17
Diphenol Oxidases, Enzyme-catalysed Browning and Plant Disease Resistance

JOHN R.L. WALKER * AND PETER H. FERRAR

Department of Plant and Microbial Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

Introduction

Enzyme-catalysed browning is a commonly observed phenomenon in the world around us. Examples of this reaction are seen regularly when fruits and vegetables are bruised or wounded, in the melanized exoskeleton of insects and the tanning of leather.

Enzymic browning, as opposed to Maillard-type non-enzymic browning, is catalysed by two major groups of enzymes: the diphenol oxidases (a loosely defined term which will be discussed later) and the peroxidases. The relative contributions of these two groups of enzymes to the resulting melanins is still not resolved clearly and is likely to differ between organisms. A generalized reaction scheme for diphenol oxidases is given in *Figure 1*, where it may be seen that mono- and di-hydroxy phenolics are oxidized to the corresponding quinones with the concomitant reduction of molecular O_2. These quinones are highly reactive and will polymerize, either with themselves, or with amino acids or proteins, to yield a complex, heterogeneous high molecular weight structure generally called 'melanin' or 'melanoidin'; this is usually brown but can also be red, black, blue or various combinations of these (Mason and Peterson, 1965; Pierpont, 1969; Walker, 1975; Walker, 1995; Walker and Ferrar, 1996).

Plants normally contain both the enzymes and the substrates for this reaction but melanin production does not become evident until the cell's internal organisation is damaged in some way. Reasons for this apparent lack of reaction include:

(i) Compartmentalization of enzyme and substrate in different parts of the cell. This often includes vacuolar compartmentalization of phenolic substances (Vaughn *et al.*, 1988; Mayer and Harel, 1979) or sequestration of phenolics in special cells (Mayer and Harel, 1979).

*To whom correspondence may be addressed (E-mail, Walker@BOTN.canterbury.ac.nz)

Biotechnology and Genetic Engineering Reviews – Vol. 15, April 1998
0264–8725/98/15/457–498 $20.00 + $0.00 © Intercept Ltd, P.O. Box 716, Andover, Hampshire SP10 1YG, UK

(ii) The enzyme is present in an inactive or latent form requiring activation (Vámos-Vigyázó, 1981; Vaughn and Duke, 1984).

(iii) The substrate present as a precursor (Robb, 1984) such as a glycoside (Baruah and Swain, 1959).

(iv) The enzyme is complexed with an inhibitor (Satô, 1980a,b)

(v) Any quinone formed being recycled at the expense of a reducing agent (Robb, 1984).

It is possible that more than one of these processes may occur simultaneously in a particular plant (Robb, 1984). Diphenol oxidases appear to be ubiquitous throughout the plant kingdom (Vámos-Vigyázo, 1981), although are absent from citrus fruits and some other higher plants (Robb, 1984).

Classification of diphenol oxidases

The specificity and classification of these enzymes is still an area of continuing confusion (Schwimmer, 1981). There are two basic types: one, variously called catecholase, catechol oxidase, *ortho*-diphenol oxidase (*o*-DPO), *o*-dihydroxyphenol oxidase, phenolase, polyphenol oxidase (PPO), or tyrosinase, will oxidize *o*-dihydroxy phenolics to the corresponding *o*-quinone (the so-called 'catecholase' or 'oxidase' activity). Some forms of this enzyme from plant sources also exhibit a monohydroxylating 'cresolase' or 'hydroxylase' activity (Zawistowski *et al.*, 1991).

The other type of diphenol oxidase catalyses the oxidation of both *o*- and *p*-dihydroxy phenols, has a much broader substrate range but does not exhibit monohydroxylase activity. This enzyme has been referred to as *p*-diphenol oxidase (*p*-DPO) but, since it also readily oxidizes *o*-diphenols, the trivial name 'laccase' is more frequently used.

The International Commission on Enzymes originally referred to two enzymes in the category of polyphenol oxidases; catechol oxidase or *o*-diphenol: oxygen oxidoreductase (EC 1.10.3.1) and laccase or *p*-diphenol: oxygen oxidoreductase (EC 1.10.3.2). The nomenclature was later revised, lumping the two enzymes under the one heading: EC 1.12.18.1 which Mayer and Harel (1979) described as 'unfortunate'. More recently the international nomenclature was changed again. Cresolase activity was

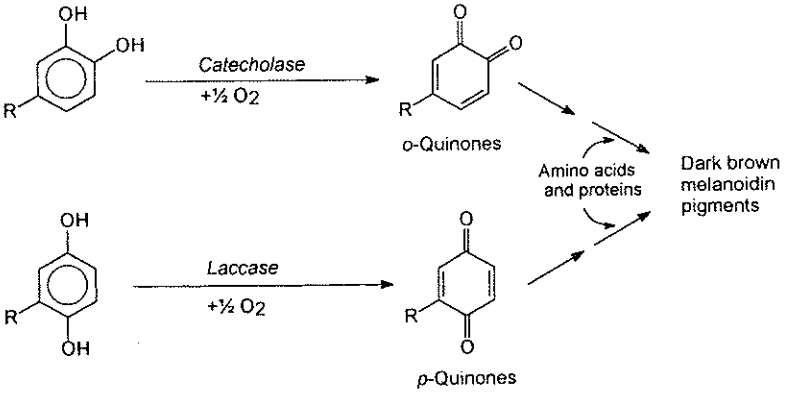

Figure 1. Generalized reaction scheme for catecholase (*o*-DPO) and laccase (*p*-DPO).

given the title monophenol monoxygenase (EC 1.14.18.1), catecholase activity became diphenol oxygen: oxidoreductase (EC 1.10.3.2) and laccase activity became labelled EC 1.10.3.1 which differentiates between cresolase and catecholase activity but does not distinguish clearly between distinct plant and fungal laccases; according to Mayer (1987) 'it is no improvement'.

In this review the terms 'catecholase' (*o*-DPO) and 'laccase' (*p*-DPO) will be used.

Reaction mechanisms and structure of diphenol oxidases

The reaction mechanisms of catecholase and laccase differ markedly and must be considered separately.

CATECHOLASE REACTION MECHANISMS AND STRUCTURE

Analysis of catecholase kinetic reactions by steady-state methods is difficult because the enzyme often involves three substrates in an irreversible reaction. However, a number of studies suggest that a sequential, rather than a ping-pong, mechanism is likely (Robb, 1984). Some early studies suggested that O_2 binds first in an ordered *bi,bi* mechanism (Matheis and Belitz, 1977) whilst others favoured a more random mechanism where O_2 does not bind first (Duckworth and Coleman, 1970; Gutteridge and Robb, 1975; Lerner and Mayer, 1976). These discrepancies could be due to use of an impure enzyme leading to secondary O_2-consuming reactions, to the inactivation of catecholase by its products, or to errors common in spectrophotometric analyses (Janovitz-Klapp *et al.*, 1990a,b). In a recent study which used a kinetically derived formula for predicting O_2 consumption from the relative concentrations and kinetic parameters of each phenolic substrate, it was concluded that O_2 did bind first in an 'ordered' reaction mechanism (Janovitz-Klapp *et al.*, 1990a,b).

Originally it was reported that catecholase may have two distinct active sites, one for cresolase (mono-hydroxylase) and one for catecholase activity (Mayer and Harel, 1979; Vámos-Vigyázó, 1981). This enzyme has also been reported to act as a peroxidase in the presence of H_2O_2 (Strothkamp and Mason, 1974) whilst Sugumaran (1986) reported that a catecholase from insects was capable of the oxidative decarboxylation of 3,4-dihydroxymandelate. Sugumaran *et al.* (1987) reported that this enzyme could also dimerize 1,2-dehydro-N-acetyl dopamine, but the former activity was due to the instability of the product quinone rather than to direct enzyme catalysis. They proposed that this was rapidly decarboxylated to generate a transient quinone methide which subsequently rearranged to yield 3,4-dihydroxybenzaldehyde (Sugumaran *et al.*, 1991; Sugumaran *et al.*, 1992). Quinone methides are structurally analogous to quinones, except that one of the carbonyl oxygens is replaced by a methylene group. Due to their highly electrophilic nature, they have been implicated in a number of biological processes including insect sclerotization and lignin formation (Thompson *et al.*, 1992).

The classical experiments of Kubowitz (1938) established copper as an essential component of catecholase activity; this was confirmed by inhibition by cyanide and the subsequent restoration of activity upon addition of Cu^{2+} (Zawistowski *et al.*, 1991). The role of Cu in this enzyme has been investigated by many workers (Kubowitz, 1939; Fling *et al.*, 1963; Gutteridge and Robb, 1975; Lerch, 1976). Recent work by

Lerch and others (Lerch, 1978, 1981; Lerch *et al.*, 1986) revealed that the active site had two anti-ferromagnetically spin-coupled copper atoms (Cu, type III) in close proximity (about 3.5Å apart). One of these appeared to be bound to three histidine residues (His-188, His-193 and His-289), the other by just one (His-306) and the structure of the active site depended on the oxidation state of the copper. Current understanding of the mechanism of the catecholase reaction has been reviewed by Zawistowski *et al* (1991) and Sánchez-Ferrer *et al.* (1995). It is suggested that the enzyme cycles through deoxy-, oxy- and met- (deoxygenated enzyme with oxidized copper) forms which allows for both cresolase and catecholase activity to occur at the same active site.

A major problem in this area are the discrepancies between different reports, which may be attributable to different sources of enzyme. These cite differences in molecular weight, number of subunits, kinetic and electrophoretic properties and pH optima of catecholases from different sources. In addition the maturity of the source and plant organ or organelle, or artefacts formed during purification, may alter the results observed (Galeazzi *et al.*, 1981; Gutteridge and Robb, 1975; Halim and Montgomery, 1978; Hasegawa and Maier, 1980; Janovitz-Klapp *et al.*, 1989; Kelly *et al.*, 1990; Lam and Ho, 1990; Leoni and Palmierri, 1990; Matheis and Belitz, 1977; Murao *et al.*, 1993; Murata *et al.*, 1992; Nakamura *et al.*, 1983; Palmer, 1963; Park and Luh, 1985; Racusen, 1969; Sato, 1982; Walker, 1964a, 1964b; Wong *et al.*, 1971; Yurkow and Laskin, 1989; Zawistowski *et al.*, 1988a,b; Zhou *et al.*, 1993). It is still not clear how reliably information obtained from one source of catecholase can be extrapolated to another.

Genetic characterization of some chloroplastic catecholases has revealed that they often have a transit peptide attached to the primary structure of the enzyme which is cleaved off once the protein is within the chloroplast membrane. For example, the biosynthesis of catecholase in grapes appears to involve the synthesis of a 67 kDa precursor protein which is imported into the chloroplast and which subsequently loses a 10.6 kDa transit peptide from the N-terminus and a 16.2 kDa peptide (this is peculiar to grapes and its function is unknown) from the C-terminus (Dry and Robinson, 1994).

LACCASE STRUCTURE AND REACTION MECHANISM

Considerable data has accumulated regarding the structure of laccases (Yaropolov *et al.*, 1994). In general these enzymes are glycoproteins containing Type I copper, which is responsible for the pale blue colour of the pure enzyme. Evidence is accumulating that the laccases of fungi and of higher plants are distinct entitities warranting separate classification since these enzymes differ in many respects, especially substrate specificity (Mayer, 1987). Unfortunately, apart from the enzyme from the Japanese lacquer tree *R. vernificera* (Nakamura, 1958), few laccases from higher plants have been isolated and described in detail.

A number of fungal laccases have been characterized in detail, including those from *Agaricus bisporus* (Wood, 1980), *Coriolus (Trametes) hirsutus* (Kojima *et al.*, 1990), *Phlebia radiata* (Karhunen *et al.*, 1990; Saloheimo *et al.*, 1991), an unidentified basidiomycete PM1 (Coll *et al.*, 1993) and *Armillaria mellea* (Billal and Thurston, 1996; Ferrar *et al.*, 1995). During the morphological development of many fungi, a number of distinct laccase isoenzymes may be produced; these differ in their substrate

Table 1. Properties of laccases

Laccase source	MW (kDa)	Glycosyl-ation (%)	No. of Cu atoms	Optimum pH	Reference
Agaricus bisporus	100	15	2	4.0–5.6	Wood (1980)
Armillaria mellea	60	19.3	?	3.75–4.0	Rehman and Thurston (1992); Ferrar *et al.* (1995), Billal and Thurston, 1996
Basidiomycete PM1	64	6.5	4	4.5	Coll *et al.* (1993)
Coriolus versicolor	63		4	4–5	Thurston (1994)
C. hirsutus	63	15	4	4.5	Kojima *et al.* (1990)
Neurospora crassa	64	11	4		Froehner and Eriksson (1974)
Phlebia radiata	64	5–15	2	4.5	Karhunen *et al.* (1990)
Rhus vernicifera (higher plant)	110	45	4,6	7.5	Nakamura (1958); Mayer and Harel (1979).

specificity as well as in the length and composition of their polypeptide chain. Microheterogenerity is often observed within isozyme types but this is probably due to variations in their carbohydrate moieties (Graziani *et al.*, 1990).

Some properties of laccases are summarized in *Table 1*.

Analysis of the primary structure of many laccases reveals considerable variation in amino acid composition, and the observed heterogeneity suggests that there are distinct families not just the result of minor evolutionary changes of an ancestral progenitor enzyme (Mayer and Harel, 1979). Alignment of the primary sequences of several different laccases with other blue copper oxidases reveals considerable homology between the conserved sequences of these enzymes (Reinhammer, 1984; Messerschmidt and Huber, 1990). In addition, comparison of the N-terminal sequences of laccase from basidiomycete PM1, *C. hirsutus* and *P. radiata* show remarkable similarities in their primary sequence. Thus cloning and sequencing of laccase genes from a variety of organisms may provide clues for the construction of phylogenetic relationships between different laccases (Coll *et al.*, 1993).

Laccases have been found to contain either two or four Cu atoms per molecule (*Table 1*). Those containing four Cu atoms include Type 1, Type 2 and coupled binuclear Type 3 centres. The Type 2 and Type 3 centres form trinuclear Cu clusters which are thought to represent the active site for the binding and multi-electron reduction of O_2 (Cole *et al.*, 1990), as seen with purified laccase from *C. consors* (Sakurai, 1990). Laccases containing two Cu atoms have also been isolated; the enzyme from the white-rot fungus *P.radiata* possessed Type 1 and Type 2 Cu atoms but, instead of a Type 3 Cu, a molecule of pyrroloquinoline quinone (PQQ) was covalently bound as cofactor (Karhunen *et al.*, 1990). PQQ is a electron-transferring group found in many oxidoreductases of plant, microbial, and animal origin (Saloheimo *et al.*, 1991).

The mechanism of the laccase reaction has been reviewed (Reinhammar, 1984; Peyratout *et al.*, 1994) and consists of a single electron oxidation of a diphenol which generates a free radical (semi-quinone) which then converts to the quinone, either by enzyme catalysis or spontaneous disproportionation. Subsequently both the semi-quinone and the full quinone may undergo other non-enzymatic reactions; the most important of these are quinone polymerisation to form melanins and the semi-quinone, single electron mediated oxidation of other compounds. The latter may be responsible

for the oxidation of the phenolic and non-phenolic moieties of lignin (Bourbonnais and Paice, 1990). The electrons gained by the oxidation appear to be retained in the laccase active site by successive reduction of the Cu atoms, until they are finally released to reduce molecular O_2.

An interesting aspect of laccases is their wide substrate range (Lonergan and Baker, 1995). Like the related catecholase, laccases are capable of oxidizing not only *o*-diphenols, but also *p*-diphenols, methoxy-substituted monophenols (e.g. guaiacol), diamines (e.g. *p*-phenylene diamine) and non-aromatics (ascorbic acid). The mechanism for many of these reactions has yet to be elucidated.

POST-OXIDATION POLYMERIZATION REACTIONS

After oxidation of dihydroxy phenolic substrates by catecholase or laccase, the quinones thus formed react further non-enzymatically. The dominant feature of quinone biochemistry is their ability to readily undergo redox reactions involving a semiquinone radical intermediate following single electron reduction (Brunmark and Cadenas, 1987). In this manner the labile quinones formed in the DPO reaction may react either together to yield 'melanoid' pigments, or with amino acids or proteins to ultimately form brown coloured complexes called 'tannins' (Mason and Peterson, 1965; Pierpont, 1969; Walker, 1975) or more generally 'melanins' or 'melanin-proteins'.

The mechanism of polymerisation is not well defined since the final melanin product, and its colour, is dependent on the reactants involved. In the best understood pathway (*Figure 2*) tyrosine is first hydroxylated to 3,4-dihydroxyphenylalanine (DOPA) which is then oxidized to dopaquinone (both steps catalysed by catecholase). This cyclizes to a leuco compound which is then oxidized to give dopachrome, an orange pigment; many catecholase assays are based on the formation of this compound. Following a series of further reactions, dopachrome then polymerizes to form melanin.

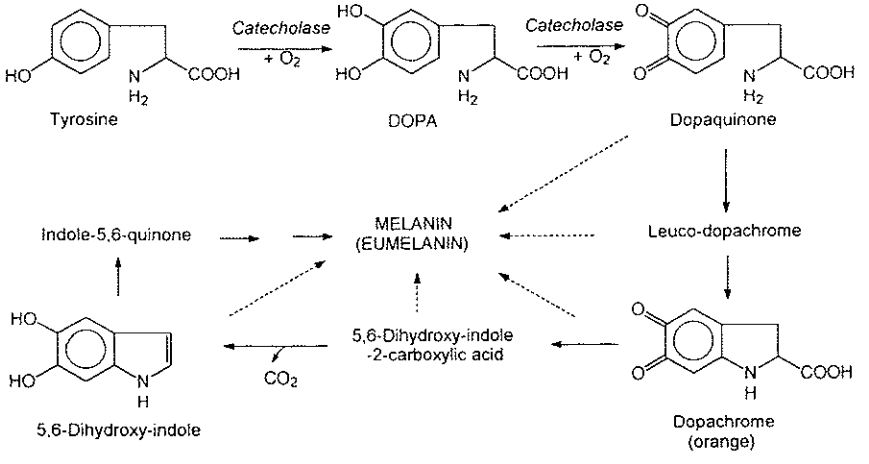

Figure 2. Pathways of melanin biosynthesis from tyrosine (modified from Robb, 1984 and Whitaker, 1985).

The term 'melanin' has now taken on a rather broad meaning to indicate brown polymeric pigments, but the more general term: 'eumelanin' is now favoured (Robb, 1984). 'Pheomelanin' is the name given to the yellow to reddish-brown pigments formed when cysteine combines with dopaquinone and the complex then polymerizes (Prota, 1980). In this chapter the general term 'melanin' includes the range of brown to black polymeric end products of DPO reactions.

ASSAY OF DIPHENOL OXIDASE ACTIVITY

In any study of enzymes and enzyme inhibition, a critical analysis of assay methods is required. Because the final product of DPO oxidation is coloured (melanins), spectrophotometric assays are used widely. One of the most common assays measures the formation of dopachrome from the oxidation of DOPA (or tyrosine) whilst other studies have used catechin (Tan and Kubo, 1990) or other chromogenic substrates. However, spectrophotometric methods which measure the end product of a sequence of reactions, rather than the inital reaction, are vulnerable to error. Furthermore, different substrates yield different final colours so valid kinetic comparisons between substrates are not possible (Walker, 1975). Coupled assays have also been used (Satô, 1980) or addition of quinone complexing agents such as Besthorn's hydrazone (Pifferi and Baldassari, 1973; Espin *et al.*, 1995). The coupled oxidation of ascorbic acid by the quinones generated by catecholase can be quantified and used as an assay.

Polarographic techniques using an O_2 electrode are often employed and, since O_2 is a primary substrate, measures its depletion as the reaction proceeds. This assay is

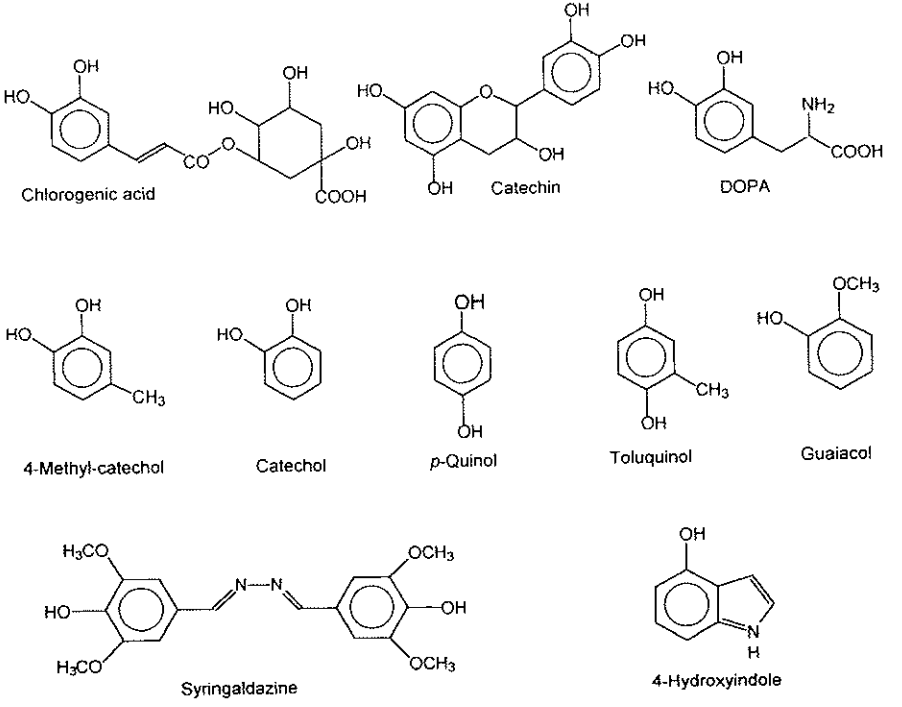

Figure 3. Some natural and artificial substrates for catecholase and laccase. superior

to most spectrophotometric methods since it measures the *initial* rate of the reaction and further reactions are ignored. It also gives significantly higher initial rate measurements compared to other methods and hence is considered the method of choice for DPO assay (Mayer *et al.*, 1966).

DIFERENTIATION OF CATECHOLASES AND LACCASES

The close similarities between catecholases and laccases creates problems for their experimental differentiation. Distinguishing between them is usually based on tests of substrate specificity and the action of selective inhibitors, although the former have limited scope because of the broad range of activities exhibited by both types of enzyme (*Figure 3*). However, the reactivity of laccases with certain substrates can be used to distinguish them; for example, laccases do not display 'cresolase'/hydroxlation activity but do oxidize quinol and toluquinol (1,4-dihydroxy-2-methyl-benzene) and guaiacol. Syringaldazine has proved to be a valuable diagnostic substrate for fungal laccases (Leonowicz and Grzywnowicz, 1981) but it is not always oxidized by laccases from higher plants. Cai *et al.* (1993) investigated a range of hydroxyindoles as substrates for the spectrophotometric assay of laccases; 4- hydroxyindole, which gives a blue oxidation product (l_{max} 615nm), proved to be the most useful and appeared to show little activity with catecholase and to display some specificity for laccases from higher plants (Harvey and Walker, unpublished results).

Selective inhibitors are a valuable tool in such work and one of the earliest examples of their use was by Lindberg (1950) who used carbon monoxide to differentiate mushroom catecholase from laccase; unfortunately its use is only possible with manometric assay systems such as the Warburg respirometer. A number of workers (McCrea and Dugglby, 1968; Walker, 1969; Walker and Wilson, 1975) have shown that cinnamic acids selectively inhibited catecholases whilst Allan and Walker (1988) showed that SHAM (salicylhydroxamic acid) only inhibited catecholase activity. Kahn and Andrawis (1985a) reported that tropolone was a powerful inhibitor of catecholase activity; this was confirmed by Ferrar and Walker (1996) who also reported that it was far less effective against laccase.

Ferrar and Walker (1996) found that 3.3 μM 4-hexyl-resorcinol (4-HR) inhibited catecholase activity, whilst at 33 μM also inhibited laccase activity, which agrees with the earlier results of Dawley and Flurkey (1993a,b) who reported the ability of 4-HR to differentiate between laccase and tyrosinase at low concentrations. However, considering the variation between catecholases, this differentiation is unlikely to be definitive since Dawley and Flurkey (1993a) used only mushroom tyrosinase – which, as shown here, is highly susceptible to inhibition by 4-HR. Again, this highlights the limitations of results obtained using only one source of catecholase.

Quaternary ammonium compounds (such as cetyl-trimethyl-ammonium bromide, CETAB) have been shown to be useful selective inhibitors for fungal laccases (Walker, 1968; Walker and McCallion, 1980; Ferrar *et al.*, 1995). More recently Murao *et al.* (1992) reported that N-hydroxy-glycine was a specific inhibitor for laccases.

Table 2 summarizes these effects and several workers (Walker and McCallion, 1980, Ferrar and Walker, 1996) have used this approach to differentiate diphenol oxidases from a range of sources.

Table 2. Differentiation of catecholase and laccase by selective substrates and inhibitors

Substrate	*Catecholase*	*Laccase*
o-Dihydroxy phenols	Oxidized	Oxidized
p-Dihydroxy phenols	May be oxidized	Oxidized
p-Phenylene diamine	Slow or nil oxidation	Oxidized
Syringaldazine		Oxidized (mauve)
Toluquinol		Oxidized
4-Hydroxy-indole		Oxidized (blue)
Guiacol		Oxidized
Inhibitors		
SHAM	Inhibition	
Tropolone	Inhibition	
Cinnamic acids	Inhibition	
Fusaric acid	Inhibition	
Polyvinylpolypyrrolidone (PVP)	Inhibition	
4-Hexyl-resorcinol (4-HR)	Inhibition	
CETAB, (and other QACs)		Inhibition
N-Hydroxy-glycine		Inhibition
Na Dodecyl sulphate (SDS)	Activation	

Functions of catecholase

At first sight, the functions of catecholase appear to be similar in all organisms, since all *o*-DPOs catalyse the oxidation of phenolics to melanins. However, its postulated roles, especially in plants, are open to question and will be considered separately.

Functions of catecholase in plants

The roles of this enzyme in plants has been a subject of speculation and controversy for some time. Two earlier reviews on this subject commented; 'The plant polyphenoloxidases (DPOs) remain enzymes in search of a function' (Mayer, 1987) and 'despite this nearly ubiquitous occurrence no function for this enzyme has been established' (Vaughn *et al.*, 1988). These statements still reflect the state of current knowledge, but evidence is accumulating to support a number of postulated roles for catecholase (Mayer and Harel, 1991, Walker, 1995).

A new factor to be considered and explained is that mutants of mung bean lacking catecholase, or plants whose synthesis of catecholase has been halted by treatment with tentoxin (a toxin secreted by *Alternaria* spp. which inhibits synthesis of cateholase) die, which implies that this enzyme must play an important role in the life of these plants (Vaughn *et al.*, 1988). However, by contrast, 'Bruce's Sport' grapes (an Australian green and white striped mutant cultivar) do not display browning in the white stripes of the grape, due to an interruption in the cleavage of an inactive 60 kDa precursor protein to yield the active 40 kDa chloroplast protein; yet are still viable.

Biosynthetic roles

Catecholase has long been considered to have a role in *o*-diphenol biosynthesis (Mayer and Harel, 1979) due to its cresolase (hydroxylase) activity but criticism has been levelled at this hypothesis since the cresolase activity of the enzyme is much

slower than the catecholase activity (Robb, 1984). In addition the enzyme is normally bound to, or associated with, plastids (Vaughn and Duke, 1984) whereas phenolics are usually either vacuolar or sequestered in special cells (Mayer and Harel, 1979); thus their localization argues against this role.

Hardening of seed coats

Another suggested role for catecholase is in the hardening of seed coats (Marbach and Mayer, 1975) where the quinones polymerize to melanins and thus harden the coat (c.f. formation of insect exoskeletons). However, Egley *et al.* (1983,1985) demonstrated that, in several species, this was due to quinones produced by peroxidases, rather than to catecholase activity.

Cyanide-insensitive respiration

It has been claimed that the cyanide-insensitive respiration of some plants may be mediated by catecholase rather than by cytochrome oxidase (Mayer and Harel, 1979), although catecholases are inhibited by cyanide. Substituted hydroxamic acids, which inhibit the cyanide-insensitive (alternate electron transfer) pathway (Schonbaum *et al.*, 1971) are powerful inhibitors of cateholase (Rich *et al.*, 1978; Mayer and Harel, 1979).

It has also been claimed that catecholase may serve as a general oxidase in respiration where an *o*-diphenol may be oxidized to the corresponding quinone, which in turn oxidizes some other cellular constituent, whilst itself being reduced (Mayer and Harel, 1979). This role could be extrapolated to include a range of redox phenomena in the cell.

Role in photosynthesis

For many years catecholase has been known to be present in chloroplasts, where it is usually tightly bound to the thylakoid membranes, and from which it may be released by detergent treatments or tryptic digestion (see Trebst and Depka, 1995). Vaughn and Duke (1984) gave some credence to a role in photosynthesis by reporting that catecholase is nuclear encoded but is still inactive until incorporated into the plastids of higher plants where it is involved in the Mehler reaction (which mediates the photoreduction of molecular O_2) in chloroplasts (Vaughn *et al.*, 1988). Lax and Vaughn (1991) demonstrated the structural association of catecholase with Photosystem II in a number of higher plants and suggested a possible role in the electron transport chain of that photosystem.

Plant disease resistance and chemical warfare

An often postulated role for this enzyme has been in the resistance of plants to disease. In the intact cell the enzyme and its phenolic substrates are kept separated with the enzyme being cytosolic or membrane bound, whilst its substrates are sequestered in the vacuole. When wounded, the vacuolar membrane is lysed and hence enzyme and substrate make contact so that reactive quinones are formed which polymerize to yield

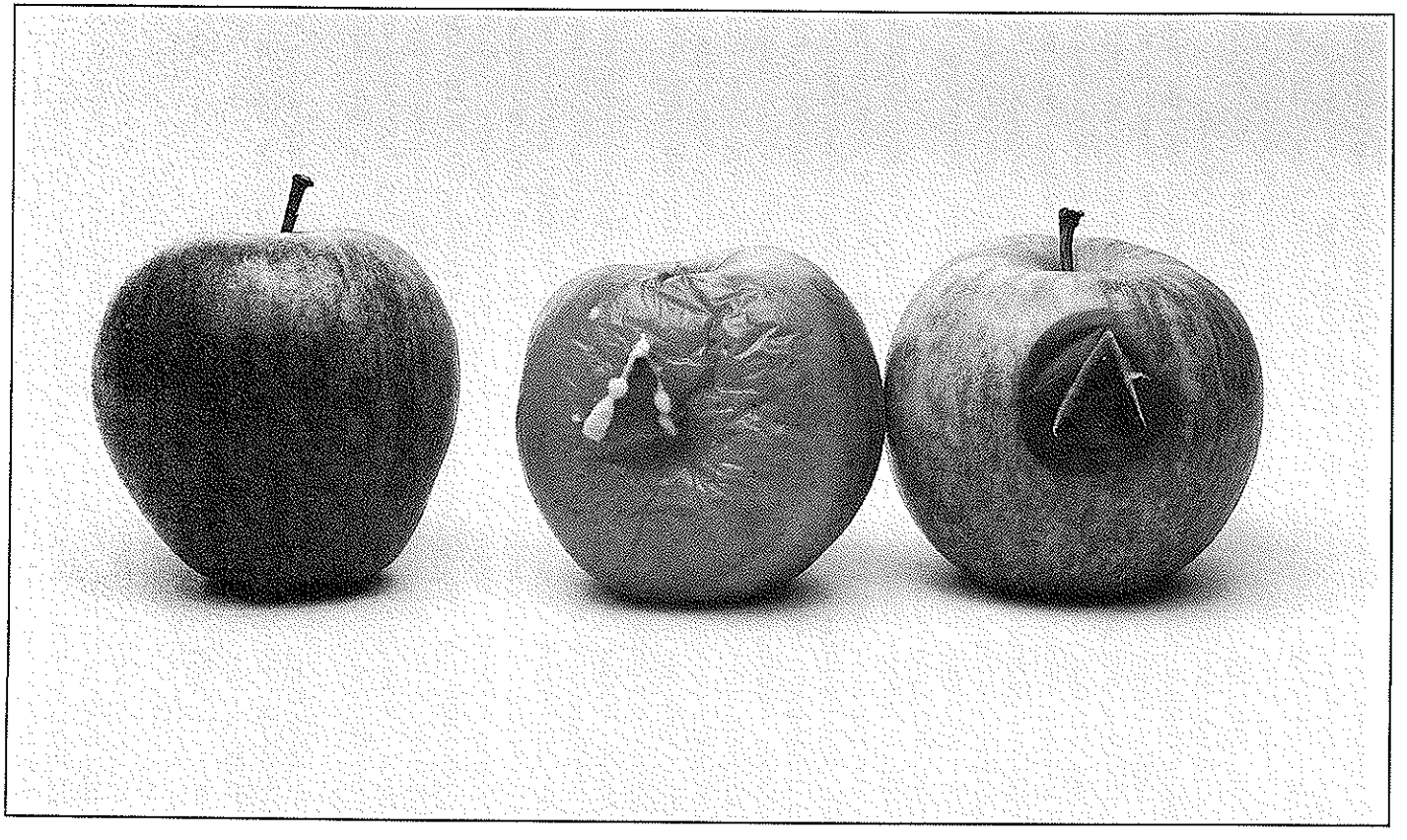

Plate 1. Infection of apples by *Penicillium expansum* ('blue mould', left) and *Glomerella cingulata* ('bitter rot', right). Note the lack of browning in the *P.expansum* infected fruit.

melanins; other reactions may also occur. Wounding also appears to trigger a shift in the plant's metabolism towards increased biosynthesis of phenolic compounds, thus providing more substrates for catecholases as well as many fungitoxic and bacteriostatic compounds (Farkas and Kiraly, 1962). Recent studies have demonstrated the induction of catecholase mRNA after wounding or the manifestation of physiological disorders in apples (Boss *et al.*, 1995) and potatoes (Thipapong *et al.*, 1995), thus providing evidence for the transcriptional control of catecholase expression.

Wounding which occurs naturally, or that induced by pathogens, has been shown to produce similar effects in plants (Moustafa and Whittenbury, 1970; Gentile *et al.*, 1988) and this is evident as enzymic browning. This may protect the plant in two ways; first the melanins thus produced seal off the site of infection or wound forming a physical barrier to further infection. Secondly, the enzymically produced quinones will polymerize with both the host's proteins but also with exo-enzymes produced by phytopathogens and hence negate their phytotoxicity. Zinkernagel (1986) has suggested that catecholase may also be involved in the oxidative detoxication of pathogen-produced phytotoxins.

In the light of recent evidence the authors believe that there is now a considerable body of evidence to support an active role for diphenol oxidases in plant disease resistance. Assuming this to be the case, the diphenol oxidase enzyme/phenolic substrate combination represents a major hurdle to successful colonization by the phytopathogen since the reactive quinones produced by this system will inactivate (tan) its enzymes and other proteins. So, if an invading organism can inactivate this biochemical defence system, it will greatly improve its chances of successful parasitism; one way to do this is by the secretion of a diphenol oxidase inhibitor to prevent the formation of the reactive quinones.

For example, Walker (1969) reported that the lack of browning observed in apples infected with *Penicillium expansum* ('blue mould') was due to conversion of the host apple's phenolics into new compounds inhibitory towards catecholase and he found elevated levels of *p*-coumaric and ferulic acid in the infected tissue. It was also found that the fungus secreted an extracellular catecholase inhibitor ('expansin') which reached a maximum concentration just prior to sporulation (Walker, 1970). Thus the apple's diphenol oxidase defence system was blocked and the fungus spread rapidly. By contrast *P.citrinum*, which infects citrus fruit, does not produce a diphenol oxidase inhibitor and this correlates with the absence of a diphenol oxidase system in citrus fruits.

More recently Ferrar and Walker (1993) demonstrated a similar suppression of browning in apples infected with *Sclerotinia sclerotiorum* and found that this fungus secreted large amounts of oxalic acid which inhibited the fruit's catecholase. By contrast *Sclerotia rolfsii* prevented enzymic browning in beans by secreting large quantities of oxalic acid which reduced the local pH from 6.6 to 3.0, a pH where the bean catecholase was inactive. Thus, in both examples, the catecholase–phenolic defence system was effectively blocked and fungal growth could proceed unimpeded. This hypothesis is also supported by further work (see below) which found that many phytopathogens secreted potent diphenol oxidase inhibitors (Ferrar, 1995; Ferrar and Walker, 1997).

Whilst this role for catecholase is generally accepted to occur in plant–pathogen interactions, the function of catecholase in plant defence still remains contentious

(Kosuge, 1969; Farkas and Kiraly, 1962; Mayer and Harel, 1979; Mayer, 1987). Many studies have correlated increased catecholase activity with a plant's resistance to a phytopathogen as compared to susceptible varieties (Mukherjee and Ghosh, 1975; Matta and Gentile, 1970; Maxemiuc-Naccache and Dietrich, 1985; Fric, 1976; Arora and Bajaj, 1985; Trajkovski, 1976). However, other studies of plant–pathogen systems have not shown such a correlation (Brueske and Dropkin, 1973; Conti *et al.*, 1982) or reported a higher catecholase activity in susceptible plants (Pollock and Drysdale, 1976). Mayer and Harel (1979) concluded that 'the present picture certainly does not preclude a role in disease resistance, but it also fails to prove such a role'.

Recently, evidence has been presented that catecholase acts as part of an integrated insect pathogen defence mechanism in *Solanum berthaultii* (the wild potato). This potato has two types of glandular trichome on its foliage; types A and B. Type B trichomes are quite simple with an ovoid gland at the tip which continually secretes a clear viscous exudate which contains sucrose esters of short chain fatty acids (King *et al.*, 1987). This induces an agitated state in a broad range of insect pest species (aphids, leafhoppers, flea beetles, spider mites and potato tuber moths) causing increased movement with subsequent accumulation of the sticky acyl sugars on their tarsi. The type A trichome is easily broken by the increased movement and stickiness of the insect tarsi and releases its contents which include a catecholase (Kowalski *et al.*, 1993) which, in turn, catalyses the oxidative polymerization of other secreted components on the mouthparts and tarsi of the insect leading to its entrapment and death. In this system, catecholase would seem to act as a plant defence protein and work is currently in progress to attempt to breed this into the common potato (*Solanum tuberosum*) since this species has fewer trichomes but is far more important commercially (Kowalski *et al.*, 1993).

FUNCTIONS OF CATECHOLASES IN MAMMALS

In mammals the function of catecholase is better understood due probably to a much simplified role with fewer ambiguities. Its main function is in skin pigmentation and its absence is usually identified with the genetic disorder albinism. Although the mammalian enzyme catalyses both tyrosine hydroxylation and DOPA oxidation, it does not appear to be involved in the synthesis of DOPA derivatives such as hormones or catecholamine neurotransmitters.

In skin, catecholase is restricted to melanosomes within specialized cells termed melanocytes (Jimbow *et al.*, 1976). Its activity is controlled by melanocyte stimulating hormone which directs an adenylate cyclase-mediated activation in premelanosomes. The usual substrate is tyrosine, which is hydroxylated to DOPA, and then oxidized to dopaquinone; the rest of the melanin synthesis (both eu- and pheo-melanin) occurs non-enzymatically.

Melanin has a variety of important functions; as a cosmetic entity participating in protective colouration and in sexual attraction within species, as a protection against ultraviolet radiation, as a scavenger of cytotoxic radicals and metabolic intermediates, and as a participant in developmental processes, particularly of the nervous system (Spritz and Hearing, 1994).

FUNCTIONS OF DIPHENOL OXIDASES IN MICRO-ORGANISMS

Diphenol oxidases have been found in most fungi investigated as well as in a number of bacteria (Mayer and Harel, 1979). However, like plants, the role in micro-organisms is still poorly understood. Possible roles include pigment formation (Kelly *et al.*, 1990), involvement in fruiting body formation (Mayer, 1987) and oxidation of extra-mitochondrial dinucleotides (Bull and Carter, 1973) although evidence for this latter role is 'not very convincing' according to Mayer and Harel (1979).

It is interesting that *Mycobacterium leprae,* the cause of leprosy, infects humans in DOPA rich areas by means of a catecholase (Prabhakaran *et al.*, 1969). Inhibition of the enzyme has been considered as a possibility for chelation therapy of patients with leprosy. (Anon, 1973).

FUNCTIONS OF DIPHENOL OXIDASES IN INSECTS

In insects both catecholase and laccase are present and their roles fall into two distinct categories: sclerotization and defence.

At each moulting of an insect's larval stage the new chitinous cuticle, laid down outside the epidermis, hardens by a process known as sclerotization (Robb, 1984). During sclerotization tanning agents are secreted by the epidermal cells into the cuticle where they are oxidized by diphenol oxidases to give reactive quinones which can polymerize with proteins and other components such as N-acetyl-dopamine (Andersen, 1985). Both catecholase and laccase have been isolated from insect epidermis and haemolymph so this function, although using a different substrate, is not too dissimilar to melanogenesis in plants or mammals.

The possibility of diphenol oxidases being involved in insect's defence against bacterial pathogens was first postulated by Taylor (1969). In a defensive role both enzymes are active in the haemocoel where invading bacteria may be lysed or agglutinated and subsequently cleared from the haemolymph in melanized clumps. The same probably occurs in the cuticle where polymerized bacterial cells may become part of the tanned cuticle itself (Ratcliffe *et al.*, 1985; Klein and Jackson, 1992).

Laccase is also active in the formation of various *p*-quinones in some species of Coleoptera, such as the bombadier beetle, as part of their defence against bacterial pathogens (Blum, 1981).

CONCLUSION

Two dominant themes reoccur regarding the roles of catecholase, irrespective of the source and despite the various hypotheses that have been forwarded. The first of these is melanin biosynthesis which may have a variety of consequences such as pigmentation, protection from damaging UV (Griffin, 1993), wound sealing and hardening of tissue. The second role seems to be defence orientated via the reactive quinones generated and which are toxic to microbial pathogens.

Functions of laccase

ROLES OF LACCASE IN FUNGI

A number of roles have been proposed for laccases in fungi. Several reports suggest

a correlation of changes in laccase activity with fruiting body development in Basidiomycetes (Wood, 1985) and in *Schizophyllum commune* fertile mycelia were shown to be associated with laccase activity which was absent in sterile mycelia (Leonard, 1971).

Laccase is involved in pigmentation of fungal tissues via the melanin-forming reactions. For example, in *Aspergillus nidulans*, conidium pigmentation was related to the appearance of a conidial laccase (Clutterbuck, 1972) whilst a different laccase was responsible for pigmentation in the huille cells and cleistothecium (Herman *et al.*, 1973). The role of laccase in the enzymic browning of *Agaricus bisporus* was investigated by Aldridge and Walker (1980) who found laccase in the mycelium and catecholase in the basidiocarp.

A number of workers have reported that laccases may play a role in phytopathogenicity (Binz and Canevescini, 1996; Wahleithner *et al.*, 1996). Laccase production has been suggested as a necessary, but not the only, requirement for infection of cucumber fruit by *Botrytis cinerea* (Bar-Nun *et al.*, 1988) whilst the cucurbitacins (cyclic triterpenoid phytoalexins characteristic of the Cucurbitaceae) repressed the induction of laccase in *B. cinerea* (Bar-Nun and Mayer, 1989; Gonen *et al.*, 1996). Laccase production by *B. cinerea* was reported to be induced, first by a phenolic substance and secondly by the products of pectin degradation (Marbach *et al.*, 1985); thus it was induced during cell wall degradation following successful infection. Laccase has also been implicated in rhizomorph (Worral *et al.*, 1986) and appresorial (Kubo *et al.*, 1982, 1983) formation. The melanins thus produced have been reported to play an important role in disease development including protection of the hyphae from cell-wall degrading enzymes (De Cal and Melgarejo, 1994).

Induction of laccase secretion by phenolics may also indicate a role in the detoxification of antifungal agents, which are often phenolic in nature, by oxidation and polymerisation. This was demonstrated by the ability of laccase to detoxify phenols toxic to *Rhizoctonia praticola* by transformation or cross-coupling reactions. In some cases detoxification required a natural phenol as an aid but some compounds remained resistant (Bollag *et al.*, 1988).

LACCASE AND LIGNIN BIODEGRADATION

The final, and most controversial, suggested role for laccase in fungi is that of lignin biodegradation (Pelaez *et al.*, 1995; Reid, 1995; Youn *et al.*, 1995). Lignin is a major component of plant secondary cell walls and is considered to be a highly complex structure produced by the random polymerisation of phenyl propanoid units. Within it, certain specific chemical bonds have been identified and these have formed the basis for the design of model compounds (soluble lignols) used to investigate lignin degradation (Eggeling, 1983; Gould, 1983; Harvey *et al.*, 1985).

The white-rot basidiomycetes display the best ability to degrade lignin and *in vitro* studies of the degradation of model compounds, have implicated three enzymes in lignin biodegradation; these are lignin peroxidase (LiP), a Mn-dependent peroxidase (MnP) and laccase. The first two are secreted under specified conditions by the much studied lignolytic fungus *Phanerochaete chrysosporium* and, since many strains of this fungus do not secrete laccase, its role in lignin breakdown has been disputed (Thurston, 1994). However, a number of other studies suggest that laccase does have

a role in lignin degradation by some white-rot fungi. For example, Ander and Eriksson (1976) demonstrated diminished ability to degrade lignin in laccase-minus mutants of *Sporotrichum pulverulentum* but this was recovered in laccase-plus revertants. Similarly, Platt *et al.* (1984) reported that laccase-deficient strains of *Pleurotus ostreatus* were unable to degrade lignin whereas Garzillo *et al.* (1992) showed that *P. ostreatus* laccase-plus strains could degrade it in the absence of lignin peroxidase activity.

Bourbonnais and Paice (1992) reported demethylation and lower lignin content (based on Kappa reduction) of Kraft wood pulp incubated with a purified laccase from *C. versicolor*. This activity was much increased in the presence of ABTS (2,2 azinobis-[3-ethylbenzthiazolone-6-sulphonate] – a so-called 'primary' substrate which generates a stable radical). Previously they had shown that the same laccase was capable of oxidizing both phenolic and non-phenolic lignin model compounds, but the latter only in the presence of 'primary' substrates (Bourbonnais and Paice, 1990). This contradicts earlier work by Evans (1985) who reported that lignin degradation continued unaffected when laccase activity was inhibited by a specific antibody in the same fungus.

For a number of organisms, lignin degradation seems to involve the synergistic action of two enzymes. In *Rigidoporus lignosus*, lignin solubilization was co-dependent upon the production of laccase and MnP (Galliano *et al.*, 1991). The latter enzyme oxidized Mn (II) to Mn (III) which, in turn, oxidized phenolic structures by a one-electron mechanism (Kuwahara *et al.*, 1984). In *Dichomitus squalens* and *Armillaria mellea* lignolysis was thought to involve the independent action of both MnP and laccase (Pétrie and Gold, 1991; Robene-Soustrade *et al.*, 1992).

Pleurotus sajor-caju degraded lignin in the absence of detectable LiP or MnP activity (Bourbonnais and Paice, 1989) and here the lignin depolymerization activity was due solely to an extracellular laccase. This may occur if the depolymerization–repolymerization equilibrium was shifted to favour depolymerization; for example, the rapid reduction of quinoid intermediates to phenols, could effectively remove them from the equilibrium reaction. In *P. sajor-caju* this is accomplished by veratryl alcohol oxidase, a flavin-containing enzyme, in the presence of a co-substrate (veratryl alcohol). In other organisms, the reducing power may be provided by glucose oxidase or cellobiose: quinone oxidoreductase (Bourbonnais and Paice, 1989).

Haars and Hüttermann (1980) reported that in *Fomes annosus* laccase activity was detrimental to lignosulphonate breakdown due to a repolymerizing activity. A number of authors (Thurston, 1994) have suggested that laccase may not be involved in lignin degradation *per se* but acts by protecting the fungal hyphae from toxic free-radical attack from the products of peroxidase activity. However, since laccase is secreted extracellularly, and not bound to the hyphal cell wall, it would seem that this function could interfere with the delignification process and hence be detremental. It seems therefore that, in a few fungi, laccase does appear to have some role in lignin breakdown (Pelaez *et al.*, 1995).

ROLES FOR LACCASE IN HIGHER PLANTS

To date, there have been relatively few reports of laccases in higher plants and little is known of their function. The most well known is the laccase from the latex of the Japanese lacquer tree *Rhus* spp. (Nakamura, 1958), which contains the phenols

urushiol and laccol, which are oxidized by laccase and polymerize to yield a protective structure (Reinhammer, 1984). Laccases have been identified in *Aesculus parviflora* leaves (Wosilait *et al.*, 1954), peach (Mayer and Harel, 1968; Harel *et al.*, 1970) and apricot fruits (Dijkstra and Walker, 1991), mangoes (Joel *et al.*, 1978) and tomatoes (Filner *et al.*, 1969).

Laccases can contribute to the same enzymatic browning process as for catecholases, and this has been suggested to play a role in plant defence. Both enzymes may be involved in fruits such as apricots and peaches (Mayer and Harel, 1968; Dijkstra and Walker, 1991).

For some time laccase has been postulated to act in the final polymerisation steps of lignin synthesis by the generation of free-radical phenolic species (Higuchi, 1957, 1985). Using the oxidation of syringaldazine or furoguaiacin as their diagnostic criteria Harkin and Obst (1973) reported a lack of laccase reaction in many tree species and therefore proposed that peroxidase was the only enzyme responsible for lignin polymerization. However, some of the genera used in their study have since been shown to contain laccases; for example, *Picea* (Walker and McCallion, 1980; Allan and Walker, 1988), *Pinus taeda* (Bao *et al.*, 1993) and *Acer* (Sterjiades *et al.*, 1992) so this hypothesis needs re-evaluation.

A number of recent studies support the hypothesis that laccase may be involved in lignification. Liu *et al.* (1994) found that in *Zinnea elegans* there was a greater correlation between lignification and laccase activity than lignification and peroxidase. Laccases from *Acer* cell cultures (Sterjiades *et al.*, 1992, 1993), or the lignifying xylem of *Pinus taeda* (Bao *et al.*, 1993) were found to be capable of oxidizing monolignols. McDougall *et al.* (1993) and Chabanet *et al.* (1994) reported a phenol oxidase present only in lignifying or lignified cell walls. This oxidase appeared to be unique since it displayed properties of *both* catecholase and laccase which made it difficult to characterize. An excellent review of this topic has been given by O'Malley *et al.* (1993). Recent work in the authors' laboratory found laccase activity bound to the cell-wall fraction from leaves and fruits (Dijkstra and Walker, 1991; Harvey and Walker unpublished results).

Despite the above report,s evidence for the involvement of laccase in lignin biosynthesis is still only circumstantial and direct evidence will probably come from isolation of the genes responsible and their specific deletion and reinsertion. If laccase is involved in lignin synthesis it opens the door for an interesting hypothesis that lignin may be both synthesized and degraded by the same enzymatic processes (free-radical, single-electron oxidation) and also by the same enzyme (laccase or peroxidase or both).

Enzymic browning in food processing

The browning which occurs due to plant DPO action is an widespread problem in the food industry (Walker, 1975, 1995) because, when plant material is cut or broken it immediately begins to melanize, which can destroy the desirable organoleptic characteristics of foods, especially colour and taste. This browning reaction in most plants is due to catecholase and perhaps peroxidase.

Enzymic browning is a major problem for fruit and vegetable marketing and in processing since damaged or cut tissue will melanize and thus appear unattractive. For

example, lettuce in salads will brown around the cut edges and fruit juices will darken, in some cases very rapidly. Some storage procedures, such as gamma irradiation, drying or cold storage, may also cause browning through disruption of intracellular membranes. The latter can become a problem if chilling injuries are sustained during thawing.

Sucrose production from sugar beet or sugar cane may be adversely affected by enzymic browning, not only altering the 'accepted' colour of the product, but also because the resultant polymers may seriously hamper recrystallization which will lower the ultimate yield (Vamos-Vigyazo, 1981).

Catecholase activity in flour may bring about browning in bread products due to the reaction with amino acids, especially lysine, resulting in an overall decrease in food nutritional quality. However, non-enzymic or Maillard browning, which results from the reactions between amino acids and reducing sugars on heating, is more of a problem in this area (Vamos-Vigyazo, 1981; McCallum and Walker, 1990). Proteins, such as casein, can undergo modification by the catecholase-catalysed oxidation of tyrosine as well as quinone polymerisation of proteins in general, both phenomena leading to decreased food quality (Mathers and Whitaker, 1984).

However catecholase action is not always a problem in the food industry and in some circumstances its action is desirable or even essential; for example, in the manufacture of black tea (Takeo, 1966; Opie *et al.*, 1995), sultanas (Grncarevic and Hawker, 1971), prunes (Vamos-Vigyazo, 1981), coffee (De Amorim and Silva, 1968) and cider (Walker, 1975, 1995).

CURRENT METHODS FOR THE CONTROL OF ENZYMIC BROWNING

Because of the detrimental effects of the browning reaction in most commercially important food products, its control is a high priority for the food processor. Present methods of control may use both physical and chemical methods, often in synergy (Almeida and Nogueira, 1995). Optimum control procedures differ for different food products so few universal control measures are available.

Physical methods

In general these involve removing from or decreasing the activity of either the enzyme or its substrates in the food product. The major physical treatment used in industry is heat, via the Pasteurisation process, which inactivates enzymes thus halting enzymic browning. Unfortunately, heat treatments are not appropriate in many systems because fruit phenolases are relatively heat stable and heat treatments may adversely affect the organoleptic qualities of the product, such as texture and colour. Freezing or refrigeration can be used to temporarily limit enzymic browning.

A variety of adsorbents have been used to remove the reactants involved in enzymic browning. Bentonite clay has protein adsorption qualities and has long been used in winemaking to reduce phenolase activity (Machiex *et al.*, 1991). Other phenolic adsorbents, such as gelatin, activated carbon and polyvinylpolypyrrolidone (PVPP, Poly-Clar AT™), have also been used to remove soluble phenolic substrates from wines and beers.

Another method of removing phenolase substrates is to limit O_2 availability, since

O_2 is a required substrate. This can be achieved by packing under vacuum or in a CO_2 or N_2 -enriched atmosphere, as has been done with pineapples (Machiex *et al.*, 1990).

One simple but effective procedure used regularly in the manufacture of 'clear' apple juice is to pulp the fruit flesh and allow the browning reaction to proceed. Because apple phenolase is located on the chloroplast, it remains in the particulate debris, and as the reaction proceeds, the particles become melanized, aggregate together and sediment out. The juice is left in settling tanks and the clear, light-amber coloured juice can be removed.

Chemical methods

Chemical methods of controlling enzymic browning involve adding browning inhibitors to the product and this is often accomplished by dipping the product (usually fruit) in a solution of the inhibitor.

Sulphites. Currently the use of sulphiting agents is the most widespread chemical approach for controlling browning (Iyengar and McEvily, 1992). Sulphiting agents can be added to foods in a number of forms, such as gaseous SO_2, sodium metabisulphite, sodium bisulphite and sodium sulphite. Sulphites may act via a number of mechanisms; they are potent non-specific reducing agents and hence can be used to inhibit both enzymic and non-enzymic browning by reduction of the quinones to the parent dihydroxy phenols (Kahn, 1985). They have also been reported to inhibit catecholase directly (Embs and Markakis, 1965) with modification of the protein structure but still retaining catecholase molecular unity (Sayavedra-Soto and Montgomery, 1986). Golan-Goldhirsh and Whitaker (1984,1985) have suggested that this may be due to a K_{cat} inactivation mechanism (where the enzyme is inactivated during turnover of substrate to product via free radical action) in addition to actual inhibition by sulphites. Finally, and probably most importantly, sulphites are known to complex with quinones forming colourless thioethers (Walker, 1975).

In addition to controlling enzymic browning, sulphites are also effective antimicrobial agents and have beeen reported to improve flavour in wines (Ough and Crowell, 1987). Thus their widespread use in foods and beverages is not surprising. However, sulphites are also known to be mildly corrosive and SO_2 is an effective bleaching agent so its use in food processing is restricted by regulatory limits. Even within these limits its use in foods is a topic of current media attention, more especially since some sulphite-sensitive individuals (usually asthmatics) have died due to large doses ingested from treated foods (Dudley and Hotchkiss, 1989). The use and labelling of sulphiting agents in foods has been restricted by new US Food and Drug Administration (USFDA) regulations and in 1986 a ban on the use of sulphites on fresh fruits and vegetables, including in restaurant salad bars, was imposed. As from 1987, the presence of sulphiting agents in any food in 'detectable amounts' (defined as 10 ppm and over) had to be declared on the label. This limitation on sulphites was based on a government study of hundreds of reports of severe allergenic reactions, mostly involving restaurant foods (Duxbury, 1986). The subject has been reviewed by Taylor and Bush (1986) and Brown (1985). Langdon (1987) concluded that 'with the present cloud of uncertainty over the use of sulphites, food processors are looking for alternatives to the use of suphiting agents to prevent browning in foods'.

Sulphur amino acids and sulphydryl compounds. Sulphur amino acids, such as cysteine, and the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, the reduced form) have been reported as effective inhibitors of browning in fruit juices (Walker, 1964a; Walker and Reddish, 1964; Montgomery, 1983; Dudley and Hotchkiss, 1989; Molnar-Perl and Friedman, 1990a,b; Richard *et al.*, 1991, Robert *et al.*, 1996) and, considering its low cost and the fact that it is a primary biometabolite, it is surprising that its use in industry has not been more widespread. Iyengar and McEvily (1992) reported that the concentration required for acceptable inhibition of enzymic browning had negative effects on taste but this is dependent on the system to be inhibited. Recently, a cysteine derivative, N-acetyl cysteine, has been reported to be as effective as sulphites in the inhibition of browning in juices and apple and potato slices (Molnar-Perl and Friedman, 1990a,b). Dithiothreitol and dithioerythritol have also been considered as control agents; Muneta (1981) demonstrated that dithiothreitol is much more effective than cysteine in controlling blackspot in potatoes.

Ascorbic acid and derivatives. Another commonly used method of controlling enzymic browning is by the addition of ascorbic acid (vitamin C). This vitamin has a lower redox potential than the quinones formed by catecholase action so it is oxidized and the quinones reduced back to their parent dihydroxyphenols, thus preventing melanogenesis. Golan-Goldhirsh and Whitaker (1985) suggested a K_{cat} inactivation mechanism for the depletion of enzyme activity by free radicals formed from quinone oxidation; however, it seems clear that it is the reducing action that is the dominant factor in prevention of browning by ascorbic acid. It also has the benefit of being an essential vitamin so its addition could be advantageous in advertising claims.

Recently, various ascorbyl derivatives have been reported to be useful antioxidants for the food industry. Sapers *et al.* (1989a) reported that ascorbyl phosphates (ascorbic acid-2-phosphate and ascorbic acid triphosphate), which release ascorbic acid when hydrolysed by acid phosphatases (Iyengar and McEvily, 1992), are useful for inhibiting browning at cut surfaces of fruit but were ineffective in juices. By contrast ascorbic acid-6-fatty acid esters were effective in juice (Sapers *et al.*, 1989b). The improved performance of these esters may be due primarily to their oxidative stability, as reflected in the longer lag times for the onset of browing obtained with these derivatives, compared with equivalent concentrations of ascorbic acid (Iyengar and McEvily, 1992).

Erythorbic acid, a stereoisomer of ascorbic acid, has been proposed as an alternative to the latter (Anon, 1977) but Sapers and Ziolkowski (1987) reported that the performance of both is dependent on the system to be inhibited and that they could not be used interchangeably. D-Araboascorbic acid is cheaper than ascorbic acid and has been suggested as an alternative when treating Spy and Jonathon apple slices prior to freezing (Santerre *et al.*, 1988).

Acidulents. One method of controlling enzymic browning, often used domestically, is the addition of lemon juice. Here citric acid chelates the Cu cofactor as well as decreasing the overall pH below the optimum for catecholase (Labell, 1983). Other acidulants that have been used include malic, tartaric and malonic acids and inorganic acids such as phosphoric acid; their main disadvantage is their higher cost and negative impact on taste (Iyengar and McEvily, 1992).

Recent alternatives to sulphite. Some phosphate compounds have been used for control of browning, including sodium acid pyrophosphate, polyphosphate and metaphosphate. Typically, these are used at levels of 0.5–2% (*w/v*) often in combination with other anti-browning agents (Iyengar and McEvily, 1992). One such compound, 'Sporix™' is an acidic polyphosphate mixture with a three-dimensional network structure, and has been patented for use as an anti-browning additive for freshly peeled fruits and vegetables (Gardner *et al.*, 1991).

Since the FDA's rulings regarding the use of sulphites a number of other non-sulphite anti-browning additives have become available commercially. These are generally mixtures of known compounds, especially citric and ascorbic acids (Iyengar and McEvily, 1992).

Cyclodextrins. Cyclodextrins are a group of complexing agents which have been patented for use as inhibitors of enzymatic browning (Hicks *et al.*, 1990). They inhibit browning by the formation of inclusion complexes with, or entrapment of, catecholase substrates or products; thus removing the source of substrate. Their effectiveness is dependent on the equilibrium between free and complexed catecholase substrates or products and the rate of complex formation (Sapers *et al.*, 1989).

Substituted resorcinols. The last group of commercially important catecholase inhibitors are the substituted resorcinols, especially 4-hexyl resorcinol (Everfresh™), which is used to control enzymatic browning in shrimps ('shrimp black spot') and has potential for application to other foods and beverages (McEvily *et al.*, 1991,1992). 4-Hexyl resorcinol does not affect the appearance, taste, texture or quality of foods and can be used at lower levels than sulphites. It can be synthesized chemically or isolated from a natural source such as fig latex and was first patented in 1991 (McEvily *et al.*, 1991). It has since been reported as a potent inhibitor of mushroom catecholase (Dawley and Flurkey, 1993) and apple browning (Monsalve-González *et al.*, 1995).

THE BROWNING PROBLEM IN WINES

The fermentation of grape juice to yield wine is an industry of enormous commercial significance. Many factors affect wine production and careful control measures are essential (Macheix *et al.*, 1990). Enzymic browning is a major problem in wine-making, since it is responsible for profound modifications of the grape phenols including the appearance of undesirable condensed tannin compounds and the elimination of certain phenolic compounds responsible for the intrinsic sensory qualities of a given grape variety. The problems and possible solutions have been comprehensively reviewed by Macheix *et al.* (1991).

The riddle in wine-making is not so much how to avoid browning but, how to avoid browning whilst still maintaining the flavour and aroma of the product. Sulphite treatment is still used widely to control browning, for example 150–200 ppm sodium metabisulphite may be added to white and red wines (Peter Evans, St. Helena vineyard, Christchurch, NZ; personal communication). However, as discussed above, sulphite alternatives are being sought due to the potentially hazardous effects of SO_2 on asthmatics. A further complication is that sulphiting actually seems to enhance, rather than debilitate, wine quality (Ough and Crowell, 1987) so its replacement could be

disadvantageous. Most other anti-browning treatments interfere somehow with phenolic substances yet these are often the very compounds which enhance wine flavour.

Recently, it has been established that grapes have their own endogenous anti-browning system. The major phenolic substances in grape juice are hydroxycinnamic acid esters, especially caftaric (caffeoyl-tartaric) and coutaric (coumaroyl-tartaric) acids (Singleton *et al.*, 1984). Singleton's group identified large concentrations of free glutathione in crushed grapes (typically 160 mg/l) and demonstrated that this interacted with caftaric (or coutaric) acid to produce a grape reaction product (called 'GRP', Singleton *et al.*, 1985) tentatively identified as 2-S-glutathionyl-caftaric acid. This assignation was confirmed later by ^{1}H-nmr studies (Cheynier *et al.*, 1986). GRP is not coloured and is only slowly oxidized by grape catecholase (Cheynier *et al.*, 1988), thus its formation acts to thwart the browning mechanism. However, there is also some evidence that GRP is oxidized indirectly by caftaric acid quinones and possibly also by hydrogen peroxide liberated by spontaneous reduction of caftaric acid quinone by peroxidase (Cheynier and Van Hulst, 1988). Procyanidins, which are not susceptible to catecholase oxidation, were also reportedly to be oxidized by caftaric acid quinones (Cheynier and da Silva, 1991). However, the final colour of the oxidized must is dependent on the molar ratio of hydroxycinnamic acid to glutathione (Cheynier *et al.*, 1990) so free glutathione would seem to be an effective endogenous anti-browning system. Indeed in 'raisining' it appears that caftaric acid and catecholase, initially separated by compartmentalization, come into contact, whereas glutathione remains compartmentalized away from the other two reactants, or in an inactive form, and so 'raisins' brown more than the must formed from the same grapes would have (Singleton *et al.*, 1985).

Thus the problem of catecholase browning in wines may be due, in part, to the indirect oxidative effects brought on by caftaric acid quinones (formed by catecholase catalysis) rather than to direct enzyme oxidation, since Valero *et al.* (1988) report that, even with this endogenous anti-browning system, enzymatic browning still occurs rapidly after crushing of grapes. Methods for the control of oxidation and browning during wine processing have been comprehensively reviewed by Machiex *et al.* (1991).

Laccase activity is often found in wine musts and comes from *Botrytis cinerea,* the fungus which causes 'grey mould' of grapes. This laccase can be a major problem since it is resistant to most of the classic catecholase inhibitors such as DIECA (diethyldithiocarbamate) and requires high levels of EDTA, cyanide or sulphite to halt its activity (Macheix *et al.*, 1991). Furthermore, laccase has been reported to oxidize GRP to the corresponding quinone, which can then react with another molecule of glutathione to form 2,5-di-S-glutathionyl-caftaric acid (Salgues *et al.*, 1986) thus depleting the available glutathione. Since the free glutathione concentration is not sufficient to allow the complete conversion of endogenous caftaric acid into GRP (Singleton *et al.*, 1984) formation of this second derivative is likely to further enhance browning. Laccase contamination of wine musts may be a more important oxidation problem than catecholase and hence its control is a real necessity.

Other inhibitors of *o*-diphenol oxidases

Apart from those inhibitors already mentioned, many compounds have been reported to be inhibitory to catecholase and some of these are reviewed here.

HALIDES

The use of common salt (NaCl) to minimize browning of fruits and vegetables has been known to cooks for many years. The inhibition of catecholase by halides was first studied by Krueger (1955) who observed that 40 mM concentrations all four halides (F^-, Cl^-, I^-, Br^-) gave approximately 50% inhibition of mushroom catecholase and this was more pronounced at low pH. This has since been confirmed by many workers (Sharon and Mayer, 1967; Ben-Shalom *et al.*, 1977; Penafiel *et al.*, 1984; Martinez *et al.*, 1986).

Rouet-Mayer and Philippon (1986) reported that, for apples, inhibition occured at acidic pH, but not at neutral pH, even with 0.4 M NaCL. Later work by Janovitz-Klapp *et al.* (1990a) with apple catecholase found the inhibition series for Na^+ halides to be $F^->Cl^->Br^->I^-$. NaF was found to be by far the most potent with an apparent K_i=0.07 mM. Martinez *et al.* (1986) compared the halide inhibition of catecholases from three different sources and found the following inhibition series:

frog epidermis	I^-	$>Br^-$	$>Cl^-$	$>>F^-$
mushroom	F^-	$>I^-$	$>Cl^-$	$>Br^-$
mouse melanoma	F^-	$>Cl^-$	$>>Br^-$	$>I^-$

Again, these results highlight the differences between catecholases from different sources.

CARBOXYLIC ACIDS

The inhibition of catecholase by carboxylic acids (both aliphatic and aromatic) has long been known. Kuttner and Wagreich (1953) studied a wide range of naturally occuring phenolic and carboxylic acids and found that benzoic acid was the strongest inhibitor having an IC_{50} of 12 μM; inhibition was associated with the protonated free acid.

Since 1953 many papers have reported inhibitory effects of carboxylic acids (Krueger, 1955; Macrae and Duggleby, 1968; Walker and McCallion, 1980; Gunata *et al.*, 1987; Janovitz-Klapp *et al.*, 1990a,b; Menon *et al.*, 1990; Iyengar and McEvily, 1992; Kermasha *et al.*, 1993). Aromatic carboxylic acids were generally better inhibitors than aliphatic acids, especially those with an electron-withdrawing group in the '4' position on the benzene ring (Pifferi *et al.*, 1974; Walker and Wilson, 1975). The conjugated double bond moiety also appears to aid inhibition, even in non-aromatic carboxylic acids. Walker (1976) demonstrated the use of cinnamic acids to control enzymic browning in fruit juices, but this has not been followed up, probably due to the general utility of ascorbic acid.

The aromatic acid inhibitors have often been considered to be acting as substrate analogues but studies have demonstrated non-competitive, mixed competitive and also uncompetitive types of inhibition (Walker and Wilson, 1975; Walker and McCallion, 1980; Selim *et al.*, 1993) so the actual mechanism involved is likely to be more complicated. Another factor involved is discrepancies between assay methods and the type of substrate used (Selim *et al.*, 1993).

HYDROXAMIC ACIDS

Hydroxamic acids have the formula R-CONHOH and have been reported as inhibitors

of catecholase (Rich *et al.*, 1978). They are aggressive metal chelators and were originally thought to inhibit catecholase by this mechanism; however Rich *et al.* (1978) demonstrated that the inhibition is both competitive and reversible. Furthermore, they were found to have inhibitor constants (K_i values) in the nanomolar range (Rich *et al.*, 1978) and are very effective inhibitors of catecholase. Salicylhydroxamic acid (SHAM) was investigated by Allan and Walker (1988) and found (in contrast to Rich *et al.*, 1978) to inhibit catecholase non-competitively with a K_i=0.2–2 μM, yet was without effect on the laccases from spruce, *Rhus vernicifera* or fungal laccases. Later work in the authors' laboratory has confirmed this differential inhibition (Ferrar and Walker, 1996).

COPPER CHELATORS

Since Cu^{2+} is the prosthetic group in catecholases, it may be chelated to inhibit the enzyme non-competitively. Many chelating agents have been shown to inhibit the enzyme including DIECA (diethyldithiocarbamate), EDTA (ethylenediaminetetraacetic acid), azide and mercaptobenzothiazole (Walker, 1975), cyanide and thiourea (Mathew and Parpia, 1971), and carbon monoxide (Albisu *et al.*, 1989).

Of the above, EDTA has been used as a food preservative (Iyengar and McEvily, 1992) and DIECA has been suggested as a chelation therapy drug for leprosy (Prabhakaran *et al.*, 1969).

PHENOLIC ADSORBENTS

Although not catecholase inhibitors in the strict sense, phenolic adsorbents are a commonly used method of minimizing enzymic browning and charcoal or PVPP (polyvinylpolypyrollidone) are often used. The latter is used widely in plant biochemical research, for example, in enzyme purification, to remove interfering phenolics.

Other compounds which inhibit enzymic browning

Various other chemicals have been reported which inhibit catecholase, often with unknown or only partially understood mechanisms (*Figure 4*). Phenylhydrazine has been reported as a powerful inhibitor (Lerner *et al.*, 1971,1974) and this activity may be due to its ability to reduce the Cu^{2+} cofactor, since Andrawis and Kahn (1990) reported this to occur in a model system.

Succinic acid-2-2-dimethylhydrazide (SADH) was reported to significantly retard the post-harvest discolouration of cultivated mushrooms. It was proposed that this occured through a two-fold mechanism: first SADH induces degradation of catecholase through an increase in proteolytic activity, and secondly it binds to quinones thereby inhibiting melanogenesis (Murr and Morris, 1974).

Tropolone (2-hydroxy-2,4,6-cycloheptatriene-1-one) and mimosine (a toxic, non-protein amino acid) are two other compounds recently reported to give catecholase inhibition. Both inhibit by non-classical mechanisms; the former involves the rapid formation of an enzyme–inhibitor complex which subsequently undergoes a relatively slow reversible reaction (Valero *et al.*, 1991), the latter is a slow binding inhibitor characterized by a prolonged transient phase (Cabanes *et al.*, 1987).

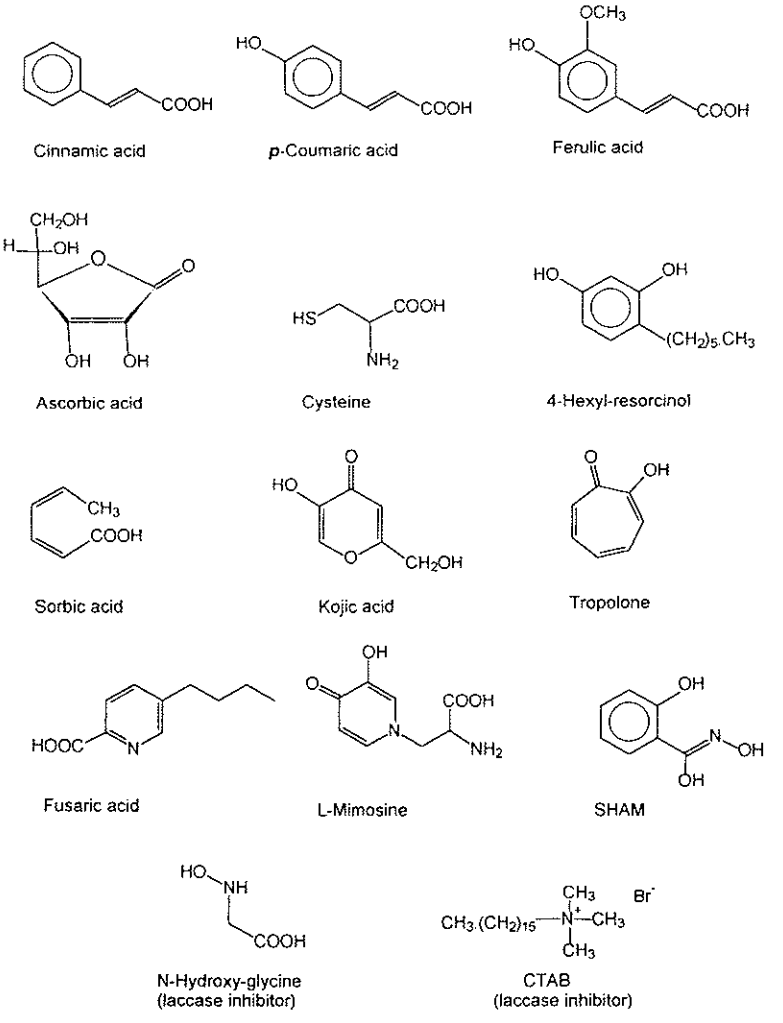

Figure 4. Structures of some of the inhibitors mentioned in this review.

Human diseases which result in disfiguring melanin hyperpigmentation, for instance, melasma and chloasma, are currently treated with melanogenesis inhibitors. The most commonly used is quinol (hydroquinone) (Lerner, 1971; Palumbo *et al.*, 1991). Other compounds which have potential in this area include 4-hydroxy-anisole (Naish and Riley, 1989) and phosphonic acid and phosphinic analogues of tyrosine and DOPA (Lejczak *et al.*, 1990).

Borates can react with *o*-dihydroxy phenols (catecholase substrates) to form complexes thus blocking oxidation by catecholase (Mathew and Parpia, 1971). Borax ($Na_2B_4O_7 \cdot 10H_2O$) has been used as a foliar spray to prevent enzymatic browning and thus increase the ascorbic acid content of potatoes (Mondy and Munshi, 1993).

Dye-sensitized photo-inactivation of catecholase has been reported to occur if it was illuminated in the presence of rose bengal or riboflavin (Lowum and Parkin,

1989). The inactivation was due to hydrogen peroxide and superoxide for the riboflavin sensitized reaction and singlet O_2 for the system containing rose bengal (Parkin and Lowum, 1990).

For any inhibitors to become useful in the food industry, they must conform to current market and regulatory demands and hence must be non-toxic, preferably not coloured nor give odours and not give any adverse side effects. Inhibitors of natural origin could be better received in view of recent opposition to 'synthetic' food additives.

NATURAL *O*-DIPHENOL OXIDASE INHIBITORS

A number of natural catecholase inhibitors have been described. The first was fusaric acid, a toxin isolated from *Fusarium lycopersici* and now known to be secreted by many Fusaria. This compound was found to inhibit catecholase competitively and was secreted by the fungus whilst infecting tomatoes (Bossi, 1959). Later, a polypeptide catecholase inhibitor was discovered in mushrooms by Karkhanis and Friedin (1961); this contained phenylalanine, glutamate and aspartate in a 1:1:1 ratio plus six other minor amino acids but sulfur amino acids were not found (Madhosingh and Sundberg, 1974). Madhosingh (1975) later identified two other low molecular weight (about 1 kDa) peptide inhibitors from mushrooms.

Avigad and Markus (1965) discovered a low molecular weight peptide inhibitor of galactose oxidase, a copper based enzyme from *Dactylium dendroides*; this inhibitor contained thiol groups. Harel *et al.* (1967) later found that this inhibitor was also active against apple catecholase and preincubating the inhibitor with enzyme, before addition of substrate, enhanced inhibition. It is surprising that, although Harel *et al.* (1967) expressed their hope that knowledge of this peptide inhibitor's structure would elucidate its mode of interaction with copper proteins, its structure has not yet been reported.

Walker (1969) reported that the lack of browning observed in apples infected with *Penicillium expansum* was due to elevated levels of *p*-coumaric and ferulic acids in the infected tissue; these acids were derived from the host's own phenolics. It was found also that the fungus secreted an extracellular catecholase inhibitor (which Walker called 'expansin') which reached a maximum concentration just prior to sporulation. Its structural characterization could not be achieved due to the instability of the compound (Walker, 1970). In a later study of some *Penicillium* species Baldwin (unpublished results) confirmed Walker's (1970) report of a catecholase inhibitor from *P.expansum* culture filtrates and also found inhibitors in culture filtrates from *P.digitatum* and *P.notatum*.

Bull and Carter (1973) reported a polypeptide inhibitor bound to, but separable from, catecholase in *Aspergillus nidulans*. This inhibitor was found to be of the same size as the 130 kDa monomer of this enzyme, which is very large compared to other reported catecholase monomers (usually 30–50 kDa, Mayer and Harel, 1978); it contained a large RNA component and inhibited non-competitively.

Satô (1979) reported that spinach catecholase was normally latent due to the presence of a low molecular weight inhibitor which he identified as oxalic acid. He later reported that inhibition increased as the pH was lowered (Satô, 1980a) and could be reversed by addition of cupric ions (Satô, 1980b).

Kojic acid (5-hydroxy-2 hydroxymethyl-γ-pyrone) was identified as the catecholase

inhibitor secreted by *Aspergillus albus* (Saruno *et al.*, 1979) is a is a fungal metabolite produced by several *Penicillium* and *Aspergillus* species. It was originally identified as a catecholase inhibitor from *A. albus* during the course of a screening program undertaken by Ikeno's group (Saruno *et al.*, 1979). Since then a patent has been taken out for a mixture of kojic and ascorbic acids as an anti-browning additive to foods (Fukusawa *et al.*, 1982). It is interesting to note that, although kojic acid has been reported to inhibit catecholase from apple, white shrimp, grass prawn, lobster, mushrooms and potatoes, with K_i values ranging from 0.03–0.7 mM (Chen, *et al.*, 1991) a catecholase isolated from edible burdock was completely unaffected by it (Murao *et al.*, 1993). This, and other results, highlight the need to fit the inhibitor to the system. Unfortunately many studies have used mushroom tyrosinase (ex Sigma) to model a fruit enzyme and this is not always valid (Ferrar and Walker, 1996).

Relatively little work regarding natural catecholase inhibitors was published until 1990–91 when five separate reports of inhibitors appeared. Oszmianski and Lee (1990) demonstrated that honey contains a small unidentified peptide inhibitor with an approximate molecular weight of 600. Tan and Kubo (1990) published their findings of an inhibitor secreted from the roots of corn seedlings simultaneously with secretion of catecholase. Ishihara *et al.* (1991) reported the discovery of melanostatin, a peptide inhibitor of melanin synthesis, found in culture filtrates of *Streptomyces clavifer* and with the molecular formula $C_{19}H_{25}N_5O_5$. However, this inhibitor did not inhibit mushroom catecholase in a routine enzyme assay. McEvily *et al.* (1991, 1992) isolated three 4-substituted resorcinols (resorcinol = 1,3-dihydroxybenzene) from commercially available ficin (a fig protease) preparations and this led to the patenting of 4-hexyl resorcinol (Everfresh™).

Thus it seems that the FDA's ban on SO_2 and sulphites has increased scientific interest in this area. Since 1991 the following reports of catecholase inhibitors have appeared:

(i) A novel cyclic tetrapeptide, cyclo(-L-pro-L-tyr-L-pro-L-val-) identified from *Lactobacillus helveticus* (Kawagishi *et al.*, 1993).

(ii) Two flavonol glycosides: kaemferol 7-(6"-*p*-coumaroyl glycoside) and isorhamnetin 7-(6"-*p*-coumaroyl glycoside) isolated from the aerial parts of *Buddleia coriacea* (Kubo and Yokokawa, 1992).

(iii) Pineapple juice contained a low MW compound, 3-(methylthio)-propionic acid methyl ester, which may be responsible for its characteristic smell and which inhibits catecholase (Lozano-de-Gonzalez *et al.*, 1993).

(iv) Pseudostellarins, a group of cyclic peptides obtained from the roots of *Pseudostellaria heterophylla* (Morita *et al.*, 1994a; Morita *et al.*, 1994b; Morita *et al.*, 1994c). (See *Table 3.*)

(v) Anacardic acids, cardols and 2-methyl-cardols from cashew fruits. The major active compounds were 6-[8(*z*),11(*z*),14-pentadecatrienyl]salicylic acid and 5-[8(*z*),11(*z*),14-pentadecatrienyl]resorcinol (Kubo *et al.*, 1994). Since both salicylic acid and resorcinol are known catecholase inhibitors, the inhibitory activity of these derivatives is not surprising.

(vi) Metallothionein from *Aspergillus niger* inhibited purified mushroom tyrosinase (Goetghebeur and Kermasha (1996).

(vii) A laccase inhibitor, N-hydroxy-glycine, has been isolated by Murao *et al.* (1992) from *Penicillium citrinum.*

Table 3. Structures of pseudostellarins and their activities against catecholase

Pseudostellarin	Structure	IC_{50} (μM)
A	Cyclo[gly-pro-tyr-leu-ala]	131
B	Cyclo[gly-ile-gly-gly-gly-pro-pro-phe]	187
C	Cyclo[gly-thr-leu-pro-ser-pro-phe-leu]	63
D	Cyclo[gly-gly-tyr-pro-leu-ile-leu]	100
E	Cyclo[gly-pro-pro-leu-gly-pro-val-ile-phe]	175
F	Cyclo[gly-gly-tyr-leu-pro-pro-leu-ser]	50
G	Cyclo[pro-phe-ser-phe-gly-pro-leu-ala]	75

From Morita *et al.*, 1994c.

POTENTIAL USES FOR DIPHENOL OXIDASE INHIBITORS

Since the exact function of the DPOs in many organisms is unclear, the utility of DPO inhibitors may be broader than is presently recognized. This section will summarise some of the general areas where DPO inhibitors may be applicable.

CATECHOLASE INHIBITORS

(i) The most apparent use of any non-toxic catecholase inhibitors is to stop enzymic browning in food products. They may be used either by themselves or in compilations.

(ii) A less considered role is the possibility of using catecholase inhibitors as insecticidal sprays, since catecholase is involved in the sclerotization of the insect cuticle.

(iii) Human tyrosinase inhibitors may have utility as a cosmetic therapy for sufferers of hyperpigmentation diseases.

(iv) Inhibitors of *Mycobacterium leprae* catecholase may be of use in leprosy treatment; for example in chelation therapy.

LACCASE INHIBITORS

(1) These could have potential for use as insecticidal sprays, as for catecholase inhibitors. Both could be used in tandem, but possible effects on plants would have to be investigated first. Since there are large variations in diphenol oxidases from different sources, it is quite likely that selective insect diphenol oxidase inhibitors could be found.

(ii) Laccase inhibitors may have use for the control of wood-rotting fungi, for example, *Armillaria mellea*, which is a major problem in *Pinus radiata* forests. Again, specific fungal laccase inhibitors may be necessary.

(iii) Laccase inhibitors may also have potential for the control of a range of phytopathogenic fungi, since it has been implicated in the infection process of some of these, for example *Colletotrichum lagenarium* (Kubo *et al.*, 1982).

(Iv) Finally, inhibitors of *Botrytis cinerea* laccase may be prove useful as additives to wine, since this laccase is not as sensitive to anti-browning treatments, such as sulphites, as is catecholase.

SCREENING FOR NATURAL INHIBITORS OF DIPHENOL OXIDASES

Over the past decade there has been a dramatic rise of interest in the search for enzyme inhibitors of 'natural' origin and several new drugs have been developed as a result of such activity-based screening programs. The search for natural inhibitors of catecholase has only recently become popular (because of the current SO_2/sulphite controversy) and little research on this theme has been reported. However, the discovery of kojic acid as an inhibitor in 1979 was documented as 'during the course of screening' (Saruno*et al.*, 1979), so some workers have been aware of the possible applications for some time; details of this survey were not published.

Likewise, the discovery of melanostatin (Ishihara *et al.*, 1991) was the result of an intensive screening program in which approximately 2000 actinomycete cultures were evaluated (Tomita *et al.*, 1990). This screen was based on the ability of *Streptomyces bikiniensis* to produce melanins in plate cultures, so assay discs containing an inhibitor gave clear zones of inhibition around the disc. However, any compound that acted as an antibiotic (thus inhibiting production of catecholase) would appear as a false positive result. From the 2000+ actinomycetes screened, five inhibitory activities were found, three of which were known protein synthesis inhibitors, one (feldamycin) was a known bacterial antibiotic and the last was melanostatin which, whilst it inhibited melanin biosynthesis in *S.bikiniensis*, had no activity on mushroom catecholase (Tomita *et al.*, 1990; Imae *et al.*, 1991).

A survey of lichens for catecholase inhibitory activities was also published recently, again by Japanese workers (Higuchi, 1993). Here the lichens were cultured axenically and the powdered tissue extracted with various solvents; activity was assayed spectrophotometrically. Of the 46 lichens tested, 45 had some activity against catecholase, although this was below 20% inhibiton in most cases; three lichens gave reasonable inhibition (39–47%, although the authors called this 'strong'). In the strongest inhibiting lichen, *Hypogymnia physodes*, the myco- and photo-bionts were separated and the former gave much higher inihibition than the latter.

More recently, the authors have undertaken intensive screening tests for catecholase and laccase inihibitors (Ferrar, 1991; Ferrar and Walker, 1996, 1997). Attention was focussed on fungal phytopathogens with the hypothesis in mind that diphenol oxidase inhibitors were more likely to be secreted by phytopathogens since this would enhance their chances for successful parasitism, assuming that diphenol oxidases play a role in plant defence. This survey utilized simple, rapid, colourimetric assays which were modified for use in microtitre plates. Over 32 fungi were screened; of these 16 produced significant (>70% inhibition) inhibitory activity against catecholase, 12 produced significant laccase inhibition, and some fungi inhibited both enzymes (Ferrar, 1996; Ferrar and Walker, 1997).

Conclusion

Hopefully, these studies will be of use in the food processor's struggle against enzymic browning, aided by our knowledge of the action and function of these enzymes. They may also add a new perspective to our view of the never-ending battle between plants and phytopathogens.

Acknowledgements

We wish to thank the New Zealand Apple and Pear Marketing Board (ENZA Processors) for financial support and the University of Canterbury for the provision of research facilities. PHF was also the recipient of a Roper Scholarship (University of Canterbury).

References

ALBISU, I., KING, R.D. AND KOVLOV, I.A. (1989). Inhibition of the catecholase activity of mushroom tyrosinase by carbon monoxide. *Journal of Science of Food and Agriculture* **37**, 775–776.

ALDRIDGE, P.D. AND WALKER, J.R.L. (1980). A study of enzymic browning in the cultivated mushroom *Agaricus bisporus*. *Mauri Ora* **8**, 35–43.

ALLAN, A.C. AND WALKER, J.R.L. (1988). The selective inhibition of catechol oxidases by salicylhydroxamic acid. *Phytochemistry* **27**, 3075–3076.

ALMEIDA, M.E.M. AND NOGUEIRA, J.N. (1995). The control of polyphenol oxidase activity in fruits and vegetables: a study of the interactions between the chemical compounds used and heat treatment. *Plant Foods for Human Nutrition* (Dordrecht). **47**, 245–256.

ANDERSEN, S.O. (1985). Sclerotization and tanning of the cuticle. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, ed. G.A. Kerkut and L.I. Gilbert, Vol.3.pp 59–74. Oxford, England: Pergamon Press.

ANDRAWIS, A. AND KAHN, V. (1990). Ability of various chemicals to reduce copper and to inactivate mushroom tyrosinase. *Journal of Food Biochemistry* **14**, 103–115.

ANON (1973). Leprosy, New Treatment? *Nature* **245**, 411.

ANON (1977). Erythorbic acid and sodium erythorbate in foods. *Data sheet 671* Pfizer Chemicals Division, New York.

ARORA, Y.K. AND BAJAJ, K.C. (1985). Peroxidase and polyphenol oxidase associated with induced resistance of mung bean to *Rhizoctonia solani* Kuhn. *PhytopathologieZeitschrift* **114**, 325–331.

AVIGAD, G. AND MARKUS, Z. (1965). Inhibition of galactose oxidase by extracts obtained from the mycelia which produce the enzyme. *Israel Journal of Chemistry* **3**, 193–196.

BAO, W., O'MALLEY, D.M., WHETTEN, R. AND SEDEROFF, R.R. (1993). A laccase associated with lignification in Loblolly Pine. *Science* **260**, 672–674.

BAR-NUN, N. AND MAYER, A.M. (1989). Cucurbitacins – repressors of laccase formation. *Phytochemistry* **28**, 193–215.

BAR-NUN, N., TAL LEV, A., HAREL, E. AND MAYER, A.M. (1988). Repression of laccase formation in *Botrytis cinerea* and its possible relation to phytopathogenicity. *Phytochemistry* **27**, 2505–2509.

BARUAH, P. AND SWAIN, T. (1959). The action of potato phenolase on flavonoid compounds. *Journal of Science of Food and Agriculture* **10**, 125–129.

BEN-SHALOM, N., KAHN, V., HAREL, E. AND MAYER, A.M. (1977). Catechol oxidase from green olives: properties and partial purification. *Phytochemistry* **16**, 1153–1158.

BILLAL, F. AND THURSTON, C.F. (1996). Purification of laccase II from *Armillaria mellea* and comparison of its properties with those of laccase I. *Mycology Research* **100**, 1099–1105.

BLUM, M.S. (1981). *Chemical Defenses of Arthropods*. pp 183–205. New York: Academic Press.

BOSS, P.K., GARDNER, R.C., JANSSEN, B.J. AND ROSS, G.S. (1995). An apple polyphenol oxidase cDNA is up-regulated in wounded tissues. *Plant Molecular Biology* **27**, 429–433.

BOSSI, R. (1959). Über die Wirkung der Fusarin säure auf die polyphenoloxydase. *PhytopathologieZeitschrift 37*, 273–316.

BOURBONNAIS, R. AND PAICE, M.G. (1989). Oxidative enzymes from the lignin degrading fungus *Pleurotus sajor-caju*. In *Plant Cell Wall Polymers*, ed. N.G. Lewis and M.G. Paice, pp. 472–481. Washington DC: American Chemical Society.

BOURBONNAIS, R. AND PAICE, M.G. (1990). Oxidation of non-phenolic substrates, an expanded role for laccase in lignin biodegradation. *FEBS Letters* **267**, 99–102.

BOURBONNAIS, R. AND PAICE, M.G. (1992). Demethylation and delignification of Kraft pulp by *Trametes versicolor* laccase in the prescence of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate). *Applied Micro Biotechnology* **36**, 823–827.

BROWN, J.L. (1985). Sulfite sensitivity. *Journal of the American Medical Association* **254**, 825.

BRUESKE, C.H. AND DROPKIN, V.H. (1973). Free phenols and root necrosis in Nematex tomato infected with the root knot nematode. *Phytopathology* **63**, 329–334.

BRUNMARK, A. AND CADENAS, E. (1987). Electronically excited state generation during reaction of *p*-benzoquinone with H_2O_2. *Free Radical Bio Medicine* **3**, 169–180.

BULL, A.T. AND CARTER, B.L.A. (1973). The isolation of tyrosinase from *Aspergillus nidulans*, its kinetic and molecular properties and some consideration of its activity *in vivo*. *Journal of General Microbiology* **75**, 61–73.

CABANES, J., GARCÍA-CÁNOVAS, F., TUDELA, J., LOZANO, J.A. AND GARCÍA-CARMONA, F. (1987). L- mimosine, a slow-binding inhibitor of mushroom tyrosinase. *Phytochemistry* **26**, 917–919.

CAI, W., MARTIN, R., LEMAURE, B., LEUBA, J-L. AND PÉTIARD, V. (1993). Hydroxyindoles: a new class of laccase substrates. *Plant Physiology Biochemistry* **31**, 441–445.

CHABANET, A., GOLDBERG, R., CATESSON, A.-M., QUINET-SZELY, M., DELAUNEY, A.-M. AND FAYE, L. (1994). Characterisation and localisation of a phenol oxidase in mung bean hypocotyl cell walls. *Plant Physiology* **106**, 1095–1102.

CHEN, J.S., WEI, C., ROLLE, R.S., OTWELL, W.S., BALABAN, M.O. AND MARSHALL, M.R. (1991).Inhibitory effect of Kojic acid on some plant and crustacean polyphenol oxidases. *Journal of Agricultural Food Chemistry* **39**, 1396–1401.

CHEYNIER, V.F. AND VAN HULST, M.W.J. (1988). Oxidation of *trans*-caftaric acid and 2-5-glutathionyl caftaric acid in model solutions. *Journal of Science of Food and Agriculture* **36**, 10–15.

CHEYNIER, V.F. AND DA SILVA, J.M.R. (1991). Oxidation of grape procyanidins in model solutions containing *trans*-caffeoyl tartaric acid and polyphenol oxidase. *Journal of Agricultural Food Chemistry* **39**, 1047–1049.

CHEYNIER, V.F., RIGAUD, J., SOUQUET, J.M., DUPRAT, F. AND MOUTOUNET, M. (1990). Must browning in relation to the behaviour of phenolic compounds during oxidation. *American Journal of Enol Viticulture* **41**, 346–349.

CHEYNIER, V.F., TROUSDALE, E.K., SINGLETON, V.L., SALGUES, M.J. AND WYLD, R. (1986). Characterisation of 2-5-glutathionylcaftaric acid and its hydrolysis in relation to grape wines. *Journal of Agricultural Food Chemistry* **34**, 217–221.

CLUTTERBUCK, A.J. (1972). Absence of laccase from yellow spored mutants of *Aspergillus nidulans*. *Journal of General Microbiology* **70**, 423–435.

COLE, L., CLARK, P.A. AND SOLOMON, E.I. (1990). Spectroscopic and chemical studies of the laccase trinuclear copper active site: geometric and electronic structure. *Journal of the American Chemical Society* **112**, 9534–9548.

COLL, P.M., FERNANDEZ-ABALOS, J.M., VILLANUEVA, J.R., SANTAMOANA, R. AND PEREZ, P. (1993). Purification and partial characterization of a phenoloxidase (laccase). from the lignin-degrading basidiomycete PM1 (CECT 2971). *Applied Environmental Microbiology* **59**, 2607–2613.

CONTI, G.G., BOCCI, A.M. AND SPROCATI, M.T. (1982). Peroxidase and polyphenol oxidase isozymes, hypersensitive reaction and systemic induced resistance in *Nicotiana glutinosa* L. infected with tobacco mosaic virus. *Rivista di Patologia Vegetale* **28**, 83–102.

DAWLEY, R.M. AND FLURKEY, W.H. (1993a). Differentiation of tyrosinase and laccase using 4- hexyl resorcinol, a tyrosinase inhibitor. *Phytochemistry* **33**(2): 281–284.

DAWLEY, R.M. AND FLURKEY, W.H. (1993b). 4-Hexyl resorcinol, a potent inhibitor of mushroom tyrosinase. *Phytochemistry* **58**, 609–610.

DE AMORIM, H.V. AND SILVA, D.M. (1968). Relationship between the polyphenol oxidase of coffee beans and the quality of the beverage. *Nature* **219**, 381–384.

DE CAL, A. AND MELGAREJO, P. (1994). Effects of *Penicillium frquentens* and its antibiotics on unmelanized hyphae of *Monilinia laxa*. *Phytopathology* **84**, 1010–1014.

DIJKSTRA, L. AND WALKER, J.R.L. (1991). Enzymic browning in apricots (*Prunus armeniacia*). *Journal of Science of Food and Agriculture* **54**, 229–234.

DRY, I.B. AND ROBINSON, S.P. (1994). Molecular cloning and characterisation of grape berry polyphenol oxidase. *Plant Molecular Biology* **26**, 495–502.

DUCKWORTH, H.W. AND COLEMAN, J.E. (1970). Physiochemical and kinetic properties of mushroom tyrosinase. *Journal of Biological Chemistry* **245**, 1613–1625.

DUDLEY, E.D. AND HOTCHKISS, J.H. (1989). Cysteine as an inhibitor of polyphenol oxidase. *Journal of Food Biochemistry* **13**, 65–75.

DUXBURY, D. (1986). Sulfite alternative blend extends fruit, vegetable freshness. *Food Processing* **47**, 64–65.

EGGELING, L. (1983). Lignin an exceptional biopolymer . . . and a rich resource? *Trends in Biotechnology* **1**, 123–127.

EGLEY, G.H., PAUL, R.N., VAUGHN, K.C. AND DUKE, S.O. (1983). Role of peroxidase in the developement of water impermeable seed coats in *Sida spinosa* L. *Planta* **157**, 224–232.

EGLEY, G.H., PAUL, R.N., DUKE, S.O. AND VAUGHN, K.C. (1985). Peroxidase involvement in lignification in water-impermeable seed coats of weedy leguminous and malvaceous species. *Plant Cell Environment* **8**, 253–260.

EMBS, R.J. AND MARKAKIS, P. (1965). The mechanism of sulfite inhibition of browning caused by polyphenol oxidase. *Journal of Food Science* **30**, 735–738.

ESPIN, J.C., MORALES, M., VARON, R., TUDELA, J. AND GARCIA-CANOVAS, F. (1995). A continuous spectrophotometric method for determining the monophenolase and diphenolase activities of apple polyphenol oxidase. *Analysis in Biochemistry* **231**, 237–246.

EVANS, C.S. (1985). Laccase activity in lignin degradation by *Coriolus versicolor*, *in vivo* and *in vitro* studies. *FEMS Microbiology Letters* **27**, 339–343.

FARKAS, G.L. AND KIRÁLY, Z. (1962). Role of phenolic compounds in the physiology of plant diseases and disease resistance. *Phytopathologie Zeitschrift* **44**, 105–150.

FERRAR, P.H. AND WALKER, J.R.L. (1993). *O*-diphenol oxidase inhibition – an additional role for oxalic acid in the phytopathogenic arsenal of *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*. *Physiology of and Molecular Plant Pathology* **43**, 415–422.

FERRAR, P.H AND WALKER, J.R.L. (1996). Inhibition of diphenol oxidases; a comparative study. *Journal of Food Biochemistry* **20**, 15–30.

FERRAR, P.H. AND WALKER, J.R.L. (1998). Phytopathogens as sources of novel diphenol oxidase inhibitors. *Journal of Food Biochemistry*, accepted for publication.

FERRAR, P.H., BARBAREL, S.I.; GINGER, M.R. AND WALKER, J.R.L. (1995). Laccase – new roles for an old enzyme. *NZ Bioscience* **3**, 7–13.

FILNER, P.H., WRAY, J.L. AND VARNER, J.E. (1969). Enzyme induction in higher plants. *Science* **165**, 358–366.

FLING, M., HOROWITZ, N.H. AND HEINEMANN, S.F. (1963). The isolation and properties of crystalline tyrosinase from *Neurospora*. *Journal of Biological Chemistry* **238**, 2045–2052.

FRIC, F. (1976). Oxidative enzymes. In *Encyclopedia of Plant Physiology*, ed. R. Heitefuss and P.H. Williams, pp. 617–627. Berlin-Heidelberg: Springer-Verlag.

FROEHNER, S.C. AND ERIKSSON, K-E.L. (1974). Purification and properties of *Neurospora crassa* laccase. *Journal of Bacteriology* **120**, 458–465.

FUKUSAWA, R., WAKABAYASHI, H. AND NATORI, T. (1982). Japanese Patent No. 5740875.

GALEAZZI, M.A.M., SGARBIERI, V.C. AND CONSTANTINIDES, S.M. (1981). Isolation, purification and physiochemical characterisation of polyphenol oxidases (PPO) from a dwarf variety of banana (*Musa cavendishii* L.). *Journal of Food Science* **46**, 150–155.

GALLIANO, H., GAS, G., SERIS, J.L. AND BOUDET, A.M. (1991). Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidising enzymes: Manganese peroxidase and laccase. *Enzymes in Micro Technology* **13**, 478–482.

GARDNER, J., MANOHAR, S. AND BORISENOK, W.S. (1991). US Patent No. 4988523.

GARZILLO, A.M.V., DI PAOLO, S., BURLA, G. AND BUONOCORE, V. (1992). Differently-induced extracellular phenoloxidases from *Pleurotus ostreatus*. *Phytochemistry* **31**, 3685–3690.

GENTILE, A., FERRARIS, L. AND MATTA, A. (1988). Variations of phenoloxidase activities as a consequence of stresses that induce resistance to *Fusarium* wilt of tomato. *Journal of Phytopathology* **12**, 45–53.

GOETGHEBEUR, M. AND KERMASHA, S. (1996). Inhibition of polyphenol oxidase by copper-metallothionein from *Aspergillus niger*. *Phytochemistry* **42**, 935–940.

GOLAN-GOLDHIRSH, A. AND WHITAKER, J.R. (1984). Effect of ascorbic acid, sodium bisulfite and thiol compounds on mushroom polyphenol oxidase. *Journal of Science of Food and Agriculture* **32**, 1003–1009.

GOLAN-GOLDHIRSCH, A. AND WHITAKER, J.R. (1985). K_{cat} inactivation of mushroom polyphenol oxidase. *Journal of Molecular Catalysis* **32**, 141–147.

GONEN, L., VITERBO, A., CANTONE, F., STAPLES, R.C. AND MAYER, A.M. (1996). Effect of cucurbitacins on mRNA coding for laccase in *Botrytis cinerea*. *Phytochemistry* **42**, 321–324.

GOULD, J.M. (1983). Probing the structure and dynamics of lignin *in situ*. *What's New in Plant Physiology* **14**, 5–8.

GRAZIANI, M.T., ANTONILLI, L., SGARGA, P., CITRO, G., MANDOVIS, B. AND ROSCI, M.A. (1990). Biochemical and immunological studies of deglycosylated *Rhus verncifera* laccase. *Biochemical International* **21**, 1113–1124.

GRIFFIN, J. (1993). UV – a burning issue. *NZ Science Monthly* Feb. 1993, 9–10.

GRNCAREVIC, M. AND HAWKER, J.S. (1971). Browning of sultana grape berries during drying. *Journal of Science of Food and Agriculture* **22**, 270–274.

GUNATA, Y.Z., SAPIS, J.C. AND MOUTOUNET, M. (1987). Substrates and aromatic carboxylic acid inhibitors of grape phenol oxidases. *Phytochemistry* **26**, 1573–1575.

GUTTERIDGE, S. AND ROBB, D.A. (1975). The catecholase activity of *Neurospora* tyrosinase. *European Journal of Biochemistry* **54**, 107–116.

HAARS, A. AND HÜTTERMANN, A. (1980). Function of laccase in the white- rot fungus *Fomes annosus*. *Archives in Microbiology* **125**, 233–237.

HALIM, D.H. AND MONTGOMERY, M.W. (1978). Polyphenol oxidase of d'Anjou pears (*Pyrus communins*, L.) *Journal of Food Science* **43**, 603–605, 608.

HAREL, E., MAYER, A.M., MARKUS, Z. AND AVIGAD, G. (1967). Inhibition of apple catechol oxidase by a galactose oxidase inhibitor from *Dactylium dendroides*. *Israel Journal of Botany* **16**, 38–41.

HAREL, E., MAYER, A.M. AND LERNER, H.R. (1970). Changes in the levels of catechol oxidase and laccase activity in developing peaches. *Journal of Science of Food and Agriculture* **21**, 542–544.

HARKIN, J.M. AND OBST, J.R. (1973b). Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. *Experentia* **29**, 381–387.

HARVEY, P.J., SCHOEMAKER, H.E. AND PALMER, J.M. (1985). Enzymic Degradation of lignin and its potential to supply chemicals. *Annals Proceedings Phytochemical Society European* **26**, 249–266.

HASEGAWA, S. AND MAIER, V.P. (1980). Polyphenol oxidase of dates. *Journal of Agricultural Food Chemistry* **28**, 891–893.

HERMAN, T.E., KURTZ, M.B. AND CHAMPE, S.P. (1973). Laccase localized in huille cells and cleistotheical primordia of *Aspergillus nidulans*. *Journal of Bacteriology* 154, 955–960.

HICKS, K.B., SAPERS, G.M. AND SEIB, P.A. (1990). US Patent No 4975293.

HIGUCHI, T. (1957). Biochemical studies of lignin formation. *Physiology of Plants* **10**, 365–370.

HIGUCHI, T. (1985). Biosynthesis of lignin. In *Biosynthesis and Biodegradation of Wood Components*, ed. T. Higuchi, pp141–160. Orlando: Academic Press.

HIGUCHI, M., MIURA, Y., BOOHENE, J., KINOSHITA, Y., YAMAMOTO, Y., YOSHIMURA, I. AND YAMADA, Y. (1993). Inhibition of tyrosinase activity by cultured lichen tissues and bionts. *Planta Medica* **59**, 253–255.

IMAE, K., KAMACHI, H., YAMASHITA, H., OKITA, T., OKUYAMA, S., TSUNO, T., YAMASAKI, T., SAWADA, Y., OHBAYASHI, M., NAITO, T. AND OKI, T. (1991). Synthesis, stereochemistry and biological properties of the depigmenting agents melanostatin, feldamycin and analogs. *Journal of Antibiotics (Tokyo)* **44**, 76–85.

ISHIHARA, Y., OKA, M., TSUNAKAWA, M., TOMITA, K., HATORI, M., YAMAMOTO, H., KIMEI, H., MIYAKI, T., KONISHI, M. AND OKI, T. (1991). Melanostatin, a new melanin synthesis inhibitor. Production, isolation, chemical properties, structure and biological activity. *Journal of Antibiotics (Tokyo)* **44**, 25–32.

IYENGAR, R. AND MCEVILY, A.J. (1992). Anti-browning agents: alternatives to the use of sulfites in foods. *Trends in Food, Science Technology* **3**, 60–64.

JANOVITZ-KLAPP, A., RICHARD, F. AND NICOLAS, J.J. (1989). Polyphenoloxidase from apple, partial purification and some properties. *Phytochemistry* **28**, 2903–2907.

JANOVITZ-KLAPP, A.H., RICHARD, F.C., GOUPY, P.M. AND NICOLAS, J.J. (1990a). Inhibition studies on apple polyphenol oxidase. *Journal of Agricultural Food Chemistry* **38**, 926–931.

JANOVITZ-KLAPP, A.H., RICHARD, F.C., GOUPY, P.M. AND NICOLAS, J.J. (1990b). Kinetic studies on apple polyphenol oxidase. *Journal of Agricultural Food Chemistry* **38**, 1437–1441.

JIMBOW, K., QUEVEDO, JR, W.L. FITZPATRICK, T.B. AND SZABO, G. (1976). Some aspects of melanin biology: 1950–1975. *Journal of Investigative Dermatology* **67**, 72–89.

JOEL, D., HARBACH, I. AND MAYER, A.M. (1978). Laccase in Anacardiaceae. *Phytochemistry* **17**, 796–800.

KAHN, V. (1985). Effect of proteins, protein hydrolyzates and amino acids on *o*- dihydroxyphenolase activity of polyphenol oxidase of mushroom, avocado and banana. *Journal of Food Science* **50**, 111–115.

KAHN, V. AND ANDRAWIS, A. (1985a). Inhibition of mushroom tyrosinase by tropolone. *Phytochemistry* **24**, 905–908.

KAHN, V. AND ANDRAWIS, A. (1985b). Tropolone as a substrate for horseradish peroxidase. *Phytochemistry* **24**, 909–914.

KAHN, V., LINDNER, P. AND ZAKIN, V. (1995). Effect of kojic acid on the oxidation of *o*-dihydroxyphenols by mushroom tyrosinase. *Journal of Food Biochemistry* **18**, 253–271.

KARHUNEN, E., NIKU-PAAVOLA, M.-L., VIIKARI, L., HALTIA, T., VAN DER MEER, R.A. AND DUINE, J.A. (1990). Cloning sequence analysis, and expression of lignolytic phenoloxidase genes of the white rot basidiomycete *Coriolus hirsutus*. *FEBS Letters* **267**, 6–8.

KARKHANIS, Y. AND FRIEDEN, E. (1961). On the induction period and a protein inhibitor of mushroom tyrosinase. *Journal of Biological Chemistry* **236**, 1–2.

KAWAGISHI, H., SOMOTO, A., KURANARI, J., KIMURA, A. AND CHIBA, S. (1993). A novel cyclotetrapeptide produced by *Lactobacillus helveticus* as a tyrosinase inhibitor. *Tetrahedral Letters* **34**, 3439–3440.

KELLY, S.K., COYNE, C.E., SLEDJESKI, D.D., CLAIBORNE FUQUA, W. AND WEINER, R.M. (1990). Identification of a tyrosinase from a periphytic marine bacterium. *FEBS Microbiology Letters* **67**, 275–280.

KERMASHA, S., GOETGHEBEUR, M., MONFETTE, A., METCHE, M. AND ROVEL, B. (1993). Inhibitory effects of cysteine and aromatic acids on tyrosinase activity. *Phytochemistry* **34**, 349–353.

KING, R.R., SINGH, R.P. AND BOUCHER, A. (1987). Variation in sucrose esters from the type B glandular trichomes of certain wild potato species. *American Potato Journal* **64**, 529–534.

KLEIN, M.G. AND JACKSON, T.A. (1992). Bacterial diseases of scarabs. In *Use of Pathogens in Scarab Pest Management*, ed. T.R. Glare and T.A. Jackson, pp. 43–61. UK: Intercept Ltd.

KOJIMA, Y., TSUKUDA, Y., KAWAI, Y., TSUKAMOTO, A., SUGIURA, J. AND KITA, Y. (1990). Cloning sequence analysis, and expression of lignolytic phenoloxidase genes of the white rot basidiomycete *Coriolus hirsutus*. *Journal of Biological Chemistry* **265**, 15224–15230.

KOSUGE, T. (1969). The role of phenolics in host response to infection. *Annual Reviews Phytopathology* **7**, 195–222.

KOWALSKI, S.P., PLAISTED, R.L. AND STEFFENS, J.C. (1993). Immunodetection of polyphenol oxidase in glandular trichomes of *S. berthaultii* and *S. tuberosum* and their hybrids. *American Potato Journal* **70**, 185–199.

KRUEGER, R.C. (1955). The inhibition of tyrosinase. *Archives in Biochemistry and Biophysics* **57**, 52–60.

KUBO, I., KINST-HORI, I.AND YOKOKAWA, Y. (1994). Tyosinase inhibitors from *Anacardium occidentale* fruits. *Journal of Natural Production* **57**, 545–551.

KUBO, Y., SUZUKI, K., FURUSAWA, I., ISHIDA, N. AND YAMAMOTO, M. (1982). Relation of appresorium pigmentation and penetration of nitrocellulose membranes by *Colletotrichum lagenarium*. *Phytopathology* **72**, 498–500.

KUBO, Y., SUZUKI, K., FURUSAWA, I. AND YAMAMOTO, M. (1983). Scytalone as a natural intermediate in melanin biosynthesis in appresoria of *Colletotrichum lagenarium*. *Experimental Mycology* **7**, 208–215.

KUBO, I. AND YOKOKAWA, Y. (1992). Two tyrosinase inhibiting flavonol glycosides from *Buddleia coriacea*. *Phytochemistry* **31**, 1075–1077.

KUBOWITZ, T. (1938). Spaltung und resynthese der polyphenoloxydase und des hamocyanins. *Biochemical Zeitschrift* **299**, 32–57.

KUTTNER, R. AND WAGREICH, H. (1953). Some inhibitors of mushroom catecholase. *Archives in Biochemistry and Biophysics* **43**, 80–87.

KUWAHARA, M, GLENN, J.K., MORGAN, M.A. AND GOLD, M.H. (1984). Separation and characterisation of two extracellular H_2O_2 dependent oxidases from lignolytic cultures of *Phanaerochaete chrysoporium*. *FEBS Letters* **169**, 247–250.

LABELL, F. (1983). Sulfite alternatives. Ascorbic, citric acids effective with proper adjustment for each fruit/vegetable application. *Food Processing* **54**, 54.

LAM, Y-L. AND HO, K-K. (1990). Purification of polyphenol oxidase from orchid leaves. *Plant Physiology and Biochemistry* **28**, 209–213.

LANGDON, T.T. (1987). Preventing of browning in fresh prepared potatoes without the use of sulfiting agents. *Food Technology* **41**, 64–67.

LAX, A.R. AND VAUGHN, K.C. (1991). Colocalization of polyphenol oxidase and photosystem II proteins. *Plant Physiology* **96**, 26–31.

LEJCZAK, B., DUS, D. AND KAFARSKI, P. (1990). Phosphonic and phosphinic acid analogues of tyrosine and 3,4-dihydroxyphenylalanine (DOPA). as potential antimelanotic agents. *Anti-cancer Drug Design* **5**, 351–358.

LEONARD, T.J. (1971). Phenoloxidase activity and fruiting body formation in *Schizophyllum commune*. *Journal of Bacteriology* **106**, 162–167.

LEONI, O. AND PALMIERI, S. (1990). Polyphenol oxidase from artichoke (*Cynara scolymus* L.). *Food Chemistry* **38**, 27–39.

LEONOWICZ, A. AND GRZYWNOWICZ, K. (1981). Quantitative estimation of laccase forms in some white rot fungi using syringaldazine as a substrate. *Enzyme Micro Technology* **3**, 55–58.

LERCH, K. (1976). *Neurospora* tyrosinase: molecular weight, copper content and spectral properties. *FEBS Letters* **69**, 157–160.

LERCH, K. (1978). Amino acid sequence of tyrosinase from *Neurospora crassa*. *Proceedings of the National Academy of Sciences, USA* 75, 3635–3639.

LERCH, K. (1981). Copper mono oxygenases: tyrosinase and dopamine b mono oxygenase. In *Metal Ions in Biological Systems. Copper Proteins*, ed. H. Sigel, pp. 143–186. Basel, New York: Marcel Dekker.

LERCH, K., HUBER, M., SCHNEIDER, H.J., DREXEL, R. AND LINZEN, B. (1986). Different origins of metal binding sites in binuclear copper proteins, tyrosinase and hemocyanin. *Journal of Inorganic Biochemistry* **26**, 213–217.

LERNER, H.R. AND MAYER, A.M. (1976). Reaction mechanism of grape catechol oxidase – a kinetic study. *Phytochemistry* **15**, 57–60.

LERNER, H.R., HAREL, E., LEHMAN, E. AND MAYER, A.M. (1971). Phenylhydrazine, a specific irreversable inhibitor of catechol oxidase. *Phytochemistry* **10**, 2637–2640.

LERNER, H.R., MAYER, A.M. AND HAREL, E. (1974). Differential effect of phenylhydrazine on the catechol oxidase and cresolase activities of *Vitis* catechol oxidase. *Phytochemistry* **13**, 397–401.

LIU, L., DEAN, J.F.D., FRIEDMAN, W.E., AND ERIKSSON, K-E.L. (1994). A laccase-like phenol oxidase is correlated with lignin biosynthesis in *Zinnea elegans* stem tissues. *The Plant Journal* **6**, 213–224.

LONERGAN, G. AND BAKER, W.L. (1995). Comparative study of substrates of fungal laccase. *Letters of Applied Microbiology* **21**, 31–33.

LOWUM, S.E. AND PARKIN, K.L. (1989). Characterisation of dye-sensitized photooxidation of mushroom tyrosinase. *Journal of Food Biochemistry* **13**, 391–401.

LOZANO-DE-GONZALEZ, P.G., BARRETT, D.M., WROLSTAD, R.E. AND DURST, R.W. (1993). Enzymatic browning inhibited in fresh and dried apple rings by pineapple juice. *Journal of Food Science* 58, 399–404.

MCCALLUM, J.A. AND WALKER, J.R.L. (1990). O-Diphenol oxidase activity, phenolic content, and colour of NZ wheats, flours and milling streams. *Journal of Cereal Science* **12**, 83–96.

MCCRAE, A.R. AND DUGGLEBY, R.G. (1968). Substrates and inhibitors of potato tuber phenolase. *Phytochemistry* **7**, 855–861.

MCDOUGAL, G.J., STEWART, D. AND MORRISON, I.M. (1993). Oxidation of coniferyl alcohol to lignin-like products by tobacco xylem cell walls. *Phytochemistry* **37**, 683–689.

MCEVILY, A.J., IYENGAR, R. AND GROSS, A.T. (1992). Inhibition of polyphenol oxidase by phenolic compounds. In *Phenolic Compounds in Food and their Effects on Health I.* ACS Symposium Series, Washington DC. **506**, 318–325.

MCEVILY, A.J., IYENGAR, R. AND OTWELL, S. (1991). Sulfite alternative prevents shrimp melanosis. *Food Technology* **45**, 80, 82–86.

MACHIEX, J.J., FLEURIET, A. AND BILLOT, J. (1990). *Fruit Phenolics.* Boca Raton, Florida: CRC Press.

MACHIEX, J.J., SAPIS, J.C. AND FLEURIET, A. (1991). Phenolic compounds and polyphenoloxidase in relation to browning in grapes and wines. *CRC Critical Reviews in Food Science and Nutrition* **30**, 441–486.

MADHOSINGH, C. (1975). Mushroom inhibitors of dopa oxidation. *Canadian Journal of Microbiology* **21**, 2108–2111

MADHOSINGH, C. AND SUNDBERG, L. (1974). Purification and properties of tyrosinase inhibitor from mushroom. *FEBS Letters* **49**, 156–158.

MARBACH, I. AND MAYER, A.M. (1975). Changes in catechol oxidase and permeability to water of seed coats of *Pisum elatius* during seed developement and maturation. *Plant Physiology* **56**, 93–96.

MARBACH, I., HAREL, E., MAYER, A.M. (1985). Pectin, a second inducer for laccase production by *Botrytis cinerea*. *Phytochemistry* **24**, 2550–2561.

MARTINEZ M.V AND WHITAKER J.R. (1995). The biochemistry and control of enzymatic browning. *Trends in Food Science Technology* **6**, 195–200.

MARTINEZ, J.H., SOLANO, F., PENAFIEL, R., GALINDO, J.D., IBORRA, J.L. AND LOZANO, J.A. (1986). Comparative study of tyrosinase from different sources: relationship between halide inhibition and the enzyme active site. *Comparative Biochemistry and Physiology* **83B**, 633–636.

MASON, H.S. AND PETERSON, E.W. (1965). Melanoproteins I. Reactions between enzyme generated quinones and amino acids. *Biochimica et Biophysica Acta* **3**, 134–146.

MATHEIS, G. AND BELITZ, H.D. (1977). Multiple forms of soluble monophenol dehydroxy-phenylalanine oxygen oxidoreductase (EC 1.14.18.1). from potato tubers (*Solanum tuberosum*). II partial characterisation of the enzyme forms with different molecular weights. *Zeit Lebensmittel Untersuch Forschung* **163**, 279–282.

MATTA, A. AND GENTILE, I.A. (1970). Differential inhibition and activation of polyphenoloxidase activity in healthy and *Fusarium* infected tomato plants. *Phytopathologia Mediterranea* **9**, 168–175.

MATTHEW, A.G. AND PARPIA, H.A.B. (1971). Food browning as a polyphenol reaction. *Advances in Food Research* **19**, 75–145.

MAXEMIUC-NACCACHE, V. AND DIETRICH, S.M.C. (1985). Changes in phenols and oxidative enzymes in resistant and susceptible *Coffea arabica* inoculated with *Hemileia vastatrix* (coffee rust). *Revta Brasilia Botany* **8**, 185–190.

MAYER, A.M. (1987). Polyphenol oxidases in plants – recent progress. *Phytochemistry* **26**, 11–20.

MAYER, A.M. AND HAREL, E. (1968). A laccase like enzyme in peaches. *Phytochemistry* **7**, 1253–1256.

MAYER, A.M. AND HAREL, E. (1979). Polyphenol oxidases in plants. *Phytochemistry* **18**, 193–214.

MAYER, A.M. AND HAREL, E. (1991). Phenoloxidases and their significance in fruits and vegetables. In *Food Enzymology*, ed. P.F. Fox, pp. 371–398. London: Elsevier.

MAYER, A.M., HAREL, E. AND BEN-SHAUL, R. (1966). Assay of catechol oxidase – a critical comparison of methods. *Phytochemistry* **5**, 783–789.

MENON, S., FLECK, R.W., YONG, G. AND STROTHKAMP, K.G. (1990). Benzoic acid inhibition of the α, β and γ isozymes of *Agaricus bisporus* tyrosinase. *Archives in Biochemistry and Biophysics* **280**, 27–32.

MESSERSCHMIDT, A. AND HUBER, R. (1990). The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin. *European Journal of Biochemistry* **187**, 341–352.

MOLNAR-PERL, I AND FRIEDMAN, M. (1990a). Inhibition of browning by sulfur amino acids. 2 Fruit juices and protein containing foods. *Journal of Agricultural Food Chemistry* **38**, 1648–1651.

MOLNAR-PERL, I. AND FRIEDMAN, M. (1990b). Inhibition of browning by sulfur amino acids. 3 Apples and potatoes. *Journal of Agricultural Food Chemistry* **38**, 1652–1656.

MONDY, N.I. AND MUNSHI, C.B. (1993). Effect of boron on enzymatic discolouration and phenolic and ascorbic acid contents of potatoes. *Journal of Science of Food and Agriculture* **41**, 554–556.

MONSALVE-GONZÁLEZ, A., BARBOSA-CÁNOVAS G.V., MCEVILY A.J. AND IYENGAR R. (1995). Inhibition of enzymatic browning in apple products by 4-hexyl resorcinol. *Food Technology* **49**, 1995, 110–118.

MONTGOMERY, M.W. (1983). Cysteine as an inhibitor of browning in pear juice concentrate. *Journal of Food Science* **18**, 951–952.

MORITA, H., KAYASHITA, T., KOBATA, H., GONDA, A., TAKEYA, K. AND ITOKAWA, H. (1994a). Cyclic peptides from higher plants 6. Pseudostellarins A-C, new tyrosinase inhibitory cyclic peptides from *Pseudostellaria heterophylla*. *Tetrahedral Letters* **50**, 6797–6804.

MORITA, H., KAYASHITA, T., KOBATA, H., GONDA, A., TAKEYA, K. AND ITOKAWA, H. (1994c). Pseudostellarins D-F, new tyrosinase inhibitory cyclic peptides from *Pseudostellaria heterophylla*. *Tetrahedral Letters* **50**, 9975–9982.

MORITA, H., KOBATA, H., TAKEYA, K. AND ITOKAWA, H. (1994b). Pseudostellarin G, a new tyrosinase inhibitory cyclic octapeptide from *Pseudostellaria heterophylla*. *Tetrahedral Letters* **35**, 3563–3564.

MOUSTAFA, F.A. AND WHITTENBURG, R. (1970). Properties which appear to allow phytopathogenic pseudomonads to counteract plant defense mechanisms. *PhytopathologieZeitschrift* **67**, 214–224.

MUKHERJEE, P.K. AND GHOSH, J.J. (1975). Phenol oxidase activity in relation to resistance of rice to infection by *Helminthosporium oryzae*. *Science and Culture* **4**, 433–435.

MUNETA, P. (1981). Comparisons of inhibitors of tyrosine oxidation in the enzymatic blackening of potatoes. *American Potato Journal* **58**, 85–92.

Murao, S., Hinode, Matsumura E., Kawai K., Ohishi H., Jin H., Oyama H. and Shin T. (1992). A novel laccase inhibitor, N-hydroxyglycine, produced by *Penicillium citrinum* YH-31. *Bioscience, Biotechnology and Biochemistry* **56**, 987–988.

MURAO, S., OYAMA, H., NOMURA, Y., TONO, T. AND SHIN, T. (1993). Purification and characterisation of *Arctium lappa* L. (edible burdock). polyphenol oxidase. *Bioscience, Biotechnology and Biochemistry* **57**, 177–180.

MURATA, M., CHIYO, K. AND HOMMA, S. (1992). Purification and some properties of chlorogenic acid oxidase from apple (*Malus pumila*). *Bioscience, Biotechnology and Biochemistry* **56**, 1705–1710.

MURR, D.P. AND MORRIS, L.L. (1974). Effect of succinic acid-2,2-dimethyl hydrazide on mushroom *o*-diphenol oxidase. *Journal of the American Society for Horticultural Science* **99**, 3–6.

NAISH, S. AND RILEY, P.A. (1989). Studies on the kinetics of oxidation of 4-hydroxy anisole by tyrosinase. *Biochemistry and Pharmacology* **38**, 1103–1107.

NAKAMURA, T. (1958). Purification and physiochemical properties of laccase. *Biochimica et Biophysica Acta* **30**, 44–52.

NAKAMURA, K., AMANO, Y. AND KAGAMI, M. (1983). Purification and some properties of a polyphenol oxidase from koshu grapes. *American Journal of Enol Viticulture* **34**, 122–127.

O'MALLEY, D.M., WHETTEN, R., BAO, W., CHEN, C-L. AND SEDEROFF, R.R. (1993). The role of laccase in lignification. *Plant Journal* **4**, 751–757.

Oszmianski, J. and Lee, C.Y. (1990). Inhibition of polyphenol oxidase activity and browning by honey. *Journal of Agricultural Food Chemistry* **38**, 1892–1895.

OUGH, C.S. AND CROWELL, E.A. (1987). Use of sulfur dioxide in winemaking. *Journal of Food Science* **52**, 386–388.

PALMER, J.K. (1963). Banana polyphenoloxidase preparation and properties. *Plant Physiology* **38**, 508–513.

PALUMBO, A., D'ISCHIA, M., MISURACA, G. AND PROTA, G. (1991). Mechanism of inhibition of melanogenisis by hydroquinone. *Biochimica et Biophysica Acta* **1073**, 85–90.

PARK, E.Y. AND LUH, B.S. (1985). Polyphenol oxidase of kiwifruit. *Journal of Food Science* **50**, 678–684.

PARKIN, K.L. AND LOWUMI, S.E. (1990). Active oxygen species involved in the dye-sensitized photoinactivation of mushroom tyrosinase. *Journal of Science of Food and Agriculture* **38**, 1297–1302.

PELAEZ, F., MARTINEZ, M.J. AND MARTINEZ, A.T. (1995). Screening of 68 species of basidiomycetes for enzymes involved in lignin degradation. *Mycology Research* **99**, 37–42.

PENAFIEL, R., GALINDO, J.D., SOLANO, F., PEDREÑO, E., IBORRA, J.L. AND LOZANO, J.A. (1984). Kinetic study of the interaction between frog epidermis tyrosinase and chlorine. *Biochimica et Biophysica Acta* **788**, 327–332.

PÉTRIE, F.H. AND GOLD, M.H. (1991). Manganese regulation of manganese peroxidase expression and lignin degradation by the white rot fungus *Dichomitus squalens*. *Applied Environmental Microbiology* 57, 2240–2245.

PEYRATOUT, C.S., SEVERENS, J.C., HOLM, S.R. AND MCMILLIN, D.R. (1994). EPR studies of ligand binding to the type 2/type 3 cluster in tree laccase. *Archives in Biochemistry and Biophysics* **314**, 405–411.

PIERPONT, W.S. (1969). *o*-Quinones formed in plant extracts, their reactions with amino acids and peptides. *Journal of Biochemistry* 112, 609–616.

PIFFERI, P.G. AND BALDASSARI, L. (1973). A separate spectrophotometric method for the determination of the catecholase activity of tyrosinase by Besthorn's hydrazone. *Analysis in Biochemistry* **52**, 325–335.

PIFFERI, P.G., LUCIANO, B. AND CULTRERA, R. (1974). Inhibition by carboxylic acids of an *o*-diphenol oxidase from *Prunus avium* fruits. *Journal of Science of Food and Agriculture* **25**, 263–270.

PLATT, M.W., HADER, Y. AND CHET, H. (1984). Fungal activities involved in lignocellullose degradation by *Pleurotus*. *Applied Environmental Microbiology* **20**, 150–155.

POLLOCK, C.J. AND DRYSDALE, R.B. (1976). The role of phenolic compounds in the resistance of tomato cultivars to *Verticillium albo-atrum*. *Phytopathologie Zeitschrift* **86**, 56–66.

PRABHAKARAN, K., HARRIS, E.B. AND KIRCHHEIMER, W.F. (1969). Effect of inhibitors on phenoloxidase of *Mycobacterium leprae*. *Journal of Bacteriology* **100**, 935–938.

PROTA, G. (1980). Recent advances in the chemistry of melanogenesis in mammals. *Journal of Investigative Dermatology* **75**, 122–128.

RACUSEN, D. (1970). The catechol oxidase of bean leaves (*Phaseolus vulgaris*). *Canadian Journal of Botany* **48**, 1029–1032.

RATCLIFFE, N.A., ROWLEY, A.F., FITZGERALD, S.W., RHODES, C.P. (1985). Invertebrate Immunity: Basic concepts and recent advances. *International Reviews of Cytology* **97**, 183–350.

REHMAN, A.U. AND THURSTON, C.F. (1992). Purification of laccase I from *Armillaria mellea*. *Journal of General Microbiology* **138**, 1251–1257.

REINHAMMER, B. (1984). Laccase. In *Copper Proteins and Copper Enzymes* Vol III, ed. R. Lontie, pp 1–35. Boca Raton, Florida: CRC Press Inc.

RICH, P.R., WIEGAND, K.K., BLUM, H., MOORE, A.L. AND BONNER JR, W.D. (1978). Studies on the mechanism of inhibition of redox enzymes by substituted hydroxamic acids. *Biochimica et Biophysica Acta* **525**, 325–337.

RICHARD, F.C., GOUPY, P.M., NICOLAS, J.J., LACOMBE, J. AND PAVIA, A.A. (1991). Cysteine as an inhibitor of enzymatic browning 1. Isolation and characterisation of addition compounds formed during oxidation of phenolics by apple polyphenol oxidase. *Journal of Agricultural Food Chemistry* **39**, 841–847.

ROBB, D.A. (1984). Tyrosinase. In *Copper Proteins and Copper Enzymes* Vol II, ed. R. Lontie, pp 207–240. Boca Raton, Florida: CRC Press.

ROBENE-SOUSTRADE, I., LUNG-ESCARMANT, B., BONO, J.J. AND TARIS, B. (1992). Identification and partial characterization of an extracellular manganese-dependent peroxidase in *Armillaria ostoyae* and *Armillaria mellea*. *European Journal of Forest Pathology* **22**, 227–236.

ROBERT, C., RICHARD, F.C., ROUCH, C., PABION, M. AND CADET, F. (1996). A kinetic study of the inhibition of palmito polyphenol oxidase by L-cysteine. *International Journal of Biochemistry and Cell Biology* **28**, 457–463.

ROUET-MAYER, M.A. AND PHILIPPON, J. (1986). Inhibition of catechol oxidases from apples by sodium chloride. *Phytochemistry* **25**, 2717–2719.

SAKURAI, T. (1991). Characterisation of coppers in *Coriolus consors* laccase. *Journal of Inorganic Biochemistry* **41**, 277–282.

SALGUES, M., CHEYNIER, V., GUNATA, Z. AND WYLD, R. (1986). Oxidation of grape juice 2-5-glutathionyl caffeoyl tartaric acid by *Botrytis cinerea* laccase and characterisation of a new substance: 2,5 di-5-glutathionyl caffeoyl tartaric acid. *Journal of Food Science* **51**, 1191–1194.

SALOHEIMO, M., NIKU-PAAVOLA, M-L. AND KNOWLES, J.K.C. (1991). Isolation and structural analysis of the laccase gene from the lignin degradating fungus *Phlebia radiata*. *Journal of General Microbiology* **137**, 1537–1544.

SÁNCHEZ-FERRER, A., RODRÍGUEZ-LÓPEZ, J.N., GARCÍA-CÁNOVAS, F. AND GARCÍA-CARMONA, F. (1995). Tyrosinase: a comprohensive review of its mechanism. *Biochimica et Biophysica Acta* **1247**, 1–11.

SANTERRE, C.R., CASH, J.N. AND VANNORMAN, D.J. (1988). Ascorbic acid/citric acid combinations in the processing of frozen apple slices. *Journal of Food Science* **53**(6), 1713–1716,1736.

SAPERS, G.M. AND ZIOLKOWSKI, M.A. (1987). Comparison of erythorbic and ascorbic acids as inhibitors of enzymatic browning in apple. *Journal of Food Science* **52**, 1732–1747.

SAPERS, G.M., HICKS, K.B., PHILLIPS, J.G., GARZARELLA, L., PONDISH, D.L., MATULAITIS, R.M., MCCORMACK, T.J., SONDEY, S.M., SEIB, P.A. AND EL-ATAWY, Y.S. (1989a). Control of enzymatic browning in apples with ascorbic acid derivatives, polyphenol oxidase inhibitors and complexing agents. *Journal of Food Science* **54**, 997–1012.

SAPERS, G.M., EL-ATAWY, Y.S., HICKS, K.B. AND GARZARELLA, L. (1989b). Effect of emulsifying agents on inhibition of enzymatic browning in apple juice by ascorbyl palmitate, laurate and decanoate. *Journal of Food Science* **54**(4), 1096–1097.

SARUNO, R., KATO, F. AND IKENO, T. (1979). Kojic acid, a tyrosinase inhibitor from *Aspergillus albus*. *Agricultural Biological Chemistry* **43**, 1337–1338.

SATÔ, M. (1979). Detection in spinach leaves of inhibitor (oxalates). and activator acting on chloroplast phenolase. *Plant Science Letters* **16**, 355–359 .

SATÔ, M. (1980a). Inhibition by oxalates of spinach chloroplast phenolase in unfrozen and frozen states. *Phytochemistry* **19**, 1613–1617.

SATÔ, M. (1980b). Reactivation by copper of phenolase pre-inactivated by oxalate. *Phytochemistry* **19**, 1931–1933.

SATÔ, M. (1982). Multiplicity of spinach root phenolase and its monophenolase activity. *Phytochemistry* **21**, 1229–1231.

SAYAVEDRA-SOTO, L.A. AND MONTGOMERY, M.W. (1986). Inhibition of polyphenol oxidase by sulfite. *Journal of Food Science* **51**, 1531–1536.

SCHONBAUM, G.R., BONNER, JR., W.D. STOREY, B.T. AND BAHR, J.T. (1971). Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. *Plant Physiology* **47**, 124–128.

SCHWIMMER, J. (1981). *Source Book of Enzymology*. New York; AVI Publishers Ltd.

SELIM, K., GOETGHEBEUR, M., MONFETTE, A., METCHE, M. AND ROVEL, B. (1993). Studies on inhibition of mushroom polyphenol oxidase using chlorogenic acid as substrate. *Journal of Agricultural Food Chemistry* **41**, 526–531.

SHARON, M. AND MAYER, A.M. (1967). The effect of sodium chloride on catechol oxidase from apples. *Israel Journal of Chemistry* **5**, 275–280.

SINGLETON, V.L., ZAYA, J., TROUSDALE, E. AND SALGUES, M. (1984). Caftaric acid in grapes and conversion to a reaction product during processing. *Vitis* **23**, 113–120.

SINGLETON, V.L., SALGUES, M., ZAYA, J. AND TROUSDALE, E. (1985). Caftaric acid disappearance and conversion to products of enzymic oxidation in grape must and wine. *American Journal of Enol Viticulture* **36**, 50–56.

SPRITZ, R.A. AND HEARING, JR., V.J. (1994). Genetic disorders in pigmentation. In *Advances in Human Genetics* **22**, ed. H. Harris and K. Hirschorn, pp. 1–45. New York: Plenum Press.

STERJIADES, R., DEAN, J.F.D AND ERIKSSON, K.E.L. (1992). Laccase from Sycamore Maple (*Acer pseudoplatanus*). polymerizes monolignols. *Plant Physiology* 99, 1162–1168.

STERJIADES, R., DEAN, J.F.D., GAMBLE, G., HIMMELSBACH, D.S. AND ERIKSSON, K.E.L. (1993). Extracellular laccases and peroxidases from sycamore maple (*Acer pseudoplatanus*). cell suspension cultures – reactions with monolignols and lignin model compounds. *Planta* **190**, 75–87.

STROTHKAMP, K.G. AND MASON, H.S. (1974). Pseudoperoxidase activity of mushroom tyrosinase. *Biochemical and Biophysical Research Communications* **61**, 827–832.

SUGUMARAN, M. (1986). Tyrosinase catalyzes an unusual oxidative decarboxlation of 3,4-dihydroxymandelate. *Biochemistry* **25**, 4489–4492.

SUGUMARAN, M., DALI, H., SEMENSI, V. AND HENNINGAN, B. (1987). Tyrosinase catalysed unusual oxidative dimerization of 1,2-dehydro-N-acetyl dopamine. *Journal of Biological Chemistry* **262**, 10546.

SUGUMARAN, M., DALI, H. AND SEMENSI, V. (1991). The mechanism of tyrosinase-catalysed oxidative decarboxylation of a-(3,4-dihydroxyphenol) lactic acid. *Journal of Biochemistry* **277**, 849–853.

SUGUMARAN, M., DALI, H. AND SEMENSI, V. (1992). Mechanistic studies on tyrosinase-catalysed oxidative decarboxylation of 3,4-dihydroxymandelic acid. *Journal of Biochemistry* **281**, 353–357.

TAKEO, T. (1966). Tea leaf polyphenol oxidase part III studies on the changes of polyphenol oxidase activity during black tea manufacture. *Agricultural and Biological Chemistry* **30**, 529–534.

TAN, K.S. AND KUBO, I. (1990). Inhibitor of the oxidation of catechin released from the roots of corn seedlings. *Experientia* **46**, 971–972.

TAYLOR, R.L. (1969). A suggested role for the polyphenol-phenoloxidase system in invertebrate immunity. *Journal of Invertebrate Pathology* 14, 427–428.

TAYLOR, S.L. AND BUSH, R.K. (1986). Sulfites as food ingredients. *Food Technology* June 47–52.

THIPYAPONG, P., HUNT, M.D. AND STEFFENS, J.C. (1995). Systemic wound induction of potato (*Solanum tuberosum*). polyphenol oxidase. *Phytochemistry* **40**, 673–676 .

THOMPSON, D.C., THOMPSON, J.A., SUGUMARAN, M. AND MOLDÉUS, P. (1992). Biological and toxicological consequences of quinone methide formation. *Chemical–Biological Interactions* **86**, 129–162.

THURSTON, C.F. (1994). The structure and function of fungal laccases. *Microbiology* **140**, 19–26.

TOMITA, K., ODA, N., OHBAYASHI, M., KAMEI, H., MIYAKI, T. AND OKI, T. (1990). A new screening method for melanin biosynthesis inhibitors using *Streptomyces bikiniensis*. *Journal of Antibiotics (Tokyo)* **43**, 1601–1605.

TRAJKOVSKI, V. (1976). Resistance to *Sphaerotheca mors-uvae* (Schw.)Berk. in *Ribes nigrum* L. *Swedish Journal of Agricultural Research* **6**, 215–223.

TREBST, A. AND DEPKA, B. (1995). Polyphenol oxidase and photosynthesis research. *Photosynthesis Research* **46**, 41–44.

VALERO, E., VARÓN, R. AND GARCIA-CARMONA, F. (1988). Characterisation of polyphenol oxidase from Airen grapes. *Journal of Food Science* **53**(5), 1482–1485.

VALERO, E., GARCIA-MORENO, M., VARÓN, R. AND GARCIA-CARMONA, F. (1991). Time dependent inhibition of grape polyphenol oxidase by tropolone. *Journal of Science of Food and Agriculture* **39**, 1043–1046.

VÁMOS-VIGYÁZÓ, L. (1981). Polyphenol oxidase and peroxidase in fruits and vegetables. *CRC Critical Reviews in Food Science Nutrition* **15**, 49–123.

VAUGHN, K.C. AND DUKE, S.O. (1984). Function of polyphenol oxidase in higher plants. *Physiology of Plants* **60**, 106–112.

VAUGHN, K.C., LAX, A.R. AND DUKE, S.O. (1988). Polyphenol oxidase: the chloroplast oxidase with no established function. *Physiology of Plants* **72**, 659–665.

WALKER, J.R.L. (1964a). Studies on the enzymic browning of apples. *Australian Journal of Biological Science* **17**, 360–371.

WALKER, J.R.L. (1964b). The polyphenol oxidase of pear fruit. *Australian Journal of Biological Science* **17**, 575–576.

WALKER, J.R.L.(1968). Studies on the diphenol oxidase of the phytopathogenic fungus *Glomerella cingulata*; inhibition by quaternary ammonium compounds. *Phytochemistry* 7; 1231–40.

WALKER, J.R.L. (1969). Inhibition of the host phenolase system through infection by *Penicillium expansum*. *Phytochemistry* **8**, 561–566.

WALKER, J.R.L. (1970). Phenolase inhibitor from cultures of *Penicillium expansum* which may play a part in fruit rotting. *Nature* **227**, 298–299.

WALKER, J.R.L. (1975). Enzymic browning in foods; a Review. *Enzyme Technology Digest* **4**, 80–100.

WALKER, J.R.L. (1976). The control of enzymic browning in fruit juices by cinnamic acids. *Journal of Food Technology* **11**, 341–345.

WALKER, J.R.L. (1995). Enzymatic browning in fruits: its biochemistry and control. In *Enzymatic Browning and its Prevention*, ed. C.Y. Lee and J.R. Whitaker, pp. 8–22. ACS Symposium Series, Washington.

WALKER, J.R.L. AND FERRAR, P.H. (1995). The control of enzymic browning in foods. *Chemistry and Industry* No.20, 836–839.

WALKER, J.R.L. AND MCCALLION, R.F. (1980). The selective inhibition of *ortho-* and *para*-diphenol oxidases. *Phytochemistry* 19, 373–377.

WALKER, J.R.L. AND REDDISH, C.E.S. (1964). Note on the use of cysteine to prevent browning in apple products. *Journal of Science of Food and Agriculture* **15**, 902–904.

WALKER, J.R.L. AND WILSON, E.L. (1975). Studies on the enzymic browning of apples. Inhibition of apple *o*-diphenol oxidase by phenolic acids. *Journal of Science of Food and Agriculture* **26**, 1825–1831.

WHITAKER, J.R. (1985). Mechanisms of oxidoreductases. In *Chemical Changes in Food during Processing*, ed. T. Richardson and J.W. Finley, pp 123–130. Connecticut, USA: AVI Publishing Co.

WONG, T.C., LUH, B.S. AND WHITAKER, J.R. (1971). Isolation and characterisation of polyphenol oxidase isozymes of clingstone peach. *Plant Physiology* **48**, 19–23.

WOOD, D.A. (1980). Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *Journal of General Microbiology* **117**, 327–338.

WOOD, D.A. (1985). In *Developmental Biology of Higher Fungi*, ed. D. Moore, L.A. Casselton, D.A.Wood and J.C. Frankland, p.162. Cambridge, UK: Cambridge University Press.

WORRAL, J.J., CHET, I. AND HÜTTERMANN, A. (1986). Association of rhizomorph formation with laccase activity in *Armillaria* spp. *Journal of General Microbiology* **132**, 2527–2531.

WOSILAIT, W.D., NASON, A. AND TERRELL, A.J. (1954). Pyridine nucleotide-quinone reductase. II, Role in electron transport. *Journal of Biological Chemistry* **206**, 271–282.

YAROPOLOV, A.I., SKOROBOGAT'KO, O.V., VARTANOV, S.S., AND VARFOLOMEYEV, S.D. (1994). Laccase: properties, catalytic mechanism, and applicability. *Applied Biochemistry and Biotechnology* **49**, 257–280.

YOUN, H.D., KIM, K.J., MAENG, J.S., HAN, Y.H., JEONG, I.B., JEONG, G., KANG, S.O., AND HAH, Y. C. (1995). Single electron transfer by an extracellular laccase from the white-rot fungus *Pleurotus ostreatus*. *Microbiology* **141**, 393–398.

YURKOW, E.J. AND LASKIN, J.D. (1989). Purification of tyrosinase to homogeneity based on its resistance to sodium dodecyl sulfate-proteinase K digestion. *Archives in Biochemistry and Biophysics* **275**, 122–129.

ZAWISTOWSKI, J., BILIADERIS, C.G. AND MURRAY, E.D. (1988a). Purification and characterisation of Jerusalem artichoke (*Helianthus tuberosus* L.) polyphenoloxidase. *Journal of Food Biochemistry* **12**, 1–22.

ZAWISTOWSKI, J., BILIADERIS, C.G. AND MURRAY, E.D. (1988b). Isolation and some properties of an acidic fraction of polyphenol oxidase from Jerusalem artichoke (*Helianthus tuberosum* L.). *Journal of Food Biochemistry* **12**, 23–35.

ZAWISTOWSKI, J., BILIADERIS, C.G. AND ESKIN, N.A.M. (1991). Polyphenol oxidase. In *Oxidative Enzymes in foods*, ed. D.S. Robinson and N.A.M. Eskin, pp. 217–273. New York: Elsevier Science Publishers Ltd.

ZHOU, P., SMITH, N.L. AND LEE, C.Y. (1993). Potential purification and some properties of Monroe apple peel polyphenol oxidase. *Journal of Agricultural Food Chemistry* **41**, 532–536.

ZINKERNAGEL, V. (1986). Untersachungen zur anfalligkeit und resistenz von kopfsalat (*Latuca sativa*) gegen falschen mehltau (*Bremia lactucae*). III Peroxydase, peroxydatische katalase und polyphenoloxydase aktivitaten. *Journal of Phytopathology* **115**, 257– 266.