozyme gene. Expression of PPO

mRNAs might be controlled in this way; a reduction in browning would be accomplished by reducing the amount of protein formed.

## Plant cell transformation

Molecular techniques and the transformation of plant cells lead to the development of transgenic plants from single transformation events. The transformation of plant tissue cultures with DNA constructs is a method of introducing foreign DNA into plant cells. There are several methods of achieving this transformation; the most commonly used one involves the plant pathogen *Agrobacterium* (both *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* are used depending on what part of the plant is infected), which inserts the desired genes into the chromosome of the plant cell. If the inserted genes are placed under the control of a constitutive promoter DNA sequence, they are expressed along with other 'native' genes that are encoded chromosomally. A summary of tissue culture and transformation procedures is shown in Fig. 5.

Some plants are more amenable than others to genetic transformation and the production of new proteins. *Arabidopsis* and tobacco are the most common model systems used experimentally because of their shorter generation times and their well-known genetic make-up. Transformation research and the production of transgenic plants in the case of both monocots and woody species is advancing more slowly. Although the frequency of stable transformation is low, the direct uptake of DNA and biolistics (the introduction of DNA-coated metal particles into living cells using a gun-like apparatus)

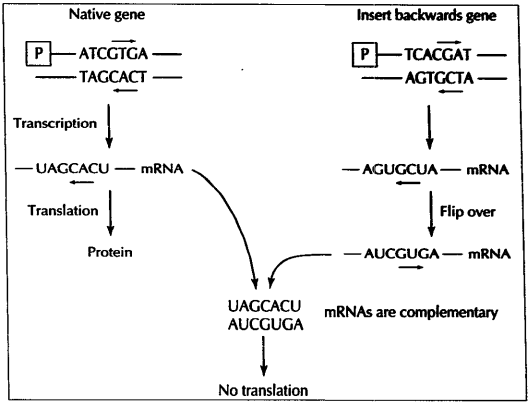


**Fig. 4**
Simplified schematic showing how antisense RNA can be used to control gene expression at the translational level (P represents the promoter).

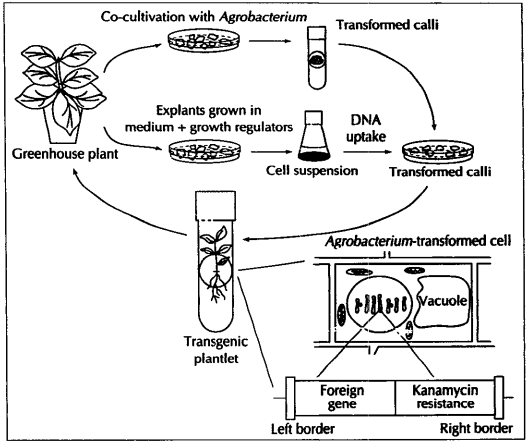


**Fig. 5**
Procedures for the transformation of existing plants with engineered genes. Any plant organ can be removed and used as an 'explant' in sterile tissue culture to produce transgenic callus cultures through several techniques such as co-cultivation with an *Agrobacterium* strain or DNA uptake through biolistic transformation. The transformed calli may produce transgenic plants if regeneration from transformed cells is possible.

are applicable to such plants[34]. DNA uptake may also be facilitated by the use of vehicles, such as liposomes, that can pass through the cell membranes[35]. There is still much work to be done before the production of transgenic woody plants is fully accomplished[36].

## Conclusions

Current approaches to the understanding and control of enzymatic browning caused by PPO have been reviewed together with the developing technologies that will make it possible to obtain crops of improved quality for marketing and storage. Some tropical crops such as papaya, mango and avocado are difficult to ship to other countries without bruising. New approaches are needed to improve the shipping and storage lives of these fruit so that they can reach far away markets; it is hoped that this will have a positive effect on the economies of tropical countries and in the year-around availability of fruit and vegetables to consumers in other countries.

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